The therapeutic effects of FGF21 on diabetic nephropathy are realized by augmenting autophagy via AMPK/mTOR signaling pathway

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Research Article

Keywords: Diabetic Nephropathy, FGF21, Autophagy, AMPK/mTOR

Posted Date: December 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-118596/v1

The therapeutic effects of FGF21 on diabetic nephropathy are realized by augmenting autophagy via AMPK/mTOR signaling pathway

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Abstract

Accumulating evidence demonstrates that FGF21 plays a preventive role in the development of diabetic nephropathy (DN). However, little is known about the therapeutical effects of FGF21 on DN and underlying mechanism. In this study, FGF21 significantly ameliorated blood glucose, HbAlc, insulin resistance, renal function and histopathological change in DN mice (BKS-Lepr^{em2Cd479}/Gpt), which develop abnormalities in renal morphology and function. Our results showed that administration of FGF21 upregulated the autophagy related genes LC3 Ⅱ and BCL-1 mRNA and protein expression levels. D-glucose was used for high glucose (HG) model in mesangial cells. The results showed that treatment with FGF21 reduced the levels of ROS, AGEs and inflammatory cytokines and significantly downregulated the protein expression of PCNA. Meanwhile, FGF21 significantly enhanced the expression of LC3 Ⅱ and BCL-1. Besides, Our studies showed that administration of FGF21 significantly upregulated the phosphorylation of AMPK and downregulated phosphorylation of mTOR. Meanwhile, the effects of FGF21 on autophagy were reversed by siRNA against β-klotho. In conclusion, The therapeutic effects of FGF21 on diabetic nephropathy are realized and FGF21 ameliorates mesangial cell glucotoxicity and abnormal proliferation *in vitro* by augmenting autophagy via AMPK/mTOR pathway. These results suggest that FGF21 can be a therapeutic target against DN.

Keyword: Diabetic Nephropathy, FGF21, Autophagy, AMPK/mTOR

Introduction

Diabetic nephropathy (DN), a microvascular complications of kidney disease, is the main cause of end-stage renal disease in diabetic patients worldwide [1, 2]. DN initiates with the thickening of the glomerular basement membrane, which is followed by mild and moderate mesangial expansion, capillary collapse in the renal tubule, epithelial cell degeneration and a gradual increase in proteinuria, and finally leads to renal fibrosis and kidney failure [3-5]. In the intrinsic cells of the kidney, the role of mesangial cell has become more and more important [6]. In the progression of DN, abnormal proliferation of mesangial cells, advanced glycation end products and ROS production were induced by prolonged hyperglycemia, finally resulting in increased glomerular volume, glomerular filtration and urinary protein excretion [4, 7]. Thus, it

affects the function of other intrinsic cells and causes the occurrence and development of DN. Recent studies have shown that autophagy is involved in the development and progression of diabetic nephropathy, which can degrade redundant proteins and harmful cytoplasmic components for cellular metabolism [8]. Autophagy is a generic term for all pathways by which cytoplasmic materials are delivered to the lysosome in animal cells or the vacuole in plant and yeast cells [9]. Besides, autophagy is also used as a recycling system that repairs tissue injury and cellular renovation and homeostasis [10, 11]. Subsequent studies demonstrated that autophagy in DN mice was more deficiency than that in normal mice [12]. The most common negative regulation signaling pathway is mTOR [13]. Therefore, identifying a treatment strategy that enhances autophagy will efficiently ameliorate DN.

FGF21 is a novel member of the fibroblast growth factor family that functions as an endocrine hormone and an important regular of energy metabolism [14, 15]. The role of FGF21 in metabolic regulation was first demonstrated as facilitating glucose uptake by adipocytes through up-regulating transcription of the glucose transporter-1 [16]. FGF21 exhibits low affinity for heparin sulfate and FGF receptors (FGFR1-4) and therefore needs co-receptor β-klotho to form the ternary complex, FGF21-FGFR-β-klotho (1:2:1), to exert its actions [17-19]. Large amounts of published studies have demonstrated that FGF21 exists beneficial effects in diabetes and its related complications by lowering blood glucose levels, stimulating lipid β-oxidation, and improving insulin sensitivity [20, 21]. Zhu et al found out that FGF21 significantly increased autophagy-related gene expression both in MSG mice and fat-loaded HepG2 cells [22]. In addition, FGF21 also activates hepatic autophagy via JMJD3/KDM6B histone demethylase [23]. Recently, it is well known that FGF21 augments auophagy in random–pattern skin flaps via AMPK/mTOR signaling pathway and improves tissue survival [24]. Besides, Kim reports that FGF21 improves insulin resistance and ameliorates renal injury in db/db mice [25]. Shao et al demonstrates an additive protection by LDR and FGF21 treatment against diabetic nephropathy in type 2 diabetic model [26, 27]. Past reports have suggested that FGF21 could prevent diabetic nephropathy. However, the therapy effects of FGF21 on diabetic nephropathy and its underlying mechanism are not reported in diabetic nephropathy mice.

In this study, we explored whether FGF21 played a substantial role in the therapeutic effects of FGF21 on diabetic nephropathy in diabetic nephropathy mice. Therefore, we examined the blood glucose, insulin resistance, renal function and histopathological change in DN mice. In

addition, the levels of ROS and AGEs, the expression levels of LC3Ⅱ, BCL-1 and PCNA were also measured in mesangial cells. Its potential mechanisms were also detected *in vivo* and *vitro.*

Results

The kidney and mesangial cells are the target for FGF21

To investigate whether the kidney and MES13 cell are the target for FGF21, we detected β-koltho and FGFR1 in the kidney of db/db mice and MES13 cells. Western blot demonstrated that FGF21 significantly upregulated β-koltho and FGFR1 expression in both MES13 cells (Fig. 1A,B) and the kidney of db/db mice (Fig. 1C,D). These results demonstrate that the kidney is the target organ for FGF21 and mesangial cell (MES13-SV40) is the target cell for FGF21.

FGF21 ameliorates diabetic nephropathy in diabetic nephropathy model

Diabetic nephropathy is a microvascular complications of kidney disease, which is associated with hyperglycemia, insulin resistance and decline in renal function [2]. The blood glucose of diabetic nephropathy mice were measured before the last injection at the fourth week's administration of Insulin or FGF21. The results demonstrated that insulin could not showed long-hypoglycemic effect and decreased the HAbc1 level compared to model control (Fig. 2A,B). However, FGF21 significantly ameliorated these effects compared to insulin or model control. The OGTT results demonstrated that insulin sensitivity was improved by FGF21 compared to insulin alone or model control. Administration of combination FGF21 with insulin showed mutual sensitization in db/db mice (Fig. 2C,D). Moreover, our studies showed that FGF21 treatment ameliorated renal dysfunction by significantly reducing kidney weight per 100g body weight (BW), urinary microalbumin (mAlb) and urine protein (UP) compared to model control (Fig. 2E,F,G). However, both treatment with FGF21 alone or combination with insulin have little influence on urine creatinine (U-Cre) compared with the model control (Fig. 2H). The reason may be that there is no significant change about U-Cre in DN [27]. Besides the renal function, plasma concentrations of AST, ALT, ALP and total bilirubin are associated with DN. The results showed that FGF21 significantly reduced the levels of AST, ALT, ALP and total bilirubin compared to model control (Table 2).

Finally, histopathological staining processed with H&E, Masson and PAS showed mesangial marix increase, basement membrance thickening, capillaries decrease, collagen accumulation and glomerulosclerosis. These pathological changes were obviously ameliorated by administration of FGF21 alone or combination with insulin groups (Fig. 3A,B) compared with insulin alone or model control. Besides, there is slightly improvement on the fibrosis of kidney compared with model control (Fig. 3C).

FGF21 augments autophagy in the kidney of diabetic nephropathy mice

Autophagy is involved in the development and progression of diabetic nephropathy [8]. The protein expression levels of LC3Ⅱ and BCL-1 are associated with autophagy [9]. Our results showed that administration of FGF21 markedly augmented the mRNA levels of LC3Ⅱ and BCL-1 compared with model control (Fig. 4A,B). The results of Western blot showed treatment with FGF21 also significantly increased the protein levels of LC3Ⅱ and BCL-1 compared with model control (Fig. 4C,D).

FGF21 attenuates glucotoxicity in high glucose-treated mesangial cells

Mesangial cell is one of the intrinsic cells of the kidney, which plays an important role in the development of DN [30]. To understand the potential mechanism that FGF21 ameliorates DN, mesangial cells were used for further research. D-glucose and D-mannitol were respectively used for high glucose (HG) model and normal osmotic pressure control (NG). The level of ROS was measured by Reactive oxygen species assay kit and the AGEs were measured using OxiSelect^{TM} advanced end product (AGE) competitive ELISA kit. The results demonstrated that the levels of ROS and AGEs were significantly reduced in administration of FGF21 groups compared with HG group (Fig. 5A,B,C). But the effects of FGF21 on mesangial cell glucotoxicity were reversed by small interfering RNA against β-klotho (see supplemental data S1). Besides, it is well known that PAI-1 and INOS are both keys of factor which reflect the levels of inflammation in mesangial cells. So we used RT-PCR and Western blot to measure the levels of PAI-1 and INOS, which were significantly reduced in administration of FGF21 groups compared with HG group (Fig. 5D-G).

FGF21 inhibits high glucose-induced mesangial cell proliferation

Mesangial cell proliferation has been proved as a pathogenic factor to glomerulosclerosis, which is a pathological change of DN [31]. To further explore the effect of FGF21 on mesangial cells, CCK-8 kit was used to measure the cell proliferation. The results showed that high glucose-induced cell proliferation was attenuated by the administration of FGF21 in a dose-dependent manner compared with high glucose treatment (Fig. 6A). The expression of PCNA further confirmed this result. The mRNA and protein levels of PCNA were decreased by treatment with FGF21 compared with HG treated treatment (Fig. 6B-D). The both results showed that FGF21 inhibited high glucose-induced cell proliferation.The effects of FGF21 on high glucose-induced mesangial cell proliferation were reversed by small interfering RNA (see supplemental data S2).

FGF21 boosts autophagy in high glucose-treated mesangial cells

Our previous studies demonstrated that FGF21 augmented autophagy in the kidney of DN mice. Kim et al reported that autophagy reduced ECM production in mesangial cells by promoting the degradation of intracellular collagen Ⅰ [8, 32]. To detected the effects of FGF21 on autophagy in mesangial cells, the mRNA and protein levels of LC3 Ⅱ and BCL-1 were detected by RT-PCR (Fig. 7A,B) and Western blot (Fig.7 C,D). The results showed that FGF21 increased the mRNA and protein expression of LC3 Ⅱ and BCL-1 *in vitro* as same as *in vivo* experiments. Besides, the effects of FGF21 on mesangial cells were reversed by small interfering RNA (siRNA-β-klotho) (Fig. 7E,F).

The effect of FGF21 on AMPK/mTOR signaling pathway

A series of researches have demonstrated that AMPK/mTOR pathway is one of the classical pathways regulating autophagy [24, 33]. To examine whether FGF21 increases auphagy via AMPK/mTOR pathway, we detected the expression of p-AMPK/AMPK and p-mTOR/mTOR at protein levels in mesangial cells and the kidney of diabetic nephropathy mice. Our results showed that FGF21 upregulated the phosphorylation level of AMPK and downregulated the phosphorylation level of mTOR in the kidney of DN mice and mesangial cells (Fig. 8A,B), but the effects of FGF21 on total protein expression level of AMPK and mTOR were not significant difference. Moreover, the effects were reversed by siRNA against β-klotho in mesangial cells (Fig. 8C,D). These results suggest that one of the mechanisms by which FGF21 augments autophagy is the AMPK/mTOR pathway (Fig. 9A).

Discussion

Diabetic nephropathy (DN) is one of the most serious public health problems worldwide [34]. About 40% of diabetes develop end-stage renal disease (ESRD). Dysregulated autophagy plays an pathogenic role in a lot of disease processes. Wang et al showed that autophagy regulated many critical aspects of disease conditions in the kidney [12]. The previous studies have demonstrated that FGF21 prevents DN via reducing blood glucose levels, enhancing insulin sensitivity, decreasing lipid levels and attenuating kidney inflammation and fibrosis [25-27, 35].However, the therapeutic effects of FGF21 have not been investigated.

FGF21 is initially isolated from a mouse embryo, which induces long-hypoglycemic effects and plays its pleiotropic functions in tissue homeostasis and metabolism [10, 27, 36]. A number of studies have demonstrated that the circulating level of FGF21 is regarded as a biomarker of progression in diabetic nephropathy [37, 38], indicating increased level of FGF21 plays a role in the kidney. The hypothesis is proved by subsequent study, which demonstrates that FGF21 ameliorates renal injury in DN mice and improves insulin resistance [25]. Besides, Shao et al shows an additive protection by LDR and FGF21 treatment against diabetic nephropathy in type 2 diabetes model [26]. Diabetic nephropathy is associated with hyperglycemia, insulin resistance, renal dysfunction. Besides, histopathological changes, including mesangial marix increase, basement membrance thickening, capillaries decrease, collagen accumulation and glomerulosclerosis, play an important role in the development of DN [8, 39]. However, the mechanism of FGF21 in the kidney of DN remain investigations. Our results demonstrated that FGF21 treatment showed long-hypoglycemic effect and decreased HbA1c level. Previous studies have demonstrated that synergistic effect between FGF21 and insulin is realized through mutual sensitization in diabetic mice [40, 41]. Our result suggested FGF21 improved insulin sensitivity and the synergistic effect was also realized in DN mice. Besides, the effects of treatment with combination FGF21 and insulin demonstrated better efficacy than treatment alone. Previous study has proved that diabetic nephropathy is associated with decline in renal function and proteinuria [8, 42]. In our study, we found administration of FGF21 potently improved renal function evaluated by reducing the excretion of UP and mAlb. In addition, HE, PAS and Masson staining revealed that treatment with FGF21 alone or combination with insulin improved pathological changes, which included mesangial marix increase, basement membrance thickening, capillaries decrease, collagen accumulation and glomerulosclerosis. All data suggest that FGF21 ameliorates diabetic nephropathy in DN mice.

Autophagy is a highly conserved "self-eating" pathway by which cells degrade and recycle macromolecules and organelles [8, 9]. Many studies have proved that down regulation of autophagy is implicated in the pathogenesis of DN [8, 32]. However, little is known about the relationship between FGF21 and autophagy in DN mice. Here, we investigated the expression of autophagy related genes LC3 Ⅱ and BCL-1. Formation of LC3 Ⅱ from LC3 Ⅰ is a critical step in autophagosome constitution [43, 44] and BCL-1 is regarded as an indicator of autophagy activity [9]. Our studies showed that FGF21 upregulated the mRNA and protein expression of LC3 Ⅱ and BCL-1 in the kidney of DN mice, indicating that autophagy is boosted by administration of FGF21 alone or combination with insulin. Abnormal proliferation of mesangial cells and hypertrophy are hallmark of diabetic nephropathy, which eventually lead to glomerulosclerosis [31, 45]. The function of autophay is not clearly in mesangial cells. Previous study shows that the reduction of autophagy contributes to diabetic kidney disease [34]. Our results showed that autophagy was impaired in high glucose-treated mesangial cells. Treatment with FGF21 significantly augmented autophagy. To prove this result, we used the small interfering RNA (siRNA) directed against β-klotho in mesangial cells. The result was consistent with our expection that the β-klotho expression was inhibited in transfected cells. we examined the effects of FGF21 on autophagy in transfected cells, which showed that the ability of augmenting autophagy was reversed. These results suggest that FGF21 significantly augments autophagy in the kidney of DN mice and high glucose-treated mesangial cells.

Previous studies have demonstrated that the level of autophagy is regulated by the phosphorylation of Atg1 via the intracellular mammalian target of rapamycin (mTOR) and adenosine monophosphate (AMP)-activated protein kinase (AMPK) [8, 32]. The mechanistic target of mTOR and AMPK are the classical nutrient-sensing pathway regulating autophgic activity [32, 46]. Therefore, we raise a hypothesis whether FGF21 ameliorates autophagy through AMPK/mTOR signaling pathway in DN. Results of Western blot demonstrated that the phosphorylation of AMPK was significantly downregulated and the phosphorylation of mTOR was significantly upregulated in the kidney of diabetic nephropathy mice and high glucose-treated mesagial cells. As anticipated, administration of FGF21 reversed the effects in both diabetic nephropathy mice and HG-treated mesangial cells. There were no significant difference on the total protein levels of AMPK and mTOR *in vivo* and *in vitro*. It was consistent with Zhou's study that FGF21 boosted autophagy in random-pattern skin flaps by AMPK/mTOR pathway [24]. In addition, the effects of FGF21 on AMPK/mTOR signaling pathway

were attenuated by si-RNA directed against β-klotho in mesangial cells. Theses results demonstrate that AMPK/mTOR signaling pathway is one of the mechanisms by which FGF21 augmentes autophagy in DN and HG-treated mesagial cells.

A number of studies have certified that mesangial cell proliferation and hypertrophy, associated with excessive ECM proteins, promote the progression of diabetic nephropathy [32, 47-49]. Lin et al showed that Smad7 alleviates glomerular mesangial cell proliferation via ROS-NF-κB pathway [48]. Ding et al reviewed that the diabetes-induced altered intracellular metabolism and cellular events, including AGEs, ROS and endoplasmic reticulum stress, modulate autophagic acitivity and contribute to the development of DN [32, 50]. Han et al has demonstrated that Triptolide suppresses glomerular mesangial cell proliferation in DN associated with inhibition of PDK1/Akt/mTOR pathway [51]. Huang et al shows that Aldosterone-induced mesangial cell proliferation is mediated by EGF receptor via Akt/mTOR pathway [47]. In this studies, we found the cell proliferation and the expression of PCNA were increased significantly in HG-treated cells. Treatment with FGF21 significantly downregulated PCNA expression and inhibited cell proliferation. The underlying mechanism may be associated with the inhibitory effect of FGF21 on mTOR. Besides, FGF21 significantly attenuated mesangial cell glucotoxicity, which included ROS, AGEs and inflammatory cytokines (PAI-1 and INOS). In high glucose milieu, the AGEs induce oxidative stress and modulate various cellular events, such as reactive oxygen species (ROS) [32]. Peng et al shows that the character of autophagic clearance of AGEs plays an important role in ameliorating diabetic vascular complications including kidney dysfunction [52]. Recombinant mouse HGF improves the endocytosis and autophagic clearance of AGEs [52]. Besides, the production of ROS is increased by high glucose concentrations in the kidney and is associated with cell dysfunction [53]. Therefore, we speculated that FGF21 could attenuate cell glucotoxicity via augmenting the autophagic clearance of AGEs in high glucose-treated mesangial cells. Furthermore, administration of FGF21 ameliorates diabetic nephropathy.

Conclusion

In this experiment, our data indicates that the therapeutic effects of FGF21 on diabetic nephropathy are realized by augmenting autophagy via AMPK/mTOR signaling pathway in diabetic nephropathy mice. Administration of FGF21 combination with insulin shows synergistic effect on the therapeutic consequence of diabetic nephropathy. Furthermore, FGF21 inhibits mesangial cell proliferation and

significantly attenuates mesangial cell glucotoxicity by boosting autophagy via AMPK/mTOR signaling pathway *in vitro*. These findings suggest that FGF21 can be a therapeutic target against diabetic nephropathy.

Materials and methods

Ethics statement

This study was approved by the ethics committee of Northeast Agriculture University. All experimental protocols involving animals followed the guidelines issued by National Institute of Health and the Institutional Animal Care and Use Committee of Northeast Agriculture University. The mice (approval number: SCXK-2018-0008) were killed under anesthesia induced by intraperitoneal injection of 1.2% avertin (Sigma, USA) at a dose of 20 μl/g body weight, and all efforts were made to minimize their suffering.

Animal model and treatment

Male C57BL/6 and db/db mice (BKS-Lepr^{em2Cd479}/Gpt) (SPF; 8 weeks old, qualified number: No 110727201101189, approval number: SCXK 2018-0008) were obtained from the experimental animal center jicui of nanajing (jiangsu, China). The db/db mice are hyperinsulinemic model of genetic diabetes that develops abnormalities in renal morphology and function that parallel those in human diabetic nephropathy [28]. Most of the db/db mice will appear diabetic nephropathy at 12th week. All mice were housed in the experimental animal center of Northeast Agriculture University at 22±2℃ with 12:12-h light-dark cycles and free access to rodent chow and tap water. After four weeks pre-feeding, the blood glucose, insulin resistance, renal function and histopathological changes were measured for evaluation indicators of DN [4, 5]. The DN model mice were divided randomly into four groups (db/db model control, insulin treatment groups, FGF21 treatment groups, the combination with insulin and FGF21 treatment groups, namely db/db, db/db+I, db/db+F, db/db+I+F). C57BL/6 mice were regarded as normal control (namely control). The mice were subcutaneously treated with saline, FGF21 and insulin daily for 4 weeks. The dose of FGF21 was 2mg/kg and the does of insulin was 1U per mice, and we selected this dose according to previous study [29]. Animals were sacrificed at the end age 16 weeks.

Measuring renal and liver function

Mice were placed in metabolic cages individually with free to tap water on the day before euthanasia to collect 24h urine samples. Total urinary

protein (UP), urinary creatinine (U-Cre) and urinary micro ablbumin (mAlb) contents were measured according to the manufacturer's instuctions as parameters of renal function using enzme-linked immunosorbent assay (ELISA) kits (AMEKO, China). The ALT, AST ALP and BIL were measured in samples of plasma. The activity was evaluated in the Dongfang hospital, Lianyungang, China.

Histological analysis

The kidney tissues were fixed in 4% paraformaldehyde at room temperature for 48h. After dehydration, the tissue blocks were embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and masson's trichrome staining. HE staining is used for general morphological examination, massion's trichrome staining is used for interstitial expansion evaluation and PAS staining is used for glomeruloslerosis evaluation. The index of sections were averaged from three mice in each group.

Mesangial cell culture and siRNA-β-klotho transfection

Murine mesangial cells (MES13-SV40) were purchased from American Type Culture Collection (ATCC, USA). The cells were grown in DMEM/F12 (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 1% penicillin/streptomycin at humidified atmosphere of 5% CO_2 at 37℃. The cells were divided into four groups in the logarithmic phase of growth: 1) the normal glucose group treated with 30mM D-mannitol (for the same osmotic pressure as high glucose group). 2) the high glucose group treated with 30mM D-glucose (Solarbio Life Science, China). 3) the high glucose group with low does of FGF21 (final concentration 0.1 μM). 4) the high glucose group with high does of FGF21 (1.0 μM), namely NG, HG, HG+FL, HG+FH. MES13 cells were seeded at a density of 1-2×10^5/ml and starved for 12h in a FBS-free medium, and then the medium was exchanged with media containing 30mM D-glucose alone or combination with FGF21 for 48 hours.

Because β-klotho is a necessary receptor for FGF21 driving its function, we next used small interfering RNA (siRNA) directed against β-klotho to investigate the effects of FGF21 on mesangial cells. The β-klotho mRNA was specifically knocked down by using commercially available siRNA oligonucleotides. The sequences of the siRNA were designed by Sangon Biotech (shanghai, China). Sense strand, 5'-CCUCUAUGACACUUUC-CCUAATT-3'; Antisense strand, 5'-UUAGGGAAAGUGUCAUAGAG-GTT-3'. The mesangial cells were maintained in DMEM/F12 with 10% FBS before transfection with 50nM siRNA using lipofectamine3000

(Invitrogen life technologies, USA) according to the manufacturer's instructions. Mesangial cells were transfected for 6h with siRNA and Opti-MEM (Gibco, USA), then the cells were cultivated in 10% FBS and antibiotic-free DMEM/F12. Cells were made quiescent for 24h and treated with 30mM high-glucose medium for 48h. The negative siRNA was used as control.

Measurement of cell proliferation

Mesangial cells were seeded at 1.5-5×10^4 cells/well in 96-well plates and incubated for 12h. Then the medium were replaced in FBS-free DMEM/F12 for cell synchronization. After 12h incubation, the cells were treated with normal and high glucose medium for 24h followed by adding FGF21. Next, the CCK-8 solution (Beyotime biotechnology, China) were added into each well plates for 2h. the absorbance was measured at 450 nm using a Flex Station 3 (Molecular Devices, USA).

Measurement the levels of ROS and AGEs

Intracellular ROS clearance activity of FGF21 was evaluated by 2',7'-Dichlorofluorescin diacetate (DCF-DA) staining (Beyotime biotechnology, China) as described previously with slight modification. The cells were managed by the previous method, then 10 μM DCF-DA was added when cells were washed three times with PBS. After incubation of cells for 20min at 37℃ the mesangial cells were washed with PBS, photographed and measured using a Flex Station 3 (Molecular Devices, USA) and flow cytometry (Thermo Fisher Scientific, USA).

The protein of the kidney tissues or cells were measured using OxiSelect™ advanced end product (AGE) competitive ELISA kit (Cell Biolabs, lnc.) in accordance with the instructions. Briefly, 50 μL of samples and 50 μl of 1000X diluted anti-AGE antibody were added in the plates and incubated at room temperature for 1 hour on an orbital shaker. After three times wash with wash buffer, 100 μl of 1000X diluted secondary antibody-HRP conjugate were added into each wells and incubated for 1 hour at room temperature. The enzyme reaction was stopped by adding 100 μl of stop solution to each well. Results were read immediately by a Flex Station 3 (Molecular Devices, USA).

RNA isolation and Real-time quantitative PCR

Total RNA from the kidney and cells were isolated with TRIzol (Invitrogen, USA), and quantitative gene expression was performed on a bio-Rad CFX manager (ABI7500, Applied biosystems, USA) using

SYBR green technology (TaKaRa, Janpan). The primer sequences are shown in Table 1.

Western blot analysis

Protein were extracted from the the renal tissues and cells using radioimmuno-precipitation assay (RIPA) buffer (Beyotime Institute of biotechnology, China) together with a protease inhibitor PMSF (Sigma, USA) and phosphatase inhibitors (Beyotime Institute of biotechnology, China). Protein concentrations were determined by the BCA quantitative kit (Beyotime Institute of biotechnology, China). Then protein was separated by sodium-dodecyl sulfate polyacrylidence gel electrophoresis (SDS-PAGE), electro-transferred to nitrocellulose filter membrance (NC membrances), blocked with Quickblock^{TM} Western block kit (Genscript, USA), and probed with the following antibodies overnight at 4℃. Rabbit monoclonal anti-PAI-1 antibody (1:1000, Abcam), Rabbit ploYclonal anti-INOS antibody (1:1000, Abcam) Rabbit ploYclonal anti-Klotho antibody (1:500, Abcam), Rabbit ploYclonal anti-FGFR1 (phosphor Y654) antibody (1:500, Abcam), Rabbit monoclonal anti-β-actin, PCNA, Beclin-1, LC3A/B, mTOR, p-mTOR (Ser2448), AMPK, p-AMPK (Thr172) antibody (1:1000, Cell Signaling), the membrane was subsequently incubated with HRP-conjugated secondary antibody (1:7500, Abcam) for 1h at room temperature. Specific signals were detected using the enhanced ECL kit (Thermo Scientific, USA). The chemiDocTM XRS+ with Image LabTM Software (BIO-RAD, USA) was used for development.

Statistical analysis

All data were performed as mean±SEM, and the results diversity conspicuousness between two groups were compared by a two-way tails student's t-test. Besides, multiple comparisons were done using one-way ANOVA on GraphPad Prism 6 software. P values <0.05 was regarded as statistically significance.

Availability of data and materials

The datasets analyzed during the current study may be available upon reasonable request.

Conflicts interest

The authors declare that there are no conflicts of interest.

Achnowledgements

This work was supported by a grant from the major prophase project of Heilongjiang development and reform commission ([2011]1570), education department of Heilongjiang province (TSTAU-R2018017) and National Key R&D program of China (2017YFD0501102, 2017YFD0501103-03).

Author' contributions statement

FR, M performed the research, and analyzed the data. YK, C. Khoso, MH and K, K participated in data collection and analysis. GP, R. W,X and DS, Li contributed to the initial and consequent project discussion, manuscript discussion and revision. All the authors approved the final version of the manuscript.

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Figure

Table 1

Sequence of PCR primers were used in this study

Species	Gene	Sequence
mice	INOS	F5'-GAAAGCGGTGTTCTTTGCTT-3'
		R5'-ATGCCCGAGTTCTTTCATCA-3'
	PAI-1	F5'-TCCTCATCCTGCCTAAGTTCTC-3'
		R5'-GTGCCGCTCTCGTTTACCTC-3'
	LC3- Ⅱ	F5'-CGGGTTGAGGAGACACACACAA-3'
		R5'-ATGAGCCGGACATCTTCCAC-3'
	BCL-1	F5'-GAATGGAGGGGTCTAAGGCG-3'
		R5'-'TCTTCCTCCTGGCTCTCTCC-3'
	β-Klotho	F5'- GAACAGAAGTCCTGCGTGTGGTC-3'
		R5'-AGCAGCAACCGAAGAAGATGAGTG-3'
	PCNA	F5'-GCGCTAGTATTTGAAGCACCAA-3'
		R5'-CGATCTTGGGAGCCAAGTAGTA-3'
	β-actin	F5'-ACATCTGCTGGAAGGTGGAC-3′
		R5'-GGTACCACCATGTACCCAGG-3

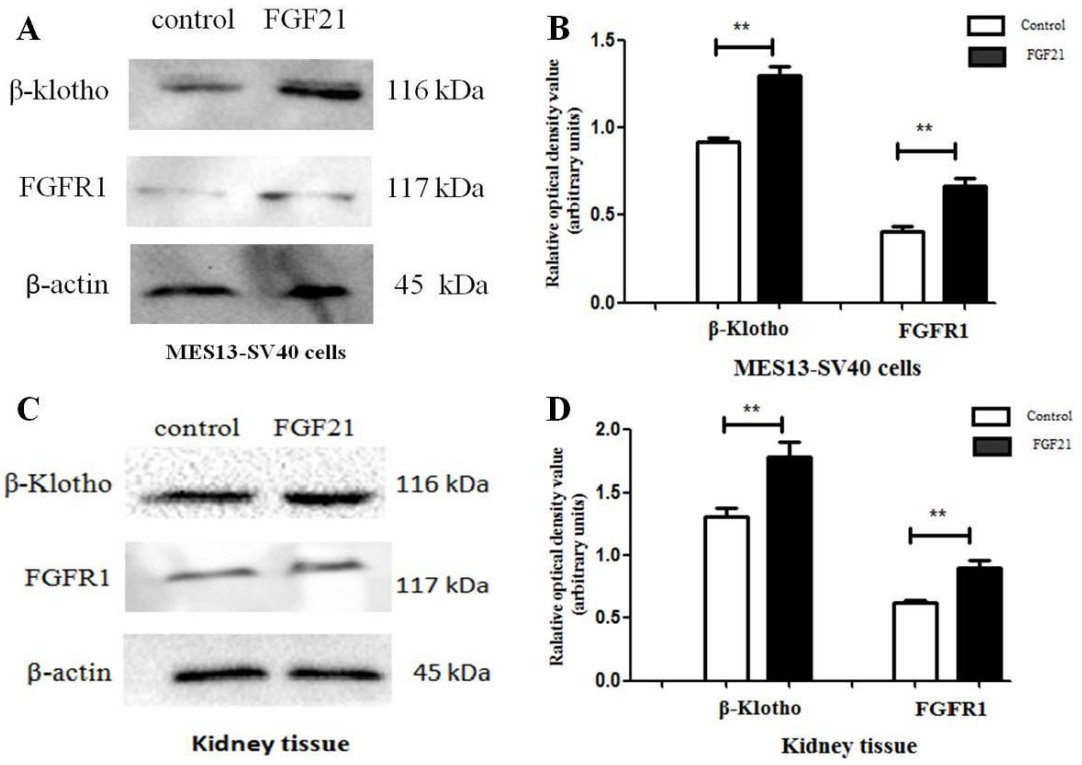
Table 2

690 Impact of FGF21 alone or combination with Insulin on ALT, AST, ALB
691 and BIL in mice.

	C57BL/6	db/db	db/db+Insulin	db/db+FGF21	db/db+Insulin+FGF21
ALP(IU/L)	38.32±3.56	85.65±6.78###	83.34±6.34	67.64±1.36**	55.31±7.9**
ALT(IU/L)	50.26±3.65	120.3±16.99###	117.06±7.3	70.34±3.65**	60.36±7.25**
AST(IU/L)	126.35±5.04	177.9±8.5##	173±6.64	140.64±3.69**	131.65±1.6**
TBIL(umol/L)	3.65±0.36	6.35±0.26##	6.16±0.065	4.86±0.042*	3.95±4.5*

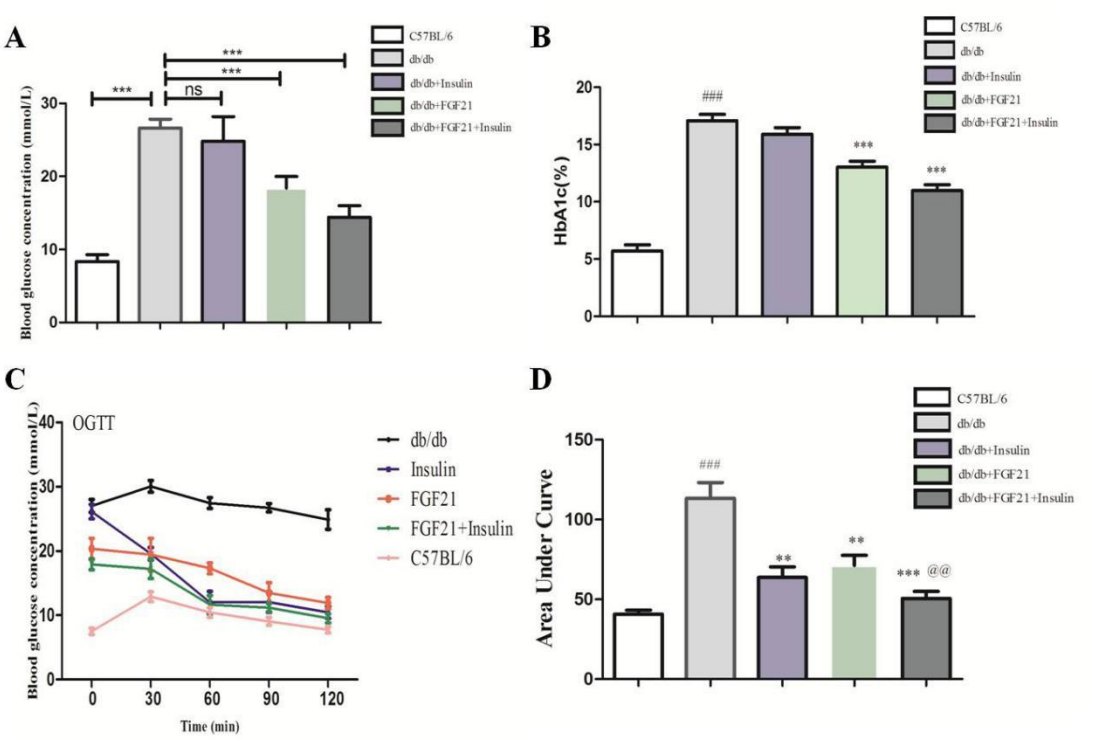
692

693 Serum of mice was collected at the end of experiment in each group. The
694 activity was evaluated in the Dongfang hospital, Lianyungang China. All
695 dates represent mean ± SEM, n=6 per group, *p<.0.05, **p<0.01
696 compared to diabetic nephropathy model mice. #p<.0.05, ###p<0.001
697 compared to control.



698

699 **Fig. 1**. The kidney and mesangial cells are the target of FGF21. The mice
700 were euthanized at 16th weeks. (**A**,**C**) Western blot analysis the protein
701 expression of β-klotho and FGFR1 in mesangial cells and the kidney of
702 diabetic nephropathy mice. (**B**,**D**) The relative β-klotho and FGFR1 levels
703 were expressed as the ratio β-klotho/β-actin and FGFR1/β-actin, which
704 were analyzed by Image J. All date represent mean ± SEM, n=6 per
705 group, *p<0.05, **p<0.01, ***p<0.01 significant as compared to control.



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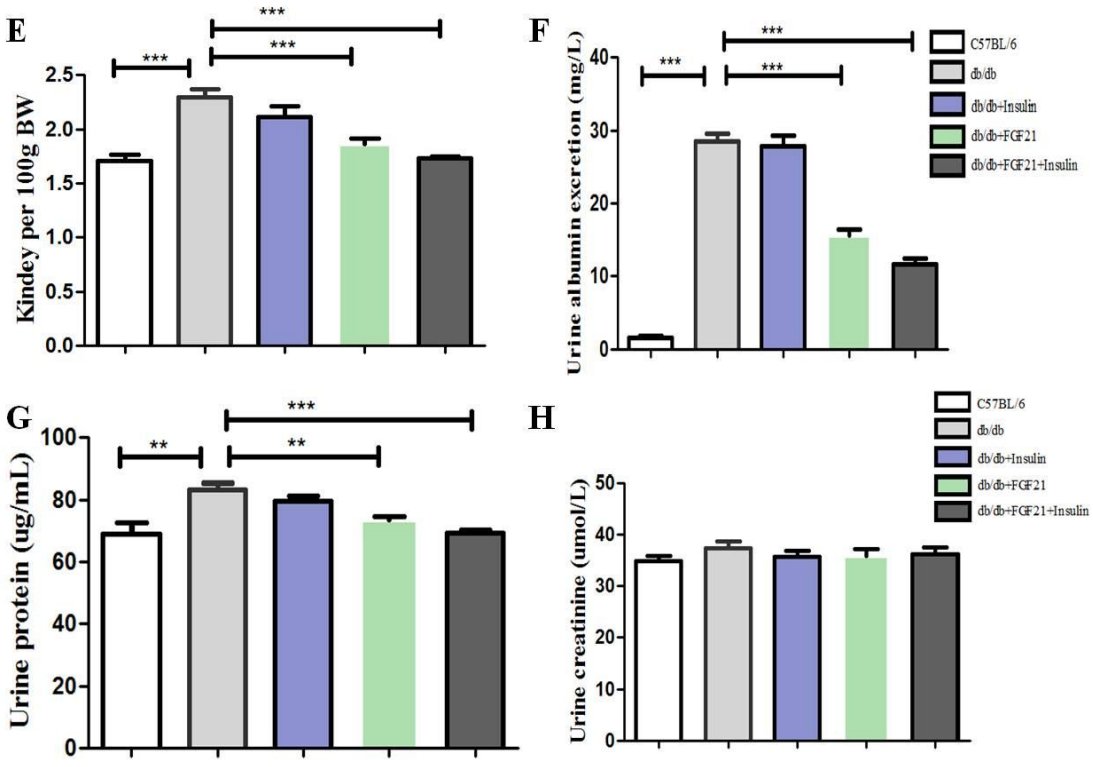


707

708 **Fig. 2**. FGF21 ameliorates the kidney tissue lesions in diabetic
709 nephropathy mice. After 4 weeks administration of FGF21 or
710 combination with insulin, blood glucose, HbA1c, OGTT, renal function
711 were measured. (**A**,**B**) The blood glucose and HbA1c of diabetic
712 nephropathy mice were measured before the last injection at the fourth
713 week's administration of Insulin or FGF21. (**C**) OGTT were performed in

all groups. (**D**) Area under the curve (AUC) value for OGTT. (**E-H**) Kidney per 100 g weight body, Urine albumin, Urine protein, Urine creatinine were measured in all groups. All data represent mean ± SEM, n=6 per group, *p<.0.05, **p<0.01, compared to diabetic nephropathy model mice. $^{\#}$p<.0.05, $^{\#\#\#}$p<0.001 compared to control. $^{@@}$p<.0.01 compared to administration of insulin alone mice.

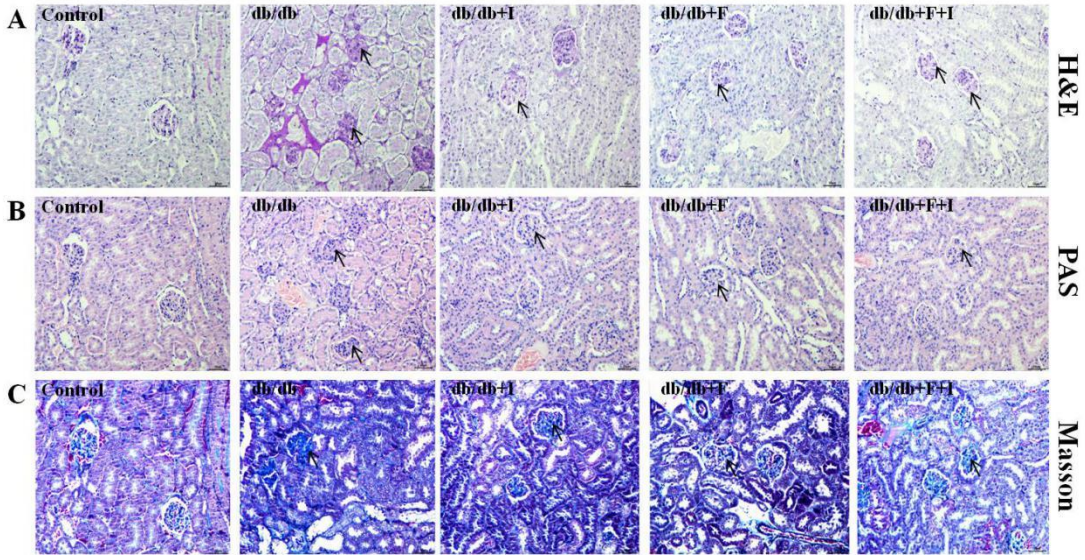


Fig. **3**. FGF21 ameliorates histopathological changes in the kidney of diabetic nephropathy mice. (**A**) Representative HE staining (×200) of kidney sections from mice. (**B**) Representative periodic acid-Schiff (PAS) (×200) of kidney sections from mice. (**C**) Representative Masson's trichrome staining (×200) of kidney sections from mice.

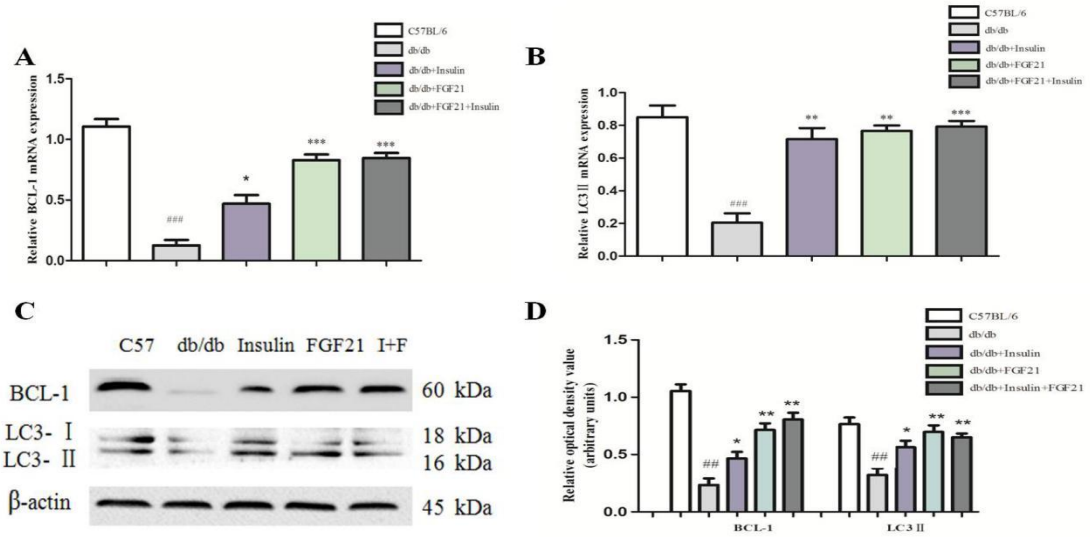
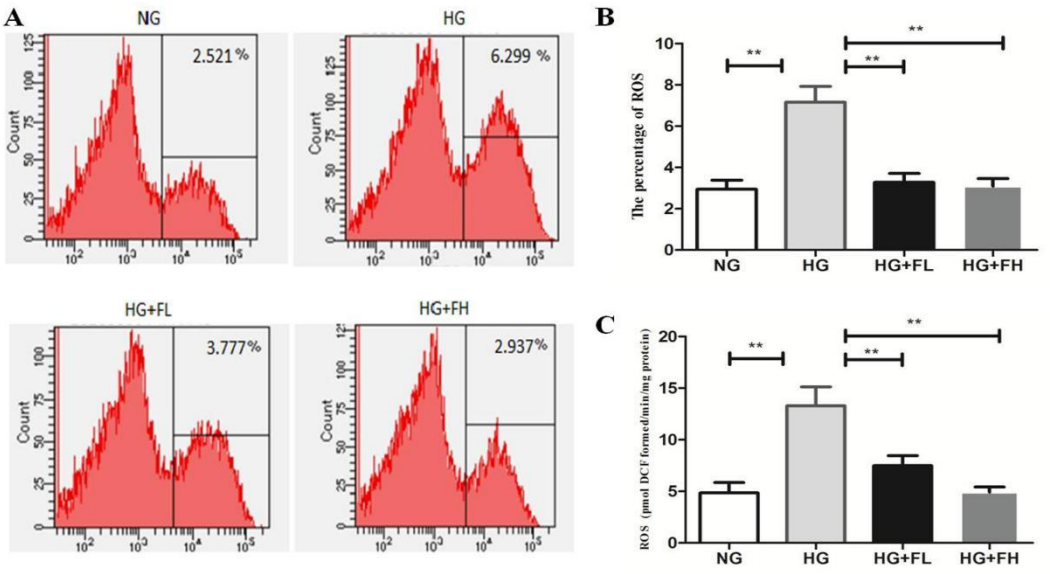


Fig. **4**. Administration of FGF21 augments autophagy in the kidney of diabetic nephropathy mice. After 4 weeks treatment of FGF21 alone or

combination with insulin, The mRNA and protein expression of LC3 Ⅱ and BCL-1 were detected by real-time PCR and Western blot. (**A**,**B**) RT-PCR analysis of LC3 Ⅱ and BCL-1. (**C**) Western blot analysis of LC3 Ⅱ and BCL-1 in the kidney. (**D**) The relative LC3 Ⅱ and BCL-1 levels. Data were performed using one-way analysis of variance (ANOVA), followed by the Student two-tail t test. Data represent the mean ± SEM, n=6 per group, significance; *p<.0.05, **p<0.01, ***p<0.001 vs diabetic nephropathy model mice. ##p<.0.01, ###p<0.001 compared to control.



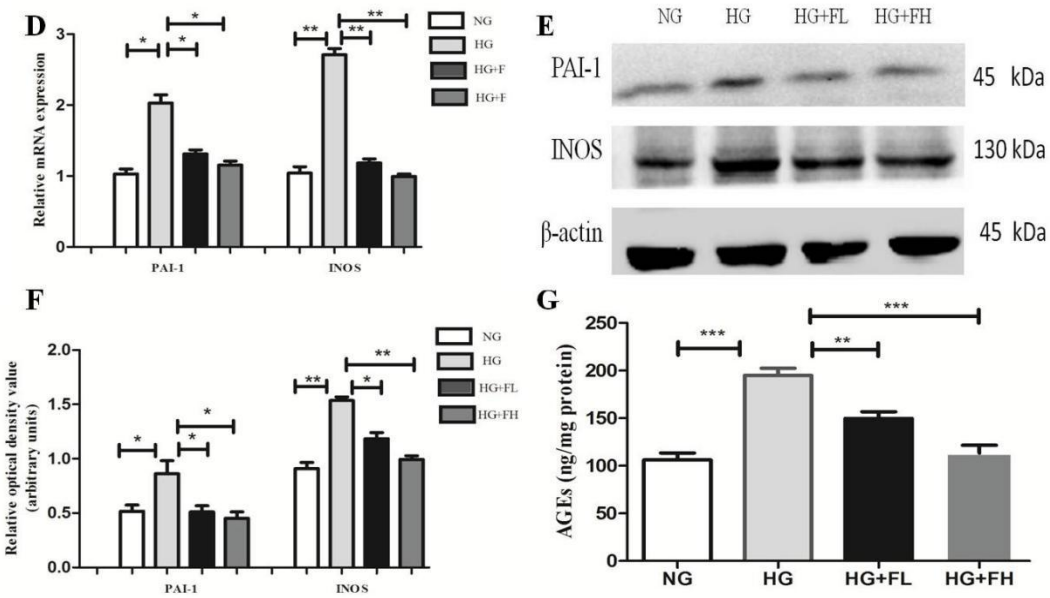


Fig. **5**. FGF21 ameliorates glucotoxicity in high glucose-treated mesangial cells. After 24h administration of FGF21, the levels of ROS, AGEs, and related inflammatory cytokines were measured. (**A**,**B**) The

levels of ROS were detected by flow cytometry and (**C**) fluorescence microplate. (D-F) The expression of PAI-1 and INOS were measured by RT-PCR and Western blot. (**G**) The level of AGEs was determined using OxiSelect^{TM} advanced end product (AGE) competitive ELISA kit. Experiment were done in triplicate. Data represent the mean ±SEM, significantly compared to high glucose group, *p<.0.05,**p<0.01, ***p<0.001.

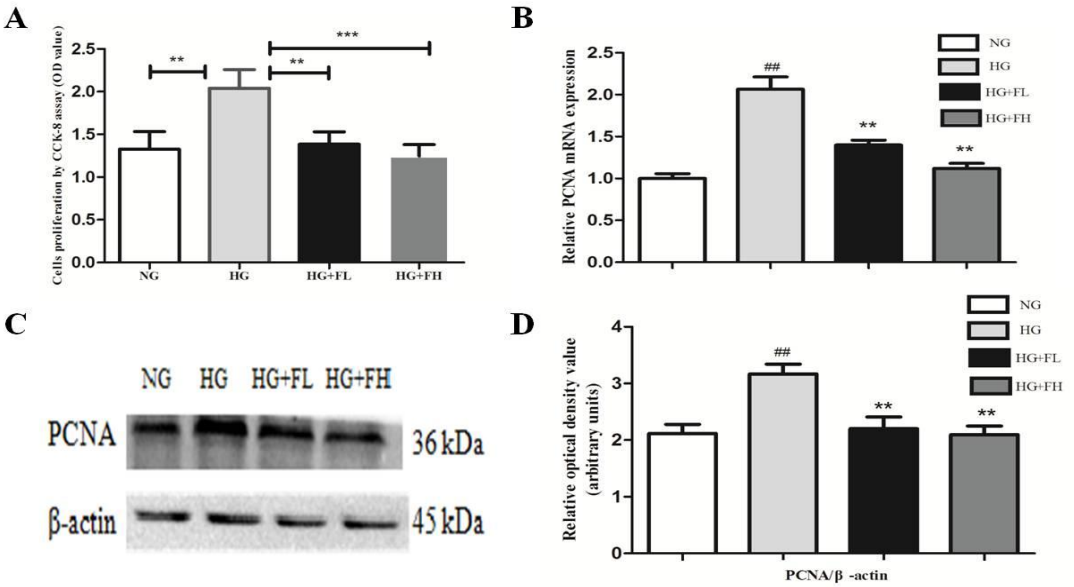
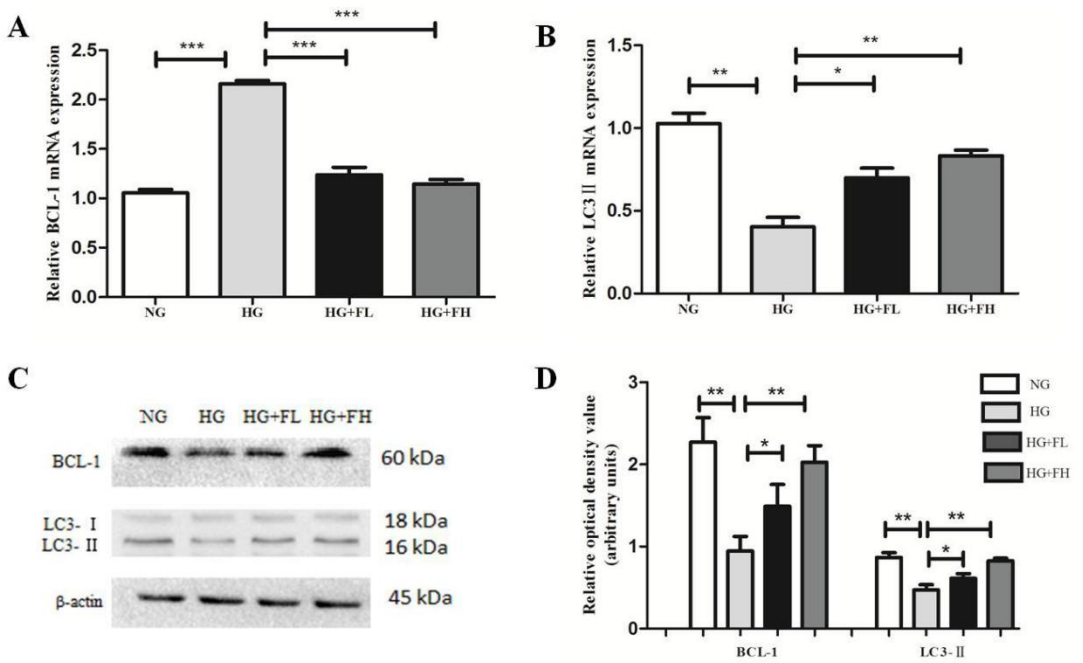


Fig. **6**. FGF21 inhibits high glucose-induced mesangial cell proliferation. (**A**). Cell proliferation were measured with CCK-8 kits and analyzed by a microplate reader using 450 nm. (**B-D**) The mRNA and protein expression of PCNA were detected by Western blot and RT-PCR. Data represent the mean ±SEM, significant as compared to control, $^{\#}$p<.0.05, $^{\#\#}$p<0.01, compared to high glucose group, *p<.0.05,**p<0.01.



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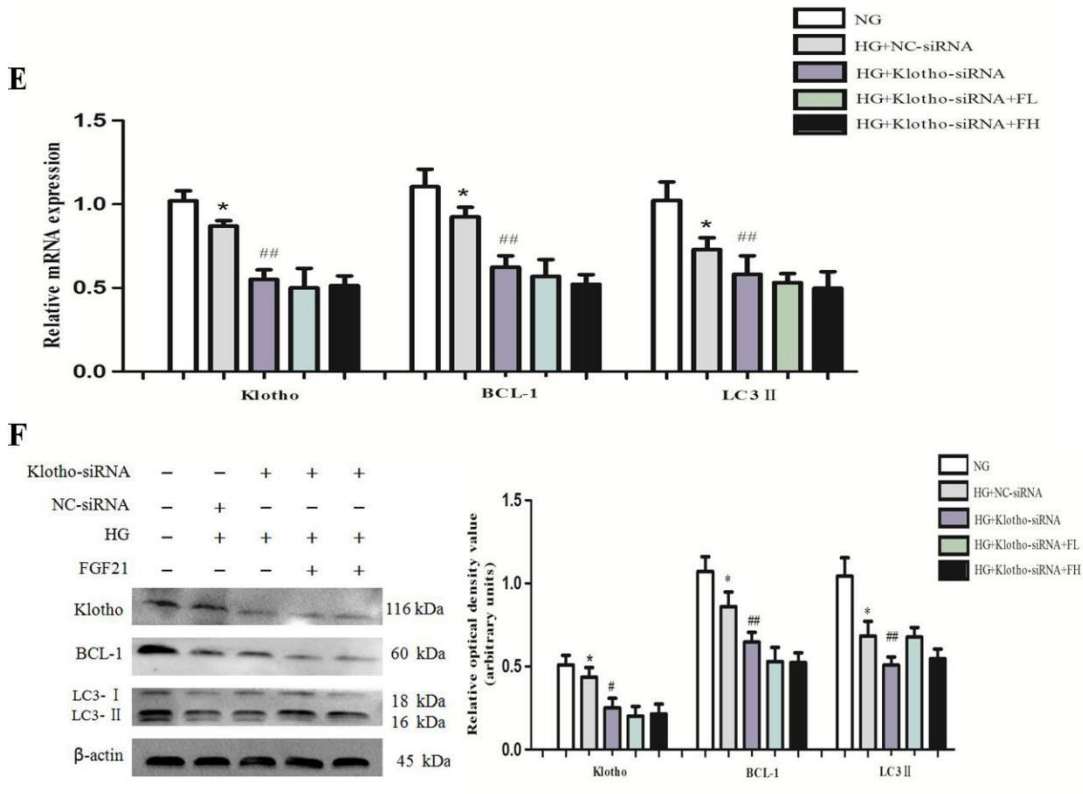
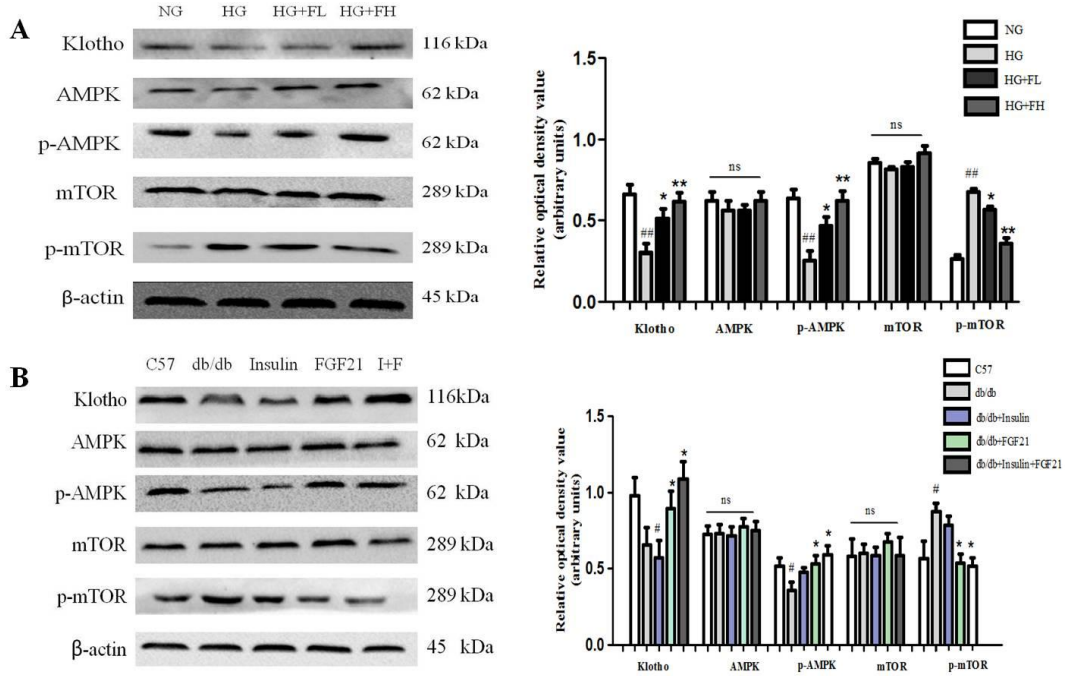


757

758 **Fig**. **7**. FGF21 augments autophagy in high glucose-treated mesangial
759 cells. After 24h administration of FGF21, (**A**,**B**) The mRNA expression
760 of LC3Ⅱ and BCL-1 were detected by real-time PCR in mesangial cells.

(**C**) The protein expression of LC3Ⅱ and BCL-1 were detected by Western blotting. (**D**) The bands were analyzed with Image J. (**E**,**F**) The autophagy relative genes of LC3Ⅱ and BCL-1 were measured by RT-PCR and Western blot in transfected mesangial cells. Data represent the mean ± SEM, significance as compared to NG control *p<.0.05, **p<0.01, ***p<0.001, $^{\#}$p<.0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001.



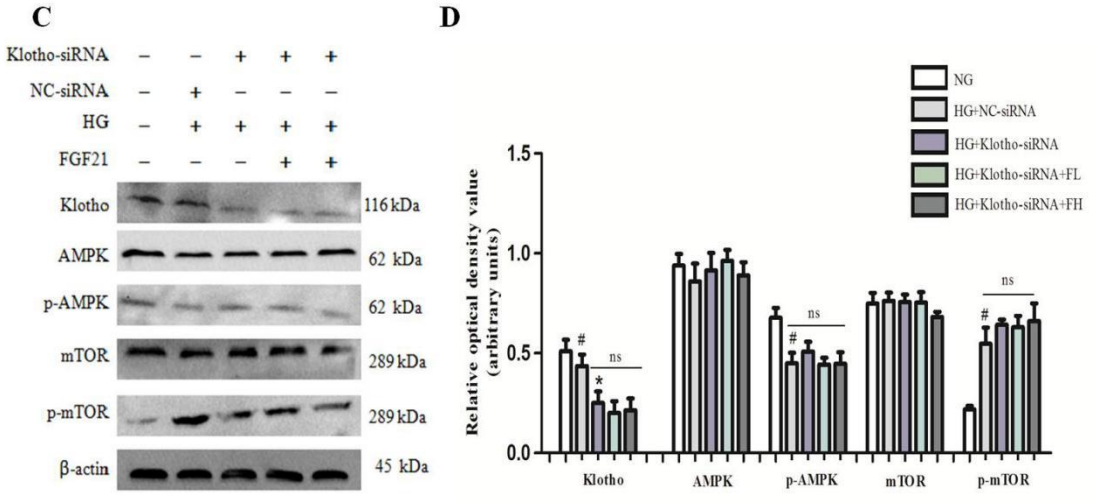


Fig. **8**. FGF21 upregulates the phosphorylation of AMPK and downregulates the phosphorylation of mTOR in the kidney of DN mice and high glucose treated mesangial cells. After high glucose-treated cells, (**A**) The expression of Klotho, AMPK, p-AMPK, mTOR, p-mTOR were

measured by Western blotting. The bands were analyzed with Image J. After 4 weeks treatment of FGF21 alone or combination with insulin in diabetic nephropathy mice, (**B**) The expression of Klotho, AMPK, p-AMPK, mTOR , p-mTOR were measured by Western blotting in the kidney. The bands were analyzed with Image J. (**C, D**) In transfected mesangial cells, The expression of Klotho, AMPK, p-AMPK, mTOR, p-mTOR were measured by Western blotting and analyzed. Experiment were done in triplicate. Data represent the mean ±SEM, significant as compared to HG group or model mice, *p<.0.05, **p<0.01, ***p<0.001, compared to NG group or control mice, $^{\#}$p<.0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, ns mean no significant differentiation.

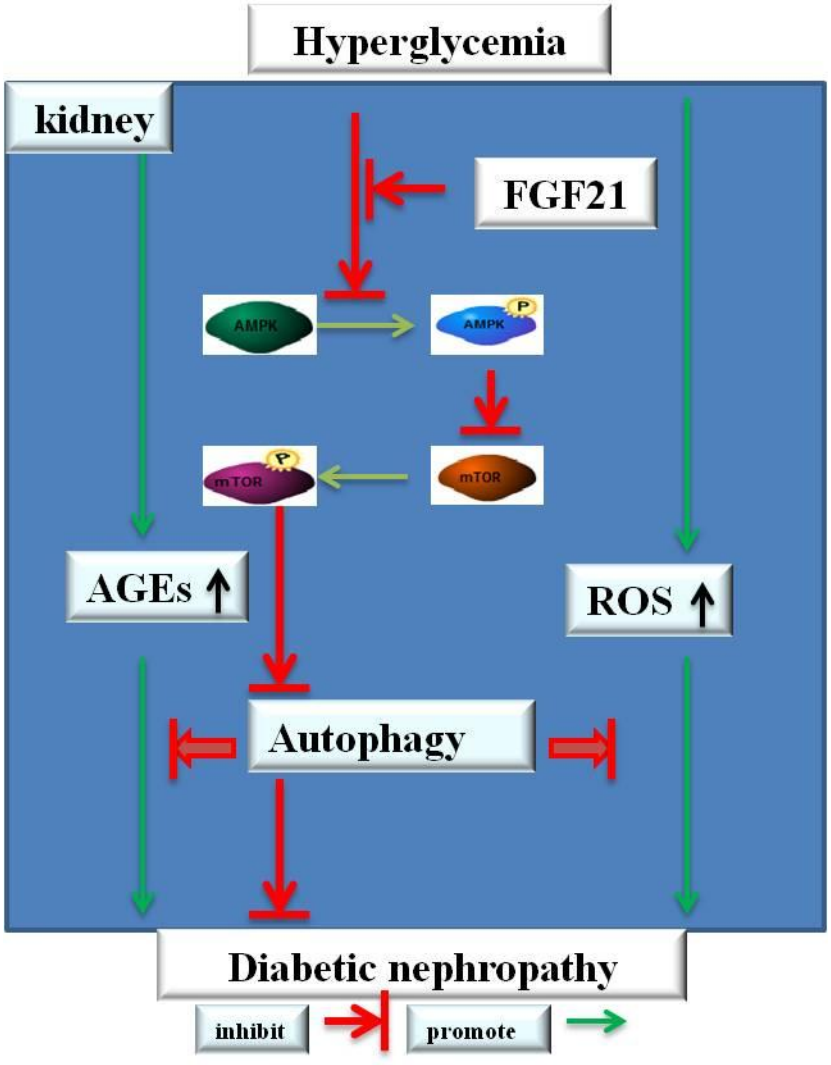
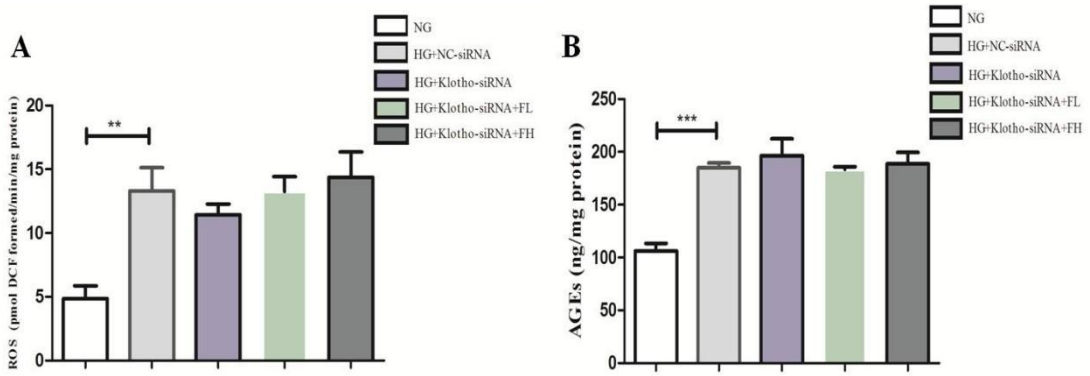
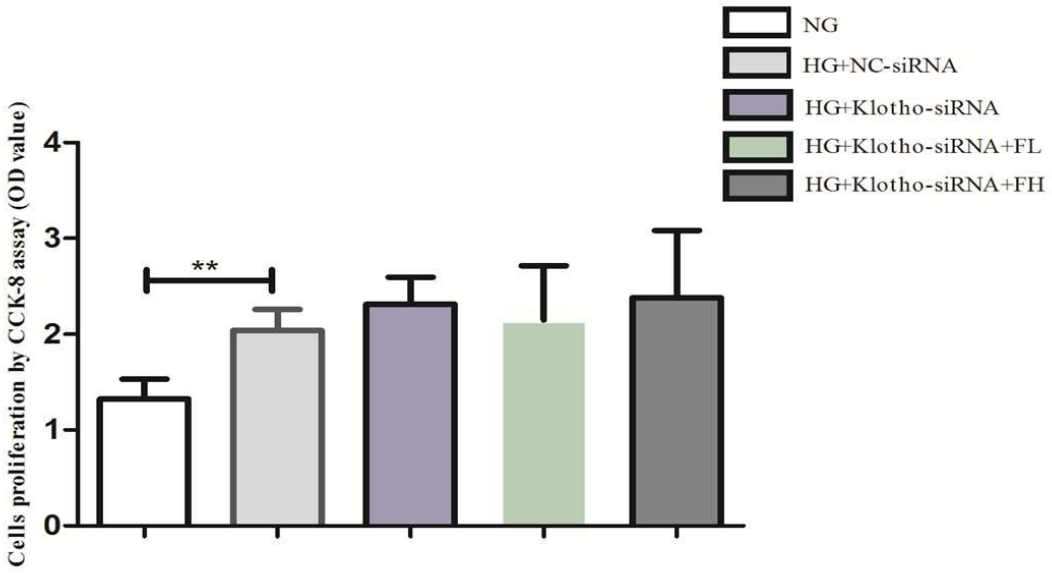


Fig. 9. The mechanism of FGF21 augments autophagy in diabetic nephropathy mice. The levels of AGEs and ROS are increased in hyperglycemia, which were attenuated by boosting autophagy. FGF21 augments autophagy by upregulating the phosphorylation of AMPK and downregulating the phosphorylation of mTOR in DN mice.

Supplemental data



S1. The effects of FGF21 on high glucose-induced mesangial cells glucotoxicity were inhibited by transfecting small interfering RNA-β-klotho. (**A**). The level of ROS was measured by fluorescence microplate. (**B**) The level of AGEs was detected by OxiSelect^{TM} AGEs ELISA kit. Data represent the mean ± SEM, significance as compared to NG control *p<.0.05, **p<0.01, ***p<0.001. No significance as compared to NC-siRNA control.



S2. The effect of FGF21 on high glucose-induced mesangial cell proliferation was reversed in transfected mesangial cells. Cell proliferation were measured with CCK-8 kits and analyzed by a microplate reader using 450 nm. Data represent the mean ± SEM, significance as compared to NG control *p<.0.05, **p<0.01, ***p<0.001. No significance as compared to NC-siRNA control.

Figures

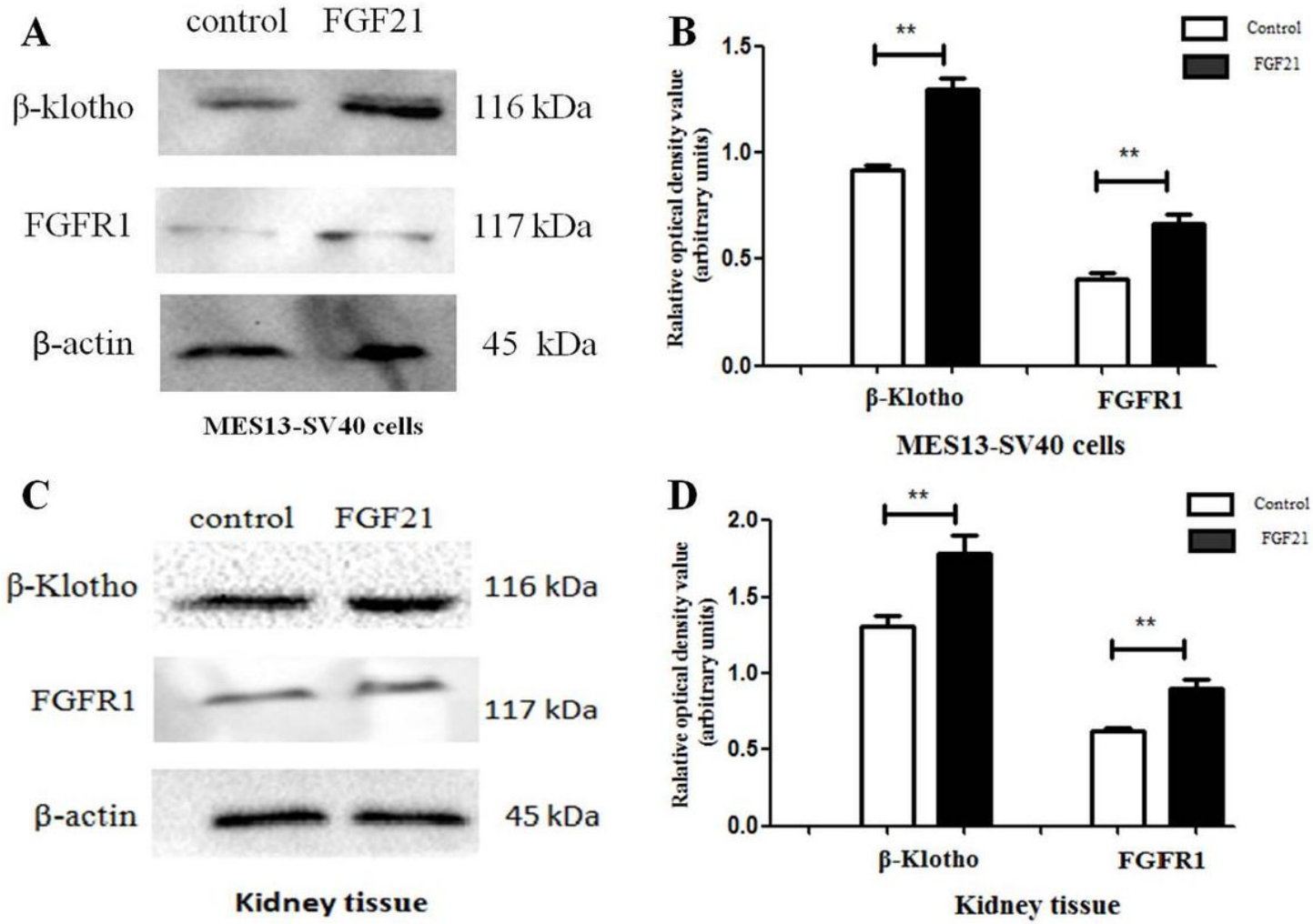


Figure 1

The kidney and mesangial cells are the target of FGF21. The mice were euthanized at 16th weeks. (A,C) Western blot analysis the protein expression of β-klotho and FGFR1 in mesangial cells and the kidney of diabetic nephropathy mice. (B,D) The relative β-klotho and FGFR1 levels were expressed as the ratio β-klotho/β-actin and FGFR1/β-actin, which were analyzed by Image J. All date represent mean ± SEM, n=6 per group, *p<0.05, **p<0.01, ***p<0.01 significant as compared to control.

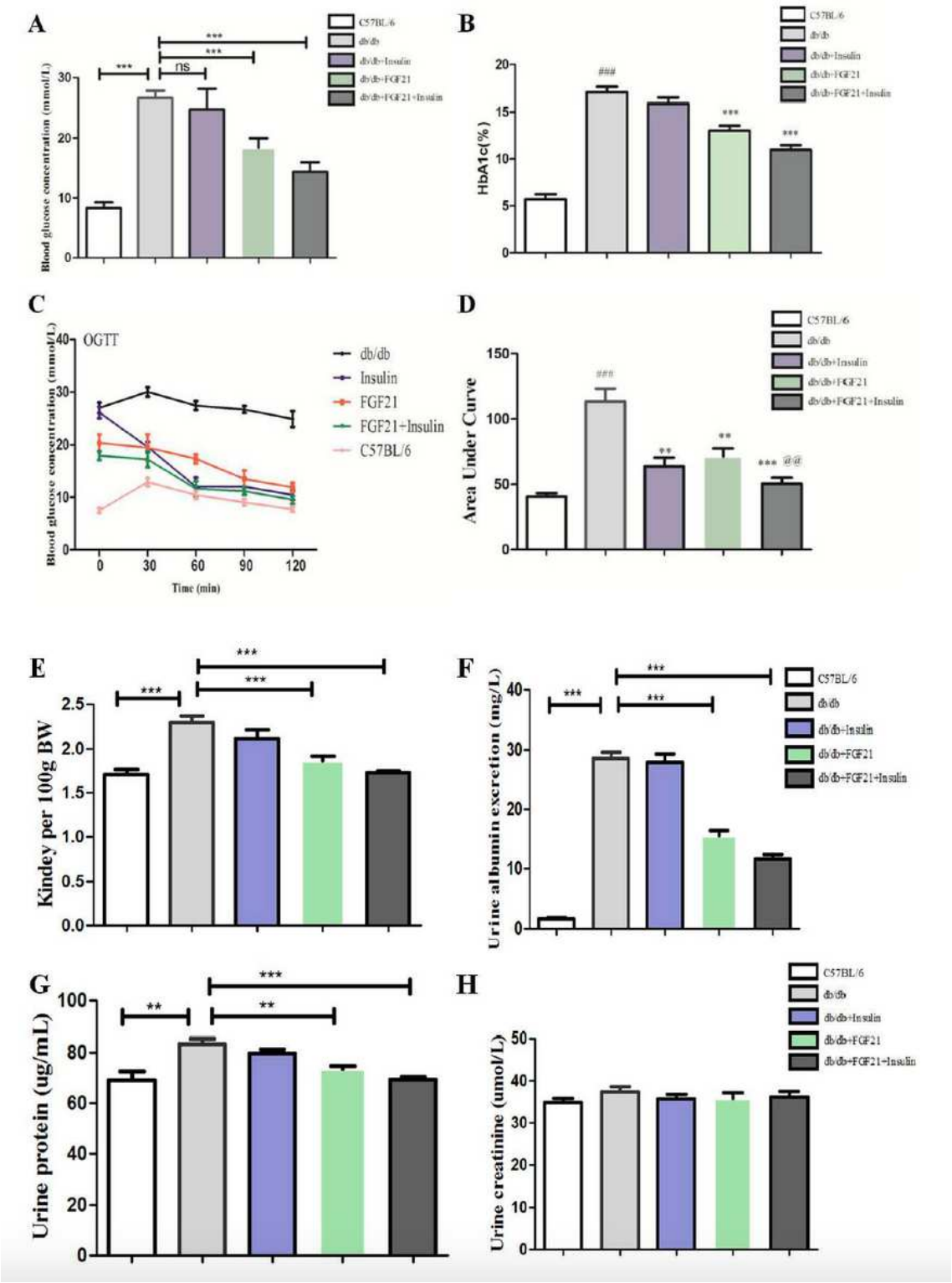


Figure 2

FGF21 ameliorates the kidney tissue lesions in diabetic nephropathy mice. After 4 weeks administration of FGF21 or combination with insulin, blood glucose, HbA1c, OGTT, renal function were measured. (A,B) The blood glucose and HbA1c of diabetic nephropathy mice were measured before the last injection at the fourth week's administration of Insulin or FGF21. (C) OGTT were performed in all groups. (D) Area under the curve (AUC) value for OGTT. (E-H) Kidney per 100 g weight body, Urine albumin, Urine protein,

Urine creatinine were measured in all groups. All data represent mean ± SEM, n=6 per group, *p<.0.05, **p<0.01, compared to diabetic nephropathy model mice. #p<.0.05, ###p<0.001 compared to control. @@p<.0.01 compared to administration of insulin alone mice.

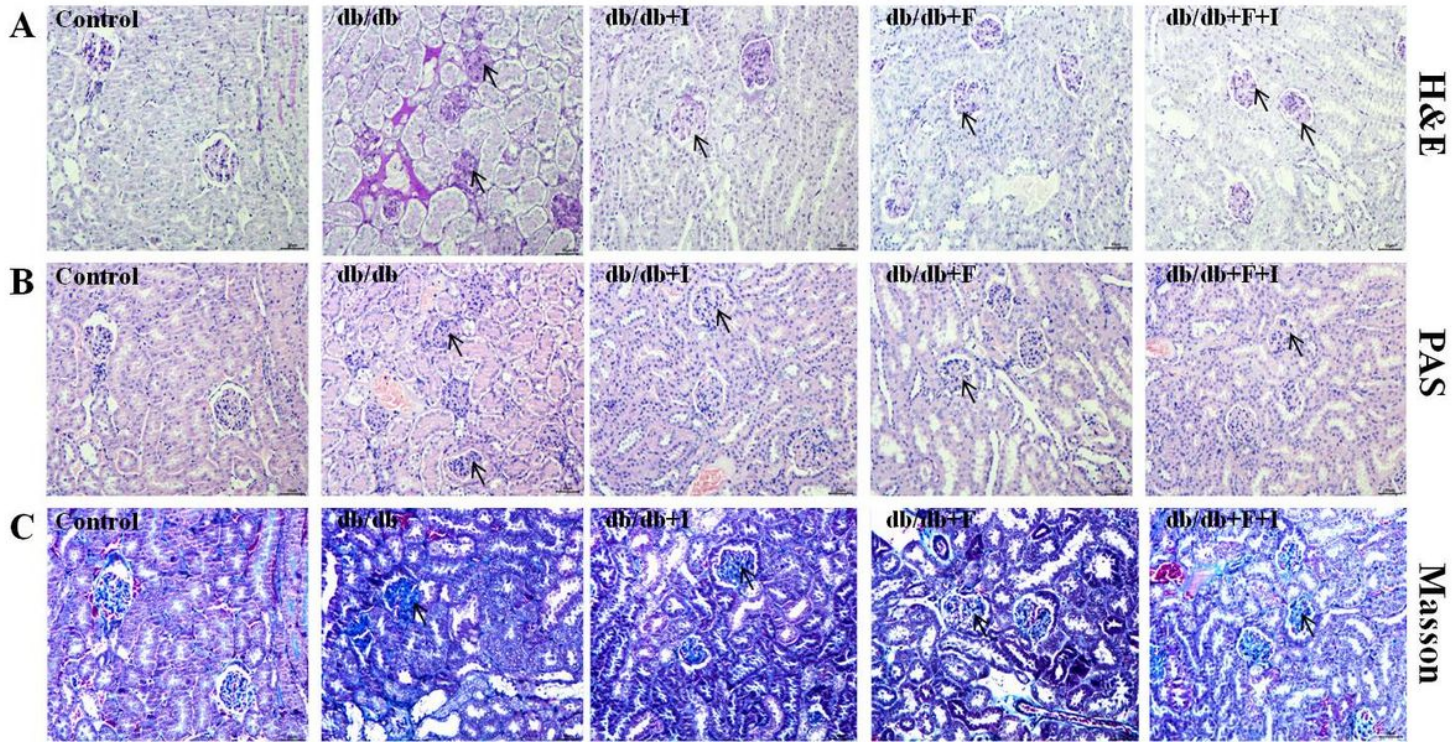


Figure 3

FGF21 ameliorates histopathological changes in the kidney of diabetic nephropathy mice. (A) Representative HE staining (×200) of kidney sections from mice. (B) Representative periodic acid-Schiff (PAS) (×200) of kidney sections from mice. (C) Representative Masson's trichrome staining (×200) of kidney sections from mice.

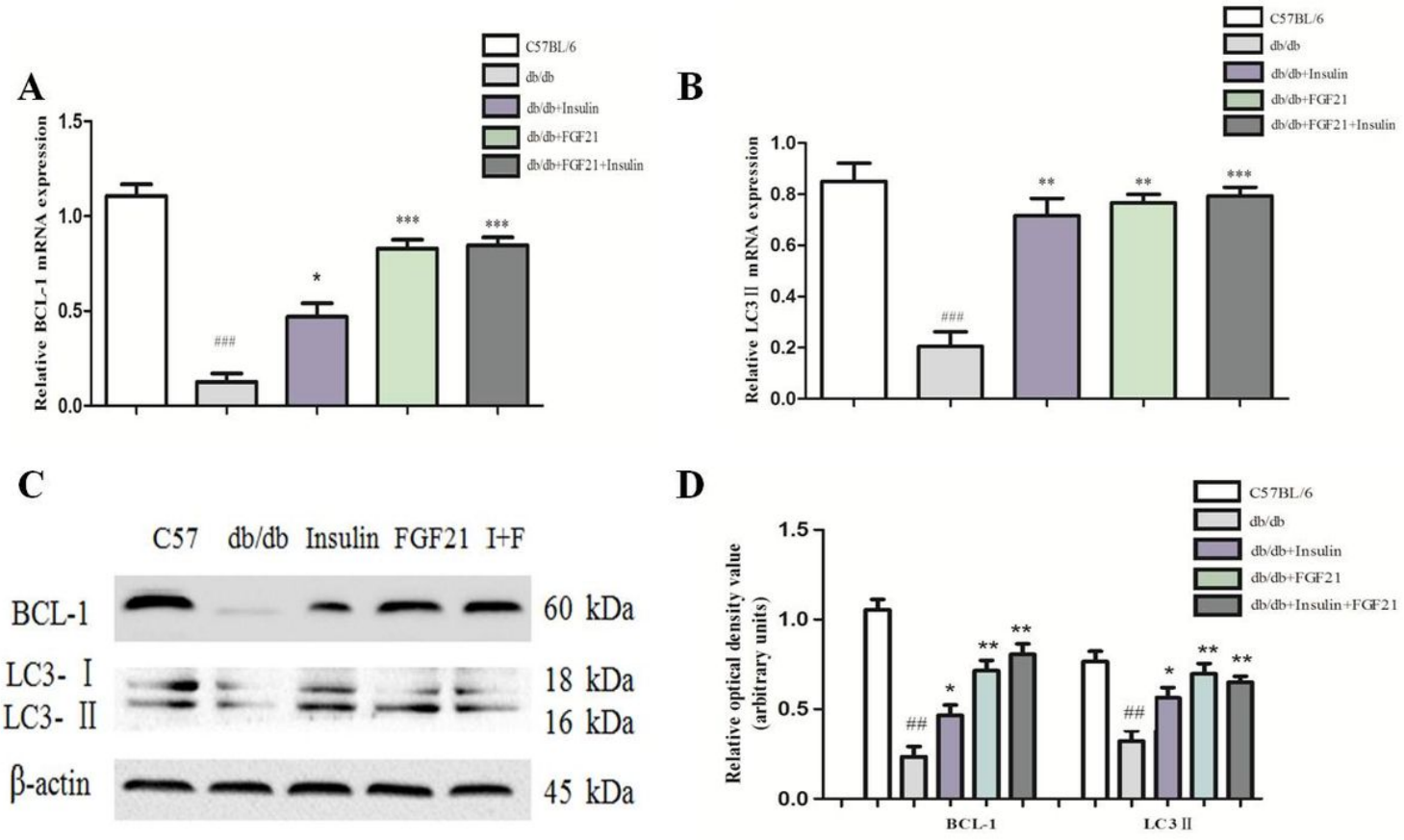


Figure 4

Administration of FGF21 augments autophagy in the kidney of diabetic nephropathy mice. After 4 weeks treatment of FGF21 alone or combination with insulin, The mRNA and protein expression of LC3Ⅱand BCL-1 were detected by real-time PCR and Western blot. (A,B) RT-PCR analysis of LC3Ⅱand BCL-1. (C) Western blot analysis of LC3Ⅱand BCL-1 in the kidney. (D) The relative LC3Ⅱand BCL-1 levels. Data were performed using one-way analysis of variance (ANOVA), followed by the Student two-tail t test. Data represent the mean ± SEM, n=6 per group, significance; *p<.0.05, **p<0.01, ***p<0.001 vs diabetic nephropathy model mice. ##p<.0.01, ###p<0.001 compared to control.

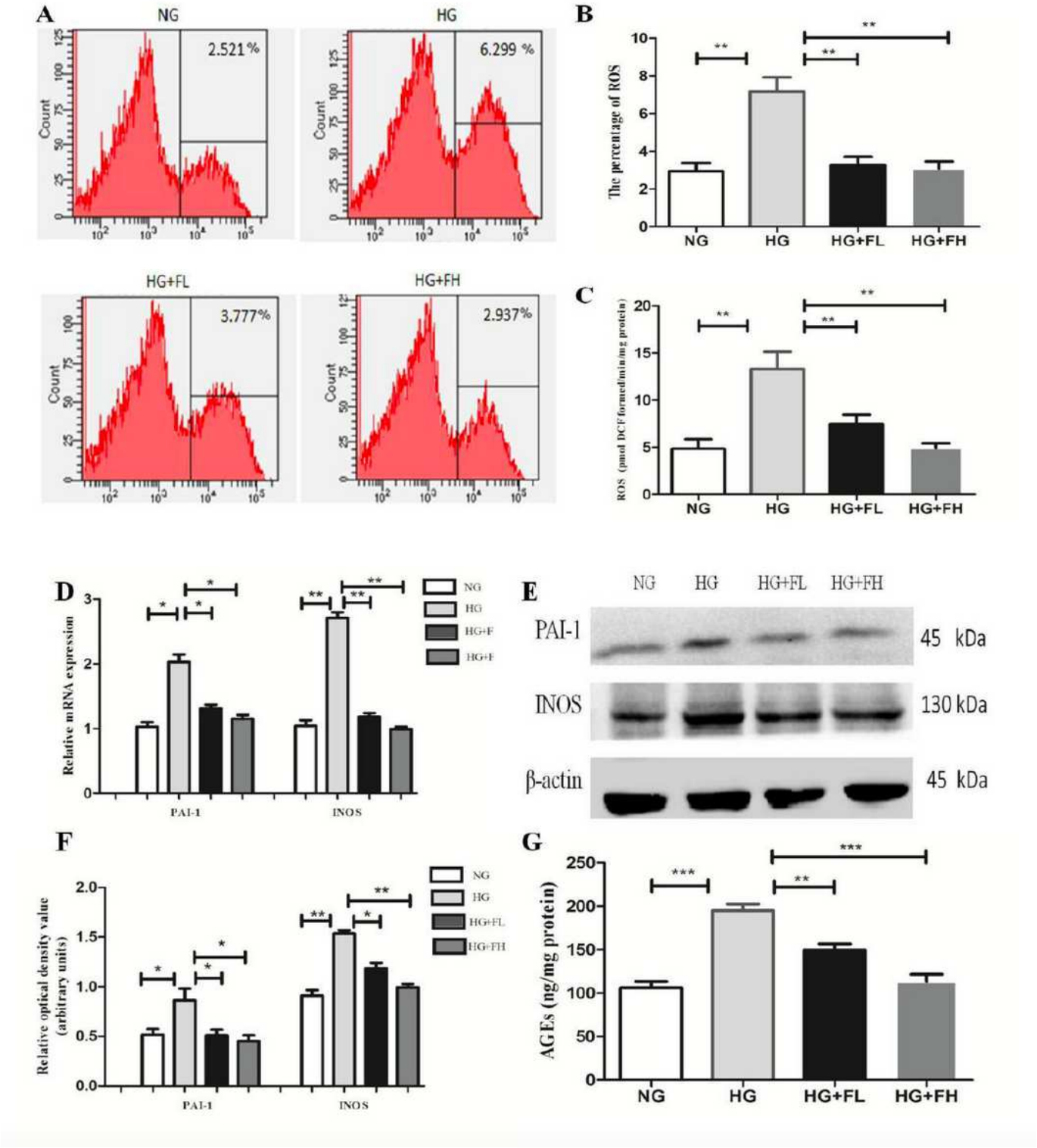


Figure 5

FGF21 ameliorates glucotoxicity in high glucose-treated mesangial cells. After 24h administration of FGF21, the levels of ROS, AGEs, and related inflammatory cytokines were measured. (A,B) The levels of ROS were detected by flow cytometry and (C) fluorescence microplate. (D-F) The expression of PAI-1 and INOS were measured by RT-PCR and Western blot. (G) The level of AGEs was determined using

OxiSelectTM advanced end product (AGE) competitive ELISA kit. Experiment were done in triplicate. Data represent the mean ±SEM, significantly compared to high glucose group, *p<.0.05,**p<0.01, ***p<0.001.

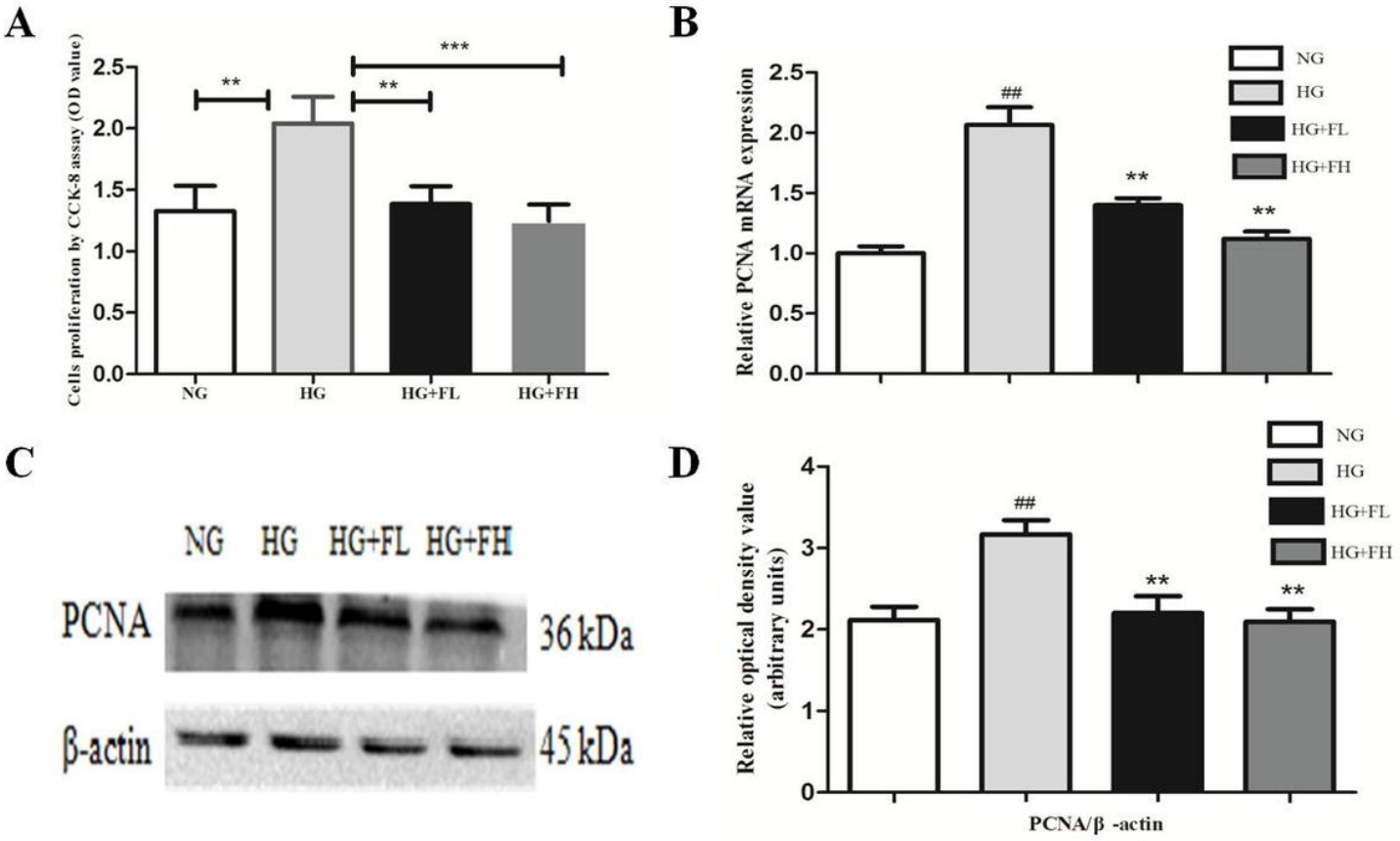


Figure 6

FGF21 inhibits high glucose-induced mesangial cell proliferation. (A). Cell proliferation were measured with CCK-8 kits and analyzed by a microplate reader using 450 nm. (B-D) The mRNA and protein expression of PCNA were detected by Western blot and RT-PCR. Data represent the mean ±SEM, significant as compared to control, #p<.0.05, ##p<0.01, compared to high glucose group, *p<.0.05,**p<0.01.

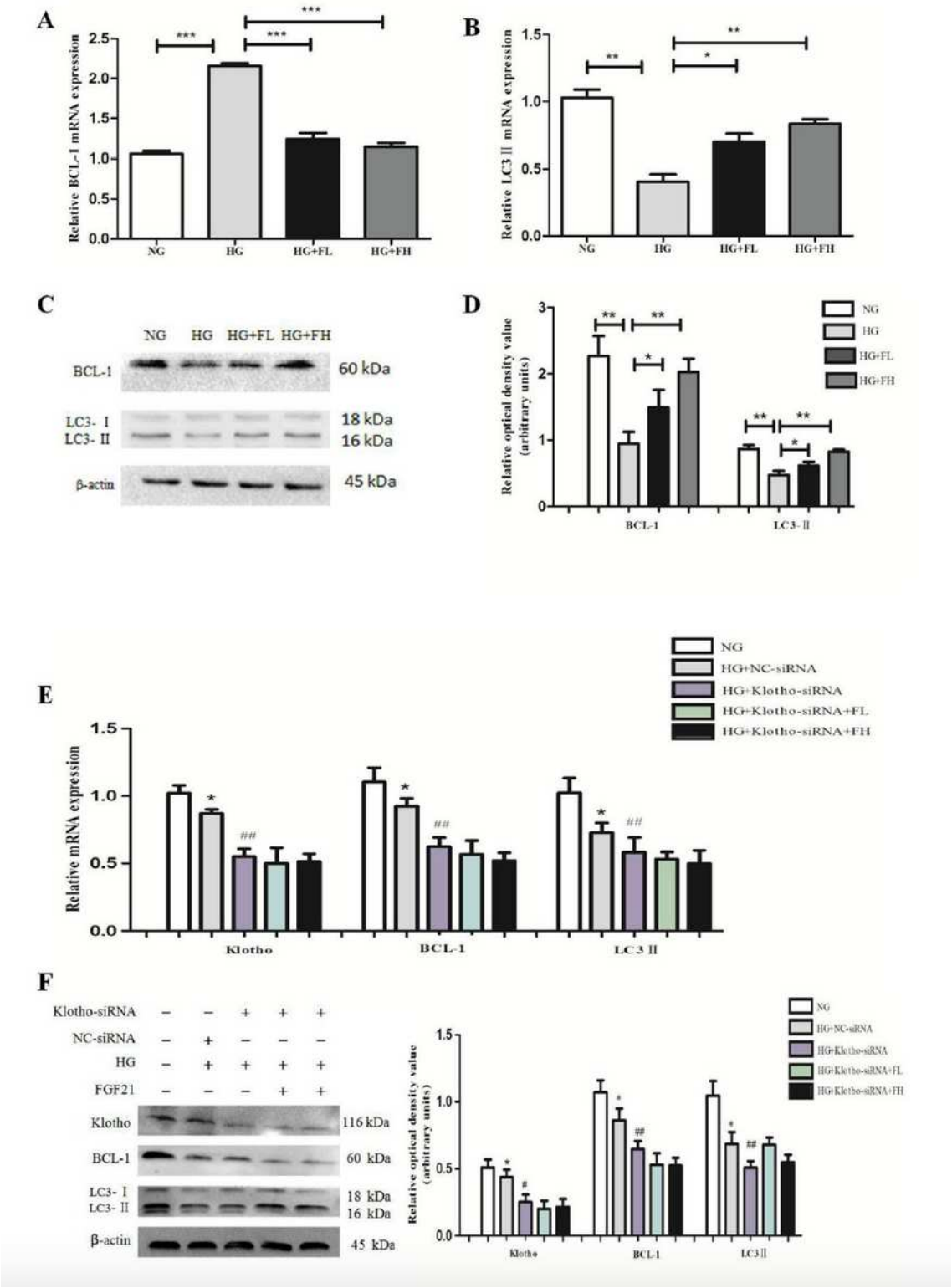


Figure 7

FGF21 augments autophagy in high glucose-treated mesangial cells. After 24h administration of FGF21, (A,B) The mRNA expression of LC3Ⅱ and BCL-1 were detected by real-time PCR in mesangial cells. (C)The protein expression of LC3Ⅱ and BCL-1 were detected byWestern blotting. (D) The bands were analyzed with Image J. (E,F) The autophagy relative genes of LC3Ⅱ and BCL-1 were measured by RT-PCR and

Western blot in transfected mesangial cells. Data represent the mean ± SEM, significance as compared to NG control *p<.0.05,**p<0.01, ***p<0.001, #p<.0.05, ##p<0.01, ###p<0.001.

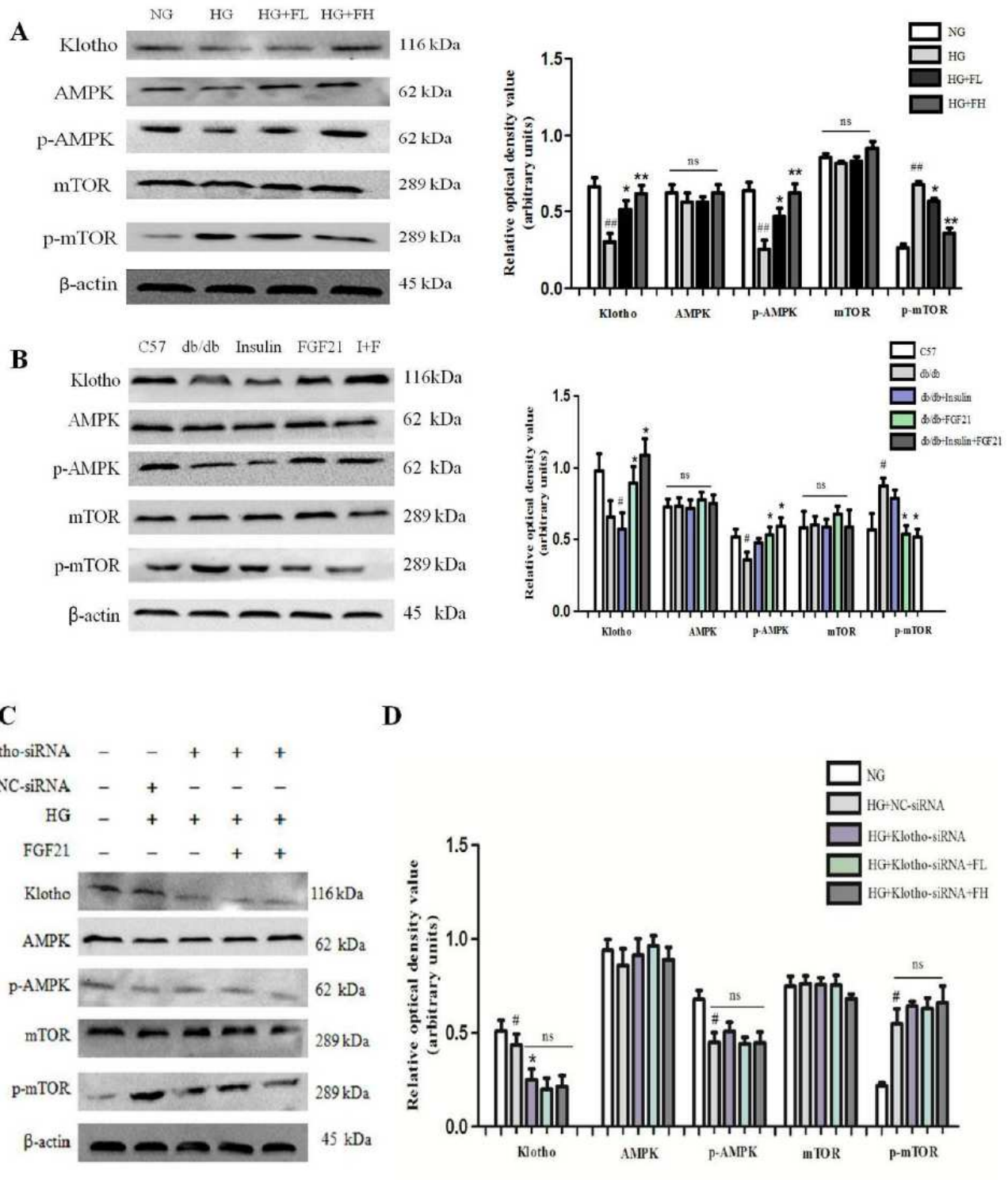


Figure 8

FGF21 upregulates the phosphorylation of AMPK and downregulates the phosphorylation of mTOR in the kidney of DN mice and high glucose treated mesangial cells. After high glucose-treated cells, (A)The expression of Klotho, AMPK, p-AMPK, mTOR, p-mTOR were measured by Western blotting. The bands

were analyzed with Image J. After 4 weeks treatment of FGF21 alone or combination with insulin in diabetic nephropathy mice, (B) The expression of Klotho, AMPK, p-AMPK, mTOR , p-mTOR were measured by Western blotting in the kidney. The bands were analyzed with Image J. (C, D) In transfected mesangial cells, The expression of Klotho, AMPK, p-AMPK, mTOR, p-mTOR were measured by Western blotting and analyzed. Experiment were done in triplicate. Data represent the mean ±SEM, significant as compared to HG group or model mice, *p<.0.05, **p<0.01, ***p<0.001, compared to NG group or control mice, #p<.0.05, ##p<0.01, ###p<0.001, ns mean no significant differentiation.

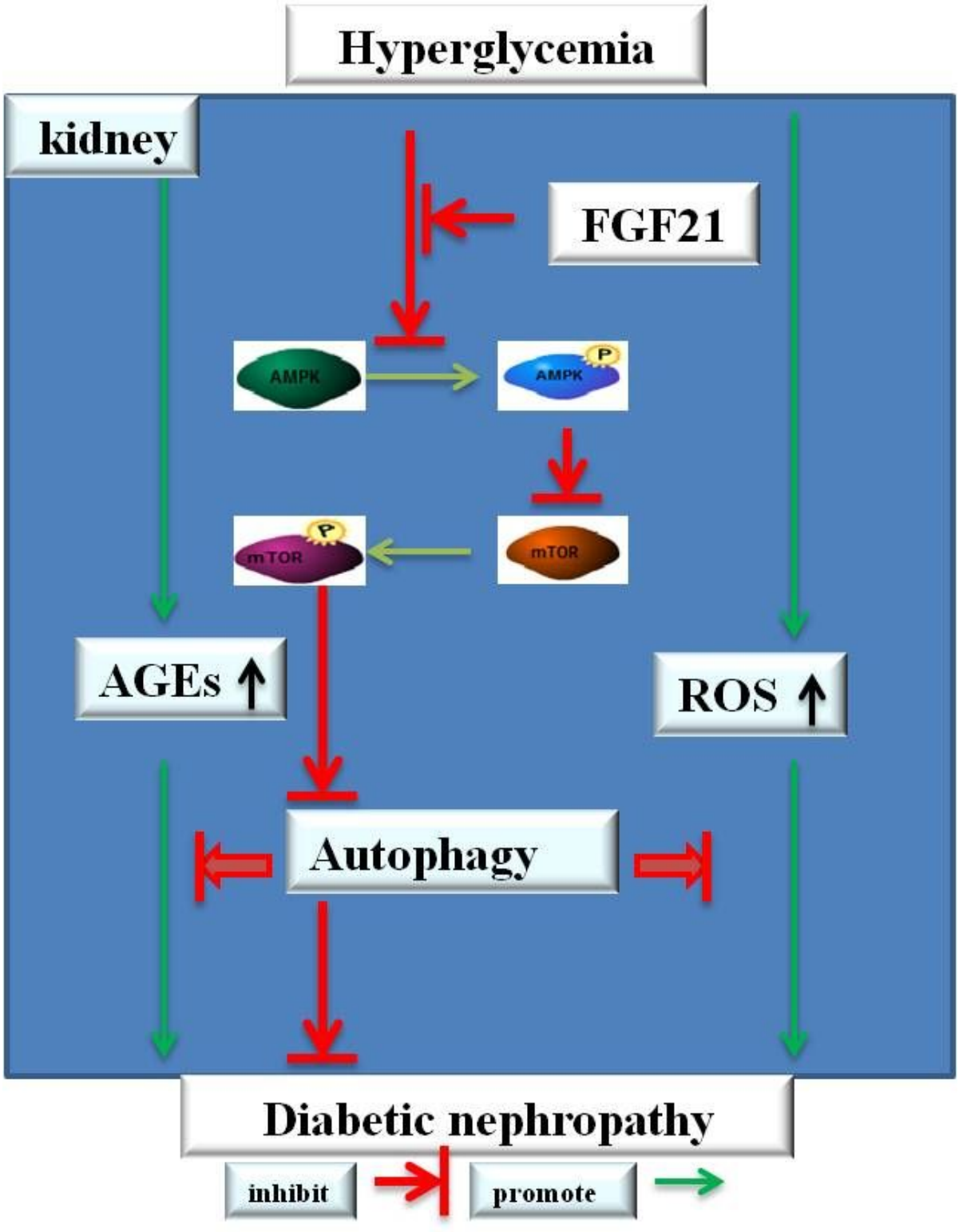


Figure 9

The mechanism of FGF21 augments autophagy in diabetic nephropathy mice. The levels of AGEs and ROS are increased in hyperglycemia, which were attenuated by boosting autophagy. FGF21 augments autophagy by upregulating the phosphorylation of AMPK and downregulating the phosphorylation of mTOR in DN mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementdata.docx
- originaldata.docx