ORIGINAL ARTICLE

Urotensin II inhibits autophagy in renal tubular epithelial cells and induces extracellular matrix production in early diabetic mice

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ABSTRACT

Aims/Introduction: Urotensin II (UII) and autophagy have been considered as important components in the pathogenesis of diabetic nephropathy. The present study explores whether UII can regulate autophagy in the kidney, and its effect in diabetes.

Materials and Methods: Immunohistochemistry and western blot were carried out on the kidney tissues of diabetic UII receptor (UT) gene knockout mice, wild-type diabetic mice and normal control mice. For the *in vitro* experiment, HK-2 cells were treated with UII (10^{-7} mol/L) in the presence or absence of UT antagonist, SB-657510, (10^{-6} mol/L) or autophagy inducer, rapamycin (10^{-3} mol/L), for 12 h. Markers for autophagy (LC3-II, p62/SQSTM1) and extracellular matrix (fibronectin, collagen IV) were analyzed.

Results: In diabetic UT knockout mice, expression of LC3-II is increased and p62 was reduced in comparison with that of the normal diabetic mice. Fibronectin and collagen IV were downregulated in diabetic UT knockout mice when compared with that of the normal diabetic mice. For the *in vitro* cell experiment, UII was shown to inhibit expression LC3-II and increase expression of p62 in comparison with that of the normal control. Treatment with SB-657510 can block UII-induced downregulation of LC3-II and upregulation of p62 while inhibiting UII-induced upregulation of fibronectin and collagen IV. Adding autophagy inducer, rapamycin, also inhibited UII-induced upregulation of fibronectin and collagen IV.

Conclusions: The present study is the first to show that UII can downregulate autophagy in the kidney while accompanying the increased production of extracellular matrix in early diabetes. Our *in vitro* study also showed that upregulation of autophagy can decrease UII-induced production of extracellular matrix in HK-2 cells.

INTRODUCTION

One of the major microvascular complications of diabetes and causes of end-stage renal disease is diabetic nephropathy (DN)[1,2]. Although many treatments are available for DN, most of the current treatments do not eradicate the disease, but only provide a form of maintenance therapy[3–5]. An alternative approach to the current treatment that can have curative potential is required. This begins by investigating novel molecules, such as urotensin II (UII), that can play a role in the development of DN[6,7].

As a regulatory neuropeptide with important vasoactive properties[8], UII has been considered as a pharmacological target for diseases such as hypertension, heart failure, diabetes and kidney diseases[9–11]. UII can only exert its physiological effect when combined with UII receptor (UT), and UT is synonymous with G protein-coupled receptor 14[9]. Regardless of its potential in medical treatments, no current study conclusively validates or negates the therapeutic value of UII and its related pharmacological developments[12,13]. This calls for further study to understand UII and its association with the pathophysiology of DN.

During the past decade, there has also been an increasing interest in using the processes of autophagy to develop therapeutic strategies for diabetic nephropathy[14]. Autophagy is a

cellular process that controls cytoplasmic quality by degrading various harmful substrates and it can be associated with the development of many diseases, such as DN[15,16]. Several important proteins are identified that monitor and regulate autophagy. Microtubule-associated protein 1 light-chain 3 (LC3) is a protein that is often used to monitor the presence of autophagy[17], and the level of LC3-II can also be used as a marker to reflect autophagy activity and progression[18]. Additionally, p62/SQSTM1 is another common protein marker used to study autophagy progression. Its quantity decreases with autophagy progression, and increases when autophagy is inhibited[17].

In our previous study, we verified that the expression of UII is positively correlated with endoplasmic reticulum stress in kidneys of patients with DN, and UII can induce endoplasmic reticulum stress in human renal tubular epithelial cells *in vitro*[19]. It is also reported that endoplasmic reticulum stress is associated with autophagy[19]; thus, UII might also have the capacity to upregulate or inhibit autophagy in renal tubular epithelial cell.

A hallmark of DN as it progresses towards end-stage renal disease is renal fibrosis, which is accompanied by irreversible loss of renal function[20]. Past research showed that reducing renal fibrosis by decreasing the synthesis of extracellular matrix (ECM) protein, such as fibronectin and collagen IV, could delay the development of DN[20]. The aim of the present study was, therefore, not only to examine the role of UII in regulating autophagy in diabetic mice *in vivo* and *in vitro*, but also to explore whether UII can affect the production of ECM proteins (fibronectin and collagen IV), which are also the markers of renal fibrosis.

MATERIALS AND METHODS
Animal model system
Experiments were carried out on 15 UT knockout male mice and 30 wild-type male mice aged between 8 and 10 weeks, and weighing between 20 and 25 g. The UT knockout mouse strain was procured from Regeneron Pharmaceuticals Inc. (Rensselaer, NY, USA), and made into live mice by the KOMP Repository (www.komp.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. Wild-type C57BL/6 mice were purchased from a local animal facility (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China). The mice were housed under standard conditions and were individually caged. The mice were divided into three groups.

A total of 15 knockout mice and 15 wild-type mice were placed in the experimental group. The remaining 15 mice were placed in the normal control group. The animals were fasted overnight before induction of diabetes with streptozotocin (STZ), but had free access to drinking water. Diabetic mice were randomly killed by cervical dislocation technique at the third week after the onset of diabetes. The experimental protocols were approved by the Biological Medical Ethics Committee of the Peking University Health Science Center (Number LA 2013-47).

UT knock-out mouse design
The UT gene knockout mouse strain used for this research project was created from ES cell clone 12922A-B6, generated by Regeneron Pharmaceuticals Inc., and made into live mice by the KOMP Repository (www.komp.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California, Davis, CA, USA. Four heterozygous UT knockout male mice were made in total. The four heterozygous UT knockout male mice were then brought to the laboratory animal facility at Peking University Third Hospital, where they were matched and rotated with wild-type female C57BL/6 mice. The mice were bred to eight generations.

The male heterozygous UT knockout mice from the Mouse Biology Program at University of California, Davis, were initially crossed with wild-type C57BL/6 females to give N1F0 offspring. The N1F0 offspring were subsequently intercrossed to generate N1F1 offspring. The N1F1 offspring were then successively backcrossed to wild-type C57BL/6 females to generate N5F0 mice. The N5F0 mice were intercrossed to create an N5F1 population. A genotype study was carried out on mice from N5F1. Genomic deoxyribonucleic acid (DNA) was isolated from the toes of these mice for polymerase chain reaction (PCR) analysis. DNA extraction from the toe tissues was carried out with Tissue DNA Kit (D3396-01; Omega Bio-tek, Norcross, GA, USA). According to the Mouse Biology Program at University of California, Davis, the forward primer and reverse primer amplicon size (bp) for genotyping are as follows: UT knockout Reg-NeoF/Reg-UTR: 756 bp; wild-type Reg-UT-wtF/Reg-UT-wtR: 63 bp. For genotyping UT knockout, the forward primer was Reg-NeoF: 5′-GCAGCCTCTGTTCCACATACACTTCA-3′; the reverse primer was Reg-UTR: 5′-CTCTCAGATCTCTCAGCTACCTGCC-3′. For genotyping the wild type, the forward primer was Reg-UT-wtF: 5′-ATTGGGCTGCTCTATATCCGTCTGG-3′; the reverse primer was Reg-UT-wtR: 5′-CTTGAAGGAAGCTTGCTGGGATAGC-3′. A PCR mixture of 25 μL reaction volume was made using 7 μL nuclease-free water, 12 μL GoTaq Green Master Mix, 2X (Promega, Madison, WI, USA), 1 μL of each primer and 2 μL of DNA sample. Cycling parameters used for PCR were: temperature 94°C for 5 min, 94°C for 15 s, 65°C for 30 s (10 cycles with decrease of 1°C/cycle), 72°C for 40 s, 94°C for 15 s, 55°C for 30 s (30 cycles), 72°C for 40 s, 72°C for 5 min and 4°C at the end of completion. The resulting PCR products were analyzed on 1% agarose gel, and visualized with Syngene Bio Imaging System and GeneSnap software (Syngene, Frederick, MD, USA).

In accordance with the Mouse Biology Program at University of California, Davis, homozygous UT knockout mice should only have DNA amplification at 756 bp, whereas wild type mice should only have DNA amplification at 63 bp. Heterozygous UT knockout mice will have DNA amplification at both 756 bp and 63 bp. Homozygous UT knockout male mice were selected for the present study.

STZ administration

Diabetes was induced by a single intraperitoneal injection of 125 mg/kg bodyweight STZ (Amresco LLC, Solon, OH, USA) that was freshly dissolved in 0.1 mol/L sodium citrate buffer at pH 4.5. All injections were completed within 30 min for two consecutive days to produce a β-cell destruction model. The plasma glucose in all diagnosed diabetic mice, examined 72 h after STZ injection, was higher than 16.7 mmol/L. Control wild-type mice were injected with an equal volume of 0.1 mol/L sodium citrate buffer as the vehicle.

Blood glucose measurement

Blood obtained from the tail vein was used for measurements of glucose levels, which were carried out using the OneTouch Ultra Blood Glucose Monitoring System (Johnson & Johnson Co., New Brunswick, NJ, USA). Mice were fasted for 5 h before blood glucose measurement.

Immunohistochemical staining

Histological studies were carried out on renal tissues of the UT knockout diabetic mice, wild-type diabetic mice and the normal wild-type control mice. The renal tissues were embedded in optimal cutting temperature compound. The tissues were sectioned at a thickness of 10 μm. For immunohistochemical analysis, 5% hydrogen peroxide was used to deplete endogenous peroxidase activity. The sections were incubated with primary rabbit or mice polyclonal anti-UII antibody (1:50), anti-LC3 antibody (1:50), anti-p62/SQSTM1 antibody (1:50), anti-fibronectin antibody (1:50) and anti-collagen IV antibody (1:50) at 4°C overnight (all antibodies were purchased from Abcam Inc., Shanghai, China, and all other chemicals were of analytical grade from commercial suppliers). The sections were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibodies (Zhongshan Gold Bridge Biotechnology Co. Ltd, Beijing, China) for 30 min at 37°C. The peroxidase was visualized by incubation with 3, 3′-diaminobenzidine in the dark for 1 min for UII, LC3, p62/SQSTM1, fibronectin and collagen IV antibody. The sections were counterstained with hematoxylin, dehydrated and observed under a light microscope. Negative controls were established using phosphate-buffered saline as a substitute for the primary antibody. Positive staining was indicated by brown deposits.

Cell culture

Renal proximal tubular epithelial cell lines HK-2 (human) were used. HK-2 cells were obtained from ATCC (Rockville, MD, USA). Cells were grown in DMEM/F12 media (Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C, and 100 U/mL antibiotics in a humidified incubator (5% CO_2 and 95% air at 37°C). The HK-2 cell suspensions were plated on tissue flasks. The cells were treated with 10^{-7} mol/L UII (Sigma-Aldrich, St. Louis, MO, USA) for 12 h in the presence or absence of either 10^{-6} mol/L UT antagonist SB-657510 (Sigma-Aldrich) or 10^{-3} mol/L autophagy inducer, rapamycin (Sigma-Aldrich). The cells were treated with UII at the concentration of 10^{-7} mol/L, because our previous studies showed that diabetic patients have an increased urinary UII at the concentration of 10^{-6}–10^{-7} mol/L[21]. We also found that there was no significant dose-dependent effect of UII on HK-2 cells from 10^{-5}–10^{-9} mol/L, and 10^{-7} mol/L was the concentration that produced the optimal effect. HK-2 cells were treated with UT antagonist, SB-657510, at the concentration of 10^{-6} mol/L in accordance with Park et al.[22].

Western blot analysis

Proteins were extracted from HK-2 cells and mice renal tissues. Western blot analysis of UII, LC3, p62/SQSTM1, fibronectin and collagen IV were carried out. The protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred onto NC membranes (Applygen Technologies Inc., Beijing, China). Membranes were subsequently incubated with primary rabbit or mice polyclonal anti-UII, anti-LC3, anti-p62/SQSTM1, anti-fibronectin, anti-collagen IV and anti-β-actin antibodies (1:500 ab194676, Abcam, Shanghai, China; 1:250 ab48394, Abcam; 1:500 ab56416; Abcam; 1:500 ab2413, Abcam; 1:250 ab6586, Abcam; 1:500 TA-09, Zhongshan Gold Bridge Biotechnology Co. Ltd Beijing, China), followed by incubation with conjugated goat (polyclonal) anti-rabbit or anti-mouse immunoglobulin G (1:10,000; IRDye 800CW, Li-Cor, Lincoln, NE, USA). Proteins were visualized using Li-Cor Odyssey Infrared Imager. Semiquantitative grayscale intensity was generated with Odyssey Software v1.2 (LI-COR Biosciences GmbH, Bad Homburg, Germany).

Statistical analysis

All analyses were carried out using Spss 16.0 (IBM SPSS Inc., Chicago, IL, USA). Results were reported as mean ± standard deviation. Independent samples t-test or ANOVA was applied in statistical analysis. A P-value <0.05 was accepted to be significant.

RESULTS

Genotyping of UT knockout mice

We generated homozygous UT knockout mice for the present study, and confirmed their genotype through DNA extraction of their toe tissues with subsequent PCR analysis (Figure 1).

There was no difference in blood pressure or heart rate between the wild-type mice and the UT knockout mice. We also did not find any difference in fasting glucose between wild-type diabetic mice and diabetic mice with UT gene knockout; however, they both had significantly higher fasting glucose in comparison with that of the control (25.6 ± 2.5 mmol/L, 24.5 ± 2.9 mmol/L, 6.4 ± 2.7 mmol/L; P = 0.001).
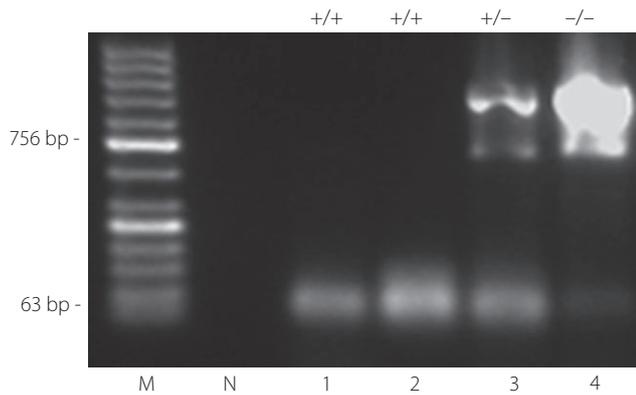
Figure 1 | Genotyping urotensin II receptor (UT) knockout mice from deoxyribonucleic acid isolation of mice toe tissues. Deoxyribonucleic acid extraction was followed by polymerase chain reaction, and genotype results were analyzed on 1% agarose gel. Lane M is the size marker. Lane N is negative control without template deoxyribonucleic acid. Lanes 1 and 2 (+/+) represent the wild-type genotype. Lane 3 (+/−) represents heterozygous urotensin II receptor knock-out genotype. Lane 4 (−/−) represents the homozygous urotensin II receptor knockout genotype.

Changes of expressions of UII and markers of autophagy in diabetic mice and diabetic mice with UT gene knockout

Immunohistochemistry analysis of renal tissues and western blot of proteins extracted from the renal tissues verified that the expression of UII is highly increased in both diabetic UT knockout mice and wild-type diabetic mice (Figure 2a,b). The control group only showed moderate UII expression (Figure 2a,b).

After establishing that UII expression is increased in the diabetic mice groups, we investigated for the presence of autophagy in all groups of mice. The present results show that the expression of autophagy is significantly increased in the diabetic UT knockout mice group when compared with the autophagy expressions in both the wild-type diabetic mice and the control mice group. In both immunohistochemistry and western blot analysis, LC3-II expression was found to be strongly enhanced in the diabetic UT knockout mice group when compared with that of the other groups of mice (Figure 3a,b). Another autophagy marker, p62, also showed increased autophagy presence in the UT knockout group in comparison with that of the other groups, as p62 expression was decreased in the UT knockout group (Figure 4a,b). This finding verified that UII inhibited autophagy in diabetic kidney tissues.

Effects of UII on ECM protein synthesis of kidney tissues in diabetic mice

ECM proteins (fibronectin and collagen IV) are known to accumulate in early DN[20]. To gain insights into the effects of UII and autophagy on ECM proteins production in diabetes, we carried out immunochemical staining of renal tissues from the diabetic UT knockout mice, diabetic wild-type mice and normal
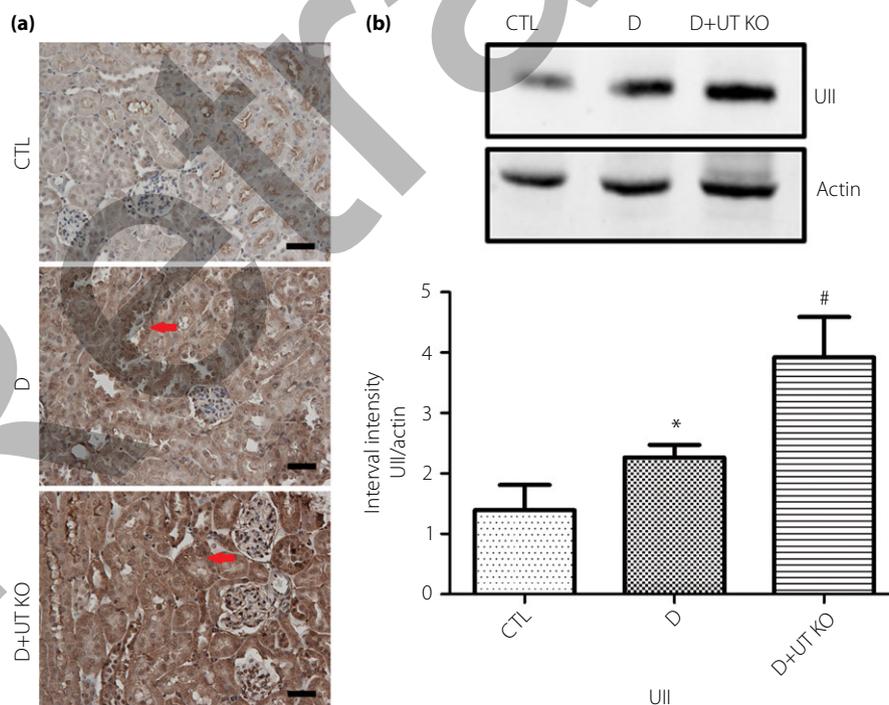

Figure 2 | (a) Urotensin II (UII) expressions in renal tissues of diabetic UII receptor (UT) knockout mice (D + UT KO), diabetic wild-type mice (D) and normal control mice (CTL), as shown by immunohistochemical staining (magnification: ×200). (b) Protein expressions of UII from renal extracts of D + UT KO, D and CTL. Data shown as mean ± standard deviation. *$P < 0.05$ vs CTL; #$P < 0.05$ vs D.
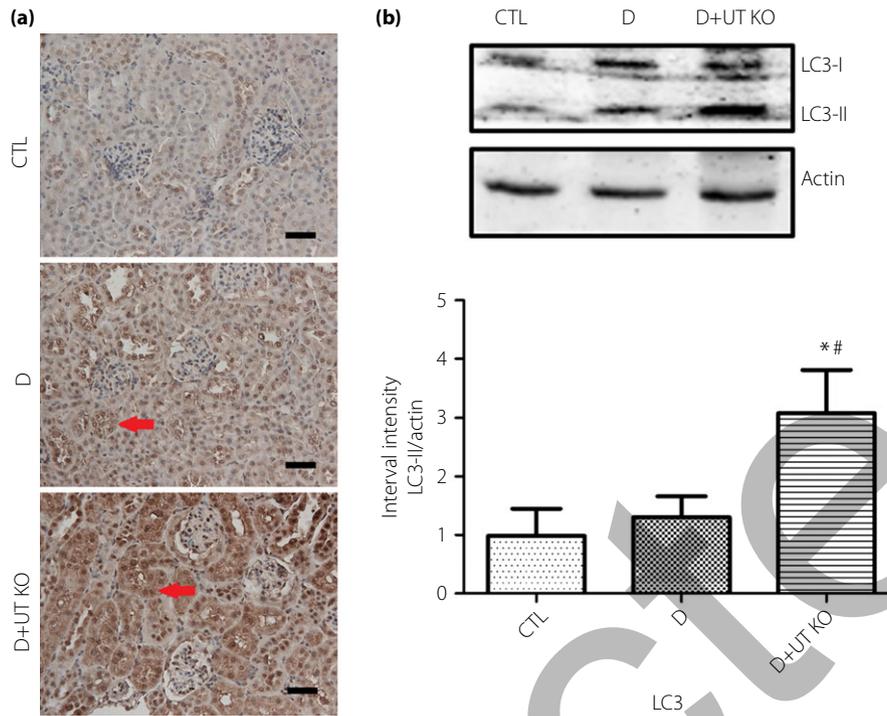
Figure 3 | (a) Microtubule-associated protein 1 light-chain 3 (LC3) expressions in renal tissues of diabetic urotensin II receptor (UT) knockout mice (D + UT KO), diabetic wild-type mice (D) and normal control mice (CTL), as shown by immunohistochemical staining (magnification: ×200). (b) Protein expressions of LC3-II from renal extracts of D+UT KO, D and CTL. Data shown as mean ± standard deviation. *P < 0.05 vs CTL; #P < 0.05 vs D.
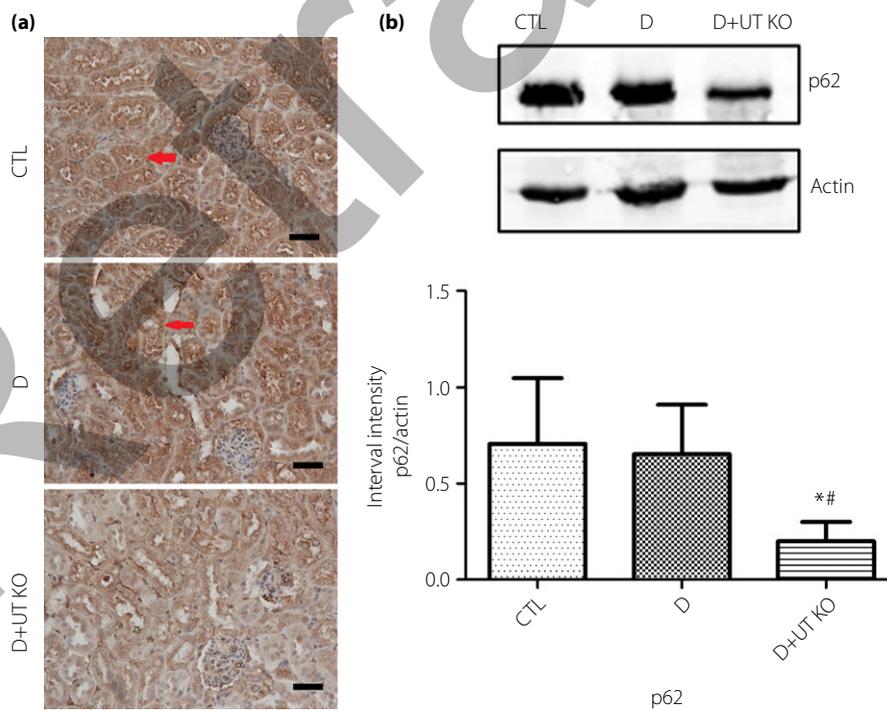

Figure 4 | (a) The p62 expressions in renal tissues of diabetic urotensin II receptor (UT) knockout mice (D + UT KO), diabetic wild-type mice (D) and normal control mice (CTL), as shown by immunohistochemical staining (magnification: ×200). (b) Protein expressions of p62 from renal extracts of D + UT KO, D and CTL. Data shown as mean ± standard deviation. *P < 0.05 vs CTL; #P < 0.05 vs D.
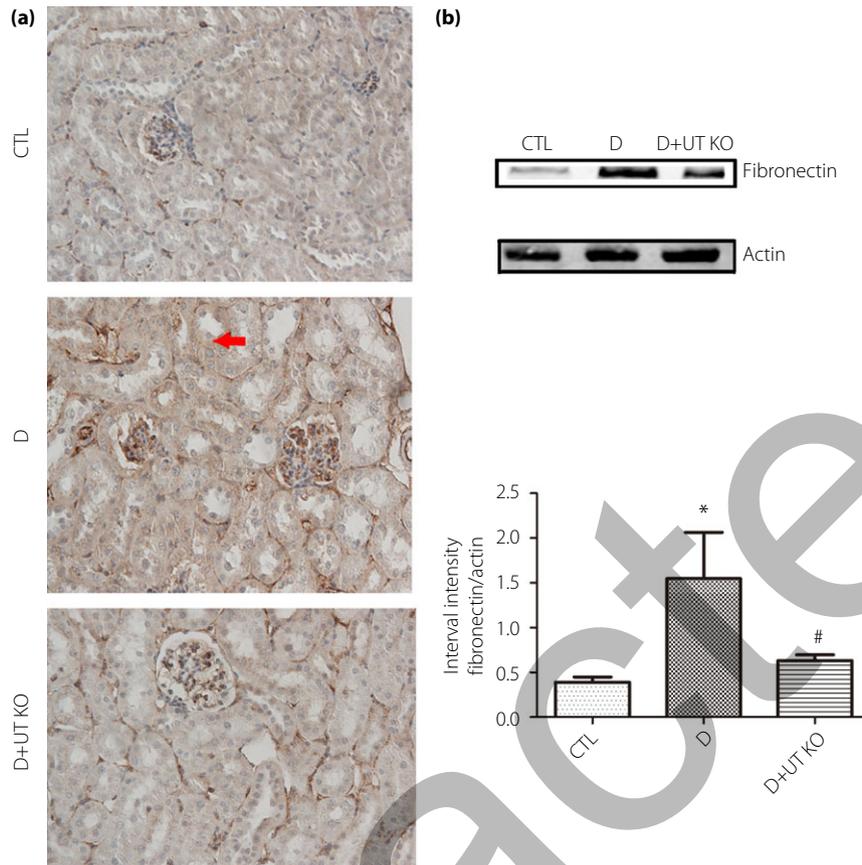
Figure 5 | (a) Fibronectin expressions in renal tissues of diabetic urotensin II receptor (UT) knockout mice (D + UT KO), diabetic wild-type mice (D) and normal control mice (CTL), as shown by immunohistochemical staining (magnification: ×200). (b) Protein expressions of fibronectin from renal extracts of D + UT KO, D and CTL. Data shown as mean ± standard deviation. *$P < 0.05$ vs CTL; #$P < 0.05$ vs D.
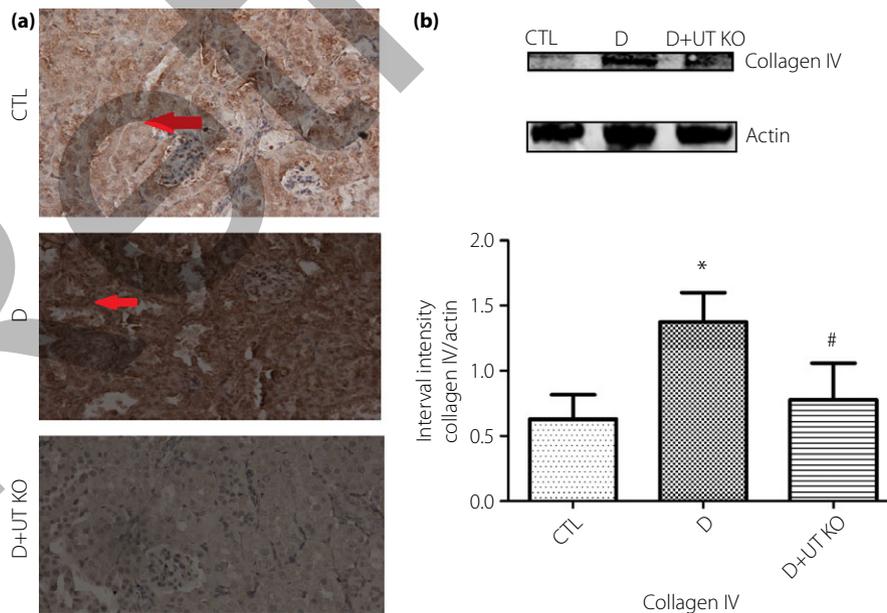

Figure 6 | (a) Collagen IV expressions in renal tissues of diabetic urotensin II receptor (UT) knockout mice (D + UT KO), diabetic wild-type mice (D) and normal control mice (CTL), as shown by immunohistochemical staining (magnification: ×200). (b) Protein expressions of collagen IV from renal extracts of D + UT KO, D and CTL. Data shown as mean ± standard deviation. *$P < 0.05$ vs CTL; #$P < 0.05$ vs D.

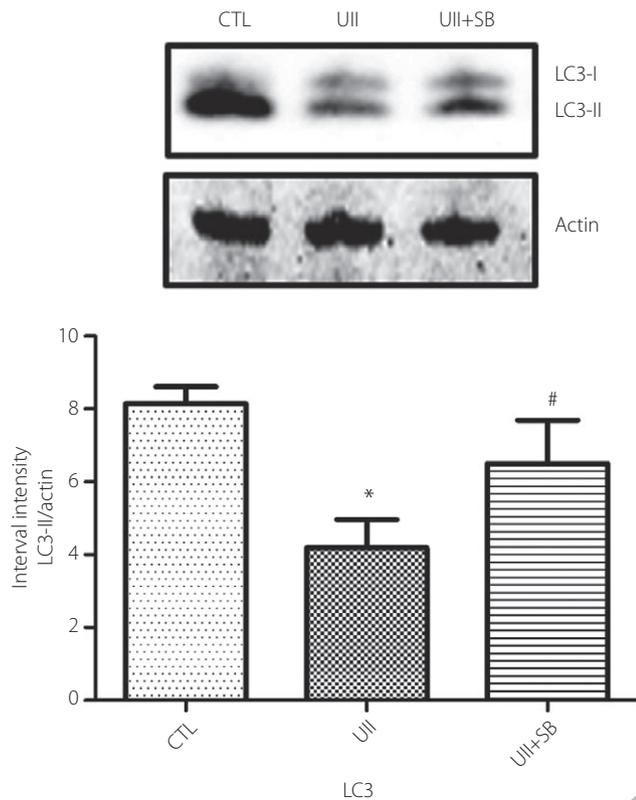**Figure 7** | Expression of Microtubule-associated protein 1 light-chain 3 (LC3)-II in HK-2 cells by western blot after urotensin II (UII) and UII + SB-657510 (SB) treatment. The expression of LC3-II was shown after HK-2 cells were exposed to 10^{-7} mol/L UII for 12 h or 10^{-6} mol/L SB + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment.
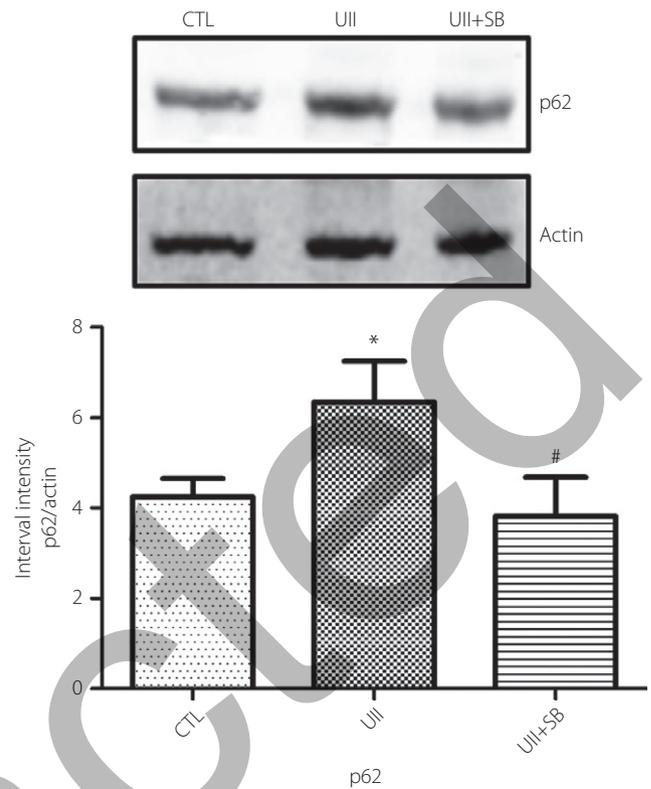
Figure 8 | Expression of p62 in HK-2 cells by western blot after urotensin II (UII) and UII + SB-657510 (SB) treatment. The expression of p62 was shown after HK-2 cells were exposed to 10^{-7} mol/L UII for 12 h or 10^{-6} mol/L SB + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment.

wild-type mice in order to investigate for the differences in ECM protein expressions among these tissues. We also used western blot analysis to examine the differences in ECM protein expressions from the renal extracts of these mice groups. The present results showed that the expression of fibronectin was significantly increased in the diabetic wild-type mice when compared with that of the normal control mice. However, the expression of fibronectin was greatly reduced in the diabetic UT knockout mice in comparison with that of the diabetic wild-type mice group (Figure 5a,b). For collagen IV, its expression was also markedly increased in the wild-type diabetic group when compared with that of the control, but significantly decreased in the diabetic UT knockout group in comparison with that of the normal diabetic group (Figure 6a, b). The present results showed that UII can increase ECM protein synthesis in diabetic kidney tissues.

Effect of UII on autophagy in UII-treated HK-2 cells
Autophagy in proximal tubular cells can become extremely active[23]. Therefore, autophagy has the potential to be used as a therapeutic target for slowing the progression of DN[14]. To

determine whether UII suppresses autophagy, we carried out western blot analysis for the autophagy markers on the protein obtained from HK-2 cells that were treated with 10^{-7} mol/L of UII in the presence or absence of UT antagonist, SB-657510.

We examined for the presence of autophagy markers (LC3-II and p62). Treatment of HK-2 cells with 10^{-7} mol/L of UII significantly decreased the expression of LC3-II in comparison with that of the control. The effect of UII was not dose-dependent, and the optimal concentration of the effect of UII was 10^{-7} mol/L. However, in the UII + SB-657510 group, LC3-II expression was significantly increased when compared with that of the HK-2 cells that were treated with only UII (Figure 7). For p62, UII significantly increased the expression of p62 in comparison with that of the control, but p62 expression was decreased in the UII + SB-657510 group when compared with that of the HK-2 cells that were treated with UII alone (Figure 8). Combining the findings of LC3-II and p62, this shows that UII inhibits autophagy activity in HK-2 cells, and this can be reversed by UT antagonist, SB-657510.
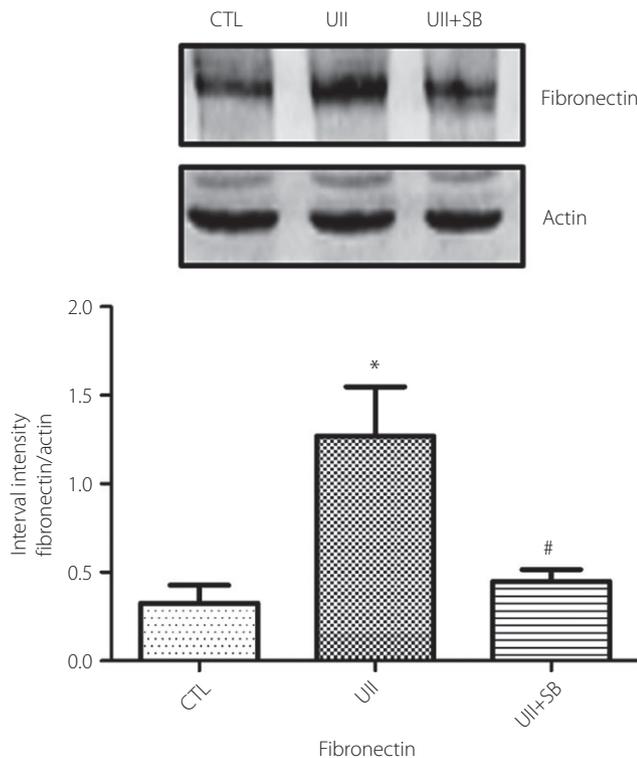
Figure 9 | Expression of fibronectin in HK-2 cells by western blot after urotensin II (UII) and UII + SB-657510 (SB) treatment. The expression of fibronectin was shown after HK-2 cells were exposed to 10^{-7} mol/L UII for 12 h or 10^{-6} mol/L SB + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment.

Figure 10 | Expression of collagen IV in HK-2 cells by western blot after urotensin II (UII) and UII + SB-657510 (SB) treatment. The expression of collagen IV was shown after HK-2 cells were exposed to 10^{-7} mol/L UII for 12 h or 10^{-6} mol/L SB + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment.

Effect of UII on ECM protein synthesis in HK-2 cells

To determine whether UII can also increase ECM protein synthesis *in vitro*, we analyzed the expressions of fibronectin and collagen IV in UII-treated HK-2 cells. Western blot results showed that UII treatment increased fibronectin protein level when compared with that of the control (Figure 9). The protein level of collagen IV also increased in UII-treated cells (Figure 10). These results confirmed that UII can increase ECM production *in vitro*. Pretreatment of the UII-treated cells with SB-657510 blocked the UII action, and SB-657510 also inhibited the upregulation of UII-induced fibronectin and collagen IV expression (Figures 9 and 10).

Upregulation of autophagy can affect UII-induced production of ECM

To further show that upregulation of autophagy can inhibit UII-induced production of ECM, HK-2 cells were treated with autophagy inducer rapamycin, UII and rapamycin + UII separately. The present results show that UII can significantly increase expressions of fibronectin and collagen IV in comparison with that of the normal control; however, rapamycin + UII can significantly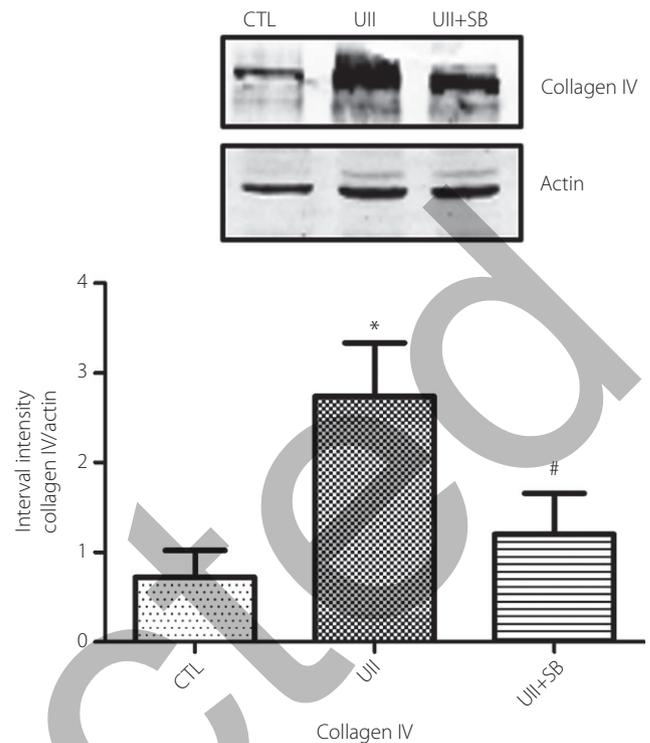 decrease the production of ECM when compared with that of the cells that were only treated with UII (Figures 11 and 12). This shows that upregulation of autophagy can inhibit UII-induced production of ECM.

DISCUSSION

DN is a leading cause of end-stage renal disease. Its pathogenesis is complex, and oxidative stress is a critical factor contributing to the progression of this disease. Autophagy is a degradation process that can respond to the rise of cellular oxidative stress, and can be linked to the development of DN[23–25]. Past studies have found that increased UII expression is associated with not only oxidative stress level induced by DN, but also the progression of DN[26,27]. A number of studies also showed that autophagy can play a renoprotective role in the progression of DN[28,29].

Our previous study showed that the expression of UII is upregulated in patients with severe pre-eclampsia. To be more specific, the expression level of UII is associated with endoplasmic reticulum stress in patients with severe pre-eclampsia. As mentioned previously and in numerous studies, endoplasmic reticulum stress can be associated with autophagy[19,30,31]. However, to the best of our knowledge, no study explored the association between UII and the process of autophagy.
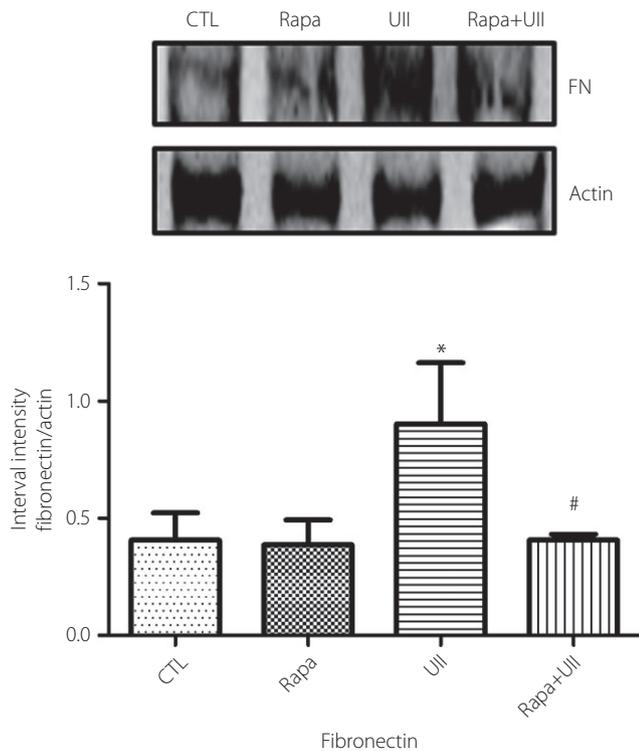
Figure 11 | Expression of fibronectin in HK-2 cells by western blot after autophagy inducer rapaymycin (Rapa), urotensin II (UII) and Rapa + UII treatment. HK-2 cells were treated with 10^{-3} mol/L Rapa, 10^{-7} mol/L UII, 10^{-3} mol/L Rapa + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment alone. FN, fibronectin.

Figure 12 | Expression of collagen IV in HK-2 cells by western blot after autophagy inducer rapaymycin (Rapa), urotensin II (UII) and Rapa + UII treatment. HK-2 cells were treated with Rapa 10^{-3} mol/L, 10^{-7} mol/L UII, 10^{-3} mol/L Rapa + 10^{-7} mol/L UII for 12 h. Data shown as mean ± standard deviation. *$P < 0.05$ vs control (CTL); #$P < 0.05$ vs UII treatment alone.

In addition to investigating the potential of UII and autophagy as novel strategies in the treatment of DN, it is also important to gain insights into the development of renal fibrosis, as it is a destructive process that can be found in all types of progressive renal diseases[32,33]. A distinctive characteristic of tubulointerstitial fibrosis is increased matrix protein synthesis (fibronectin and collagen IV) and excessive matrix accumulation, which leads to obliteration of tubules and peritubular capillaries. As a result, glomerular filtration declines with the reduction of intact nephrons[32].

We provide here the first evidence to show that UII can inhibit autophagy in renal tubular epithelial cells under the setting of diabetic mellitus. For the present study, we carried out experiments *in vivo* and *in vitro* in order to analyze the association between UII and the process of autophagy. Through immunochemistry staining and western blot, we were able to observe UII upregulated in the kidney tissues of both diabetic UT knockout mice and diabetic wild-type mice in comparison with that of the healthy control mice. LC3-II expression was increased while p62 expression was decreased in the kidney tissues of the diabetic UT knockout mice group when compared with that of the diabetic wild-type mice and wild-type normal
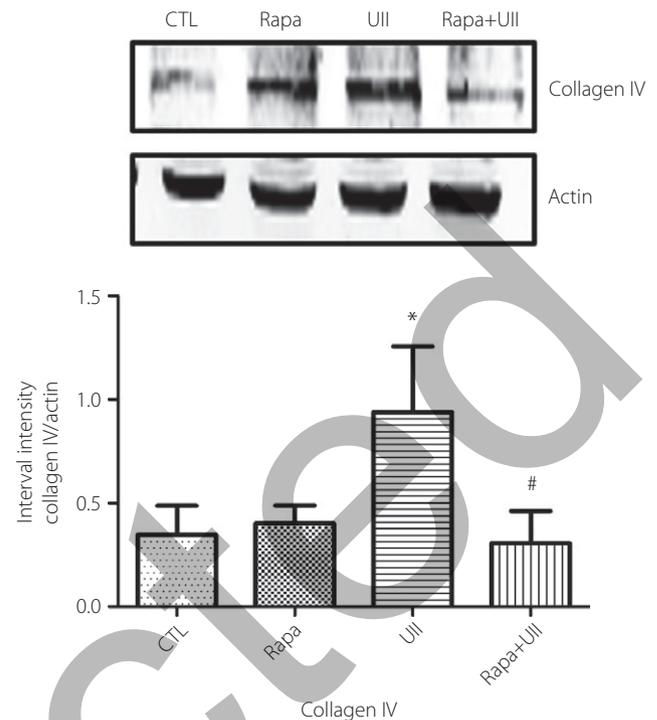
mice groups. The results of LC3-II and p62 show that autophagy activity is increased when UII is inhibited. Additionally, expressions of UII, LC3-II and p62 were predominantly in the renal tubular epithelial cell. The present results are the first to verify that UII can downregulate autophagy in the renal tubular epithelium of diabetic mice. Previous studies already showed that autophagy is a mechanism that cellular systems deploy to slow the progression of DN[14,23]. It is, therefore, plausible to believe that a natural mechanism to ameliorate the condition of DN involves the process of autophagy. Past studies also showed that UII-receptor antagonist can be effective in the treatment of renal diseases[10,11].

In our *in vitro* study, we verified that UII downregulated the expression of LC3-II and upregulated the expression of p62, which showed that UII inhibits autophagy. With the addition of UT antagonist, SB-657510, inhibition of autophagy by UII is reversed. Our *in vitro* study results are consistent with our results from the *in vivo* study. Taken together, our findings show that the enhancement of autophagy through inhibiting the effect of UII might provide an effective alternative to the current treatment for DN.

In our current study, we also found that the expressions for the markers of renal fibrosis (fibronectin and collagen IV) in the diabetic UT knockout mice were significantly decreased in

comparison with that of the wild-type diabetic mice. Our *in vitro* results showed that UII induced upregulation of fibronectin and collagen IV in human renal tubular epithelial cells. However, in the presence of UT antagonist, SB-657510, these renal fibrosis markers were significantly downregulated. As mentioned previously, our results showed that autophagy is upregulated in the renal tubular epithelium when UII is inhibited. Taken together, these results suggested that blocking UII can enhance autophagy and attenuate ECM proteins synthesis, which are markers for renal fibrosis. Recently, another study also supported the role of autophagy in reducing renal fibrosis, as it is found that metformin, an inducer of autophagy, can prevent renal fibrosis in mice with unilateral ureteral obstruction[33].

We verified that UII inhibits autophagy and increases the production of ECM. In order to verify that the inhibition of autophagy and the increase production of ECM by UII are not two separate phenomena, but a linked process, we carried out a further study to test for the changes in ECM production when HK-2 cells were treated with autophagy inducer rapamycin, UII or rapamycin combined with UII. We found that the expressions of ECM decreased in the presence of rapamycin + UII when compared with that of the cells that were treated with UII alone. The present results are the first to show that UII-induced production of ECM might involve inhibiting autophagy in early diabetic kidney.

We showed that blocking UII can exert a protective effect on the diabetic kidneys through attenuation of ECM proteins production. To date, autophagy has been recognized as a cellular process that not only responds to nephrotoxic stress, but also a potential therapeutic target for DN.

In summary, both our *in vivo* and *in vitro* study are the first to show that UII can downregulate autophagy in the kidney, and the downregulation of autophagy can accompany an increased production of extracellular matrix in early diabetes. The present study also showed that upregulation of autophagy can decrease UII-induced production of ECM in HK-2 cells.

DISCLOSURE
The authors declare no conflict of interest.

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