Journal of Medical Systems (2019) 43: 266
https://doi.org/10.1007/s10916-019-1412-4

PATIENT FACING SYSTEMS

Basic Research in Diabetic Nephropathy Health Care: A study of the Renoprotective Mechanism of Metformin

Jing Xue [1,2] · Liang Wang [1] · Zhuxing Sun [1] · Changying Xing [3]

Received: 12 May 2019 / Accepted: 28 June 2019 / Published online: 4 July 2019

Abstract

The anti-aging gene klotho is closely related to kidney disease, and an increase in the level of the klotho protein inhibits the progression of various kidney diseases. According to clinical studies, dimethyl-biguanide hydrochloride (DMBG) reduces the urinary protein level in patients with diabetic nephropathy to protect the kidney, but the specific renoprotective mechanism remains unclear. In this study, the application of DMBG partially alleviates the pathological changes in the kidneys of db/db mice, increases the level of the klotho protein in the blood, urine and kidney tissues of the mice, and reduces the levels of the mTOR and p-mTOR proteins. The effects of high glucose and DMBG on klotho and the mTOR pathway in MDCK cells were analyzed at the cellular level. High glucose stimulation activates mTOR pathway and decreases the activity of MDCK cells. DMBG decreases the level of the mTOR protein and reverses the effect of hyperglycaemic stimulation on the activity of MDCK cells. After inhibiting the expression of the klotho protein, DMBG is unable to decrease the level of the mTOR protein. Therefore, klotho plays an important role in the mechanism by which DMBG inhibits the mTOR pathway to protect renal function.

Keywords Dimethyl-biguanide hydrochloride · Diabetic nephropathy · Klotho · mTOR pathway · MDCK cells

Introduction

Diabetic nephropathy (DN) caused by diabetes has become the main secondary kidney disease in China [1]. Even with strict control of blood sugar levels and blood pressure, 30-50% of patients with diabetes still progress to DN [2, 3]. DN also imposes a substantial economic burden in terms of healthcare costs, which increases as the disease progresses. Therefore, the situation of DN control is grim. China has a high incidence of diabetes, with 114 million people living with diabetes, according to 2017 data from the World Diabetes Federation. According to epidemiological studies, chronic kidney disease (CKD) also has a high incidence in China, with 120 million patients diagnosed with CKD [4]. DN has become the main cause of hospitalization of patients with CKD in China and one of the main causes of dialysis in patients with end-stage renal disease (ESRD) in China [5].

The klotho gene was first discovered by Kuro-o et al. in 1997 as an anti-aging gene [6]. Klotho is expressed in a variety of tissues and organs, but it is expressed at the highest levels in the kidney. Klotho mutant mice suffer from a variety of diseases that are associated with chronic renal failure, such as a shorter life span, osteoporosis, calcium and phosphorus metabolism disorders, atherosclerosis, coronary calcification, and abnormal glucose metabolism [7]. Overexpression of the klotho gene can be induced to reverse the ageing phenotype, and the life span of female and male mice overexpressing klotho is increased by 20% and 30%, respectively [8]. Recently, studies have found that klotho gene expression is significantly decreased in patients with chronic renal insufficiency [9, 10]; therefore, the klotho gene is closely related to renal disease. However, researchers have not clearly determined whether an increase in the expression of klotho gene protects the kidney and delays the progression of kidney

This article is part of the Topical Collection on *Patient Facing Systems*

✉ Changying Xing
cyxing62_nm@163.com

[1] Department of Nephrology, the Affiliated Wuxi People's Hospital of Nanjing Medical University, 299 Qingyang Rd, Wuxi, Jiangsu 214023, People's Republic of China

[2] Jiangsu Jiangda Smart Science and Technology Ltd., No. 18 Shannan Road, Chengbei Park, Jingjiang, Jiangsu 214500, People's Republic of China

[3] Department of Nephrology, the First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Rd, Nanjing, Jiangsu 210029, People's Republic of China

disease. As shown in previous animal studies [11], klotho mutant mice exhibit aggravated streptozotocin (STZ)-induced renal hypertrophy due to the lack of the klotho gene, but the mechanism was not clear. The purpose of this study is to explore the effect and mechanism of DMBG on the levels of the klotho protein in db/db mice and Madin-Darby canine kidney cells (MDCK) cells stimulated with high glucose to identify a new target for the treatment of DN.

Materials and Methods

Schematic Showing the Flow of the Experiment using MDCK Cells

In this study, the cell-based experiments were performed according to the flow chart shown in Fig. 1. MDCK cells were cultured in vitro and subjected to hyperglycaemic stimulation for different periods. Cell morphology was observed and MTT and Hoechst 33258 staining were used to examine viability. The levels of the mTOR, p-mTOR and klotho proteins in MDCK cells were detected using Western blotting. Meanwhile, an siRNA was used to inhibit the expression of the klotho protein, and Western blotting was used to detect the mTOR and p-mTOR levels in the cells of the high glucose group and the DMBG intervention group.

Experimental Procedures

The mouse experimental procedures include four steps.

1) Modelling and grouping: Fifteen 8-week-old male SPF-grade mice were purchased from the animal centre of Wuxi Institute of Blood Control, including 10 db/db mice and 5 db/m mice. The experiment was initiated after one week of adaptive feeding. Ten db/db mice were randomly divided into a DN group and DMBG treatment group, and 5 db/m mice were used as a normal control group. In the DMBG treatment group, 0.0042 g/kg/d of DMBG (Sigma, BCBT7573) was administered by gavage every day, while the DN and the normal control groups were administered the same dose of normal saline by gavage for 8 weeks.
2) Biochemical indicators: Blood was collected from the tail vein of mice, and blood glucose levels were detected with a blood glucose metre. Blood was collected from the orbital vein, and blood urea nitrogen and creatinine levels were measured using automatic biochemical analyser. The 24 h urine microalbumin levels were detected using an ELISA.
3) Kidney tissue staining: Paraffin-embedded mouse kidney tissues were sectioned and dehydrated for HE and PASM staining to observe the changes in renal microstructure during renal pathology.
4) Western blotting was used to detect the levels of the klotho, mTOR and p-mTOR proteins in kidney tissue homogenates, and the experiment was independently repeated three times.

Culture of MDCK cells and cell grouping.

The experimental procedures using MDCK cells mainly consisted of the six parts listed below.

1) Experimental setting

MDCK cells were purchased from ACTT and stored in liquid nitrogen in the laboratory until further use. MDCK cells were cultured and inoculated in the logarithmic growth stage.

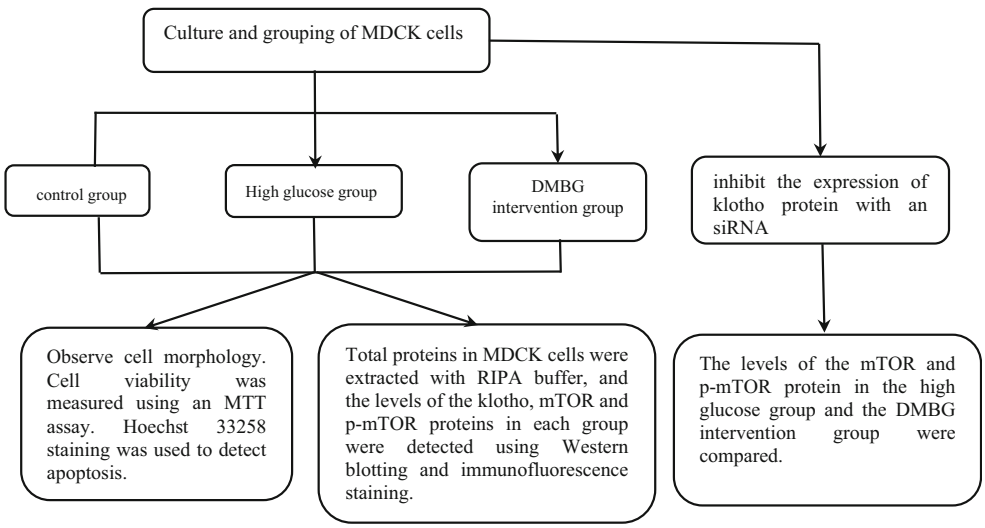


Fig. 1 Schematic showing the flow of the experiment using MDCK cells

Table 1 The primer sequences used in the experiment

The primer	The sequence (5′ to 3′)	The length of the amplified fragment
GAPDH F	TGGGGTGATGCTGGTGCTGAGTA	127 bp
GAPDH R	GGGCATCAGCAGAAGGAGCAGAG	
klotho F	GGGGCAGCAACGTTCCTTTTAAA	138 bp
klotho R	CGGGCCTGGAGACCTCAAAAGT	

Cells were passaged with 0.25% trypsin upon reaching 80% confluence. Cells at passages 5-13 were used in the experiment.

2) Experimental grouping

The experiment was divided into a normal control group (MEM + mannitol), high glucose group (DMEM with a glucose concentration of 30 mmol/L), and DMBG intervention group (high glucose +DMBG).

3) Cell morphology observations and activity determination

Morphological changes in the MDCK cells in each group were observed using an inverted microscope. The MTT assay and Hoechst 33258 staining were used to determine the activity of MDCK cells in each group. Hoechst 33258 staining showed that the nuclei of the surviving cells were clearly demarcated with uniform light blue chromatin distribution, while the nuclei of the apoptotic cells showed uneven distribution of chromatin, and some of the nuclei were lobular and fragmented, with a bright blue colour.

4) siRNA transfection

Two millilitres of normal growth medium without antibiotics were added to each well of a 6-well plate, and 2×10^5 cells/wells were seeded. Experiments were carried out when the cells reached 60-80% confluence. The diluted transfection reagents were mixed with the double-stranded siRNA and incubated at room temperature for 15-45 min. The cells were washed once with 2 mL of siRNA transfection medium. Next, 0.8 ml of siRNA transfection medium was added to the mixture of siRNA and transfection reagents, and the mixture was added to the washed cells. After a 5-7 h incubation, 1 ml of normal growth medium containing twice the concentrations of serum and antibiotics was added without removing the transfection mixture. The cells were incubated for 18 to 24 h, and then the medium was aspirated and replaced with a fresh normal growth medium. Cells were examined 24 to 72 h after the addition of fresh medium.

5) Real-time fluorescence quantitative PCR (qPCR)

Total RNA extraction: After lysis, 0.2 ml of chloroform was added to the cells. After oscillation and centrifugation, the upper aqueous phase was transferred to a new tube without precipitation. Isopropanol was added to precipitate the RNA, and colloidal precipitates were observed on the bottom and sidewall after centrifugation. After cleaning and drying, the precipitate was dissolved in water without RNase, and the RNA was obtained.

Reverse transcription assay: The methods used in this study are described below. 1) The following reaction components were added to an RNase-free centrifuge tube in an ice-bath: 1-5 μg of total RNA or 50-500 ng of mRNA, 2 pmol of gene-specific primers, which are listed in Table 1, supplemented with RNase-free ddH_2O to 14.5 L. 2) The mixture was incubated at 70 °C for 2 min, rapidly cooled on ice for 5 min, briefly centrifuged, and the supernatant was collected and mixed with the following components: 4 mu of 5x MMLV Buffer, 1 mu of dNTPs, 0.5 mu of RNasin L and 1 L mu of M–MLV. 3) The mixture was incubated in a 42 °C water bath 60 min. If random primers were used, the tube was incubated in a 25 °C water bath for 10 min, followed by a 42 °C water bath 60 min. 4) The samples were heated to 95 °C for 5 min to terminate the reaction, and then used in a subsequent

Table 2 Biochemical parameters and blood and urine levels of the klotho protein in mice

	The normal control group	The non-DMBG group	The DMBG group
Fasting blood glucose level (mmol/l)	9.96 ± 0.77	32.12 ± 3.29[a]	27.98 ± 3.56[ab]
Serum creatinine level (μmol/l)	6.94 ± 0.24	21.98 ± 1.39[a]	16.00 ± 0.58[ab]
Urine protein level (μg)	67.02 ± 0.81	130.14 ± 1.28[a]	94.86 ± 0.91[ab]
Serum klotho level (pg/ml)	56.99 ± 8.22	17.79 ± 3.38[a]	41.86 ± 4.29[ab]
Urine klotho level (pg/ml)	27.73 ± 5.34	15.17 ± 1.88[a]	22.79 ± 6.06[ab]

Note: [a] $p < 0.05$ compared with the normal control group, [b] $p < 0.05$ compared with the non-DMBG group

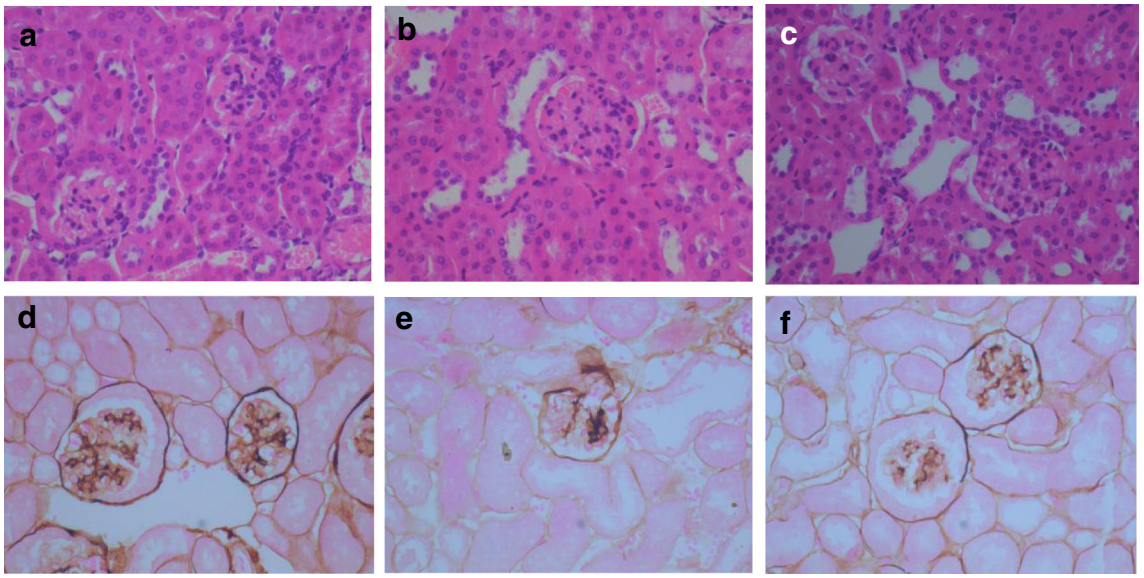


Fig. 2 Images of HE and PASM staining in kidney tissues from each group (400X magnification). (**a**) HE staining in the normal control group. (**b**) HE staining in the non-DMBG group. (**c**) HE staining in the DMBG group. (**d**) PASM staining in the normal control group. (**e**) PASM staining in the non-DMBG group. (**f**) PASM staining in the DMBG group

experiment or stored at −20 °C. In addition, for subsequent PCR amplification, the reaction system was diluted to 50 L with RNase-free ddH2O, and 2-5 L was used as the PCR template.

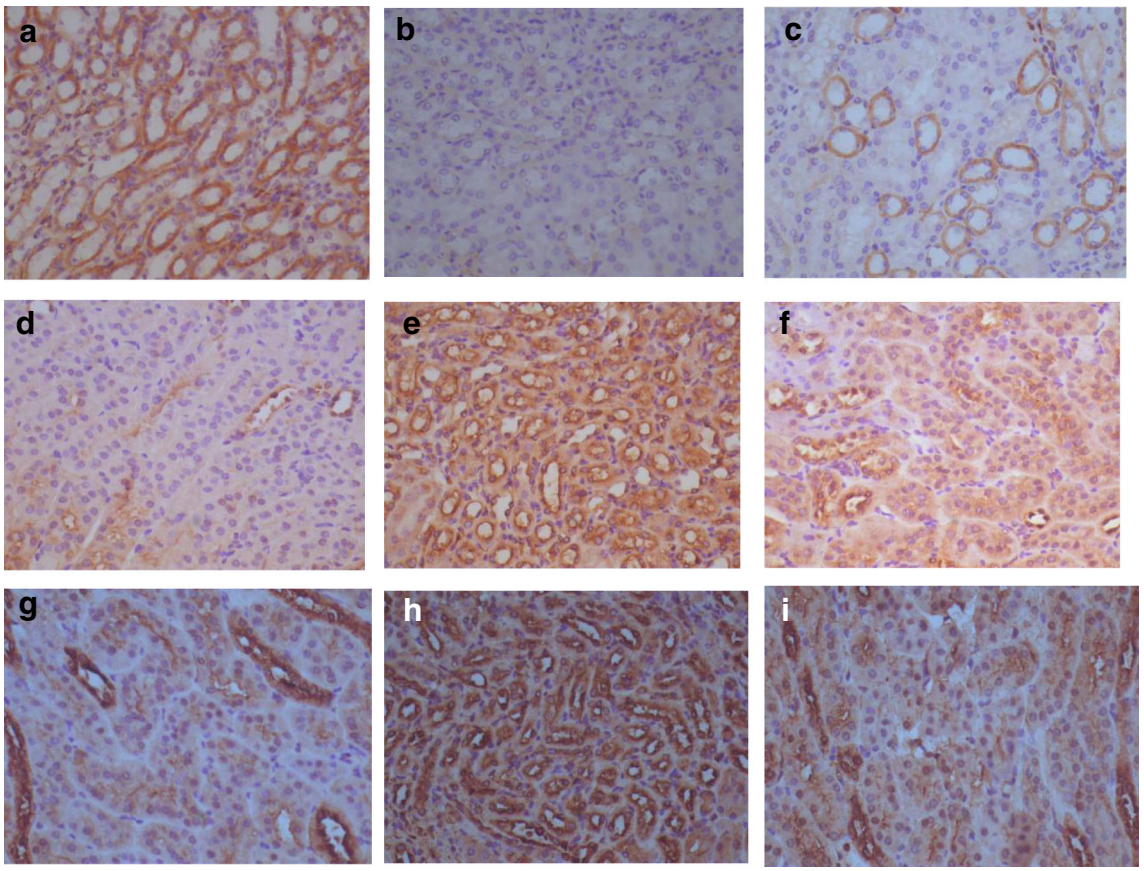


Fig. 3 Immunohistochemical staining for the klotho, mTOR and p-mTOR proteins in each group (400X magnification). Levels of the klotho protein in (**a**) the normal control group, (**b**) the non-DMBG group, and (**c**) the DMBG group. Levels of the mTOR protein in the (**d**) normal control group, (**e**) the non-DMBG group, and (**f**) the DMBG group. Levels of the p-mTOR protein in the (**g**) normal control group, (**h**) non-DMBG group, and (**i**) DMBG group

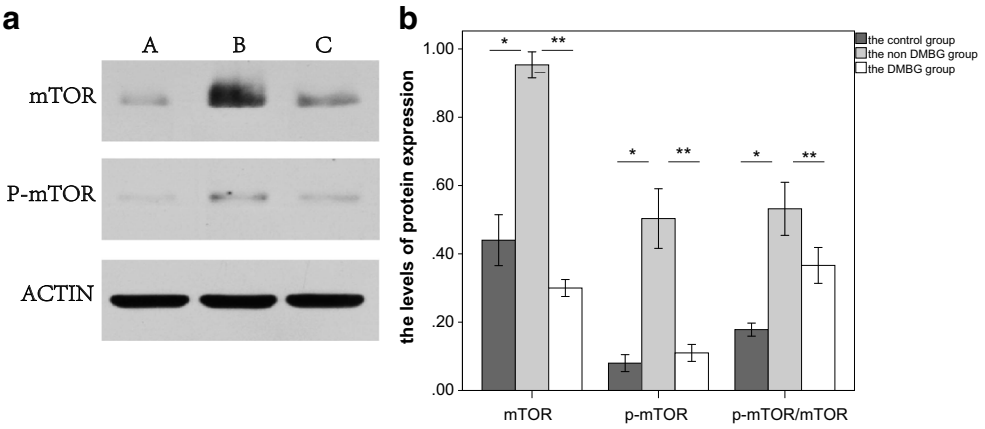


Fig. 4 The levels of the mTOR and p-mTOR proteins in kidney tissues from mice in each group were detected using Western blotting. (**a**) indicates the normal control group, (**b**) indicates the non-DMBG group, and (**c**) indicates the DMBG group. Notes: * $p < 0.001$ and ** $p < 0.05$

6) Western blot analysis

Western blotting was used to detect the levels of the klotho, mTOR and p-mTOR proteins in MDCK cells. Total proteins were extracted using lysis buffer (1 mM PMSF). Proteins were separated using SDS-PAGE, electrotransferred and immobilized on nitrocellulose membranes. Membranes were incubated with 5% skim milk for 2 h. Then, after washing, the membrane was incubated with a primary antibody or (1:0) beta actin (1:4000) antibody at 4 °C overnight with shaking. Next, the membrane was hybridized with the diluted secondary antibody for 1 h at room temperature. Antibody and antigen complexes were detected using an ECL kit. The membrane was scanned or exposed to X-ray film.

Statistical Methods

Data are presented as the means±standard deviations. One-way ANOVA was used to compare the data between multiple groups, a t test was used to compare data between two groups, and SPSS 19.0 statistical software was used for statistical analyses. All tests were two-sided, and $p < 0.05$ was defined as statistically significant.

Results

Biochemical Parameters and Levels of the Klotho Protein in each Group of Mice

Compared with the normal control group, the fasting blood glucose level of mice in the non-DMBG group increased significantly ($p < 0.001$), indicating the successful establishment of the diabetes model. Compared with the non-DMBG group, the fasting blood glucose level of mice in the DMBG group was reduced, but the difference was not statistically significant ($p = 0.093$), suggesting that the DMBG intervention can reduce the blood glucose level of diabetic mice to a certain extent. Compared with the normal control group, serum creatinine and urinary microalbumin levels were significantly increased in the non-DMBG group ($p < 0.001$, Table 2), suggesting that diabetic mice developed renal damage and subsequently developed DN. Compared with the non-DMBG group, the serum creatinine and urinary microalbumin levels were significantly decreased in mice in the DMBG group ($p < 0.05$, Table 2), suggesting that the DMBG treatment improved the renal function of DN mice. Serum and urine klotho levels were significantly reduced in the non-DMBG group compared with the normal control group (p

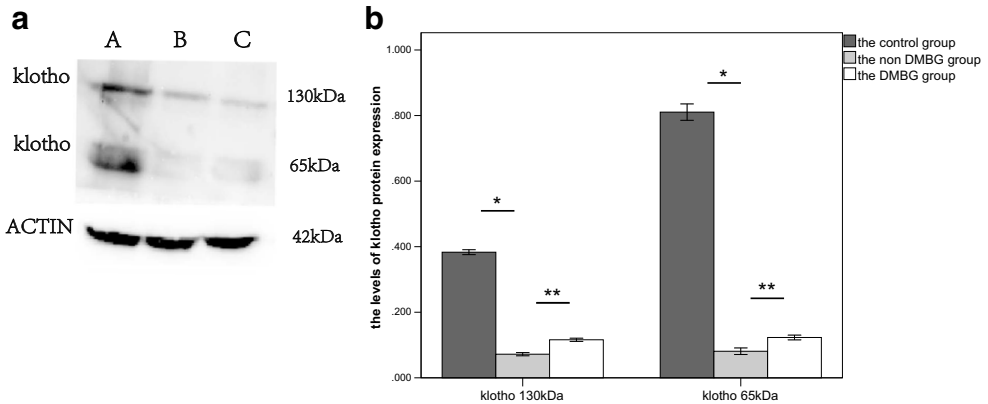


Fig. 5 The levels of the klotho protein in kidney tissues from mice in each group were detected using Western blotting. (**a**) indicates the normal control group, (**b**) indicates the non-DMBG group, and (**c**) indicates the DMBG group. Notes: * $p < 0.001$ and ** $p < 0.05$

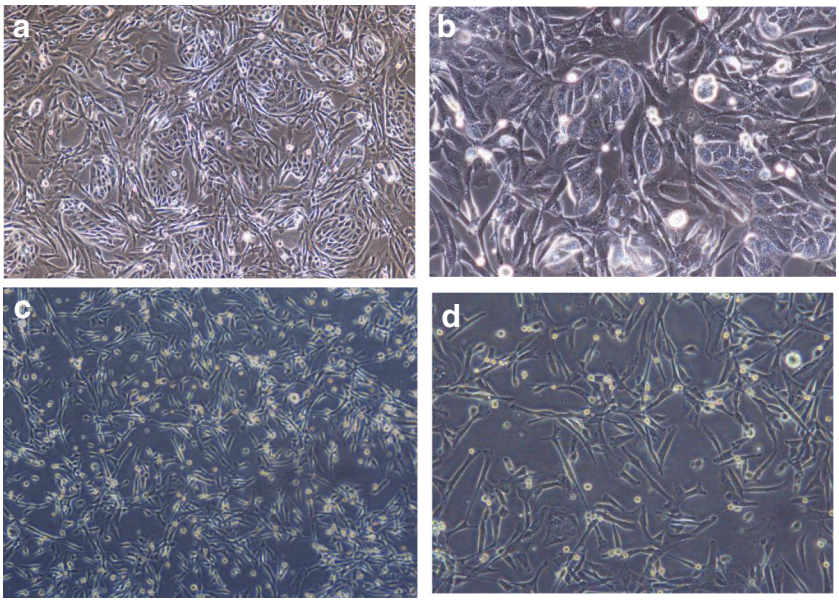


Fig. 6 Morphology of MDCK cells in the normal control group and the high glucose group. (**a**) Images of the normal control group at 100X magnification, (**b**) image of the normal control group at 200X magnification, (**c**) image of the 48 h hyperglycaemia group at 100X magnification, and (**d**) image of the 48 h hyperglycaemia group at 200X magnification

$_{blood} < 0.001$, $p_{urine} = 0.001$, Table 2). Compared with the non-DMBG group, the serum and urine klotho levels were significantly increased in the DMBG group ($p_{blood} < 0.001$, $p_{urine} = 0.028$, Table 2).

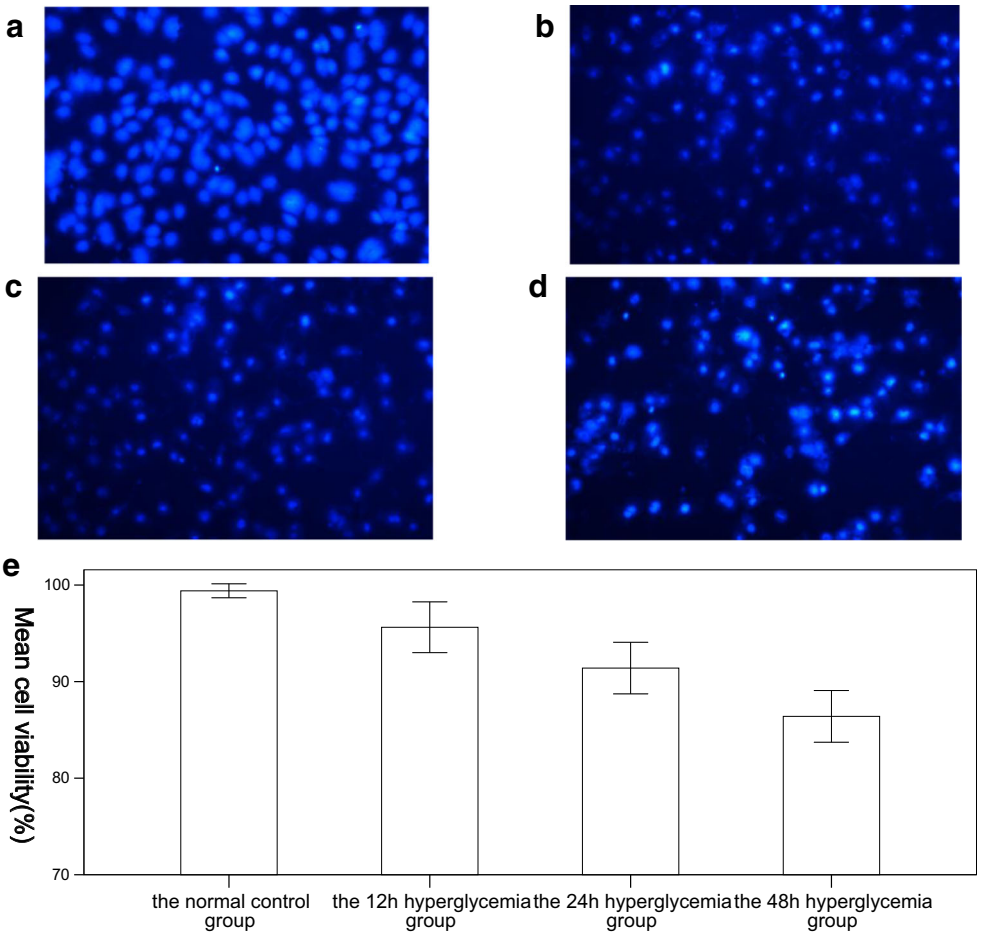


Fig. 7 Analysis of Hoechst 33258 staining in MDCK cells in the control group and the high glucose group (200X magnification). (**a**) The normal control group, (**b**) the 12 h hyperglycaemia group, (**c**) the 24 h hyperglycaemia group, (**d**) the 48 h hyperglycaemia group, and (**e**) cell viability measured in each group

Table 3 Effects of the high glucose stimulation on the activity of MDCK cells at different time points

Descriptions	Absorbance	Cell survival rate (%)	*P* value
the blank group	0.05 ± 0.002	0	
the control group	1.70 ± 0.02	100 ± 1.11	p < 0.001
the high glucose group analyzed at 12 h	1.63 ± 0.01	95.49 ± 0.64	
the high glucose group analyzed at 24 h	1.50 ± 0.009	91.19 ± 0.35	
the high glucose group analyzed at 48 h	1.98 ± 0.04	86.23 ± 5.03	

Changes in the Renal Microstructures of each Group of Mice

Observations and comparisons of HE and PASM staining in renal tissues from each group under a light microscope revealed an increase in the renal glomerular volume in the non-DMBG group; the renal tubules were separated by a brush border, the basement membrane was rigid, the mesangial area was widened and the matrix was increased. Compared with the non-DMBG group, these pathological changes were alleviated in the kidneys of the DMBG group to some extent (Fig. 2).

Levels of the mTOR, p-mTOR and Klotho Proteins in Pathological Kidney Sections from each Group

Immunohistochemistry was used to detect the expression of the klotho protein, which was mainly detected in distant tubules in the mouse kidney tissues from the normal control group, non-DMBG group and DMBG group. Compared with the control group, the expression of klotho protein was significantly decreased in the renal tubules of mice in the non-DMBG group, and the levels of klotho protein increased after the DMBG intervention (Fig. 3A-C). The mTOR and p-mTOR proteins were widely distributed in glomeruli and renal tubules, and the levels of mTOR and p-mTOR proteins were significantly increased in mice in the non-DMBG group compared with the normal control group. After the DMBG treatment, the levels of mTOR and p-mTOR proteins decreased (Fig. 3D-I).

Levels of the mTOR, p-mTOR and Klotho Proteins in Kidney Tissues from Mice in each Group

Levels of the mTOR and p-mTOR proteins in the kidney tissues from mice in the normal control group, non-DMBG group and DMBG group were detected using Western blotting. Levels of the mTOR and p-mTOR proteins and the p-mTOR/mTOR ratio were noticeably increased in the DMBG group compared with the normal control group ($p < 0.001$). After the DMBG intervention, the levels of the mTOR and p-mTOR proteins decreased ($p < 0.05$, Fig. 4), suggesting that DMBG inhibited the activation of the mTOR pathway.

The expression levels of klotho protein in mice's kidney tissues in the normal control group, the non-DMBG group and the DMBG group were detected by Western blot method. The results showed that two klotho protein bands with molecular weight of 65 kDa and 130 kDa could be detected in kidney tissues of mice, and the expression levels of two klotho protein bands showed similar changes in each group. The expression of klotho protein in mice's kidney tissues in the non-DMBG group was significantly lower than that in the normal control group ($p < 0.001$). After the intervention of DMBG, the expression level of klotho protein in kidney tissues of mice was increased ($p < 0.05$, see Fig. 5), suggesting that DMBG can upregulate the expression of klotho protein.

Effects of High Glucose Stimulation on MDCK Cells

Under an inverted microscope, MDCK cells grew in cell culture flask as an adherent monolayer and displayed a polygonal

Table 4 Effects of different concentrations of DMBG on the viability of MDCK cells

Descriptions	Absorbance	Cell survival rate (%)	p value
The blank group	0.04 ± 0.001	0	
The control group	3.09 ± 0.07	100 ± 1.11	p < 0.001
The hyperglycaemia group	2.69 ± 0.02	86.78 ± 0.30	
The hyperglycaemia+0.6 μg/mL DMBG group	2.79 ± 0.04	90.29 ± 0.65	
The hyperglycaemia+0.3 μg/mL DMBG group	2.87 ± 0.01	92.91 ± 0.11	
The hyperglycaemia+0.15 μg/mL DMBG group	2.78 ± 0.01	90.00 ± 0.16	
The hyperglycaemia+0.075 μg/mL DMBG group	2.73 ± 0.09	88.09 ± 0.15	

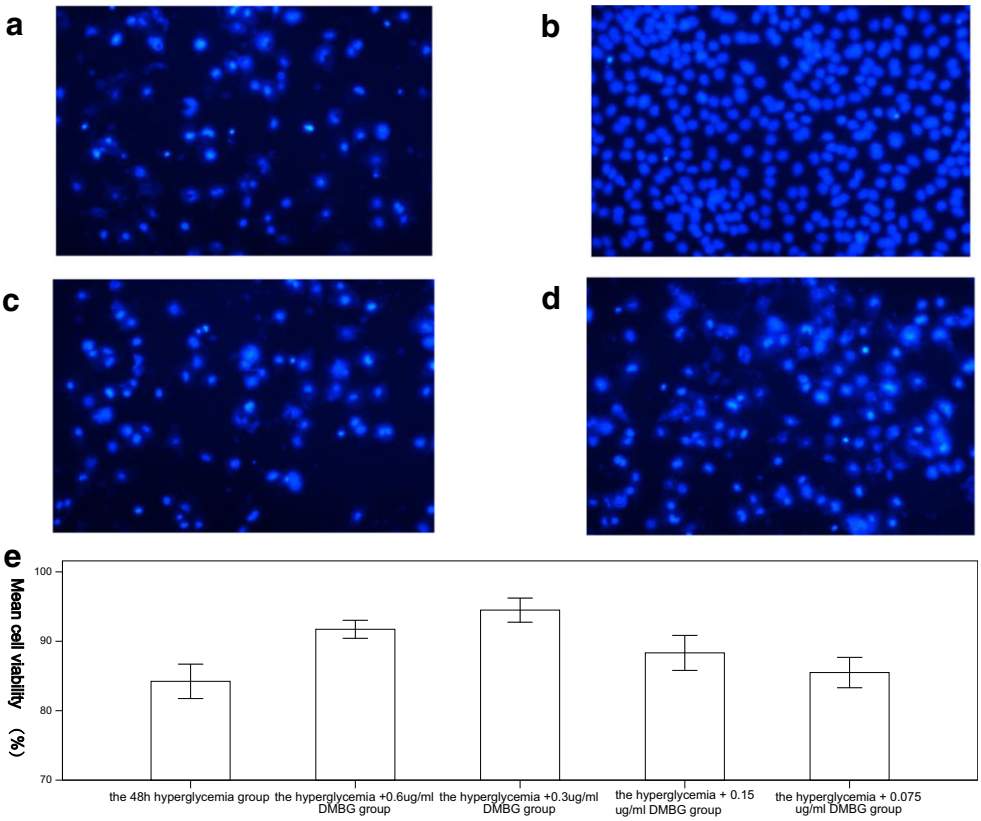


Fig. 8 Analysis of Hoechst 33258 staining in MDCK cells in the DMBG group at 48 h (200X magnification). (**a**) The 48 h hyperglycaemia +0.6 μg/ml DMBG group, (**b**) the 48 h hyperglycaemia +0.3 μg/ml DMBG group, (**c**) the 48 h hyperglycaemia +0.15 μg/ml DMBG group, (**d**) the 48 h hyperglycaemia +0.075 μg/ml DMBG group, and (**e**) cell viabilities of each group

shape. After 48 h of stimulation with 30% high glucose medium, MDCK cells lost their characteristic morphology and displayed a long spindle shape (Fig. 6). Hoechst 33258 staining was used to observe the activity of MDCK cells in each group under a fluorescence microscope. The nuclei of the normal control group were clearly demarcated and exhibited a uniform distribution of light blue-stained chromatin, while the nuclei of MDCK cells stimulated with high glucose showed an uneven chromatin distribution, and some of the nuclei were lobed, fragmented and bright blue (Fig. 7A-D). In a comparison of the cell viability after high glucose stimulation for different periods, the lowest cell viability was observed in the 48 h hyperglycaemic group (Fig. 7E). The viability of MDCK cells in each group was detected using the MTT assay and gradually decreased with time. MDCK cells were most damaged after 48 h of hyperglycaemic stimulation, and the average cell survival rate was 86.23% ($p < 0.001$, Table 3), suggesting that hyperglycaemia produced increasing damage in MDCK cells over time.

Effects of DMBG on the MDCK Cells Cultured with High Glucose

In order to observe the effect of DMBG on the high glucose culture of MDCK, the viability and apoptosis of MDCK cells were detected by establishing a concentration gradient of DMBG of 0.6, 0.3, 0.15 and 0.075 μg/mL. The results showed that DMBG increased the survival rate of MDCK cells stimulated with high glucose, with the highest viability observed in the 0.3 μg/mL DMBG group ($p < 0.001$, Table 4). Based on the Hoechst 33258 staining, the highest activity was observed in MDCK cells treated with 0.3 μg/mL DMBG group, and the cells were characterized by a well-defined nucleus and a uniform

Table 5 Relative levels of the mTOR and p-mTOR proteins in the high glucose group measured at different times

Descriptions	Relative level of the mTOR protein	Relative level of the p-mTOR protein	p value
control group at 12 h	0.90 ± 0.01	0.55 ± 0.01	$p < 0.001$
high glucose group at 12 h	0.96 ± 0.02	0.79 ± 0.003	
control group at 24 h	0.84 ± 0.01	0.55 ± 0.01	$p < 0.001$
high glucose group at 24 h	1.02 ± 0.02	0.83 ± 0.01	
control group at 48 h	0.86 ± 0.01	0.58 ± 0.006	$p < 0.001$
high glucose group at 48 h	1.14 ± 0.005	1.12 ± 0.014	

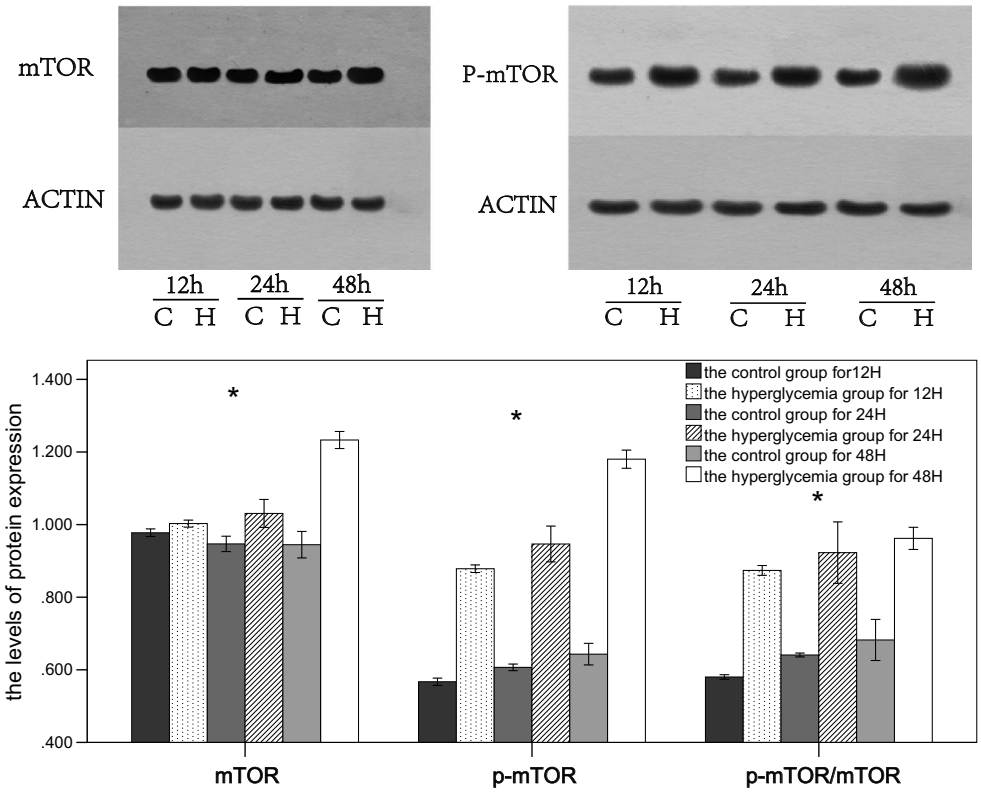


Fig. 9 Relative levels of the mTOR and p-mTOR proteins in the control group and the high glucose group at different time points. In Western blots, C indicates the control group and H indicates the hyperglycaemia group. Note: * $p < 0.001$

chromatin distribution with light blue staining. The groups treated with the other concentrations of DMBG presented nuclei with an uneven distribution of chromatin, a disrupted nuclear morphology, and bright blue staining (Fig. 8).

Levels of the mTOR, p-mTOR and Klotho Proteins in Cells from the DMBG Intervention Group

Western blotting was used to detect the levels of the mTOR and p-mTOR proteins in MDCK cells cultured with high glucose for

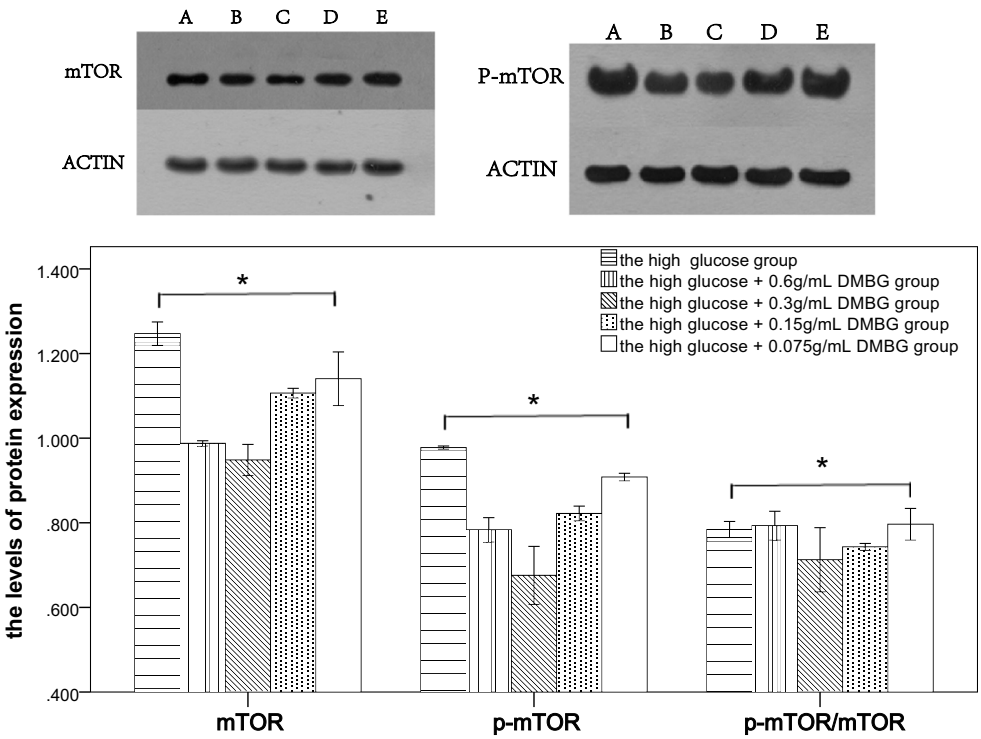
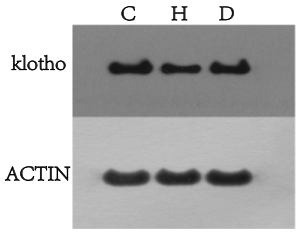


Fig. 10 Effects of different concentrations of DMBG on the levels of the mTOR and p-mTOR proteins. In Western blots, A indicates the high glucose group, B indicates the high glucose+ 0.6 μg/ml DBMG group, C indicates the high glucose+ 0.3 μg/ml DBMG group, D indicates the high glucose +0.15 μg/ml DBMG group and E indicates the high glucose+ 0.075 μg/ml DBMG group. Note: * $p < 0.05$



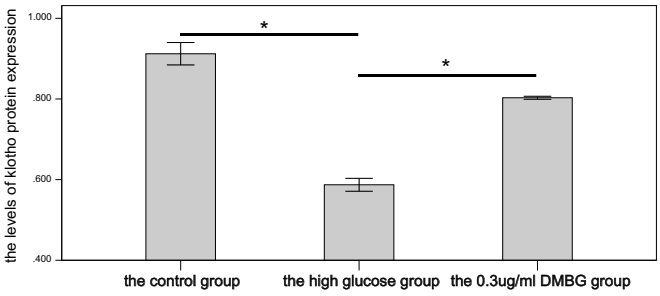


Fig. 11 The relative levels of the klotho protein in each group were detected using Western blotting. In Western blots, C indicates the control group, H indicates the high glucose group, and D indicates the 0.3 μg/ml DMBG group

different times, and the levels of the mTOR and p-mTOR proteins gradually increased in MDCK cells over time ($p < 0.001$, Table 5, Figs. 9 and 10), suggesting that the high glucose stimulation activated the mTOR pathway in MDCK cells.

The Western blot analysis of klotho protein levels in the control group, hyperglycaemia group and DMBG group revealed significantly lower levels of the klotho protein in the hyperglycaemia group than in the normal control group (1.1344 ± 0.0013 vs 1.2264 ± 0.005). However, DMBG significantly increased the levels of the klotho protein in high glucose-stimulated cells (1.21 ± 0.003 vs 1.00 ± 0.008, $p < 0.05$, Fig. 11).

Note: * $p < 0.05$.

Effects of DMBG on the Levels of the mTOR and p-mTOR Proteins after the Inhibition of Klotho Protein Expression

An siRNA was used to inhibit the expression of the klotho protein in MDCK cells, and fluorescently labelled cells were successfully transfected with the siRNA, as visualized under a fluorescence microscope (Fig. 12). Western blotting and qPCR were used to verify the inhibitory effect on klotho, and the levels of the klotho protein were significantly decreased in transfected MDCK cells decreased ($p < 0.05$, Fig. 13). After siRNA-mediated inhibition of klotho expression, significant differences in the levels of the mTOR and p-mTOR proteins were not observed between the high glucose culture group and the DMBG intervention group ($p > 0.05$, Fig. 14), suggesting that klotho plays an important role in the mechanism by which DMBG inhibits the mTOR pathway to protect renal function.

Note: * $p < 0.001$.

Discussion

In China, DN is the second major cause of end-stage renal disease; patients with this disease are susceptible to cardiovascular and cerebrovascular accidents and display a high

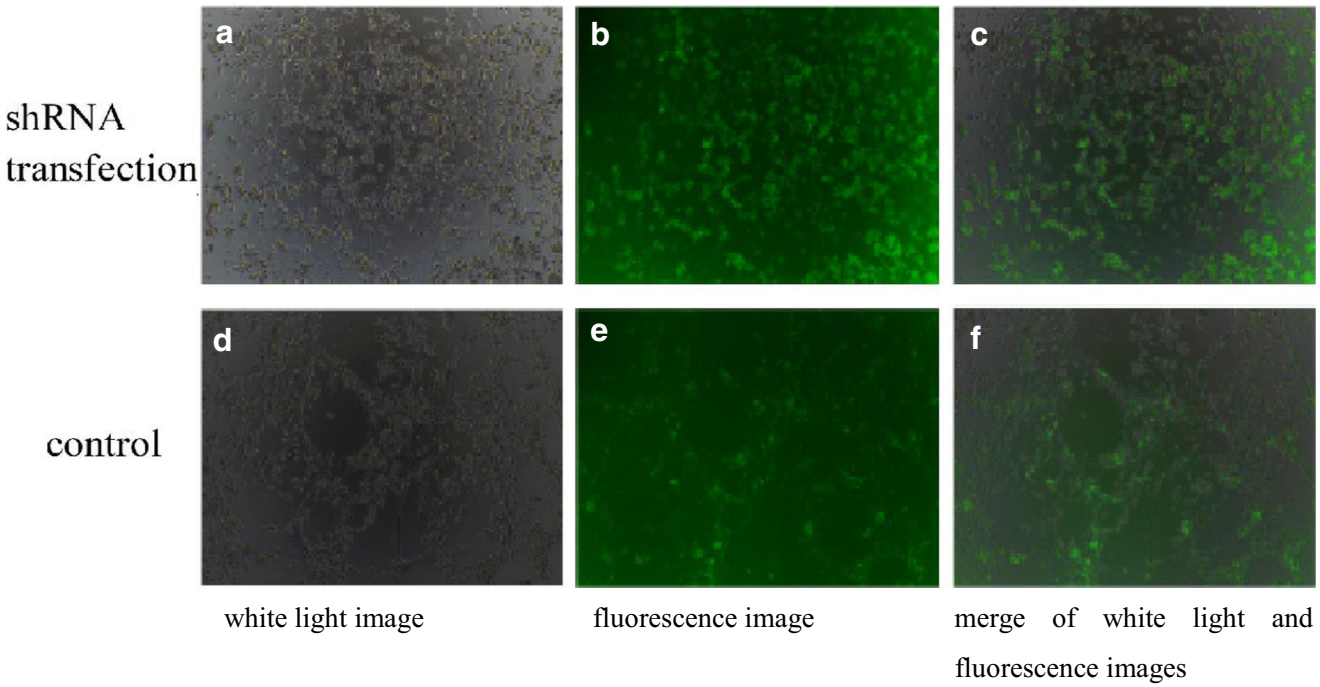


Fig. 12 Results of siRNA transfection. Images of cells shRNA-transfected cells captured with (**a**) white light, (**b**) fluorescence, and (**c**) merged signals. Images of the control group captured with (**d**) white light, (**e**) fluorescence, and (**f**) merged signals

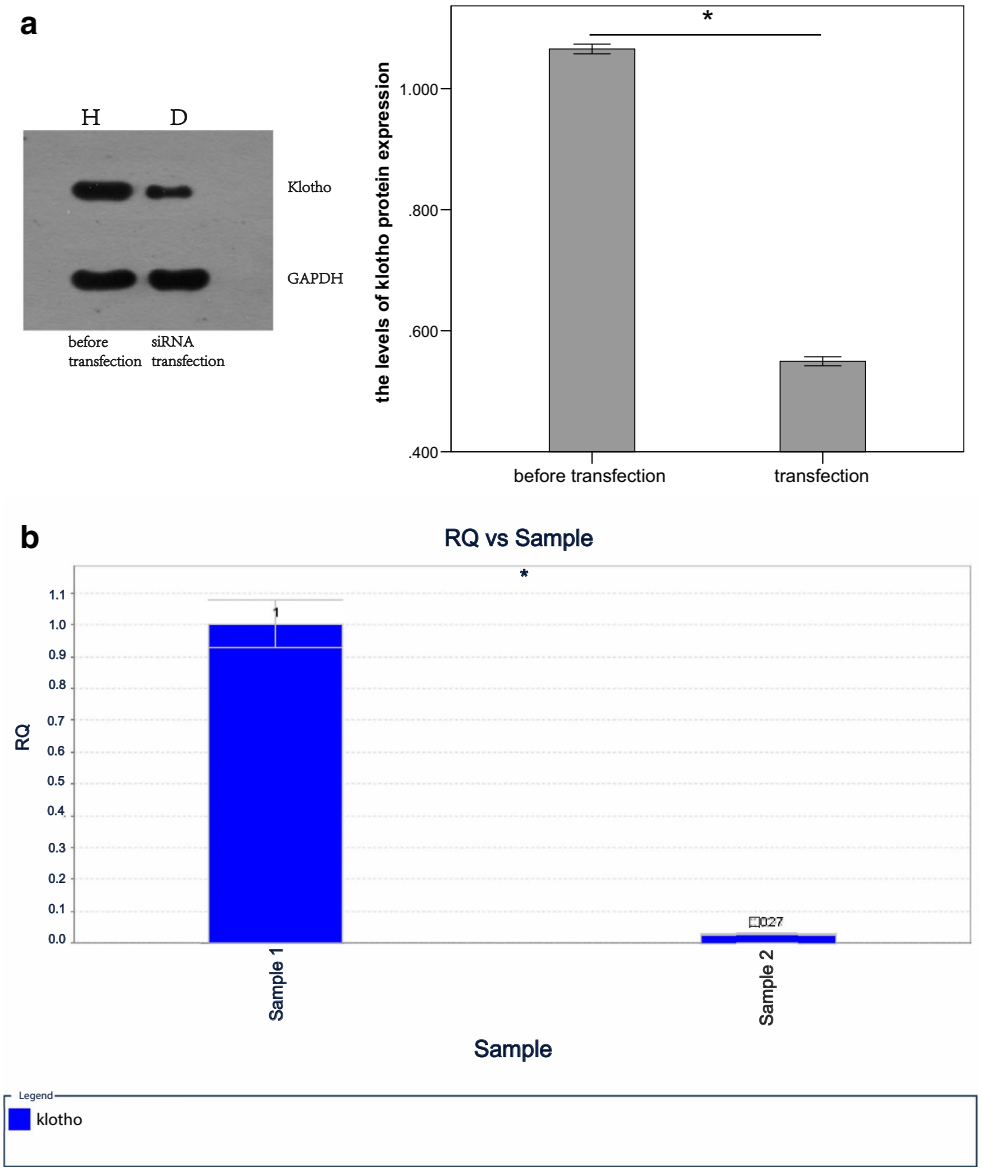


Fig. 13 Western blot and qPCR verified the effect of the siRNA on transfected cells

mortality rate [1, 12]. The pathogenesis of DN has mainly been attributed to be the glomerular theory, but the traditional glomerular centre theory does not explain the aetiology of all patients. On the one hand, only a portion of patients with diabetes complicated with microalbuminuria progress to clinical nephropathy, while the disease is stable or reversed in a

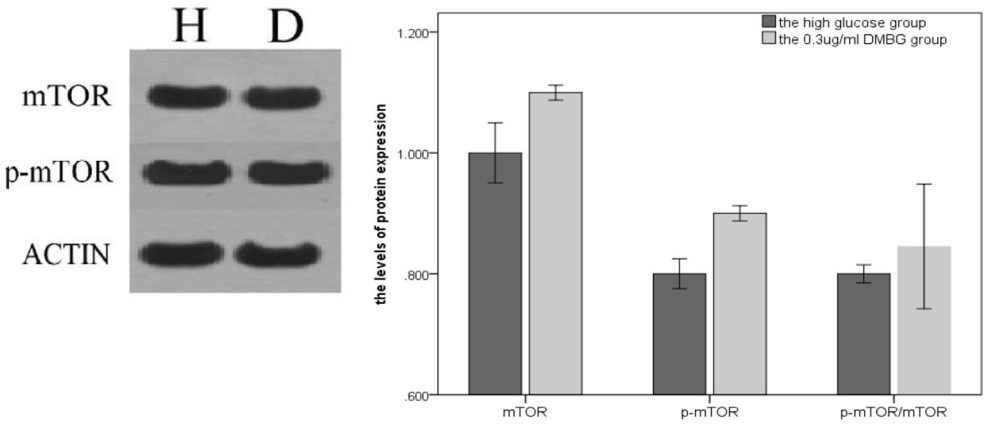


Fig. 14 Relative levels of the mTOR and p-mTOR proteins in the high glucose group and DMBG group after siRNA-mediated interference (the left band represents the high glucose group and the right band represents the high glucose+ 0.3 μg/ml DMBG group)

considerable portion of patients. On the other hand, the proportion of patients with type 2 diabetes mellitus and microalbuminuria presenting severe renal tubular lesions is higher than the proportion presenting typical glomerular structural changes, suggesting that renal tubular injury is also an important pathological change associated with DN. Based on the results from related studies, renal tubular injury may be the driving force of DN development and progression. In patients with DN, renal tubule hypertrophy and hypoxia, bulb feedback abnormalities, and damage to renal tubule absorption function lead to proteinuria. Subsequently, renal tubules release chemotactic and inflammatory factors that promote renal interstitial injury, renal tubule interstitial differentiation promotes renal interstitial fibrosis, and renal tubular epithelial cell injury negatively affects the glomeruli [13]. Compared with the "glomerular centre theory", the "tubular centre theory" may better explain the occurrence and development of DN. Renal tubular epithelial cells play an important role in the progression of DN and the process of renal fibrosis, which is expected to be a new target for DN prevention and treatment. DN not only reduces renal tubular injury caused by hypoglycaemia, high blood pressure, hyperlipidaemia and proteinuria but also reduces renal fibrosis and renal inflammation through anti-fibrosis and anti-inflammatory effects. Klotho, which is located in renal tubular epithelial cells, is expected to be a new target for DN treatment. In this experiment, levels of the klotho protein were increased in the blood, urine and kidney tissues of diabetic mice after treatment with DMBG, and DMBG increased the levels of the klotho protein and the survival rate of MDCK cells stimulated with high glucose stimulation, suggesting that DMBG increased klotho expression to protect kidney function.

Hemodynamic disorders and various metabolic pathways have recently been shown to be involved in the pathogenesis of DN [14]. Excess glucose stimulation activates cell signalling pathways, including the mTOR pathway, diacylglycerol (DAG)-protein kinase C (PKC) pathway, advanced glycosylation end products (AGE), polyol pathway, hexose amine pathways and oxidative stress, and these pathways interact to activate inflammation, leading to the development of diabetic glomerulus sclerosis [15, 16]. The mTOR protein is a highly conserved serine/threonine protein kinase that plays a role in regulating cell functions by forming mTORC1 and mTORC2. The mTORC1 signalling pathway is involved in biosynthesis, autophagy, and immune-mediated processes. Notably, the mTORC1 protein complex includes mTOR, mTOR protein involved in regulating Raptor, mammalian homologues of the LST8 mLST8 proteins, a proline-rich AKT substrate containing 1-PRAS40 and DEP structure domain mTOR DEPTOR interacting proteins; its main functions are to promote cell biosynthesis, cell growth, proliferation, the absorption of nutrients, inhibit cell autophagy etc. The complex is activated by rapamycin, insulin, growth factors and other regulatory factors [17]. The lack of the klotho gene in klotho mutant mice exacerbates STZ-induced renal hypertrophy and increases the phosphorylation of mTOR and S6 [11], suggesting that the lack of klotho gene activates mTOR signalling pathway and aggravates the damage in animals with early DN. The results of this that study showed that DMBG intervention increased the levels of the klotho protein in the blood, urine and kidney tissues of mice with DN, and reduced the levels of the mTOR and p-mTOR proteins in kidney tissues, suggesting that the renoprotective effect of DMBG may be related to the klotho protein and mTOR pathway. In addition, high glucose decreased the levels of the klotho protein in MDCK cells and increased the levels of the mTOR and p-mTOR protein over time, suggesting that high glucose stimulation activated the mTOR pathway in MDCK cells through a mechanism dependent on the expression of the klotho protein. The siRNA inhibited the expression of the klotho protein and weakened the effect of DMBG on the mTOR pathway, suggesting that DMBG regulated the activation of the mTOR pathway through klotho to protect renal tubular cells, thereby delaying the progression of renal dysfunction in patients with diabetes. Further studies are needed to determine whether DMBG increases the levels of the klotho protein through other pathways in addition to the mTOR pathway.

Conclusions

DMBG slows the progression of increased urinary protein and serum creatinine levels in mice with DM, increases the levels of the klotho protein in serum, urine and kidney tissues, and reduces the levels of the mTOR and p-mTOR proteins in kidney tissues to protect renal function. High glucose decreases the level of the klotho protein in MDCK cells, activates the mTOR signalling pathway, and eventually damages MDCK cells. DMBG partially reversed the high glucose-induced damage to MDCK cells, and the underlying mechanism may be related to the increase in the level of the klotho protein and the inhibition of the mTOR pathway. These results identify a new target for DMBG in the treatment of DN.

Acknowledgements This study was partially supported by the Youth Foundation of the Commission of Health and Family Planning of Wuxi under grant Q201654.

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest with the contents of this paper to declare.

Human studies This article does not contain any studies with human participants performed by any of the authors.

References

1. Wan, Q., Xu, Y., and Dong, E., Diabetic nephropathy research in China: Data analysis and review from the National Natural Science Foundation of China. Journal of Diabetes 7:307–314, 2015.
2. Wang, L., Gao, P., Zhang, M. et al., Prevalence and ethnic pattern of diabetes and prediabetes in China in 2013. JAMA 317:2515–2523, 2017.
3. Alicic, R. Z., Rooney, M. T., and Tuttle, K. R., Diabetic kidney disease: Challenges, progress, and possibilities. Clinical Journal of the American Society of Nephrology 12:2032–2045, 2017.
4. Allison, S. J., and disease, C. k., Study warns of burgeoning health burden of CKD in China. Nature Reviews Nephrology 8:251, 2012.
5. De Boer, I. H., Afkarian, M., and Tuttle, K. R., The surging tide of diabetes: Implications for nephrology. American Journal of Kidney Diseases 67:364–366, 2016.
6. Kuro-o, M., Matsumura, Y., Aizawa, H. et al., Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390:45–51, 1997.
7. Nabeshima, Y. I., Toward a better understanding of klotho. Science of Aging Knowledge Environment 2006:pe11, 2006.
8. Kurosu, H., Yamamoto, M., Clark, J. D. et al., Suppression of aging in mice by the hormone Klotho. Science 309:1829–1833, 2005.
9. Xu, Y., Peng, H., and Ke, B., α-klotho and anemia in patients with chronic kidney disease patients: A new perspective. Experimental and Therapeutic Medicine 14:5691–5695, 2017.
10. Zou, D., Wu, W., He, Y. et al., The role of klotho in chronic kidney disease. BMC Nephrology 19:285, 2018.
11. Lin, Y., Kuro-o, M., and Sun, Z., Genetic deficiency of anti-aging gene Klotho exacerbates early nephropathy in STZ-induced diabetes in male mice. Endocrinology 154:3855–3863, 2013.
12. Ahmad, J., Management of diabetic nephropathy: Recent progress and future perspective. Diabetes & Metabolic Syndrome: Clinical Research & Reviews 9:343–358, 2015.
13. Ina, K., Kitamura, H., Nagai, K. et al., Ultrastructural and functional changes of the proximal tubular epithelial cells in the renal cortex from spontaneously diabetic KKAy mice. Microscopy 48:443–448, 1999.
14. Thomas, M. C., Brownlee, M., Susztak, K. et al., Diabetic kidney disease. Nature Reviews Disease Primers 1:15018, 2015.
15. Shahbazian, H., and Rezaii, I., Diabetic kidney disease; review of the current knowledge. Journal of Renal Injury Prevention 2:73–80, 2013.
16. Alicic, R. Z., Johnson, E. J., and Tuttle, K. R., Inflammatory mechanisms as new biomarkers and therapeutic targets for diabetic kidney disease. Advances in Chronic Kidney Disease 25:181–191, 2018.
17. Bhattacharjee, N., Barma, S., Konwar, N. et al., Mechanistic insight of diabetic nephropathy and its pharmacotherapeutic targets: An update. European Journal of Pharmacology 791:8–24, 2016.