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ORIGINAL ARTICLE

METTL3/YTHDF2 m6A axis mediates the progression of diabetic nephropathy through epigenetically suppressing PINK1 and mitophagy

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ABSTRACT

Aims: This research aimed to investigate the specific mechanism of methyltransferase like 3 (METTL3) in the progression of diabetic kidney disease (DKD).

Materials and Methods: The model of diabetic kidney disease was established with HK-2 cells and mice *in vitro* and *in vivo*. The N6 methyladenosine (m6A) contents in the cells and tissues were detected with a commercial kit and the m6A levels of PTEN induced putative kinase 1 (PINK2) were detected with a MeRIP kit. The mRNA and protein levels were determined with RT-qPCR and western blot. The ROS, TNF-α, and IL-6 levels were assessed with ELISA. The cell proliferative ability was measured by a CCK-8 assay and cell apoptosis was determined with TUNEL staining. The HE and Masson staining was performed to observe the renal morphology. The RIP assay was conducted to detect the interaction between METTL3/YTHDF2 and PINK1.

Results: The m6A content and METTL3 levels were prominently elevated in diabetic kidney disease. METTL3 silencing promoted the cell growth and the expression of LC3 II, PINK1, and Parkin, while inhibiting the cell apoptosis and the expression of LC3 I and p62 in the high glucose (HG) stimulated HK-2 cells. METTL3 silencing also decreased the ROS, TNF-α, and IL-6 levels in diabetic kidney disease. PINK1 silencing neutralized the function of sh-METTL3 in the HG stimulated HK-2 cells. The HE and Masson staining showed that METTL3 silencing alleviated the kidney injury induced by DKD. METTL3 silencing decreased the m6A levels of PINK1, while increased the mRNA levels of PINK1 which depended on YTHDF2.

Conclusions: METTL3 silencing could inhibit the progression of diabetic nephropathy *in vivo* and *in vitro* by regulating the m6A modification of PINK1, which depends on YTHDF2. Our research lays the theoretical foundation for the precise treatment of diabetic kidney disease and the development of targeted drugs in the future.

INTRODUCTION

Diabetic kidney disease (DKD) is a microvascular complication of diabetes[1]. DKD is characterized by the increased formation of extracellular matrix and the disappearance of foot process, which eventually leads to increased urinary protein and a decreased estimated glomerular filtration rate[2]. Previous studies have shown that DKD is the result of the interaction of hemodynamic disorders and metabolic abnormalities[3]. However, increasing evidence has determined that the local inflammatory state of the kidney leads to the occurrence and progression of DKD[4]. In patients with DKD, the driving effect of hyperglycemia, an increase of toxic metabolites (such as advanced glycation), and the vascular mechanical changes caused by disturbance of hemodynamics will

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lead to an inflammatory response *in vivo*, which further affects the proliferation, hypertrophy, aging, and apoptosis of renal cells[5–8].

In recent years, epigenetics, a branch of genetics, has developed rapidly. Epigenetics mainly regulates gene expression through gene modification, without changing the nucleotide sequence of the gene[9]. RNA modification widely affects the structure, function, and stability of RNA, which plays a critical role in the regulation of various cell process[10]. Among all RNA modifications, N6 methyladenosine (m6A) is the most common modification form in eukaryotic mRNA, which is also one of the most studied RNA modifications at present[11]. Methyltransferase like 3 (METTL3) is a central enzyme in the process of methylation modification, which was confirmed to be closely related to the progression of many diseases[12]. Additionally, abnormal METTL3 levels have been demonstrated to participate in the regulation of inflammation and cell death, which are critical mechanisms in the malignant development of DKD[13]. However, whether METTL3 modulates the development of DKD and its potential mechanism are largely unknown.

PTEN induced putative kinase 1 (PINK1) and Parkin protein constitute the autophagy pathway and are key regulatory proteins of mitophagy[14]. After oxidative stress is activated, the mitochondrial membrane potential will be changed. Meanwhile, activated Parkin is recruited to the outer membrane of damaged mitochondria to participate in the autophagy of damaged mitochondria[15]. PINK1, as an important molecular detector for sensing changes in the damaged mitochondrial membrane potential, acts on upstream of Parkin and regulates its translocation activity[16]. A recent study reported that PINK1/Parkin mediated mitophagy might be a potential therapeutic strategy for DKD[17]. However, the role of PINK1 mediated mitophagy in the progression of DKD remains to be elucidated.

Therefore, this study was carried out to explore the effect and mechanism of METTL3 in the development of DKD. We hypothesized that METTL3 is involved in the progression of DKD by regulating the PINK1/Parkin axis mediated mitophagy.

MATERIALS AND METHODS

Sample collection

This research was approved by the Ethics Committee of the School of Basic Medical Sciences, Jiamusi University (No. JMSU-248). The blood samples of 20 patients with diabetic kidney disease (five cases in stage I, three cases in stage II, five cases in stage III, three cases in stage IV, and four cases in stage V) were collected from the School of Basic Medical Sciences, Jiamusi University. Additionally, 20 diabetes patients were recruited as controls. All the patients signed the informed consent.

Cell culture and treatment

Human renal tubular epithelial cells (HK-2) were provided by Shanghai Mingjin Biotechnology Co., Ltd (Shanghai, China). The cells were maintained in the endothelial culture medium supplemented with 5.6 mmol/L glucose and 10% fetal bovine serum (FBS; Gibco). In order to induce the DKD model, HK-2 cells were treated with 20 mmol/L D-glucose, named the high glucose (HG) group[18]. In addition, HK-2 cells were treated with 5.6 mmol/L D-glucose in the normal glucose (NG) group.

Cell transfection

The short hairpin RNA METTL3 (sh-METTL3), YTHDF2 (sh-YTHDF2), PINK1 (sh-PINK1), and controls (sh-nc), METTTL3 overexpression vector (oe-METTL3) and empty vector (oe-nc) were provided by GenePharma (Shanghai, China) and transfected into the HK-2 cells using Lipofectamine 3000 (Invitrogen, USA).

RT-qPCR

The total RNA was extracted with Trizol kits (Beyotime, Shanghai, China), and the total RNA was reverse transcribed into cDNA according to the instructions of the PrimeScript RT reagent kit (Takara, Japan). Synthetic cDNA was used as the template for the RT-qPCR reaction with a Fast SYBRGREEN PCR kit (Takara), and in a ABIPRISM 7300 RT-PCR system (Applied Biosystems). In this experiment, GAPDH was used as the internal control of genes. A relative quantification method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the relative levels of related genes.

RNA stability detection

For the determination of mRNA stability of PINK1, the cells were treated with actinomycin D for 2, 4, 6, 8 h. After treatment for different times, the mRNA levels of PINK1 were detected using RT-qPCR as mentioned above.

CCK-8 assay

A CCK8 detection kit (Shanghai Liji Biotechnology Co., Ltd, Shanghai, China) was used to detect the cell viability. 100 μL of cell suspension was seeded in a 96-well plate at a density of 5×10^3/well. At 24 h, 48 h, and 72 h after seeding, 10 μL of CCK8 solution was added to each well, and mixed gently without bubbles. After being incubated in a 5% CO_2 incubator at 37°C for 4 h, the absorbance of each well at 450 nm was measured with a microplate reader.

Transmission electron microscopy (TEM)

After washing with PBS three times, Karnovsky's fixative was added to the cells followed by fixing, infiltration, dewatering, and embedding. Subsequently, the cells were sectioned at 70–80 nm with an ultramicrotome. After staining with uranyl acetate and lead citrate, the sections were observed and photographed under a TEM (Zeiss, Germany).

Western blot

The cultured cells were collected and the supernatant was discarded. Then the cells were lysed with enhanced RIPA lysate (Beyotime, Shanghai, China), and the protein concentration

was determined with a BCA protein quantitative kit (Beyotime). Proteins were separated with 10% SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were blocked in 5% skimmed milk at room temperature for 1 h. Then the blots were treated with primary antibodies at 4°C overnight. On the next day, after washing, the membranes were treated with HRP labeled secondary antibody for 1 h. Thereafter, the membranes were visualized with ECL solution (Biomiga, USA) for 1 min at room temperature. ImageJ software was used to quantify the gray scale of each band. GAPDH was used as the internal control.

M6A dot blot assay

The total RNA was extracted from the blood, cells, and tissues by Trizol (Beyotime). The m6A content relative to the total mRNA level was measured by an EpiQuik M6A RNA methylation quantification kit (Colorimetric) (AmyJet Scientific, Wuhan, China) according to the instructions. Briefly, 4 μg of total RNA was purified using the RNeasy Mini Kit (Qiagen). Then the RNA was separated on 1.2% formaldehyde-agarose gels and transferred onto Hybond N + membranes (Amersham). Then the membranes were incubated with m6A antibody overnight and then treated with horseradish peroxidase conjugate anti-rabbit immunoglobulin G. Finally, after washing, the blots were developed on PhosphorImager screens and quantitated with ImageQuant.

MeRIP qPCR experiment

The Magna RIPTM RNA binding protein immunoprediction kit was purchased from Millipore (MA, USA) and the experiment was carried out according to the instructions. After the cells were treated with Rip lysate, anti-m6a antibody was used to incubate the cell lysate. Then the mixture was incubated with magnetic beads. After that, protease K was used to remove proteins. Finally, the RNA was purified and reverse transcribed into cDNA, and the expression of target genes was detected by RT-qPCR.

RNA-binding protein immunoprecipitation (RIP) experiment

The Rip Kit (Millipore, USA) was used to detect the binding of METTL3 to PINK1 protein. The HK-2 cells were washed twice with cold PBS and collected. The cells were then resuspended with RIPA lysate (Beyotime) and centrifuged (14,000 rpm, 4°C) for 10 min to collect the supernatant. The experimental procedures were as follows: 50 μL of magnetic beads was washed and then resuspended in 100 μL of RIP wash buffer, and then incubated with 5 μg of anti-METTL3 or IgG. The magnetic bead–antibody complexes were washed and resuspended in 900 μL RIP wash buffer, and then incubated with 100 μL cell extract at 4°C overnight. The sample was then placed on a magnetic holder to collect the magnetic bead–protein complexes. The complex pulled down were digested with proteinase K to extract RNA, and the expression level of PINK1 was detected by RT-qPCR as mentioned above.

Double luciferase reporter assay

The wild-type and mutant fragments of PINK1 were constructed and inserted into the pmirGLO reporter vector using endonuclease sites Spe I and Hind III, named as PINK1-WT and PINK1-MUT, respectively. These reporter plasmids along with sh-nc or sh-METTL3 were co-transfected into HK-2 cells using the LipofectamineTM3000 reagent. After 48 h, the cells were collected and fully lysed. The firefly luciferase activity was finally measured and normalized to the renin luciferase activity.

Animal experiment

Five week old male C57bl/6 mice (20 ± 2 g) were divided into the DKD model group and the control group. The fasting blood glucose of all the mice was measured before the experiment, and was required to be less than 7 mmol/L. The mice in the DKD model group were fed with a high-fat and high-sugar diet for 8 weeks. Then, 1 day after restoring the high-fat and high-sugar diet, the mice in the DKD model group were fasted for 16 h and injected with STZ at 50 mg/kg per day for 5 days. The fasting blood glucose was measured 2 weeks after the last injection. If the fasting blood glucose was greater than 16 mmol/L, the medium-term diabetes model had been established successfully. Then, the rat urine was collected and the ratio of urinary albumin to urinary muscle intoxication (AIb/Cr) was measured. The AIb/Cr was greater than 30 mg/g, indicating a diagnosis of DKD. The mice in the control group received standard chow and were injected with the same amount of normal saline. Subsequently, all DKD mice were randomly divided into the sh-NC group and the sh-METTL3 group. After establishment of the DKD model, the lentiviruses carrying sh-NC or sh-METTL3 (MOI = 50) were injected into the caudal vein at a dose of 1 μg/g according to the weight of mice, once a week for 8 weeks.

Sample collection

At the end of the study, the body weight (BW) of the mice in each group was recorded. Then, the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 mL/kg), and blood was collected from the apex of the heart. Urine protein was measured by the Automated Chemistry Analyzer (BK200, BIOBASE). After blood collection, the kidney weight (KW) was weighed by laparotomy and perfused with normal saline through the abdominal aorta. The kidney hypertrophy index (KHI) was obtained from the ratio of KW (mg) to BW (g).

HE and Masson staining

The kidneys of the mice were collected and fixed in 4% paraformaldehyde. After fixation, dehydration, and paraffin embedding, the paraffin sections were cut into 2 μm thick sections. The HE staining and Masson staining kits were purchased from Shanghai GEFAN Biotechnology Co., Ltd (Shanghai, China). The sections were stained according to the instructions of the kits. The pathological changes of renal glomerulus, basement membrane, and mesangial matrix were observed under the microscope.

Determination of ROS, TNF-α, and IL-6 levels

The ROS, TNF-α, and IL-6 levels in the kidneys of the mice and in the HK-2 cells were tested by corresponding enzyme linked immunosorbent (ELISA) kits purchased from Jiancheng Bio (Nanjing, China). All operations were carried out according to the operation instructions of the kits.

Statistical analysis

SPSS 22.0 software was applied for data analysis. The results were expressed as mean ± SD. The independent sample *t*-test was used for comparison between the two groups, and the one-way ANOVA was used for comparison between multiple groups; The m6A methylation sites of PINK1 were predicted using the SRAMP database (http://www.cuilab.cn/sramp). A value of $P < 0.05$ was statistically significant.

RESULTS

M6A content was elevated in DKD

In the cell and mouse model of diabetic kidney disease, it was found that the m6A content was prominently elevated in the HG stimulated HK-2 cells, and in the kidneys of the DKD mice (Figure 1a,b). In addition, the m6A level was detected in the serum of the patients with DKD. Interestingly, the m6A level in the serum of DKD patients was notably higher than that in the normal controls (Figure 1c). Then, we tried to determine which m6A related regulators lead to the abnormal m6A level of DKD. The results suggested that the METTL3, FTO, and METTL14 were prominently enhanced in the HG stimulated HK-2 cells (Figure 1d). Through further analysis in the DKD mice and patients, METTL3 was prominently elevated in the kidneys of DKD mice (Figure 1e) and in the serum of patients (Figure 1f). Thus, we selected METTL3 for further study.

METTL3 knockdown alleviated the injury in HK-2 cells induced by HG treatment

Then, we constructed shRNA targeting METTL3. The qPCR results confirmed that sh-METTL3 successfully inhibited the METTL3 expression in HK-2 cells (Figure 2a,b). The high glucose treatment significantly suppressed the cell proliferative ability of the HK-2 cells, while sh-METTL3 transfection significantly enhanced it (Figure 2c). Besides, the HG treatment dramatically elevated the ROS, TNF-α, and IL-6 (Figure 2d–f) levels in the HK-2 cells. Sh-METTL3 transfection significantly decreased them. Previous studies have confirmed that mitophagy plays a crucial role in the progression of DKD. Thus, after apoptosis detection, we further explored the mitophagy of the HK-2 cells in each group. Interestingly, TEM images of the HG-stimulated HK-2 cells demonstrated that mitochondria damage was induced, while METTL3 knockdown dramatically relieved the mitochondria damage (Figure 2g). We found that the protein expressions of LC3 II, PINK1, and Parkin was prominently attenuated, while LC3 I and p62 was prominently up-regulated in the HG-stimulated HK-2 cells. Again, sh-METTL3 transfection significantly reversed these effects of HG treatment (Figure 2h). These results indicated that oxidative stress, inflammation, and mitophagy might all be involved in the development of DKD.

METTL3 knockdown inhibited the progression of DKD *in vivo*

After sh-METTL3 treatment, the METTL3 levels were prominently decreased in the kidney tissues (Figure 3a). The 24 h urine protein level was prominently enhanced and sh-METTL3 treatment prominently decreased it in the DKD mice (Figure 3b). In addition, we found that in the kidney of the DKD mice, the BW was dramatically reduced and the KW and KHI were dramatically elevated. Sh-METTL3 treatment prominently increased the BW while decreased the KW and KHI in the kidneys of the DKD mice (Figure 3c–e). Additionally, the ROS (Figure 3f), TNF-α (Figure 3g), and IL-6 (Figure 3h) levels were prominently elevated in the kidney of the DKD mice, while sh-METTL3 treatment prominently decreased them. What is more, the HE and Masson staining results exhibited that in the control group the kidney tissue was stained evenly, the structure was clear, the morphology of glomerulus and kidney tubules was normal, the kidney tubular epithelial cells were in an orderly arrangement, and there was no inflammatory infiltration. However, in the DKD group, there were focal lesions in the kidney tubule renal interstitium, the lumen of the kidney tubules became larger, the vacuolization was obvious, the cytoplasmic staining became lighter, the glomerular volume became smaller, and the nuclei of the renal tubular epithelium became pyknotic. Interestingly, the above kidney lesions of DKD mice were relieved after sh-METTL3 treatment (Figure 3i,j).

METTL3 regulated m6A modification of PINK1

As the critical regulator of mitophagy, we wondered whether METTL3 exerts its role by regulating PINK1. As expected, after sh-METLL3 transfection, the mRNA levels of PINK1 were prominently elevated in the HK-2 cells, while the mRNA of Parkin levels showed no difference (Figure 4a). Additionally, after sh-METLL3 transfection, the protein levels of PINK1 and Parkin were prominently elevated in the HK-2 cells (Figure 4b). Furthermore, the RIP assay showed that the METTL3 antibody could enrich the PINK1 mRNA in the HK-2 cells (Figure 4c). After METTL3 knockdown, the m6A levels of PINK1 were prominently decreased (Figure 4d). Then, SRAMP, a sequence-based m6A modification site predictor, was applied to predict the potential theoretical binding sites of PINK1, and there is a site for m6A modification located at 1707–1711 bp (Figure 4e,f). Afterwards, whether the site can play the m6A modification function was verified by the base mutation of the binding site. The luciferase assay indicated that Sh-METTL3 prominently increased the luciferase activity of wt-PINK1 (Figure 4g). Besides, after METTL3 knockdown, the mature PINK1 levels were prominently elevated, while the precursor PINK1 levels showed no differences (Figure 4h). It indicated that the METTL3 mediated m6A modification of PINK1 might influence the stability and degradation of PINK1 RNA.

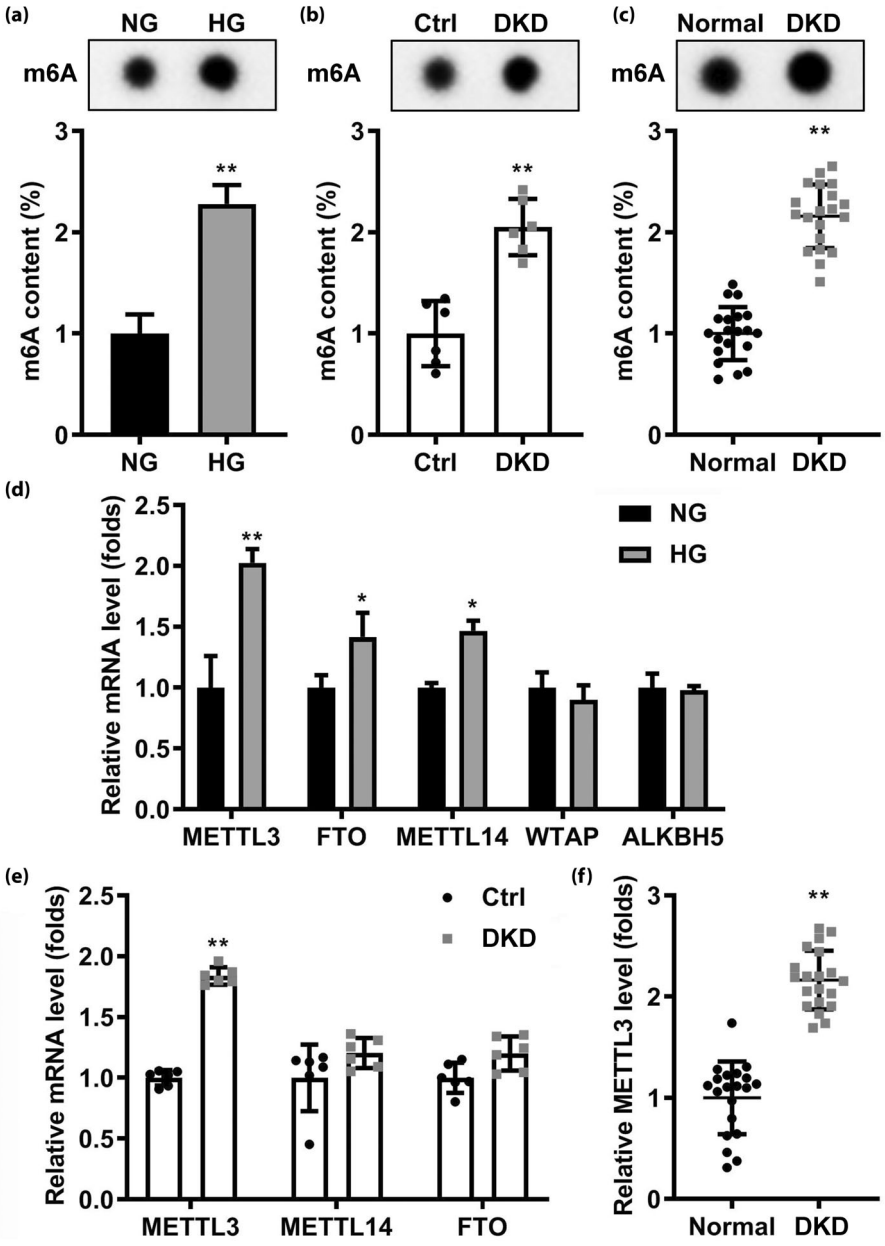

Figure 1 | The M6A content was elevated in DKD. The m6A content was measured in the HG stimulated HK-2 cells (a), kidneys of the DKD mice (b), and serum of DKD patients (c). (d) m6A methylase levels in the HG stimulated HK-2 cells were assessed by RT-qPCR. (e) METTL3, METTL14, and FTO levels in the DKD mice were assessed by RT-qPCR. (f) METTL3 levels in the DKD patients were assessed by RT-qPCR. *$P < 0.05$, **$P < 0.01$.

YTHDF2 degraded PINK1 mRNAs in an m6A dependent manner

The above findings demonstrated that METTL3 induced significant changes of the m6A content and mRNA expressions of PINK1. As we know, the m6A modification should be read by the 'readers' to exert its regulatory function. YTHDF2 was demonstrated to be a reader that plays a degradation role in m6A methylation modification[19]. The RIP assay showed that the YTHDF2 antibody specifically enriched the PINK1 mRNA in HK-2 cells (Figure 5a). After sh-YTHDF2 transfection, the YTHDF2 levels were prominently decreased while the PINK1 level was elevated (Figure 5b,c). Besides, we found that METTL3 overexpression prominently suppressed the mRNA expressions (Figure 5d) and stability (Figure 5e), and protein (Figure 5f) of PINK1 in HK-2 cells, while YTHDF2 knockdown elevated them.

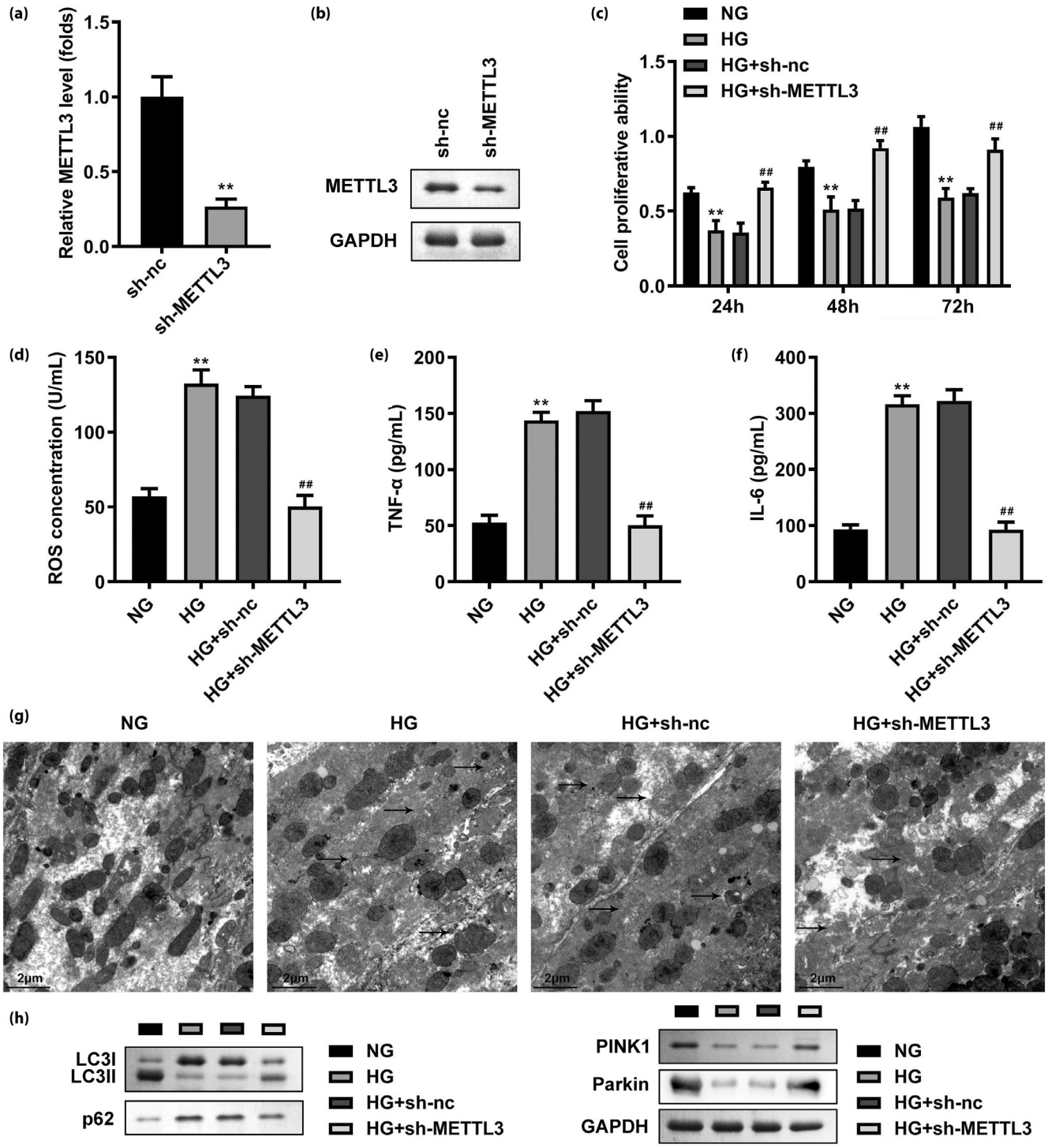

Figure 2 | METTL3 knockdown moderated the injury in the HK-2 cells induced by HG treatment. Transfection efficiency of sh-METTL3 was verified by RT-qPCR (a) and western blot (b) assays. (c) The cell proliferative ability was tested with CCK-8 assay. The ROS (d), TNF-α (e), and IL-6 (f) levels were analyzed with Elisa kits. (g) TEM was carried out to determine the mitochondria damage. (h) The LC3 I and II, p62, PINK1, and Parkin levels were measured by western blot. $^{**}P < 0.01$ *vs* NG group. $^{\#\#}P < 0.01$ *vs* HG + sh-nc group.

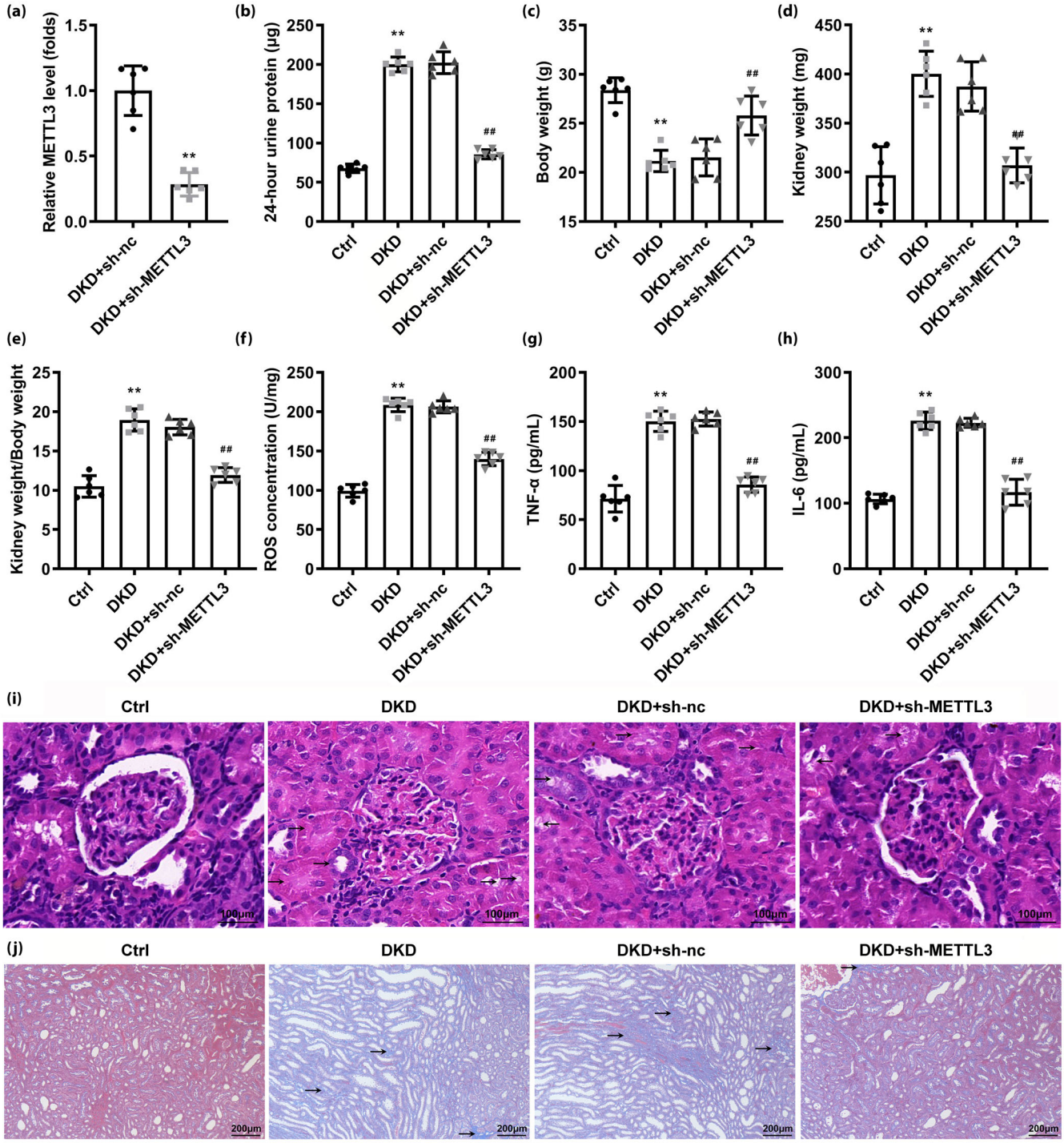

Figure 3 | METTL3 knockdown moderated the DKD progression *in vivo*. (a) The METTL3 levels in the kidney of the DKD mice were tested by RT-qPCR. The 24 h urine protein level (b), body weight (c), kidney weight (d), and the ratio of kidney weight to body weight (e). The ROS (f), TNF-α (g), and IL-6 (h) levels in the kidney of the DKD mice were analyzed with Elisa kits. The HE (i) and Masson (j) staining of the kidney. $^{**}P < 0.01$ *vs* NG group. $^{\#\#}P < 0.01$ *vs* HG + sh-nc group.

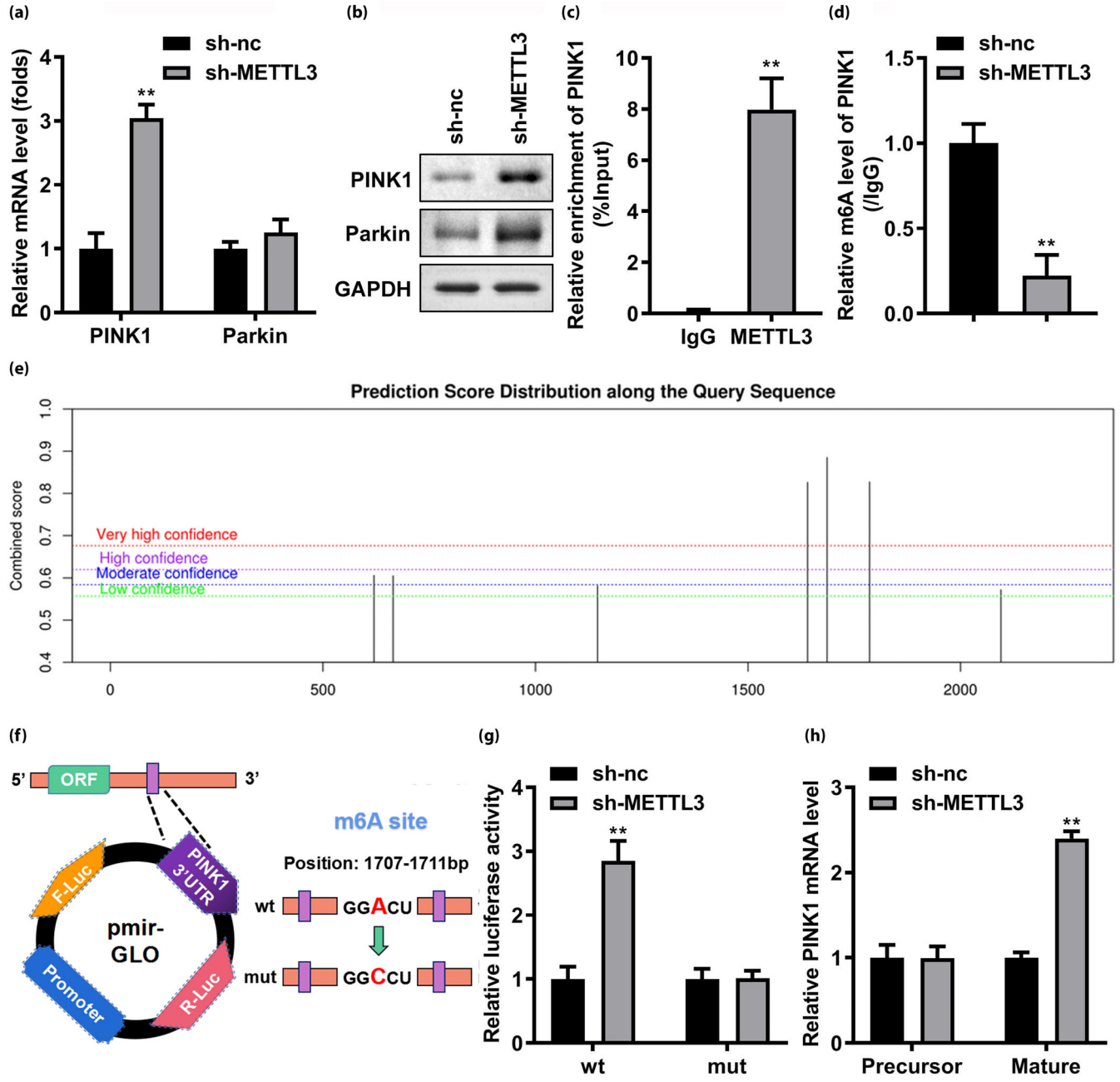

Figure 4 | METTL3 regulated m6A modification of PINK1. The PINK1 and Parkin levels were measured by RT-qPCR (a) and western blot (b) assays after METTL3 knockdown. (c) RIP assay was carried out to confirmed the PINK1 mRNA enrichment by METTL3. (d) The m6A levels of PINK1 were assessed by MeRIP assay after METTL3 knockdown. (e, f) The m6(A) methylation sites of PINK1 were predicted using the SRAMP database. (g) Luciferase assay was performed to verify the relationship between PINK1 and METTL3. (h) The mRNA levels of the precursor and mature PINK1 were assessed by RT-qPCR assay after METTL3 knockdown. $^{**}P < 0.01$.

PINK1 knockdown neutralized the function of sh-METTL3 in the HG stimulated HK-2 cells

Finally, to confirm the interaction between METTL3 and PINK1, the rescue experiments were conducted. We found that sh-PINK1 prominently down-regulated the mRNA expression of PINK1 in HK-2 cells (Figure 6a,b). PINK1 knockdown prominently decreased the cell proliferative ability (Figure 6c), elevated the ROS, TNF-α, and IL-6 (Figure 6d–f) levels and the mitochondria damage (Figure 6g) in the HG stimulated HK-2 cells treated with sh-METTL3. Additionally, after PINK1

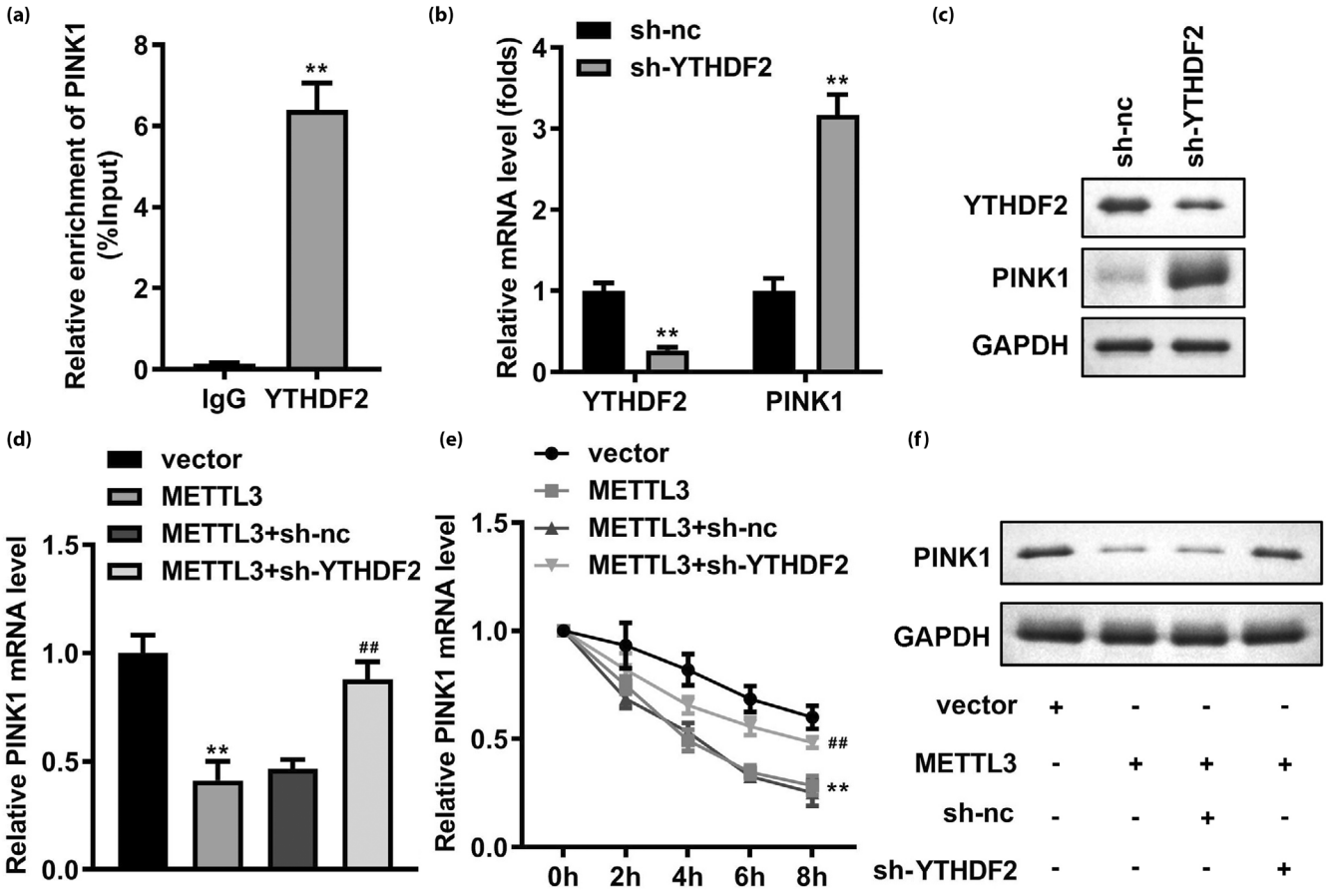

Figure 5 | YTHDF2 degraded PINK1 mRNAs in an m6A dependent manner. (a) RIP assay was carried out to confirm the PINK1 mRNA enrichment by YTHDF2. The levels of YTHDF2 and PINK1 were determined with RT-qPCR (b) and western blot assay (c) after YTHDF2 knockdown. The mRNA expressions (d) and stability (e), and protein levels (f) of PINK1 were detected by RT-qPCR and western blot assays after METTL3 and sh-YTHDF2 transfection. **$P < 0.01$ *vs* control group. ##$P < 0.01$ *vs* METTL3 + sh-nc group.

knockdown, the protein expressions of LC3 II, PINK1, and Parkin was prominently decreased, while LC3 I and p62 was prominently up-regulated in the HG-stimulated HK-2 cells transfected with sh-METTL3 (Figure 6h).

DISCUSSION

In the present research, it was demonstrated that METTL3 epigenetically inhibited the PINK1 expression in an m6A-YTHDF2-dependent manner. Additionally, we illustrated that METTL3 was prominently highly expressed in HK-2 cells treated by high glucose, in kidneys of DKD mice as well as in the serum of DKD patients. METTL3 silencing relieved the injury of HK-2 cells induced by HG treatment and repressed the kidney injury in the DKD mice. This research is the first to investigate the function of METTL3 on the PINK1/Parkin signaling pathway in the progression of DKD.

M6A methylation is confirmed to be the most common RNA modification in mammals and is involved in the RNA metabolism processes including RNA biosynthesis and decay[20,21]. Methylase METTL3, as an important m6A regulator, has been highlighted to participate in various diabetic complications, including microvascular complication[22], diabetic cataract[23], diabetic foot ulcers[24]. Due to the different modification sites and m6A binding readers, the function of m6A modification is complex. For instance, Xu *et al.*[25] found that methylase METTL14 levels were prominently depleted in the HK-2 cells after HG treatment, which would further inhibit the histone deacetylase 5 and TGF-β1 expression levels. Eventually affecting the EMT of renal tubular cells in diabetic kidney disease. However, Li *et al.*[26] found that METTL14 levels were dramatically up-regulated in the kidneys of DKD, which was further demonstrated to elevate the ROS, TNF-α, and IL-6 levels and decreased the α-klotho levels depending on the m6A levels. Here, we confirmed that METTL3 was highly expressed in DKD. METTL3 silencing promoted the cell growth while inhibiting the cell death and inflammatory reaction *in vivo* and *in vitro*. Our results were similar to the previous report of Jiang *et al.*[14], who also confirmed that METTL3 silencing effectively relieved the podocyte injury and

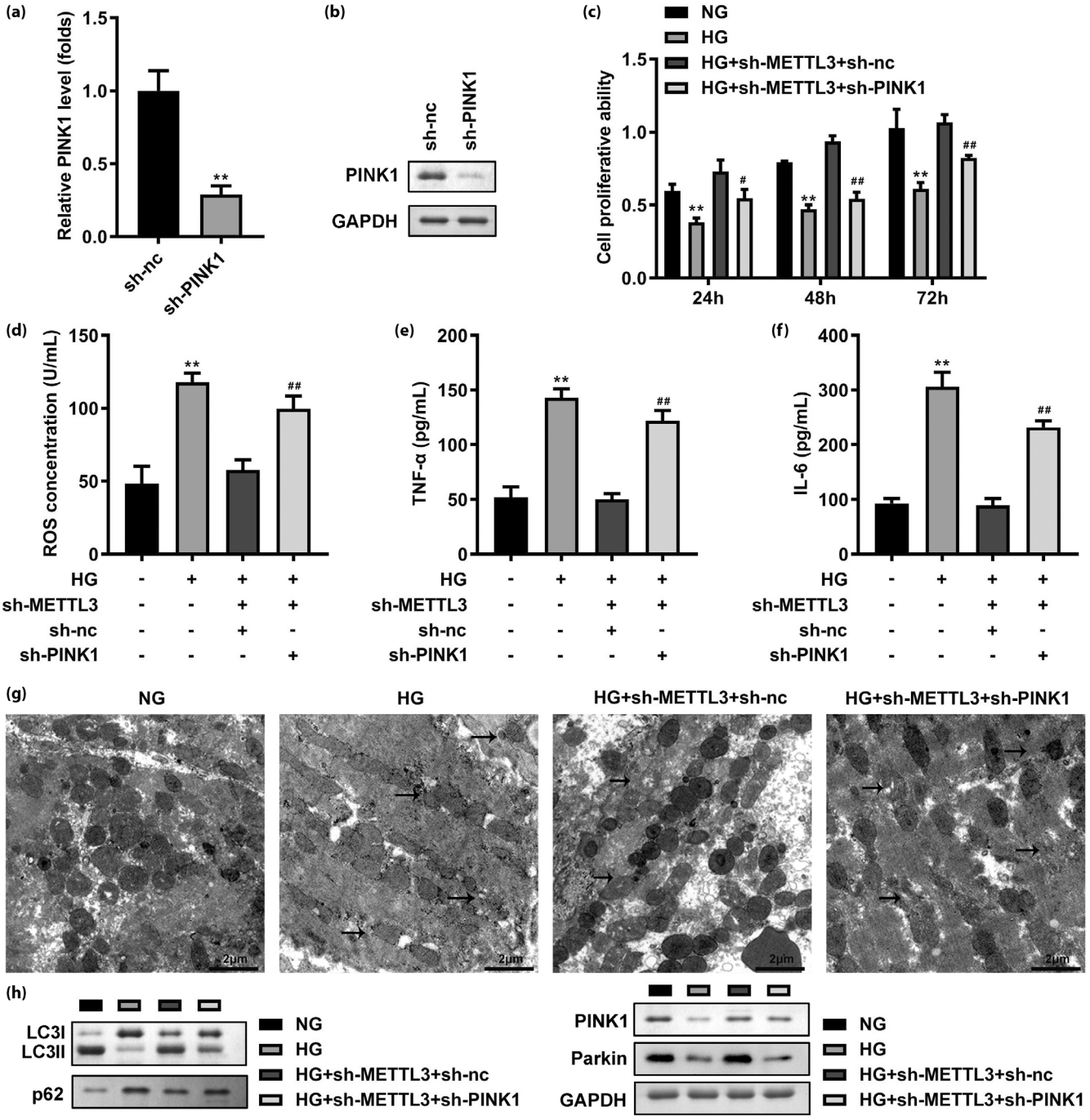

Figure 6 | PINK1 knockdown neutralized the role of sh-METTL3 in the HG stimulated HK-2 cells. Transfection efficiency of sh-PINK1 was verified by RT-qPCR (a) and western blot (b) assays. (c) The cell proliferative ability was tested with CCK-8 assay. The ROS (d), TNF-α (e), and IL-6 (f) levels were analyzed with Elisa kits. (g) TUNEL staining was carried out to determine the mitochondria damage. (h) The LC3 I and II, p62, PINK1, and Parkin levels were measured by western blot. $^{**}P < 0.01$ *vs* control group. $^{\#\#}P < 0.01$ *vs* HG + sh-METTL3 + sh-nc group.

albuminuria in DKD mice. All these results implied that METTL3-mediated m6A modification might be a critical factor in the progression of DKD. We found that METTL3 was dysregulated in the serum of DKD patients. But we did not evaluate whether METTL3 could be a biomarker for the diagnosis of DKD. It will be studied in our further research.

Autophagy is a protective mechanism for maintaining cellular homeostasis, and impairment of autophagy can aggravate

renal cell dysfunction and apoptosis[27]. In the microenvironment of diabetes, a deficiency of autophagy can induce pathological changes of different renal cells and further promote the progression of renal disease[28,29]. Mitochondrial autophagy, termed mitophagy, was discovered to reduce the release of ROS, promote apoptotic substances, and reduce apoptosis[30]. Recently, mitophagy mediated by PINK1/Parkin signaling pathway has been proved to participate in various diseases and is the main pathway for the removal of damaged mitochondria[31,32]. In this study, the western blot results revealed that in the HG-stimulated HK-2 cells, the ratio of LC3-II/LC3-I was prominently down-regulated, while p62 was up-regulated. METTL3 silencing reversed these effects of HG treatment. Additionally, we found that the PINK1/Parkin signaling pathway was inhibited in the HG-stimulated HK-2 cells. After METTL3 silencing, it was activated. Therefore, we speculate whether METTL3 regulates the signaling pathway by regulating the m6A methylation modification of PINK1.

Interestingly, through the RIP and double luciferase report assay, we confirmed the interaction between METTL3 and PINK1. And METTL3 silencing prominently decreased the m6A levels of PINK1 and increased the production of PINK1 mature mRNA levels. Furthermore, PINK1 silencing neutralized the functions of sh-METTL3 in the HG-stimulated HK-2 cells. These results indicated that METTL3 participated in the progression of DKD through modulating the PINK1/Parkin signaling pathway.

Studies have shown that in the process of m6A methylation modification, different m6A readers determine the different destinies of mRNA. Then m6A readers recognize and bind m6A modification sites to induce the degradation of the target RNA or make them more stable. As previous research reported, the METTL3/YTHDF2 m6A axis degraded the SETD7 and KLF4 levels in bladder cancer progression, which led to malignant behavior of tumor cells[33]. Additionally, Zhou *et al.*[34] demonstrated that METTL3 inhibited the YPEL5 expression in colorectal cancer through the recognition of METTL3 mediated m6A modification sites in the YPEL5 by YTHDF2. In this study, we found that YTHDF2, as a m6A reader, promotes the degradation of PINK1. YTHDF2 silencing neutralized the effects of METTL3 overexpression on the mRNA expression and stability of PINK1. The RIP assay also further confirmed the combination relationship between YTHDF2 and PINK1.

However, there were still some limitations in this study. Our study is basic research, and there is no in-depth exploration of the role of METTL3 mediated m6A modification in the clinical treatment of DKD patients. In addition, our research focuses on human renal tubular epithelial cells, and more research is needed to demonstrate whether this molecular mechanism also exists in DKD models of other cells.

To sum up, our study determined that METTL3 mediates the progression of DKD through regulating the apoptosis and mitophagy of renal tubular epithelial cells. Mechanistically, METTL3 regulates the m6A modification and expression of PINK1 dependent on YTHDF2. Our research lays a theoretical foundation for the precise treatment of DKD and the development of targeted drugs in the future.

DISCLOSURE

The authors confirm that no conflicts of interest exist in this work.

Approval of the research protocol: The study was approved by the School of Basic Medical Sciences, Jiamusi University.

Informed consent: N/A.

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Animal studies: This research was approved by the Ethics Committee of the School of Basic Medical Sciences, Jiamusi University.

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DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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