The Role of Transcription Factor Ap1 in The Activation of Nrf2/ARE Pathway Through TET1 in Diabetic Nephropathy

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Research article

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Abstract

Background: TET1 abnormal expression is related to tumorigenesis, but its role in diabetic nephropathy (DN), the most common diabetic complication, is unclear. We attempted to probe the possible mechanism of TET1 in DN.

Methods: The DN rat model was established and verified by marker detection and histopathological observation. The *in vitro* model was established in human mesangial cells (HMCs) induced by high glucose (HG), and levels of IL-6, TNF- α , IV-C and FN were examined. The differentially expressed mRNAs were screened out by microarray analysis. The most differentially expressed mRNA (TET1) was overexpressed in DN rats and HMCs to evaluate inflammation, cell viability and apoptosis, biochemical indexes and renal injury. The upstream transcription factor of TET1 was verified, and overexpressed with/without TET1 to access its role in inflammation and renal injury. The downstream gene and pathway were also verified.

Results: TET1 was poorly expressed in DN rats and HG-HMCs. High expression of TET1 decreased biochemical indexes, and renal injury of DN rats, decreased the activity, fibrosis and inflammation of HG-HMCs. Ap1 inhibited TET1 expression, and enhanced secretion of inflammatory factors in HG-HMCs and renal injury in DN rats. TET1 overexpression inhibited the effect of Ap1 on DN. TET1 promoted the transcription of Nrf2. The Ap1/Tet1 axis mediated the Nrf2/ARE pathway activity.

Conclusion: Ap1 inhibits TET1 expression and activates the Nrf2/ARE pathway in DN, thus aggravating inflammation and renal injury.

Background

Diabetic nephropathy (DN) is the most common diabetic complication, which often develops into end-stage renal disease [1]. DN is featured with thickening glomerular basement membranes, glomerular capillary damage, inflammation and oxidative stress [2]. Oxidative stress and activated inflammatory processes in diabetes mellitus activate fibrotic pathways, leading to DN [3]. Hyperfiltration is the early presentation of DN and a risk factor for DN clinically [4]. Although DN takes several years to develop, it is almost irreversible and still a major suffering for diabetic patients [5]. DN is also a risk factor for major vascular diseases, like stroke, atherosclerosis and hypertension [6]. Kidney-associated diseases including DN further complicates a timely diagnosis, thus demanding an early biomarker [1]. Since DN pathogenesis has not been fully comprehended, the current therapies are mainly based on the management of hyperglycemia and blood pressure [7]. Therefore, the urgency at present in the field of DN is to search for effective early biomarkers for DN diagnosis and to develop novel treatment approaches for DN patients.

Prior studies reveal the association of DNA methylation levels of genes and diabetes and DN [8, 9]. So, we suppose that DN may be related to demethylation of some genes. DNA methylation is an epigenetic event in gene expression known in cancers and chemotherapy resistance, which can be reversed by DNA

demethylases, ten-eleven translocation enzymes (TETs) [10]. TET1 activities affect the DNA damage signaling, cell cycle and cell death [11]. Since the unveiling of TET1 in DNA demethylation, the study on TET proteins has attracted extensive attention. In mammary tumors, TET1 is documented as a tumor suppressor, and its reduction stimulates breast cancer progression and metastasis [12]. TET1 has the ability to eliminate DNA methylation and plays key roles in the transcriptional regulation of target genes [13]. However, the underlying role of TET1 in DN has been less studied. Previous findings revealed the TET1-mediated transcriptional regulation in prostate cancer, and offered evidence for the mechanism of hypomethylation and abnormal transcriptional level of some genes in cancers [14]. Based on the above information, we hypothesize that TET1 incorporates with some transcription factors to participate in the development of DN.

Methods

Animal treatment and grouping

Sprague Dawley male rats (175-225 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). After one week of adaptive feeding, the rats were randomly divided into normal control (NC) group (normal saline + normal diet group, n = 6) and DN group (high glucose (HG) and high fat diet group, n = 60). Rats in the DN group were intraperitoneally injected with streptozotocin (STZ, 0.1 mol/L citric acid buffer, 30 mg/kg). After 72 hours, the blood glucose concentration was measured by a glucometer (Roche Diagnostics, Indianapolis, IN, USA). When the blood glucose level was equal to or higher than 16.7 mmol/L for three consecutive times, the diabetes model was established successfully (if the blood glucose level of rats was lower than 16.7 mmol/L, STZ was injected repeatedly, and the amount of each injection was 20 mg/kg). The successful DN model was randomly assigned into 10 groups, 6 rats in each group: activator protein 1 (Ap1)-NC group, Ap1-overexpression (OE) group, TET1-NC group, TET1-OE group, Nrf2-NC group, si-Nrf2 group, Ap1-OE + TET1-NC group, Ap1-OE + TET1-OE group, TET1-OE + Nrf2-nc group, TET1-OE + si-Nrf2 group. Except for the NC group with normal diet, rats in the other groups were all fed with high fat diet, with free diet during the whole experimental period, and all rats were measured for weight and blood glucose at a fixed time every week. The following analysis was conducted 10 weeks later.

Biochemical analysis

At the end of the 10th week, the rats in each group were placed in the metabolic cages, and urine within 24 hours was collected from 6:00 am to 6:00 am of the next day. The concentration of microprotein in 24-hour urine was detected by using UAE assay kits (Nanjing Jing Bioengineering Institute, China) and colorimetry. One mL of tail vein blood was taken for measurement of serum creatinine (Src) and blood urea nitrogen (BUN) by Hitachi 7180 automatic biochemical analyzer (Hitachi, Japan).

Periodic acid-schiff (PAS) staining

After fasting for 12 hours, rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (90 mg/kg). The left kidney of rats was dissected, and the renal vein was washed with precooled normal saline. After the left kidney became white, it was taken out and fixed for 2 hours with 4% paraformaldehyde. The renal tissue was embedded in paraffin and sliced at 4 μ m. PAS kit (BaSO diagnostics Inc., China) was applied to evaluate the renal pathological changes. The proliferation of mesangial cells and glomerular basement membrane were observed under the microscope (BX51, Olympus, Tokyo, Japan). Then the rats were euthanized by intraperitoneal injection of 1% pentobarbital sodium (150 mg/kg).

Cell culture and treatment

Human mesangial cells (HMCs) were purchased from Cell Bank of Chinese Academy of Sciences (catalog number TCHu 104, Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher, USA) containing 10% fetal bovine serum (Australian Biosearch, Australia) and 1% antibiotic (Thermo Fisher) at 37°C with 5% CO₂, followed by Hg treatment. HMCs were cultured in 25 mM D-glucose (Amresco, Solon, OH, USA) for 48 hours and used for subsequent analysis.

Enzyme-linked immunosorbent assay (ELISA)

After 48 hours of transfection, the cells were seeded into 24-well microplates with 1×10^6 cells/well at 37°C with 5% CO₂ for 24 hours. After centrifugation at 1800 g for 1 minute, the supernatant was collected. Rat IL-6 ELISA Kit (ab100772, Abcam) and rat TNF- α ELISA Kit (ab100785, Abcam) were used to detect the expression of TNF- α and IL-6 in the supernatant. The antibody concentration was adjusted to 10 μ g/mL and added to the microplates overnight at 4°C. Then cells were supplemented with 0.2 mL diluted plasma for 1 hour at 37°C, followed by 3 times of PBS washes. Afterwards, cells were treated with 0.2 mL enzyme-labeled antibody solution at 37°C for 1 hour, 0.2 mL substrate at 37°C for 30 minutes, and finally added with 0.05 mL H₂SO₄ to terminate the treatment. Optical density (OD) value was determined by a microplate reader (Thermo Fisher).

Western blot (WB)

Total protein of tissues and cells was extracted by RIPA lysis buffer, and the protein concentration was examined by a BCA protein quantitative kit (Beyotime Institute of Biotechnology). After denaturation by boiling, the protein samples (40 μ g per lane) were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (DuPont NEN, USA). After being sealed with 5% skimmed milk for 1 hour, the membranes were incubated for 16 hours at 4°C with anti-fibronectin (ab45688, 1:400, Abcam, UK), anti-collagen IV (bs1072, 1:400, BioWorld Technology Inc., USA), anti-TET1 (Abcam, 1:1000), anti-Ap1 (Abcam, 1:5000), and anti-Nrf2 (Abcam, 1:2000) antibodies, and then probed with goat anti-rabbit IgG antibody (ab205718, 1:5000) for 4 hours. Protein bands were soaked in enhanced chemiluminescence (Pierce Biotechnology, Bonn, Germany) and analyzed using ChemiDoc™ XRS imaging system (Bio-rad, Hercules, CA, USA).

Microarray analysis

The kidney tissues of NC rats and DN rats were homogenized in TRIzol, and then extracted with chloroform. After chloroform extraction and centrifugation, a small amount of aqueous phase (1.2 mL) was adjusted to 35% ethanol and added to RNeasy column. The RNA was eluted according to the RNeasy kit (Qiagen, CA), and the integrity of RNA was checked by electrophoresis, and the concentration was determined by an ultraviolet spectrophotometer. cDNA was synthesized by the Superscript[™] III reverse transcription (Invitrogen) and hybridized with Affymetrix Rat Genome 430 2.0 array (Affymetrix, CA). A 200 µL mixture containing 15 µg cRNA was loaded onto the chip. The chip was hybridized at 45°C for 16 hours and then put into Affymetrix GeneChip scanner for scanning analysis. The differential expression genes were screened and a heatmap was plotted according to |FoldChange| > 2 and P < 0.01.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA of tissue and HMCs was prepared by TRIzol reagent (Invitrogen). The Superscript[™] III reverse transcription (Invitrogen) was used for reverse transcription. The Universal SYBR Green Master kit (Roche, Tokyo, Japan) was utilized for RT-qPCR. The expression was calculated by the $2^{-\Delta\Delta CT}$ method. The forward primer of TET1 was TGAGAACTGTCCTTACGTGACC, and the reverse primer was AGAGACCAAGCGGCTC. The forward primer of Ap1 was 5'-AGGGTACTACAAGAGAC-3', and the reverse primer was 5'-TCAGGCGAGCGATAACC-3'. The forward primer of Nrf2 was 5'-GCACCGCATTTACACCAATG-3', and the reverse primer was 5'-TGCTTGCTGATCCACATCTG-3'. The forward primer of β -actin (loading control) was 5'-GATAAAGACCTACAGGG-3' and the reverse primer was 5'-CATCCGTCTCTATGCCAAC-3'.

Cell counting kit-8 (CCK-8) assay

Each well in the 96-well microplate was added with 100 µL media containing 10% FBS. Cells in logarithmic growth period were seeded into the microplate at 1×10^3 cells/well at 37°C with 5% CO₂ for 24 hours. CCK-8 kit (AbMole) was used to determine the cell activity. Each well was supplemented with 10 µL CCK8 solution at 37°C for 1 hour. The absorbance at 450 nm was measured by a microplate reader.

Flow cytometry

After 48 hours of transfection, cells were detached using 0.25% trypsin (excluding EDTA) (Shanghai Yubo, China) and collected into the flow tubes. After three times of cold PBS washes, the cells were treated with annexin V-FITC/PI retaining kit (BD Biosciences, USA). After that, 1×10^6 cells were resuspended in every 100 µL staining solution, and the cells were vibrated evenly. After incubation for 15 minutes, they were placed on FACScan flow cytometer (BD Biosciences) to detect apoptosis.

Chromatin immunoprecipitation (ChIP) assay

HMCs were cultured to 70-80% confluency, and fixed for 10 minutes by 4% paraformaldehyde to make the DNA and protein fixed and cross-linked. After cross-linking, they were randomly fragmented into pieces of

appropriate sizes using a sonicator, and centrifuged at 13000 rpm at 4°C for 5 minutes to collect supernatant. The supernatant was divided into two parts and incubated overnight at 4°C with rabbit anti-IgG (ab109489, 1:100, Abcam, Cambridge, UK) and target protein specific antibodies Ap1 (1:1000, ab21981, Abcam), TET1 (1:2000, ab220867, Abcam). The endogenous DNA protein complex was precipitated by protein agarose/Sepharose, and the nonspecific complex was washed. The DNA was de-crosslinked overnight at 65°C and purified by phenol/chloroform. The enrichment of Ap1 in TET1 promoter and the enrichment of TET1 in Nrf2 promoter were examined.

Statistical analysis

SPSS 22.0 statistical software (IBM Corp. Armonk, NY, USA) was used to process the data. All experiments were repeated 3 times independently. All the data were presented as mean $\pm$ standard deviation. The comparisons between two groups were done by the unpaired *t* test and the comparisons among multiple groups were conducted by the one-way or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A probability value of $P < 0.05$ indicated the difference was statistically significant.

Results

Establishment of DN rat model and cell model

To preliminarily understand the pathological process of DN in rats and whether the model has been successfully established, we examined the changes of body weight, blood glucose level, BUN, Scr and urinary albumin excretion (UAE) of rats in the NC and DN group. The weight of rats in DN group (Fig.1A), blood glucose (Fig.1B), BUN level (Fig.1C), Scr (Fig.1D), and UAE in 24 hours were increased (Fig.1E). The renal tissues of NC and DN rats were stained with PAS, which showed that the glomerular structure of normal control rats was normal, mesangial cells were not hypertrophied, matrix was not proliferated; mesangial cells were hypertrophied, mesangial matrix was significantly proliferated and expanded in DN rats (Fig.1F). In HG-treated HMCs, the expression of IL-6 and TNF- α (Fig.1G), and IV-C and FN protein levels increased significantly (Fig.1H). HG treatment increases inflammatory factors and fibrosis of HMCs, which is the main feature of diabetes. Through the above experiments, we found that DN mice and cells were successfully induced.

TET1 relieved inflammation and renal injury in DN rats

The renal tissues of NC and DN rats were taken for mRNA microarray analysis, and the mRNA with different expression was screened out (Fig.2A). Their expression patterns in kidney tissues were verified by RT-qPCR (Fig.2B). Among all the differentially expressed mRNAs, TET1 was significantly downregulated in DN rats and hyperglycated HMCs (Fig.2C). So, we guess that TET1 plays an important role in DN. TET1 was highly expressed in the kidney of DN rats and HG-HMCs, and the successful transfection of TET1-OE fragment was verified by detecting the TET1 mRNA and protein expression in the kidney of DN rats and HG-HMCs (Fig.2D/E). The effect of TET1 on hyperglycated HMCs was detected. As

the CCK8 method (Fig.2F), flow cytometry (Fig.2G), ELISA (Fig.2H) and WB detection (Fig.2I) indicated, TET1 increased the proliferative activity of HMCs, reduced apoptosis, reduced the contents of IL-6 and TNF- α , IV-C and FN protein, and cell fibrosis. The changes of TET1 on DN rats were detected. The body weight, blood glucose, BUN, Scr and UAE levels of DN rats were decreased (Fig.2J). PAS staining area decreased, the degree of glomerular mesangial cell hypertrophy decreased, and the matrix hyperplasia decreased (Fig.2K). These results confirm that TET1 involvement in DN, which is related to the activity of HMCs and renal damage.

Ap1 downregulated the activity of demethylase TET1

To explore the mechanism of TET1 in DN, we first looked for the upstream factors of TET1. TET1 and transcription factors can regulate the activity of each other [13]. So, we wonder if TET1 is regulated by a transcription factor. After obtaining the TET1 promoter sequence from UCSC Genome Browser, we predicted the transcription factors regulating TET1 in ALGGEN. Ap1 has a binding site with TET1 (Fig.3A). In the above mRNA microarray results, Ap1 was significantly upregulated in DN rats and HG-HMCs (Fig.3B). Then, we overexpressed Ap1 in DN rats and HG-HMCs. With the increase of Ap1 expression, TET1 decreased significantly (Fig.3C). The binding rate of Ap1 and TET1 promoter sequence -181 to -188 was higher in the prediction of microarray. Then, we used ChIP experiment to detect whether Ap1 is enriched in the TET1 promoter region in Ap1-overexpressed cells. The enrichment of Ap1 in this area was obviously increased (Fig.3D). Ap1 increased the secretion of inflammatory factors in HMCs and renal injury in DN rats (Fig.3E ~F).

TET1 upregulation inhibited the effect of Ap1 on DN rats

To explore the effect of TET1 on AP1, we overexpressed Ap1 and TET1 simultaneously (Fig.4A). TET1 reduced the level of inflammation and fibrosis induced by overexpressing Ap1 in varying degrees (Fig.4B ~C), and decreased the body weight, blood glucose, BUN, Scr and UAE levels in DN rats overexpressing Ap1 (Fig.4D). In addition, the renal tissue damage of DN rats was also greatly reduced (Fig.4E). To sum up, TET1 upregulation effectively inhibits the role of Ap1 in DN.

Nrf2 downregulation inhibited the protective effect of TET1 on DN rats

Next, we explored the downstream mechanism of TET1. TET1 is one of demethylase, so we consider there is a gene demethylated by TET1 and playing a role in DN. TET1 enhanced the activity of Nrf2 by demethylation of Nrf2 protein [10]. Nrf2 is important in DN [15]. Therefore, we hypothesized that the upregulation of Nrf2 in DN was due to the activation of TET1. To test this hypothesis, we detected the expression of Nrf2 in DN rats and HG-HMCs, and found that Nrf2 decreased significantly (Fig.5A). High expression of TET1 increased Nrf2 contents (Fig.5B). ChIP experiment showed that the enrichment of TET1 in Nrf2 promoter increased obviously (Fig.5C). Poor Nrf2 expression was delivered into rats and cells (Fig.5D), which increased inflammatory factors and aggravated renal injury (Fig.5E/F). Therefore, TET1 promoted Nrf2 expression by DNA demethylation in DN. Nrf2 expression was downregulated in rats and cells overexpressing TET1 (Fig.5G). Compared with pure high expression of TET1, the inflammatory

factors in HG-HMCs increased (Fig.5H), fibrosis related protein increased (Fig.5I), the body weight and other related indexes in DN rats increased (Fig.5J), and the renal injury aggravated (Fig.5K). These indicate that Nrf2 downregulation stimulates DN progression, and inhibits the protective effect of TET1 on DN rats.

Nrf2/ARE pathway is a downstream effector of Ap1/TET1 axis

Because TET1 can effectively demethylate Nrf2 and increase its active expression, we think that TET1 affects DN progression by mediating the Nrf2/ARE pathway. The activity of the Nrf2/ARE pathway in DN rats and cells was examined. Ap1 overexpression effectively inhibited Nrf2 protein expression in DN rats and HG-HMCs, while TET1 overexpression effectively promoted the activity of Nrf2/ARE pathway (Fig.6A). We also detected the protein changes of DN rats and HG-HMCs after transfection of Ap1-OE + TET1-OE, TET1-OE + si-Nrf2 and the corresponding control plasmids. We found TET1 upregulation reduced the inhibition of Ap1 on Nrf2/ARE pathway activity, while downregulation of Nrf2 reduced the promotion of TET1 on Nrf2/ARE pathway activity (Fig.6B). This also provides some evidence for the targeting relationship of Ap1/TET1/Nrf2 axis.

Discussion

DN is still a common and independent risk factor for kidney and cardiovascular diseases, and is associated with significant incidence rate and mortality [16, 17]. The abnormal expression of TET1 is related to tumorigenesis and can be used as a potential biomarker for tumor therapy [18]. In this study, we unveiled that overexpression of TET1 weakened the inhibitory effect of Ap1 on the Nrf2/ARE pathway, thus reducing inflammation and renal injury in DN rats.

Through mRNA microarray analysis and RT-qPCR detection, we verified TET1 was significantly downregulated in DN rats and hyperglycated HMCs among all the differentially expressed mRNAs. Reduction in TET1 is believed to be associated with the progression of preeclampsia and gastric cancer [19, 20]. So, we supposed upregulated TET1 may be protective of DN rats. Then TET1 was overexpressed in DN rats and hyperglycated HMCs, and results showed that TET1 increased the proliferative activity of HMCs, reduced apoptosis, the contents of IL-6 and TNF- α significantly reduced, IV-C and FN protein decreased, and cell fibrosis decreased. Inflammatory cytokines, such as IL-6 and TNF- α , participate in DN pathogenesis [21]. IL-6 and TNF- α could induce mouse mesangial cell proliferation in HG conditions [22]. In other words, upregulated TET1 inhibited the inflammation responses in DN rats. Additionally, the body weight, blood glucose, BUN, Scr and UAE levels of DN rats were decreased. PAS staining area, the degree of glomerular mesangial cell hypertrophy, and the matrix hyperplasia decreased. Elevated levels of Scr and BUN levels are typical markers of DN development, while UAE and albumin are symbols of glomerular dysfunction and renal tubular damage [23]. These results confirmed that TET1 upregulation mitigated renal damage.

Then we looked for the upstream factors of TET1. A former study highlighted that TET1 and Sin3a, another transcription factor, can regulate the activity of each other [13]. Through our website prediction,

we found Ap1 has a binding site with TET1. In the above mRNA microarray results, Ap1 was significantly upregulated in DN rats and HG-HMCs. Ap1 is pivotal in response to stimulants, like cellular stress, infections, and inflammatory cytokines [24]. With the increase of Ap1 contents, TET1 expression decreased significantly. Ap1 is activated after HG stimulation [25]. More attention has been paid to the role of Ap1 in the inflammatory response, which lays a foundation for new applications of anti-diabetic drugs [26]. Ap1 increased the secretion of inflammatory factors in HMCs and renal injury in DN rats. Ap1 stimulates the transcription of inflammatory cytokines (TNF- α , ILs), whose uncontrolled production leads to cytotoxic damage and dysfunction in DN [27]. However, there is little study on the correlation between Ap1 and TET1. To sum up, we got a novel finding that Ap1 regulated the activity of demethylase TET1. To explore the effect of TET1 on AP1, we co-overexpressed Ap1 and TET1. TET1 reduced the level of inflammation and fibrosis induced by overexpressing Ap1, and the renal tissue damage of DN rats was also greatly reduced.

Then we turned to explore the downstream mechanism of TET1. TET1 is one of demethylase, so we consider there is a gene demethylated by TET1 and playing a role in DN. TET1 enhanced Nrf2 activity by demethylation of Nrf2 protein [10]. Nrf2 expression decreased significantly in DN rats and HG-HMCs. Recently, the activation of Nrf-2 in response to HG-induced reactive oxygen species appears to be a protective pathway from oxidative damage and mouse mesangial cell dysfunction [28]. We observed in the current work that TET1 promoted Nrf2 expression by DNA demethylation in DN. Poor Nrf2 expression increased inflammatory factors and aggravated renal injury. Nrf2 knockout mice with DN suffered from greater renal damage, had enhanced FN production, more oxidative damage and severe glomerular injury [15]. Nrf2 downregulation stimulates DN progression, and inhibits the protective effect of TET1 on DN rats.

The Nrf2/ARE pathway is protective of central nervous system diseases [29] and is a key defense against oxidative stress and inflammation [30]. Because TET1 can demethylate Nrf2 and increase its active expression, we think that TET1 may affect DN by mediating the Nrf2/ARE pathway. Vinpocetine can confer protection to rat renal cells by activating the Nrf2/ARE signaling pathway, suggesting that activation of the Nrf2/ARE pathway may be beneficial for relieving renal injury [31]. Ap1 overexpression inhibited Nrf2 expression, while TET1 overexpression promoted the activity of the Nrf2/ARE pathway. TET1 upregulation reduced the inhibition of Ap1 on Nrf2/ARE pathway activity, while downregulation of Nrf2 reduced the promotion of TET1 on Nrf2/ARE pathway activity. The relationships among Ap1/TET1/Nrf2 were further evidenced.

Conclusions

In summary, our data indicated that that overexpression of TET1 weakened the effect of Ap1 on the Nrf2/ARE pathway (Fig. 7), thus reducing inflammation and renal injury in DN rats. The study might hint the possibility of TET1 as a potential therapeutic target for DN patients. More work should be done to validate the application value in clinic.

Abbreviations

ANOVA, analysis of variance; Ap1, activator protein 1; BUN, blood urea nitrogen; CCK-8, Cell counting kit-8; ChIP, Chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; DN, diabetic nephropathy; ELISA, Enzyme-linked immunosorbent assay; HG, high glucose; HMCs, NC, human mesangial cells; normal control; OD, Optical density; OE, overexpression; RT-qPCR, Quantitative real-time polymerase chain reaction; Src, STZ, serum creatinine; streptozotocin; TETs, ten-eleven translocation enzymes; UAE, urinary albumin excretion; WB, Western blot.

Declarations

Ethics approval and consent to participate

This study was approved and supervised by the ethics committee of Jinan City People's Hospital. All experiments and procedures were conducted in accordance with the laboratory animal care and use guidelines of National Institutes of Health, making every effort to reduce the pain of animals and the number of animals used.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interest.

Funding

Not applicable.

Authors' contributions

YST is the guarantor of integrity of the entire study and contributed to the concepts; YST and HMC contributed to the design and definition of intellectual content of this study; YST, HMC, QFL and JJS contributed to the experimental reports, data attainment and statistical analysis; QFL and JJS contributed to the manuscript preparation. All authors read and accepted the final manuscript.

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Figures

Figure 1

Establishment of DN rats and cells. A-E, the changes of biochemical indexes in NC and DN rats were measured; F, PAS staining detected the renal injury; G, ELISA detected the level of inflammatory factors IL-6 and TNF-α; H, the level of IV-C and FN protein detected by WB. *P < 0.05. Data in panels G were processed using the two-way ANOVA, and data in panels A-E, and H were processed using the unpaired t test.

Figure 2

TET1 relieved inflammation and renal injury in DN rats. A. Differential expression of mRNAs screened by microarray analysis in kidney tissues of DN and NC rats; B. mRNA expression of differentially expressed mRNAs detected by RT-qPCR; C, RT-qPCR detected the expression of TET1 in HG-HMCs; D-E, RT-qPCR and WB detected the change of TET1 mRNA and protein expression in the kidney and HMCs of rats after transfection of TET1-OE; F. CCK-8 method for cell viability; G. Flow cytometry for apoptosis; H. ELISA detected the content of inflammatory factors; I. WB detection of fibrosis related protein expression; J. the

changes of biochemical indexes in urine and blood of rats; K. Detection of renal injury by PAS staining. *P < 0.05. Data in panels B/D/E/F/H/J were processed using the two-way ANOVA, and data in panels C/G/I were processed using the unpaired t test.

Figure 3

Ap1 downregulated the activity of demethylase TET1. A, the targeted binding site of Ap1s and TET1; B, the expression of Ap1 detected by RT-qPCR; C, RT-qPCR and WB detected the levels of TET1 and Ap1; D, ChIP detected the enrichment of Ap1 in the TET1 promoter; E, Detection of inflammatory factors in HMCs by ELISA; F, PAS staining observed the renal tissue of rats. *P < 0.05. Data in panels B-E were processed using the two-way ANOVA.

Figure 4

TET1 upregulation inhibited the effect of Ap1 on DN rats. A. Detection of TET1 by RT-qPCR; B-C, WB detected FN protein and IV-C protein; D, Biochemical analysis of rats; E, PAS staining observed the kidney of rats. * $P < 0.05$. Data in panels A/D were processed using the two-way ANOVA, and data in panels B/C were analyzed by the unpaired t test.

Figure 5

Nrf2 downregulation inhibited the protective effect of TET1 on DN rats. A, RT-qPCR and WB detected Nrf2 levels; B, RT-qPCR detected Nrf2 expression after the overexpression of TET1; C, ChIP experiment detected the combination of TET1 and Nrf2 promoter; D, RT-qPCR detect Nrf2 expression after Nrf2 expression was inhibited; E, ELISA detected the change of inflammatory factors after inhibiting Nrf2; F, PAS staining for measurement of renal injury; G, Nrf2 expression after the overexpression of TET1 and inhibition of Nrf2;

H, detection of inflammatory factors in HMCs by ELISA; I, WB detection of fibrosis related protein expression; J, Biochemical analysis of the changes of DN-related factors in rats, K, PAS staining detected the kidney of rats. *P < 0.05. Data in panels A/B/C/D/E/G/H/J were processed using the two-way ANOVA, and data in panel I were analyzed by the unpaired t test.

Figure 6

Nrf2/ARE pathway was a downstream effector of Ap1/TET1 axis. A-B, Nrf2 protein levels detected by WB. *P < 0.05 according to the two-way ANOVA.

Figure 7

Mechanism chart. Ap1 can inhibit the expression of Tet1, and Tet1 can demethyl Nrf2 and activate the Nrf2/ARE pathway activity, and inhibit the progression of DN in DN rats, and inflammatory response and fibrosis of HMCs.

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