ORIGINAL PAPER

Down-regulation of SETD6 protects podocyte against high glucose and palmitic acid-induced apoptosis, and mitochondrial dysfunction via activating Nrf2-Keap1 signaling pathway in diabetic nephropathy

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Abstract

Diabetic nephropathy (DN), a serious complication of hyperglycemia, is one of the most common causes of end-stage renal disease (ESRD). Glomerular podocyte injury is a major mechanism that leads to DN. However, the mechanisms underlying podocyte injury are ambiguous. In this study, we sought to investigate the contribution of SET domain-containing protein 6 (SETD6) to the pathogenesis of podocyte injury induced by glucose (GLU) and palmitic acid (PA), as well as the underlying mechanisms. Our results showed that GLU and PA treatment significantly decreased SETD6 expression in mouse podocytes. Besides, Cell Counting Kit-8 (CCK-8) and flow cytometry assay demonstrated that silencing of SETD6 silence obviously enhanced cell viability, and suppressed apoptosis in GLU and PA-induced podocytes. We also discovered that downregulation of SETD6 suppressed GLU and PA-induced ROS generation and podocyte mitochondrial dysfunction. Nrf2-Keap1 signaling pathway was involved in the effect of SETD6 on mitochondrial dysfunction. Taken together, silencing of SETD6 protected mouse podocyte against apoptosis and mitochondrial dysfunction through activating Nrf2-Keap1 signaling pathway. Therefore these data provide new insights into new potential therapeutic targets for DN treatment.

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Graphic abstract
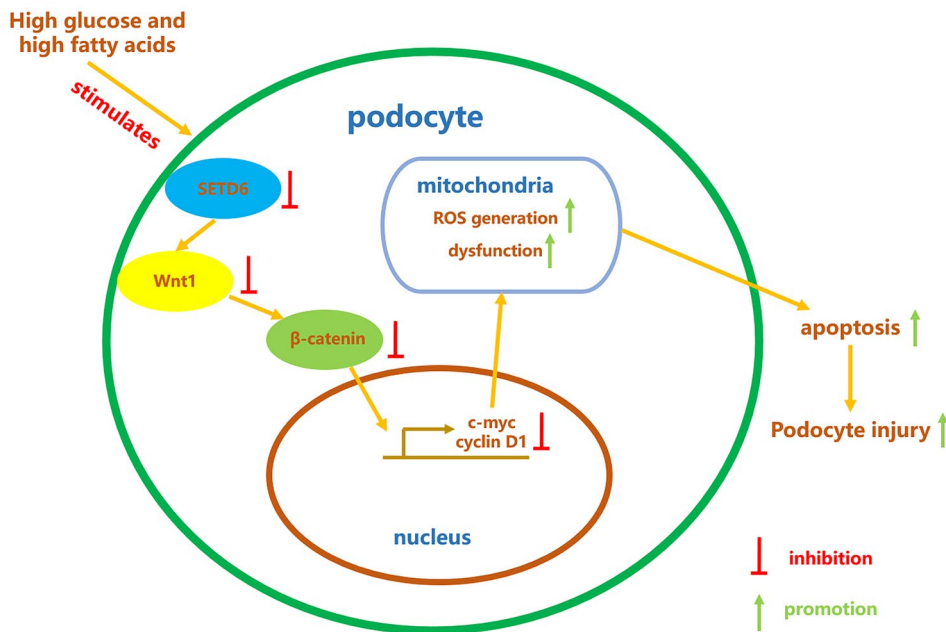
Introduction

Diabetic nephropathy (DN) is one of the major chronic complications of diabetes (Cao and Cooper 2011). It is widely acknowledged that DN is the leading cause of end-stage renal disease (ESRD) (Bommer 2001). Hyperglycemia and hyperlipidemia can cause glomerular basement membrane thickening, mesangial expansion, and extracellular matrix hyperplasia in DN patients, further leading to glomerular hyperfiltration, proteinuria, and finally progressing to ESRD (Ahmad 2015). Podocytes are terminally differentiated glomerular visceral epithelial cells that maintains the integrity of the glomerular filtration barrier (GFB) and prevents urinary protein loss (Belinda et al. 2012). Glucose (GLU) and palmitic acid (PA)-induced podocyte injury, such as podocyte apoptosis and mitochondrial dysfunction, has been demonstrated to intimately associated with GFB destruction and proteinuria in DN (Michele et al. 2003; Katalin et al. 2006; Siu et al. 2006). Thus, Searching the target to preventing podocyte injury will provide a novel target for DN therapy.

SET domain-containing protein 6 (SETD6), an important member of the lysine methyltransferase family (Chang et al. 2011), was recently identified as an important regulator of multiple signaling pathways through methylating protein substrates (Vershinin et al. 2016; Mukherjee et al. 2017).

On the other hand, it can also interact with nuclear receptor signaling factors and transcriptional regulators to stimulate transcriptional activity, affecting cell proliferation, inflammation, and oxidative stress response (Chen et al. 2016; Binda et al. 2013). Notably, several studies demonstrated that SETD protein was abnormally expressed in mice with high-carbohydrate diet (Villeneuve et al. 2010), which was closely related to occurrence and development of diabetes (Adamska-Patruno et al. 2019; Korsmo-Haugen et al. 2019). Nevertheless, there is no related studies focused on the role of SETD6 in DN.

Nuclear factor-erythroid-2-related factor 2 (Nrf2) is universally involved in diabetic renal injury (Cheng et al. 2019), mainly expressed in mesangial cell (Lin et al. 2006), renal tubular epithelial cell (Du et al. 2018), and podocyte injury (Wang et al. 2011, 2014). These cell damage can further cause glomerular sclerosis and renal interstitial fibrosis, which were the pathological features of DN (Bose et al. 2017). Aberrant expression of Nrf2 was found in mice with diabetic retinopathy (Xu et al. 2014). Induction of Nrf2 enhanced the antioxidant response during mitochondrial stress (Shih et al. 2005). Recently, Nrf2 was revealed to be regulated by SETD6, and enhance the expression of antioxidant genes under oxidative stress (Chen et al. 2016). In our research, we investigated the function of SETD6 in GLU and PA-induced podocyte apoptosis and mitochondrial

dysfunction. And we further explored whether Nrf2-Keap1 pathway was involved in the mediation of GLU and PA-induced podocyte injury. Our findings are expected to provide some theoretical basis to search for a new solution for the DN treatment.

Materials and methods

Cell culture and treatments

High glucose and high fatty acids was widely used to mimic a pathological state of early podocyte injury in human DN (Sanjay et al. 2011; Hou-Yong et al. 2011; Wang et al. 2019), and the injury of mouse podocytes was induced by GLU and PA in this study. Mouse podocytes,which were previously reported to express podocytes-specific markers(Liu et al. 2018), were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Briefly, podocytes were incubated at humidified incubator (33 °C, 5% CO_2) in RPMI-1640 (Sigma, St. Louis, MO, USA) medium which consisted of 10% Fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 1× insulin transferrin-selenium (ITS) (Sigma, St. Louis, MO, USA). When podocytes reached 70–80% confluence, the cells were induced to differentiate using ITS-free medium for 10–14 days. Then the podocytes were treated with normal-gulcose(5.5 mmol/L) or high-glucose (30 mmol/L, GLU) (Ha et al. 2013) and different concentrations of palmitic acid (50, 100, 200, 300 and 400 μmol/L, PA) for various periods of time (12, 24, and 48 h).

Plasmids

Plasmids used for suppression in podocytes were SETD6 siRNA (si-SETD6). The siRNAs with sequences that do not target any gene productwas a negative control (Vector, 1 μg/mL). The si-SETD6 (1 μg/mL) was transfected into immortalized mouse podocytes (6-well plate, $1.0 \times 10^6/cm^2$). After transfection for 24 h, the podocytes were treated with 30 mmol/L GLU and 200 μmol/L PA for 24 h.

Western blot analysis

At the end of experiments, podocytes were lysed on ice for 15 min in RIPA lysis buffer (Sigma, St. Louis, MO, USA). The supernatants were collected after centrifugation at $12,000 \times g$ at 4 °C for 20 min. Total protein concentration from podocyte lysate was detected by BCA protein concentration detection kit (Solarbio, Beijing, China). Then total protein was separated by 8% SDS polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% zero fat milk powder and incubated with corresponding target protein antibodies and rabbit anti-β-actin monoclonal antibody. Then, the membranes were incubated with a goat anti-rabbit HRP antibody (Sigma, St. Louis, MO, USA). The protein complexes were detected by the enhanced chemiluminescence reagents (Abcam, Cambridge, UK). Primary antibodies used were as Table 1.

CCK-8 proliferation vitality assay

Mouse podocyte proliferation was measured using the Cell Counting Kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). The cell viability was measured at specified time points using microplate reader (Thermo Fisher Scientific, Massachusetts, USA) by spectrophotometry at 450 nm.

Apoptosis assay

Mouse podocyte apoptosis was detected using the Cell Apoptosis Detection Kit (Qiagen, Valencia, CA, USA). After 24 h cultured with 30 mmol/L GLU and 200 μmol/L PA, si-SETD6-infected podocytes were incubated with 10 μL FITC Annexin-V and propidiumiodide (Becton, Heidelberg, Germany) at room temperature for 15 min. Then, flow cytometry was performed according to the manufacturer's instructions.

Table 1 Antibodies information

Antibody name	Company and item number	Dilution concentration
SETD6	GeneTex, SC, USA; GTX629891	1:1000
caspase 3	CST, Boston, MA, USA; cat. no.9662	1:1000
BAX	CST, Boston, MA, USA; cat. no.2774	1:2000
BCL-2	Abcam, Cambridge, UK; cat. abno. ab185002	1:1500
SOD1	CST, Boston, MA, USA; cat. no.2770	1:1000
SOD2	CST, Boston, MA, USA; cat. no.13,141	1:1000
GPX1	Abcam, Cambridge, UK; cat. no.ab22604	1:1000
GCLC	Abcam, Cambridge, UK; cat. no.ab207777	1:1000
Wnt1	Abcam, Cambridge, UK; cat. no.ab15251	1:1000
β-catenin	CST, Boston, MA, USA; cat. no.8480	1:1000
c-myc	CST, Boston, MA, USA; cat. no.5605	1:1000
cyclin D1	CST, Boston, MA, USA; cat. no.2978	1:1000
β-actin	Abcam, Cambridge, UK; cat. no.ab3280	1:2000

Mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) measurement

The MMP of podocytes was monitored using Tetrechloro-tetraethylbenzimidazol carbocyanine iodide (JC-1, Beyotime Biotechnology, Shanghai, China). And the generation of ROS in podocytes was evaluated by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe. Briefly, podocytes were washed twice with PBS, and incubated in the dark with JC-1 (2 μmol/L, 30 min at 37 °C) or DCFH-DA (10 μM, 20 min at 37 °C). Then, the cells were fixed using 4% poly-formaldehyde (Beyotime Biotechnology, Shanghai, China) for 10 min. JC-1 (590/520 nm) and DCFH-DA (520 nm) fluorescence levels were detected by confocal microscopy and flow cytometry.

Statistical analysis

The data were represented as means ± SEM (standard error of mean) and each experiment was performed in triplicate in this study. One-way ANOVA (analysis of variance) and Student's unpaired t test were used to analyze statistical significance. p value < 0.05 were considered statistically significant.

Results

SETD6 was downregulated in GLU and PA-induced mouse podocytes

Podocytes were treated with GLU (30 mmol/L) and PA (50, 100, 200, 300 and 400 μmol/L) to establish a podocyte injury model. CCK-8 assay evidenced that GLU and PA decreased the viability of podocytes in a dose-dependent and time-dependent manner (Fig. 1a–c). What's more, the result showed that SETD6 protein level was reduced by GLU and PA treatment (Fig. 1d).

Downregulation of SETD6 inhibited GLU and PA-induced podocyte apoptosis

To investigate the effect of SETD6 on the cell growth in GLU and PA-induced podocytes, podocytes were transfected with SETD6 siRNA (si-SETD6) or negative control (Vector) for 24 h and then the cells were treated with or without 30 mmol/L GLU and 200 μmol/L PA for 24 h. Our Western blotting results displayed the efficiency of si-SETD6 (Fig. 2a, b). As expected, CCK-8 and flow cytometry analysis respectively demonstrated that transfection of si-SETD6 obviously enhanced cell viability and suppressed apoptosis of podocytes (Fig. 2c, d). Meanwhile, the results evidenced that SETD6 silence significantly reduced caspase-3 and

Bax (Bcl-2 Assaciated X protein) expression and enhanced BCL-2 (B-cell lymphoma-2) expression in GLU and PA-induced podocytes (Fig. 2e–h).

Downregulation of SETD6 suppressed GLU and PA-induced ROS generation and podocyte mitochondrial dysfunction

Several studies have indicated podocyte apoptosis usually manifests mitochondrial dysfunction and abundant generation of ROS (reactive oxygen species) (Herrera et al. 2001; Fan et al. 2019). Thus we then investigate the effect of SETD6 on mitochondrial dysfunction in GLU and PA-induced podocyte. As shown in Fig. 3a, the level of MMP was reduced in the presence of GLU and PA, which was strongly reversed by SETD6 downregulation. Strikingly, our findings also revealed that silencing of SETD6 markedly decreased ROS generation (Fig. 3b). Consistent with this result, SETD6 deletion significantly facilitated the expression of SOD1/2, GPX1, and GCLC in GLU and PA-induced podocytes (Fig. 3c–g).

Downregulation of SETD6 protected against podocyte apoptosis and mitochondrial dysfunction through activating Nrf2-Keap1 signaling pathway

Considering that Nrf2 signaling alleviates podocyte injury (Shen et al. 2019), and SETD6 has a negative effect on Nrf2 expression (Chen et al. 2016). We wonder that Nrf2 signaling pathway maybe participate in the mediation of SETD6-induced podocyte injury. The results showed that GLU and PA treatment decreased the expression of Nrf2, and increased the expression of Keap-1, which were inversed by silencing of SETD6 (Fig. 4a–c). Then we study the effect of Nrf2 signaling pathway blockage on podocyte apoptosis and mitochondrial dysfunction. The deactivating of Nrf2-Keap1 pathway was established by brusatol (Selleck, USA) treatment. Strikingly, it was observed that Nrf2 blockage strongly attenuated the protection effect of SETD6 on GLU and PA-induced podocyte apoptosis (Fig. 4d, e). At the same time, ROS reduction and MMP elevation induced by SETD6 downregulation were damaged through Nrf2 inhibitor co-treatment (Fig. 4f, g).

Discussion

Podocytes serve as a pivotal role in the DN progression and the development of proteinuria (Mundel and Reiser 2010). Both basic and clinical findings indicated that DN patients presented with long-term hyperglycemia (Tagawa et al. 2016). High glucose resulted in glomerular podocyte injury,
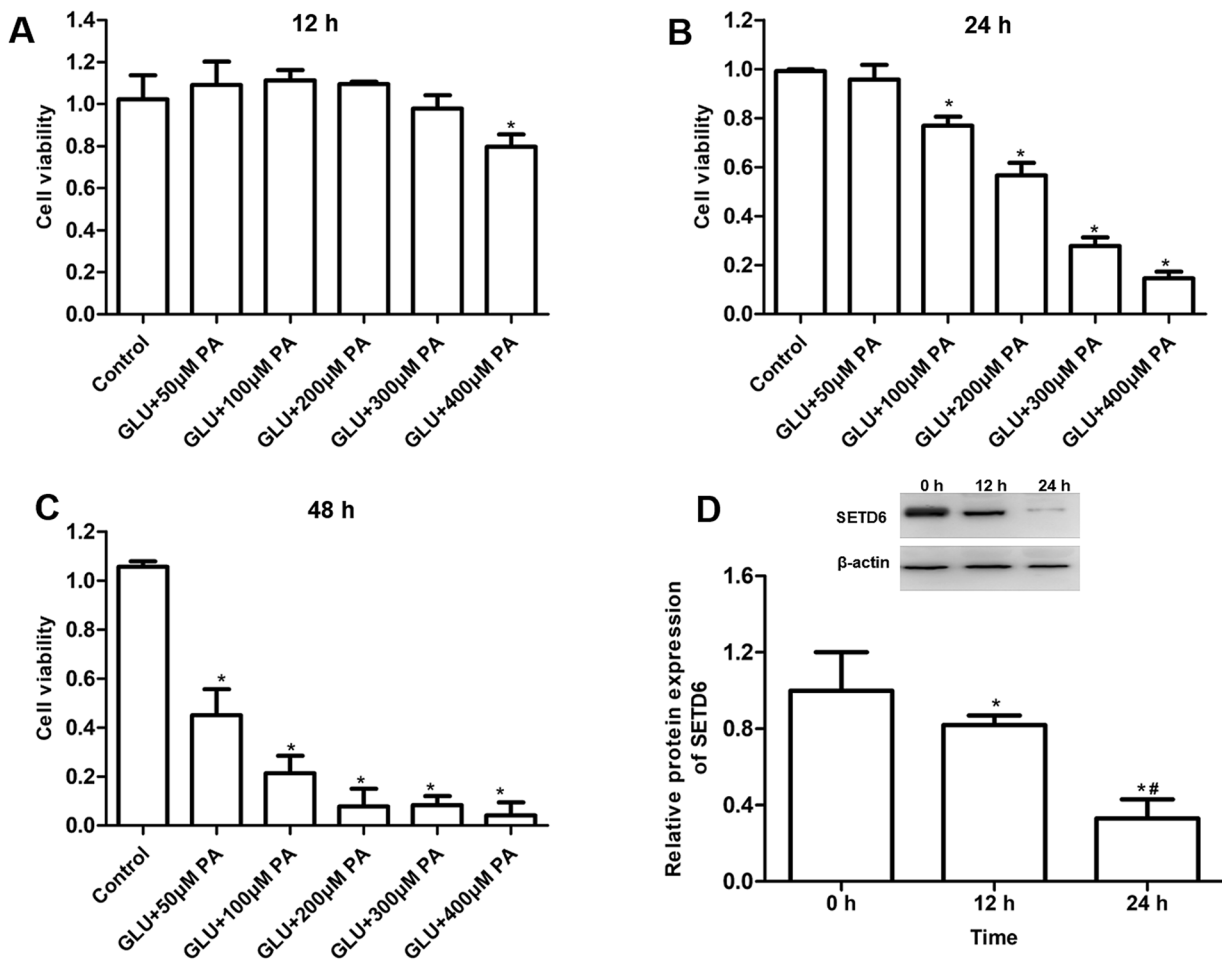
Fig. 1 SETD6 was downregulated in GLU and PA-induced mouse podocytes. Mouse podocytes were treated with 30 mmol/L glucose (GLU) and different concentrations of palmitic acid (50, 100, 200, 300 and 400 μmol/L, PA) for the indicated time points (12 h, 24 or 48 h). **a–c** The vibility of podocytes was measured by CCK-8 assay.

$*p < 0.05$ (vs. Control). **d** Western blot analysis was used to detect the SETD6 expression in podocytes treated with 30 mmol/L GLU and 200 μmol/L PA for 24 h. β-actin was used as an internal control. $*p < 0.05$ (vs. 0 h). $*^{\#}p < 0.05$ (vs. 12 h). The columns were presented as the mean ± SEM (n ≥ 03)

and the apoptosis and detachment of podocytes eventually led to the appearance of proteinuria (Khaled et al. 2012; Eid et al. 2009; Choel et al. 2009). Reducing or blocking the apoptosis of podocytes that involved in the DN development (Zhang et al. 2019) is an effective treatment strategy for DN. In this study, we reported that SETD6 was significantly downregulated in podocytes treated with GLU and PA treatment, and down-regulation of SETD6 rescued the podocyte apoptosis.

SETD6, a lysine methyltransferase, express a high level in liver and kidney (Chen et al. 2015). Previous studies report that SETD6 was a negative regulator of antioxidant stress response (Chen et al. 2016), and dysfunction of redox homeostasis may lead to mitochondria damage. Our further study demonstrated that SETD6 prevents podocytes against mitochondrial dysfunction by inhibiting the accumulation

of ROS. As a critical factor in apoptosis, Mitochondrial oxidative stress and ROS are increased in HG-treated podocytes(Qiao et al. 2018). Maintaining the balance of MMP and suppressing intracellular ROS production is beneficial to prevent podocyte against apoptosis, and thus have protective effects on DN (Fan et al. 2019; Cai et al. 2016). Thus SETD6 may protect podocytes against apoptosis via mediating mitochondrial dysfunction.

SETD proteins usually function as a methyltransferase to monomethylated H2AZ at lysine 7 (H2AZK7me1) and finally maintained embryonic stem cell self-renewal (Binda et al. 2013). Similarly, inhibition of SET domain–containing lysine methyltransferase 7/9, two other members of the lysine methyltransferase family, suppressed methylation of lysine 4 of histone H3 (H3K4) and finally ameliorated renal fibrosis (Sasaki et al. 2016). SETD6 also methylated
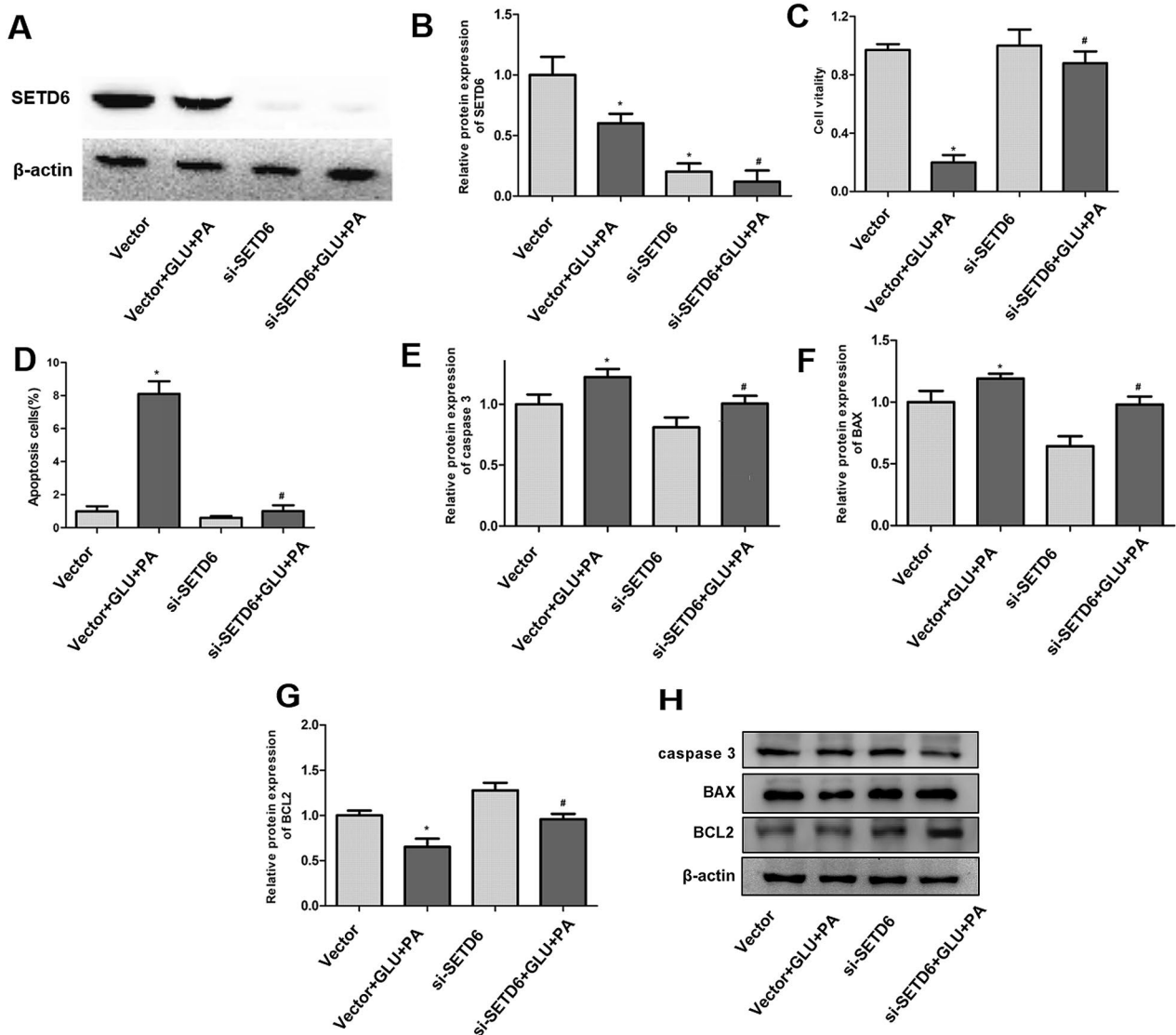
Fig. 2 Down-regulation of SETD6 inhibited GLU and PA-induced podocyte apoptosis. Mouse podocytes $(1.0 \times 10^6/cm^2)$ were transfected with SETD6 siRNA (si-SETD6, 1 μg/mL) or negative control (Vector, 1 μg/mL) for 24 h and then the cells were treated with or without 30 mmol/L GLU and 200 μmol/L PA for 24 h. **a** and **b** Western blot analysis of SETD6 expression in podocytes. **c** The viabil-ity of podocytes was evaluated by CCK-8 assay. **d** The apoptosis of podocytes was detected by flow cytometry analysis. **e–h** The expression of caspase-3, Bax, and BCL2 were assayed by Western blot analysis. β-actin was used as an internal control. *$p < 0.05$ (vs. vector). #$p < 0.05$ (vs. vector + GLU + PA). The columns were presented as the mean ± SEM (n ≥ 03)

the lysine at position 310 of RelA (p65) protein, which restrained p65 phosphorylation. Recently, Nrf2 was revealed to be regulated by SETD6, and SETD6 down-regulation increased the Nrf2 protein expression (Chen et al. 2016). Consistent with previous reports, we uncovered novel findings that the Nrf2-Keap1 pathway were activated by GLU and PA in podocytes. The Nrf2-Keap1 pathway plays a vital role in regulation of nephron development, glomerular podocyte motility, adhesion, as well as apoptosis. In normal conditions, actin binding repressor protein-Keap1 make sure that Nrf2 located in the cytoplasm. Once oxidative stress occurs, Nrf2 easily unwinds bound with Keap1, translocates into the nucleus, combine with antioxidant response genes (Palsamy and Subramanian 2011; Nakai et al. 2013). Our further study confirmed that silencing of SETD6 induced the activation of Nrf2 signaling pathway, preventing the over-accumulation of ROS and mitochondrial dysfunction, eventually inhibiting apoptosis.
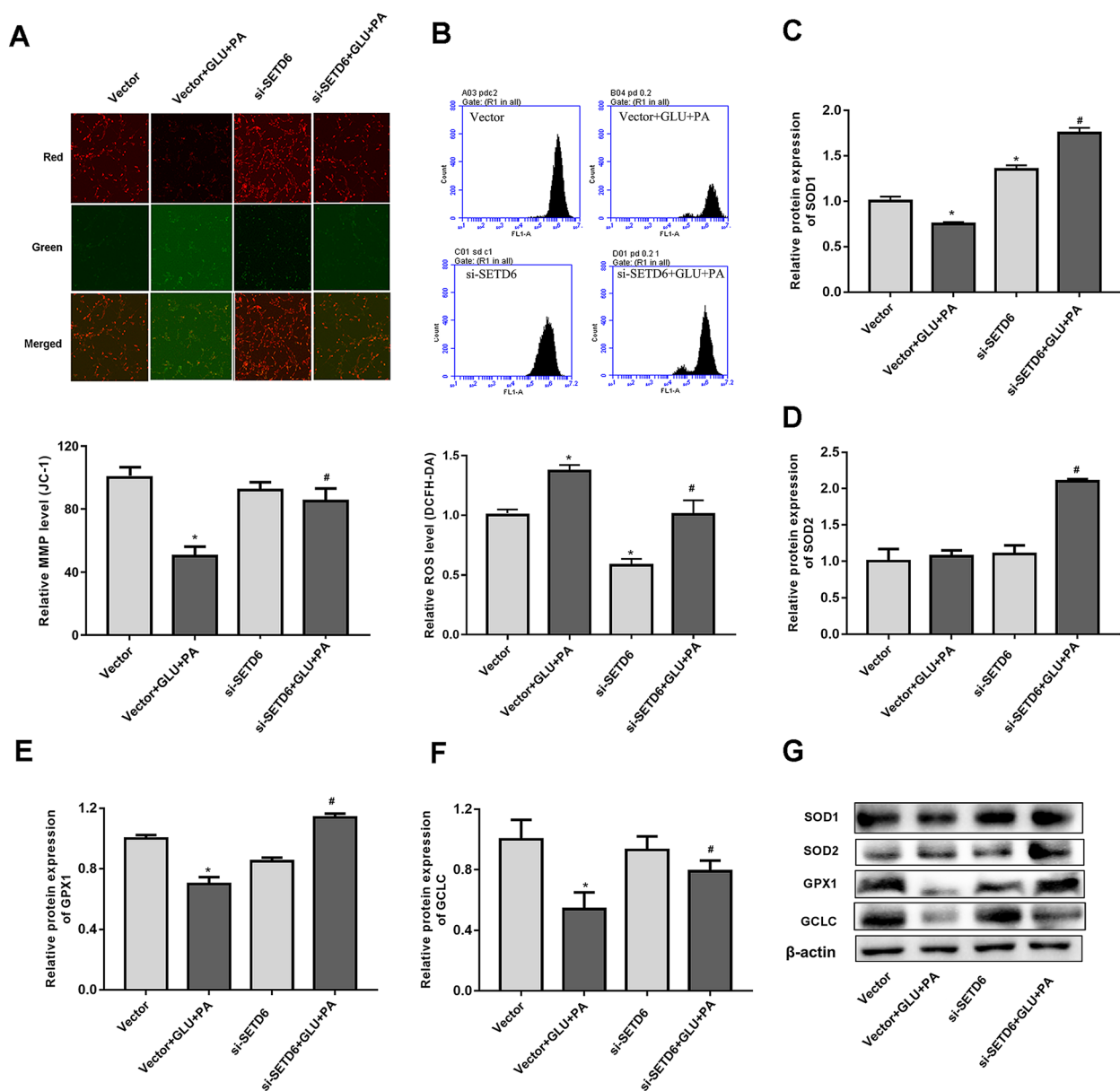
Fig. 3 Down-regulation of SETD6 suppressed GLU and PA-induced podocyte mitochondrial dysfunction and ROS generation. Mouse podocytes ($1.0 \times 10^6/cm^2$) were transfected with SETD6 siRNA (si-SETD6, 1 μg/mL) or negative control (Vector, 1 μg/mL) for 24 h and then the cells were treated with or not 30 mmol/L GLU and 200 μmol/L PA for 24 h. **a** Manifests mitochondrial membrane potential was measured by JC-1 using fluorescence microscope (origi-nal magnification, ×400). **b** Endogenous ROS content was assayed via DCFH-DA fluorescent probe. **c–g** Western blot analysis of the SOD1/2, GPX1 and GCLC expression in podocytes. β-actin was used as an internal control. *$p < 0.05$ (vs. vector). #$p < 0.05$ (vs. vec-tor+GLU+PA). The columns were presented as the mean ± SEM (n ≥ 03).

In conclusion, we showed that SETD6 expression was decreased in mouse podocytes treated with GLU and PA. SETD6 silencing obviously suppressed apoptosis of GLU and PA-induced mouse podocytes. Meanwhile, down-reg-ulation of SETD6 suppressed GLU and PA-induced mouse podocyte mitochondrial dysfunction and ROS generation. Additionally, Down-regulation of SETD6 protected against GLU and PA-induced mouse podocyte apoptosis and mito-chondrial dysfunction through activating the Nrf2-Keap1 pathway.
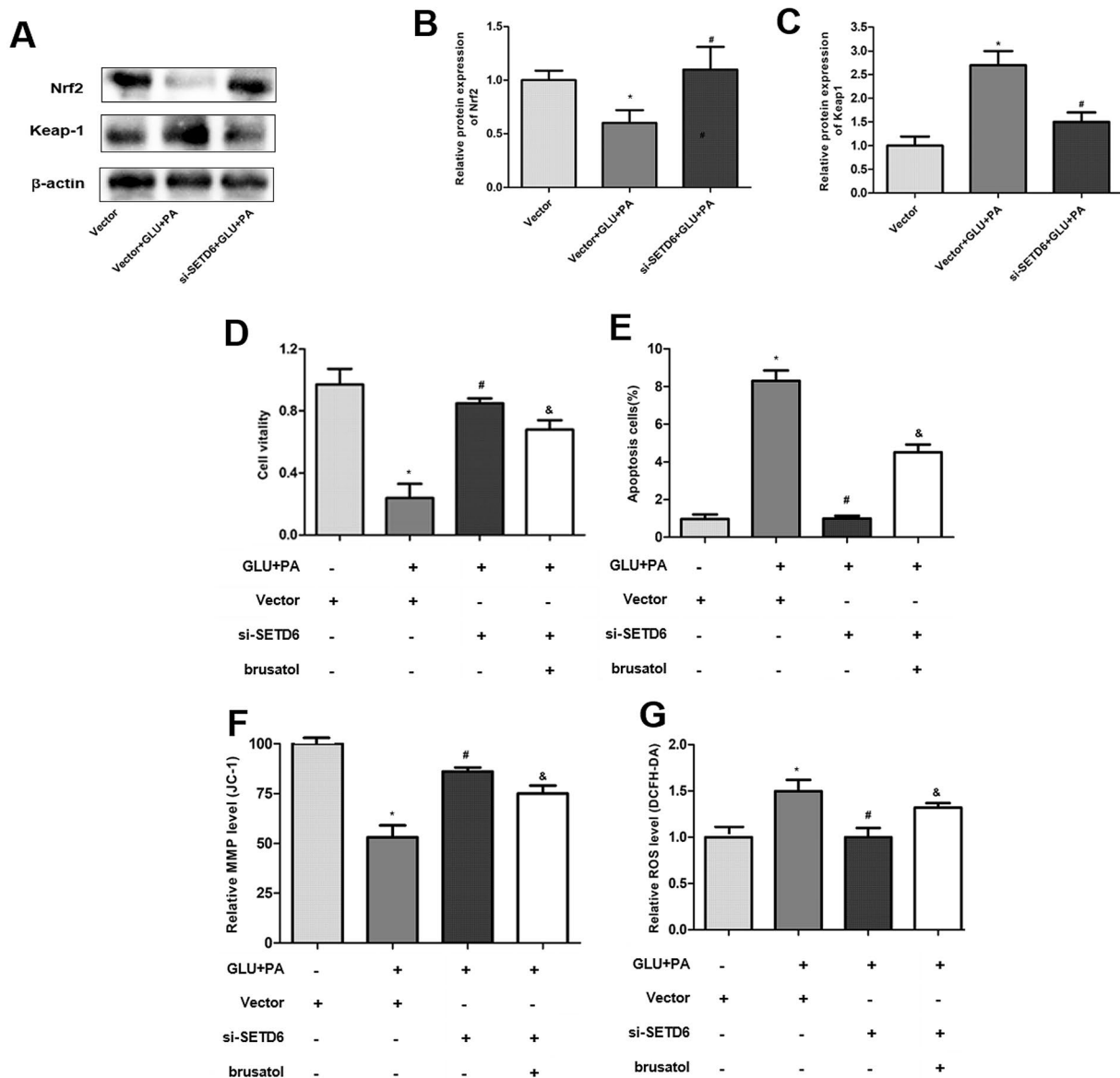
Fig. 4 SETD6 silence protected against podocyte apoptosis and mitochondrial dysfunction through activating Nrf2-Keap1 pathway. Mouse podocytes ($1.0 \times 10^6/\text{cm}^2$) were transfected with SETD6 siRNA (si-SETD6, 1 μg/mL) or negative control (Vector, 1 μg/mL) or treated with Nrf2 inhibitor brusatol (10 μM) for 24 h. And then the cells were treated with or not 30 mmol/L GLU and 200 μmol/L PA for 24 h. **a–c** Western blot analysis of the Nrf2 and Keap1 expression in podocytes. β-actin was used as an internal control. **d** The vibility of podocytes was measured by CCK-8 assay. e The apoptosis of podocytes was detected by flow cytometry analysis. **f** Manifests mitochondrial membrane potential was measured by JC-1 fluorescent probe. **g** Endogenous ROS content was assayed via DCFH-DA fluorescent probe. $*p < 0.05$ (vs. vector). $^{\#}p < 0.05$ (vs. vector+GLU+PA). $^{\&}p < 0.05$ (vs. si-SETD6+GLU+PA). The columns were presented as the mean ± SEM (n ≥ 03)

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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