Inflammation Research (2019) 68:325–336
https://doi.org/10.1007/s00011-019-01221-3

Inflammation Research

ORIGINAL RESEARCH PAPER

EZH2 plays a crucial role in ischemia/reperfusion-induced acute kidney injury by regulating p38 signaling

Hua Liang[1] · Qiong Huang[2] · Mei-juan Liao[1] · Feng Xu[1] · Tao Zhang[1] · Jian He[1] · Lei Zhang[1] · Hong-zhen Liu[1]

Received: 21 November 2018 / Revised: 19 January 2019 / Accepted: 21 February 2019 / Published online: 28 February 2019

Abstract

Objective and design Renal ischemia–reperfusion (IR)-induced acute kidney injury (AKI) remains a major challenge in clinic. The histone methyltransferases enhancer of zest homolog-2 (EZH2) is associated with the development of renal injury. However, the molecular mechanism has not been fully elucidated.

Materials AKI in C57BL/6 mice was generated by renal IR.

Treatments The 3-deazaneplanocin A (DZNeP), a selective EZH2 inhibitor, or vehicle was administrated in mice after IR. HK-2 cells were exposed to hypoxia-reoxygenation (H/R) stress.

Methods Apoptosis was detected by TUNEL assay or flow cytometry. EZH2, caspase-3, p38, $F4/80^+$ macrophages, and $CD3^+$ T cells were examined by immunohistochemistry or Western blot. Tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, IL-6, and IL-18 were measured using RT-PCR.

Results Mice treated with DZNeP exhibited less severe renal dysfunction and tubular injury following IR. EZH2 inhibition decreased apoptotic cells while reducing activation of caspase-3 in kidneys under IR condition. Moreover, EZH2 inhibition impaired the recruitment of $CD3^+$ T cells and $F4/80^+$ cells in kidneys with IR. Administration of DZNeP suppressed the production of TNF-α, MCP-1, IL-6, and IL-18 in IR-treated kidneys. Of note, EZH2 inhibition reduced p38 phosphorylation in kidneys after IR. In H/R-treated HK-2 cells, DZNeP treatment or EZH2 knockdown reduced apoptosis. EZH2 inhibition inactivated p38 resulting in reduction of active caspase-3 and proinflammatory molecules. By contrast, EZH2 overexpression induced p38 phosphorylation, caspase-3 activation, and production of proinflammatory molecules, which was reversed by SB203580.

Conclusions EZH2 plays a crucial role in IR-induced AKI via modulation of p38 signaling. Targeting EZH2/p38 signaling pathway may offer novel strategies to protect kidneys from acute kidney injury induced by ischemia–reperfusion.

Keywords EZH2 · Renal ischemia–reperfusion·p38 · Inflammation · Apoptosis

Introduction

Acute kidney injury (AKI) is a constant health issues, which characterized by a rapid decline of kidney function. AKI often occurs in patients undergoing cardiovascular surgery, organ transplantation, trauma, and hypovolemic shock [1, 2]. To date, AKI is rising at alarming rates worldwide and is associated with unacceptably morbidity and mortality [3]. It is now universally recognized that AKI predisposes to the transition to chronic kidney disease and increases the likelihood of end-stage renal disease [4]. As such, identification of effective therapeutic interventions for this life-threatening disorder is a top clinical priority.

Renal ischemia–reperfusion (IR) injury is commonly regarded as a main cause of AKI [5]. Extensive evidences

Responsible Editor: John Di Battista.

✉ Hua Liang
lhlh2003@126.com

✉ Qiong Huang
390925660@qq.com

[1] Department of Anesthesiology, Affiliated Foshan Hospital of Sun Yat-sen University, Foshan 528000, China

[2] Department of Medical Statistics, Foshan Chancheng Central Hospital, Foshan 528000, China

show that the pathophysiological of IR-induced AKI is featured by the damage of renal tubules, apoptosis, and a robust inflammation [6–8]. Despite the pathological characterization, the detailed mechanism underlying IR-induced AKI remains largely unexplored [9]. Elucidating the modulation of apoptosis and inflammation during IR-induced AKI will not only further enhance our understanding of the pathogenesis of AKI, but also provide potential targets for novel therapeutic strategies.

Epigenetic modifications have been implicated in the development of some autoimmune disorders, which are mainly represented by posttranslational modifications of histone [10, 11]. Recent advances show an emerging role of epigenetic modifications in AKI [12]. The enhancer of zeste homolog 2 (EZH2), a histone-lysine N-methyltransferase, is a key component of polycomb repressive complex 2. EZH2 catalyzes the trimethylation of histone H3 at lysine 27 (H3K27me3) and mediates gene silencing of the target genes [13]. A study has demonstrated that EZH2 inhibition exerts a protective role against acute kidney injury via attenuation of the Raf-1/ERK1/2 pathway [14]. However, its molecular mechanism has not been fully elucidated. Recently, it has been reported that EZH2 regulates the activation of p38 signaling pathway in cancer cells [15]. Moreover, EZH2 deficiency reduces the cytokine secretion and decreases inflammatory response in colitis and experimental autoimmune encephalomyelitis [16, 17]. Based on these findings, we hypothesized that EZH2 plays a role in IR-induced AKI through regulating p38 signaling pathway, leading to the inhibition of apoptosis and inflammatory response.

In the present work, we demonstrated that pharmacological inhibition of EZH2 or EZH2 knockdown alleviates tubular cells injury through inactivating p38 signaling, resulting in reducing apoptosis and inflammation.

Materials and methods

Animals

Male C57BL/6 mice (8–10 weeks old, weighing 20–30 g) were obtained from the Sun Yat-Sen University Laboratory Animal Center. The animals were maintained with filtered water and food ad libitum in a 12-h dark–light cycle. All procedures involving mice followed the animal care protocols of the Institutional Animal Care and Use Committee of the Sun Yat-Sen University.

Renal IR injury model and treatment

Renal ischemia of mice was induced similar as we described previously [18]. Mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg). IR-induced AKI model was generated by bilateral ischemia–reperfusion injury. The renal pedicle was bluntly dissected and exposed using a dorsal lumbotomy incision. A non-traumatic microaneurysm clamp was applied to the renal artery for 30 min clamping. Reperfusion was confirmed by the disappearance of organ cyanosis after clamps were removed. Body temperature of mice was maintained at 36.5–37.5 °C throughout the surgery. The DZNeP (2 mg/kg, dissolved in 0.9%NaCl, Tocris, France), a selective EZH2 inhibitor, or vehicle was administrated intraperitoneally immediately after IR treatment. All mice were killed at 48 h after IR or sham surgery.

Assessment of kidney function

Serum blood urea nitrogen (BUN) and creatinine were assessed to determined kidney function using a Quantichrom assay kit (BioAssay Systems) according to the manufacturer's protocol.

Evaluation of renal injury

Renal injury was evaluated histologically with hematoxylin and eosin (H&E) staining and with Periodic Acid-Schiff (PAS) staining under light microscope. All histologic examinations were performed by a pathologist in a blinded fashion. The pathological abnormalities in the kidney were graded based on the presence and severity of component abnormalities (i.e., tubular necrosis or damage, loss of brush border, tubular dilatation, cell lysis, and tubular casts formation) in the sample: 0 = normal kidney (no damage); 1 = minimal damage (< 25% damage); 2 = mild damage (25–50% damage); 3 = moderate damage (50–75% damage); and 4 = severe damage (> 75% damage) similar as we previously described [18, 19].

TUNEL assay

A terminal transferase dUTP nick-end labeling assay was performed to detect apoptosis using an ApopTag plus Peroxidase in situ Apoptosis Detection Kit (Millipore) according to the manufacturer's protocol. The number of terminal transferase dUTP nick-end labeling-positive cells per high-power field was counted and analyzed in a blinded manner under Nikon microscope.

Immunohistochemistry

Immunohistochemistry was performed similar as we described previously [20, 21]. Deparaffinized and hydrated sections were incubated with antigen unmasking solution or proteinase K for antigen retrieval. The sections were exposed to 3% H_2O_2 for 10 min. After washing, sections were incubated with serum diluted in PBS for 1 h, and then

incubated with avidin D and biotin for 15 min. EZH2 antibody (1:200, #5246, Cell Signaling Technology), CD3 antibody (1:400,SAB4700041, Calbiochem, USA), and F4/80 antibody (1:400, MCA497GA, AbD Serotec, UK) were applied to kidney tissue. The slides were stored overnight at 4 °C in humidified chamber. Diluted appropriate biotinylated secondary antibodies were covered sections for 1 h at room temperature and then incubated with ABC reagent (Vector Laboratories, USA) for 30 min. Diaminobenzidine solution was applied to slides. To counterstain nuclei, hematoxylin staining was performed. Kidney slices were visualized using a microscope equipped with a digital camera (Nikon). The positive cells were counted under x400 magnification observing ten consecutive non- overlapping fields per animal in a blinded manner.

Western blot

The protein expression in kidney tissue was determined by Western blot. Proteins were lysed with RIPA buffer-containing cocktail proteinase inhibitors (Thermo Fisher). An equivalent amount of protein from each sample was mixed with a loading buffer and loaded onto separate lanes on 10% sodium dodecyl sulfate–polyacrylamide gel. Proteins were electrotransferred onto polyvinylidene fluoride membranes and blotted with EZH2 antibody (1:500, #5246, Cell Signaling Technology), cleaved caspase-3 antibody (1:500, ab32351, Abcam), p38 antibody (1:500, SC-7972, Santa Cruz), p-p38 antibody (1:500, SC-7973, Santa Cruz), and GAPDH (1:1000, SC-32233, Santa Cruz) antibodies, followed by incubation with appropriate fluorescence-conjugated secondary antibodies. Protein bands were visualized using Odyssey infrared imaging system (LICOR). Densitometry analysis was performed using National Institutes of Health (NIH) ImageJ software and the densitometric intensity corresponding to each band was normalized against GAPDH expression.

RT-PCR

Total RNA from the HK-2 cells or kidneys was isolated using TRIzol reagent (Invitrogen; Thermo Fisher) and cDNA was synthesized with the M-MLV Reverse Transcription Kit (Promega) according to the manufacturer's protocol. Quantitative Real-Time PCR was performed using IQ SYBR green supermix reagent (Bio-Rad) with a Bio-Rad real-time PCR machine according to the manufacturer's instructions. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The expression levels of the target genes were normalized to GAPDH level in each sample. The primer sequences were as follows: TNF-α—forward, 5′-CCCGGAATGTCGATGCCTGAGTG-3′, reverse, 5′-CGCCCCGGCCTTCCAAATAAAT-3′; MCP-1—forward, 5′-AGCATCCACGTGCTGTCTC-3′, reverse, 5′-GATCATCTTGCCAGTGAATGAG-3′; IL-6—forward, 5′-TCTCGAGCCCACCAGGAACGA-3′, reverse, 5′-AGGGAAGGCAGTGGCTGTCA-3′; IL-18—forward, 5′-GACCTGGAATCAGACAACTTTGG-3, reverse, 5′-GCC TCGGGTATTCTGTTATGGA-3′and GAPDH—forward, 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse, 5′- TGTAGA CCATGTAGTTGAGGTCA-3′.

Cell culture and treatment

Human renal proximal tubular epithelial cells, HK-2 (Chinese Academy of Science, Shanghai, China), were cultured in DMEM supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) in a humidified atmosphere containing 5% CO_2 at 37 °C. When cells reached 80% confluence, an HK-2 cell-based hypoxia-reoxygenation (H/R) model was established to simulate in vivo IR. The cells in H/R group were cultured for 12 h under $1\%O_2/94\%N_2/5\%CO_2$ conditions and in serum/glucose-free medium, followed by reoxygenation in normal complete medium and normoxic condition for 12 h. The cells in DZNeP-treated groups and SB203580-treated group were pretreated with DZNeP (10 μM) or SB203580 (10 μM), a selective inhibitor of p38 MAPK, for 12 h before the H/R treatment, respectively.

Apoptosis analysis

Apoptosis was measured using an annexin V-FITC/ propidium iodide (PI) staining kit and flow cytometry (Becton Dickinson). Cells were harvested, washed with ice-cold PBS, and fixed with 70% ethanol at − 20 °C for 1 h. Then, cells were incubated with fluorescein-conjugated annexin V and PI in the dark for 15 min at room temperature. The samples were analyzed by flow cytometry and apoptosis was quantified.

EZH2 knockdown with siRNA transfection

For RNAi-mediated knockdown of EZH2, HK-2 cells were grown to 60% confluence in a 6-well plates at a density of 2×10^5 cells/well and transiently transfected with siRNA targeting EZH2 using lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The other groups were transfected with blank siRNA as control. Thereafter, cells were supplemented with complete medium and incubated for 48 h.

EZH2 overexpression plasmid construction and cell transfection

The overexpression plasmid of EZH2 gene was synthesized by Mingshanshang Medical Biotechnology Corporation

(Guangzhou, China). The recombinant vectors were confirmed by the digestion analysis of restriction endonuclease. All the constructed plasmids were verified by DNA sequencing. The cells were seeded into 65 mm dishes (5×10^5 cells per well) and transfected at 80% confluence with pcDNA3.1-EZH2 (overexpression of EZH2 gene) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol.

Statistical analysis

Data were expressed as mean ± SEM. Multiple group comparisons were performed by ANOVA followed by the Bonferroni procedure for comparison of means. A P value < 0.05 was considered statistically significant.

Results

EZH2 is induced in the kidneys of mice following IR stress

A recent study has demonstrated that high expression of EZH2 in fibrotic kidneys of mice with unilateral ureteral obstruction [22]. Therefore, we first examined the level of EZH2 expression in kidneys tissue of mice following IR stress. We show that the protein levels of EZH2 were substantially induced in the kidneys of mice with IR compared with sham controls. On the contrary, DZNeP treatment markedly down-regulated the protein levels of EZH2 in the IR-treated kidneys of mice (Fig. 1a, b). Immunohistochemistry staining shows that EZH2 is localized in tubular epithelial cells after IR treatment (Fig. 1c).

Inhibition of EZH2 reduces IR-induced renal injury

DZNeP, a specific inhibitor of EZH2, is capable of inducing EZH2 degradation [23, 24]. We next investigated whether pharmacological inhibition EZH2 by DZNeP reduces renal dysfunction and tubular injury. We found that the BUN and creatinine were profoundly decreased in DZNeP-treated mice under IR condition compared with vehicle-treated mice (Fig. 2a, b). Consistent with the data of kidney function, treatment with DZNeP markedly reduced IR-induced renal histological injury of mice (Fig. 2c–e). These data suggest that inhibition of EZH2 alleviates IR-induced kidney dysfunction and reduces renal injury.

Inhibition of EZH2 represses apoptosis of tubular cells and caspase-3 activation

We next observed whether inhibition of EZH2 represses IR-induced tubular cells apoptosis. Using TUNEL assay and Western blot, we showed that the number of apoptotic cells and levels of cleaved caspase-3 was markedly elevated in the kidneys of mice following IR challenge. Conversely, administration of DZNeP significantly decreased the number of apoptotic cells and caspase-3 activation in the kidneys of mice with IR injury (Fig. 3a–d). These data suggest that inhibition of EZH2 protects renal tubular cells from IR-induced apoptosis by suppressing caspase-3 activation.

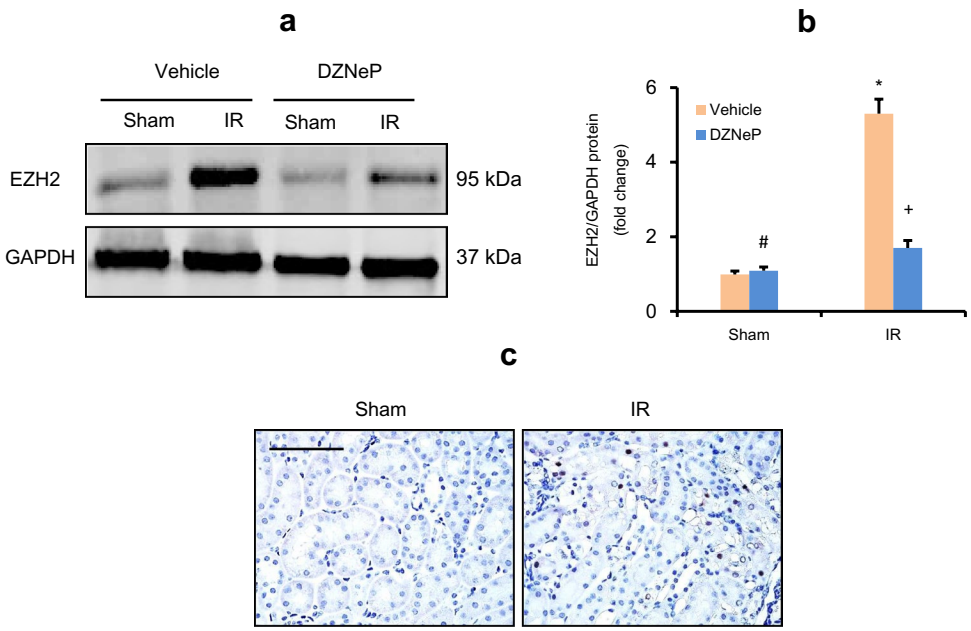

Fig. 1 EZH2 is induced in the kidneys after IR stress. **a** Representative Western blot shows EZH2 protein levels in the kidneys of vehicle-treated or DZNeP-treated mice after sham or IR treatment. **b** Quantitative analysis of EZH2 protein expression. **c** Representative photomicrographs of kidney sections stained for EZH2 (brown) and counterstained with hematoxylin (blue) in the kidneys of sham-treated or IR-treated mice. $^*P < 0.05$ vs. vehicle-sham, $^{\#}P < 0.05$ vs. DZNeP-IR; $^{+}P < 0.05$ vs. vehicle-IR. $n = 6$ in each group. IR, ischemia–reperfusion injury; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Scale bar: 50 μm. (Color figure online)

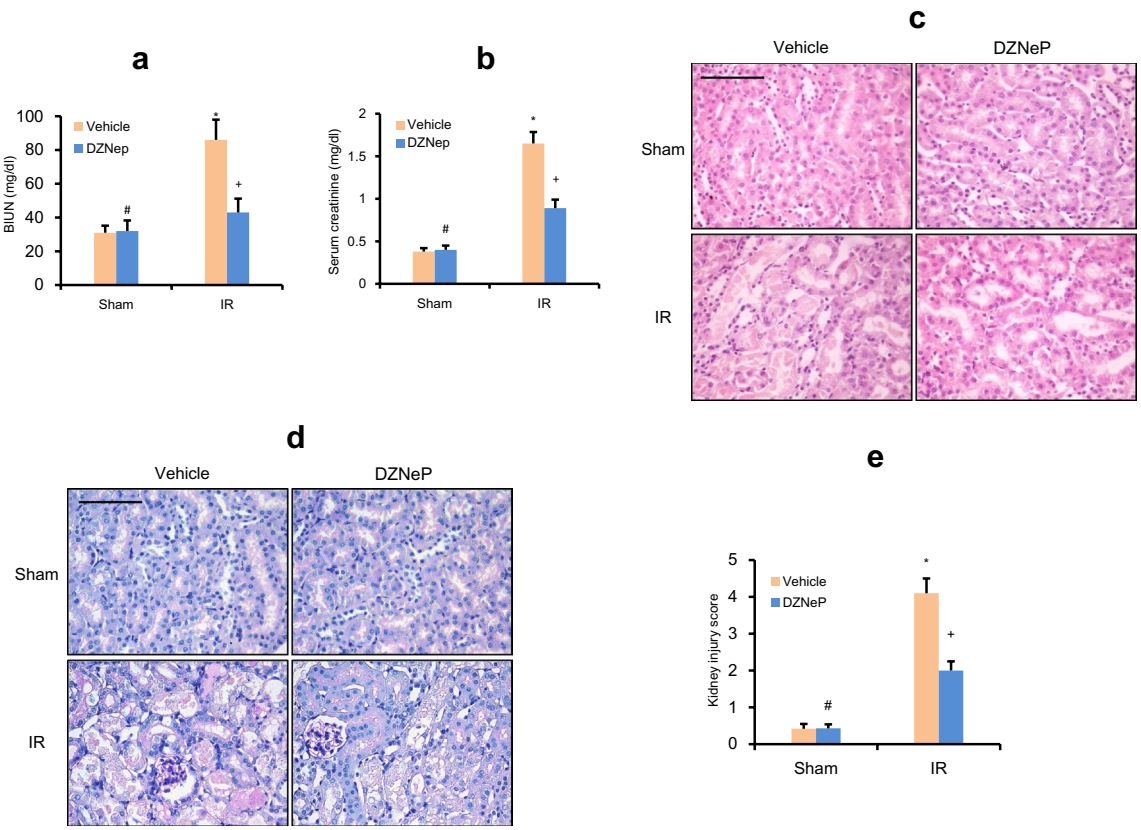

Fig. 2 Inhibition of EZH2 reduces IR-induced renal injury. **a, b** The levels of serum BUN and creatinine in vehicle-treated or DZNeP-treated mice after sham or IR treatment. **c, d** Representative photomicrographs of H&E staining and PAS staining for kidney sections from vehicle-treated or DZNeP-treated mice after sham or IR treatment. **e** Quantitative assessment of renal tubular injury. $^{*}P<0.05$ vs. vehicle-sham, $^{\#}P<0.05$ vs. DZNeP-IR; $^{+}P<0.05$ vs. vehicle-IR. n = 6 in each group. IR, ischemia–reperfusion injury. Scale bar: 50 μm

Inhibition of EZH2 impairs inflammation cells infiltration and proinflammatory molecules release

Inflammatory response is a major contributor to IR-induced AKI [19, 25]. After DZNeP treatment, the IR-treated kidneys of mice displayed a significant reduction of CD3 positive cells and F4/80-positive cells (Fig. 4a–d). In addition, treatment with DZNeP considerably decreased the mRNA levels of TNF-α, MCP-1, IL-6, and IL-18 in the kidneys of mice under IR condition (Fig. 4e-h). These data indicate that EZH2 inhibition impairs inflammatory response in the IR-treated kidneys of mice.

Inhibition of EZH2 decreases p38 phosphorylation in IR-treated kidneys

A study shows that EZH2 inhibition decreases p38 signaling [15]. We then investigated whether EZH2 inhibition decreases p38 phosphorylation. We showed that the levels of p38 phosphorylation in IR-treated kidneys of mice were significantly decreased after DZNeP administration compared with vehicle-treated mice (Fig. 5a, b). These data suggest that inhibition of EZH2 suppresses IR-induced p38 phosphorylation in kidneys.

DZNeP treatment and EZH2 knockdown reduce apoptosis of HK-2 cells induced by H/R

We show that H/R treatment obviously increased apoptosis rate of HK-2 cells, whereas DZNeP treatment considerably decreased cells apoptosis induced by H/R injury (Fig. 6a, b). In agreement with the results of DZNeP, we reveal that EZH2 knockdown significantly reduced cell apoptosis (Fig. 6c, d). These data suggest that the inhibition of EZH2 suppresses IR-induced cells apoptosis.

DZNeP treatment and EZH2 knockdown inhibit p38 phosphorylation, caspase-3 activation, and proinflammatoryproinflammatory molecules release

Our results show that DZNeP treatment down-regulated the levels of EZH2 protein (Fig. 7a, b). In agreement with

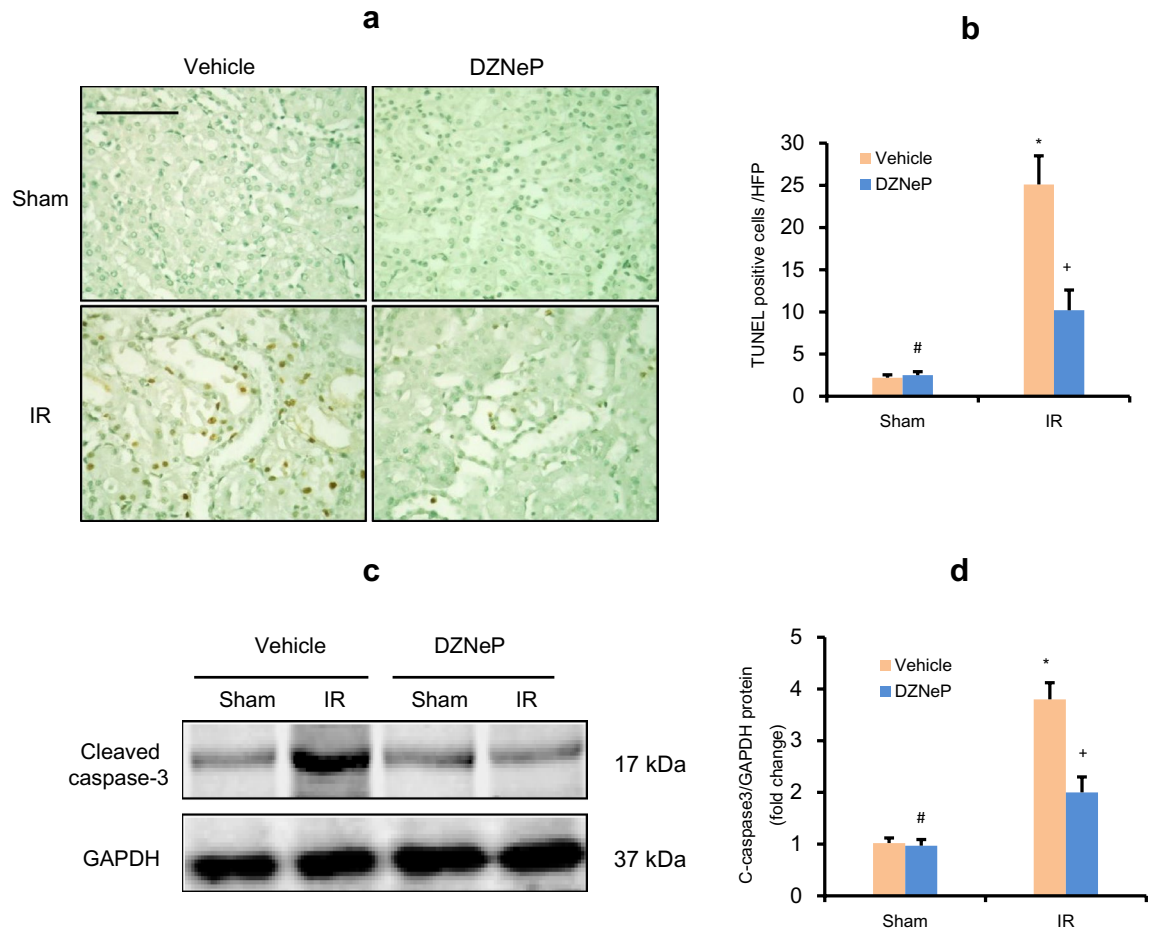

Fig. 3 Inhibition of EZH2 suppresses apoptosis of tubular cells and the activation of caspase-3. **a** Representative photomicrographs of kidney sections stained for apoptotic cells (brown) and counterstained with methylgreen (green) in the kidneys of vehicle-treated or DZNeP-treated mice after sham treatment or IR treatment. **b** Quantitative analysis of apoptotic cells. **c** Representative Western blot shows cleaved caspase-3 protein levels in vehicle-treated or DZNeP-treated mice after sham or IR treatment. **d** Quantitative analysis of cleaved caspase-3 expression. $^{*}P<0.05$ vs. vehicle–sham, $^{\#}P<0.05$ vs. DZNeP-IR; $^{+}P<0.05$ vs. vehicle-IR. $n=6$ in each group. IR, ischemia–reperfusion injury; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Scale bar: 50 μm. (Color figure online)

mouse model, we revealed that H/R treatment profoundly increased the levels of p38 phosphorylation, whereas DZNeP treatment markedly reduced p38 phosphorylation in H/R-treated HK-2 cells (Fig. 7a, c). We also show that EZH2 knockdown substantially decreased EZH2 protein expression and p38 phosphorylation in H/R-treated HK-2 cells (Fig. 7e–g). Our results revealed that administration of DZNeP suppressed caspase-3 activation and proinflammatory molecules release in H/R-treated HK-2 cells (Fig. 7a, c, d). Consistent with the results of DZNeP treatment, EZH2 knockdown inhibited cleaved caspase-3 up-regulation and proinflammatory molecules production in H/R-treated HK-2 cells (Fig. 7e, g, h). These findings suggest that inhibition of EZH2 suppresses IR-induced p38 phosphorylation, caspase-3 activation, and proinflammatory molecules release.

EZH2 regulates caspase-3 activation and proinflammatory molecules released by targeting p38 in H/R-induced in HK-2 cells

To clarify whether EZH2 regulates apoptosis and inflammation by targeting p38 signaling, we next investigated the effects of EZH2 overexpression on p38 signaling in H/R-treated HK-2 cells. The expression of EZH2 in cells treated with pcDNA-EZH2 was determined using Western blot analysis (Fig. 8a, b). We showed that EZH2 overexpression up-regulated the levels of p38 phosphorylation in H/R-treated HK-2 cells (Fig. 8c, d). Of note, SB203580 treatment abolished H/R-induced caspase-3 activation in the context of EZH2 overexpression (Fig. 8e, f). We also show that EZH2 overexpression increased the production of TNF-α and IL-6 in H/R-treated HK-2 cells, whereas administration of SB203580 reversed proinflammatory molecules release

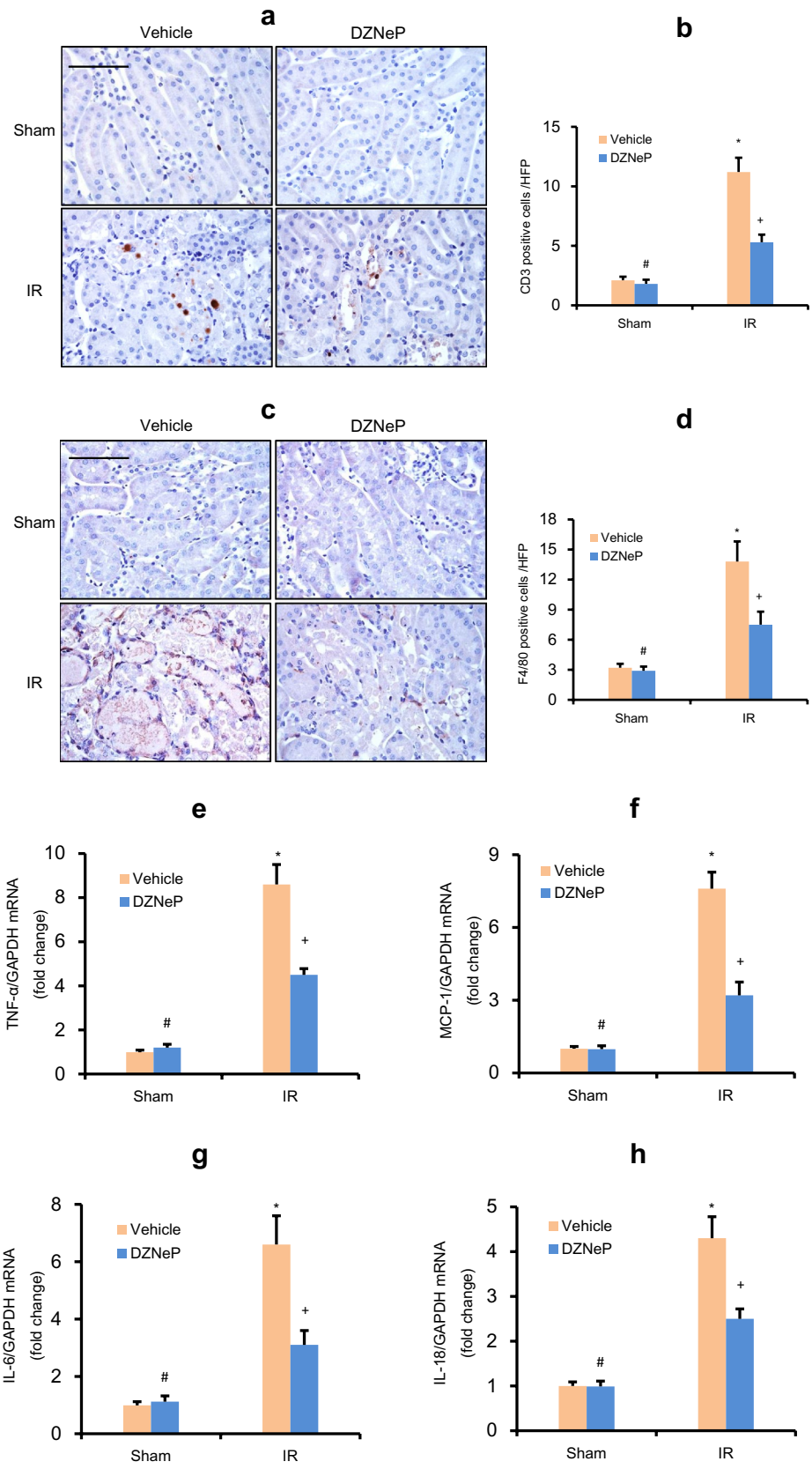

Fig. 4 Inhibition of EZH2 impairs inflammation cells infiltration and down-regulates proinflammatory gene expression. **a** Representative photomicrographs of kidney sections stained for CD3 (brown) and counterstained with hematoxylin (blue) in vehicle-treated or DZNeP-treated mice after sham or IR treatments. **b** Quantitative analysis of $CD3^+$-positive T cells. **c** Representative photomicrographs of kidney sections stained for F4/80 (brown) and counterstained with hematoxylin (blue) in the kidneys of vehicle-treated or DZNeP-treated mice after sham or IR treatment. **d** Quantitative analysis of F4/80 positive macrophages. **e–h** Quantitative analysis of TNF-α, MCP-1, IL-6, and IL-18 mRNA expression in the kidneys. $^*P<0.05$ vs. vehicle–sham, $^{\#}P<0.05$ vs. DZNeP-IR; $^{+}P<0.05$ vs. vehicle-IR. $n=6$ in each group. IR, ischemia–reperfusion injury; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Scale bar: 50 μm

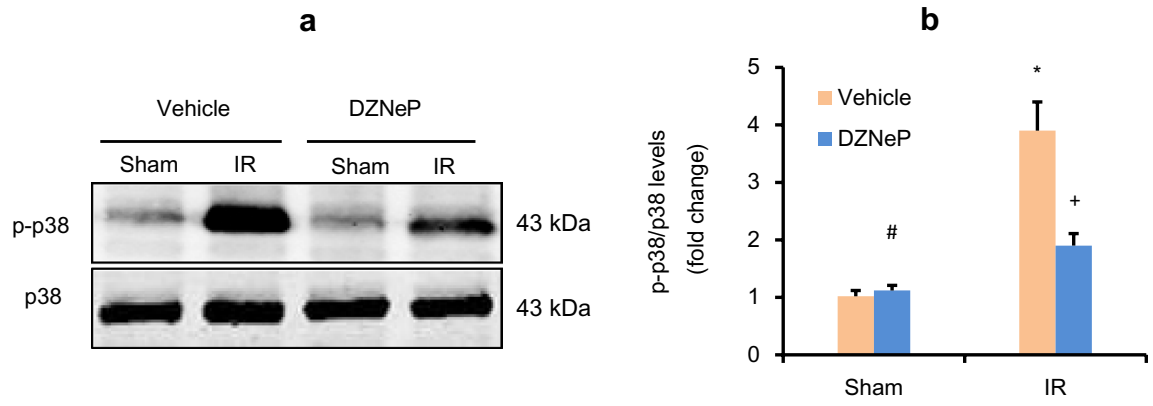

Fig. 5 Inhibition of EZH2 decreases p38 phosphorylation in IR-treated kidneys. **a** Representative western blot shows p38 phosphorylation in the kidneys of vehicle-treated or DZNeP-treated mice after sham or IR treatments. **b** Quantitative analysis of the levels of p38 phosphorylation. $^{*}P<0.05$ vs. vehicle–sham, $^{\#}P<0.05$ vs. DZNeP-IR; $^{+}P<0.05$ vs. vehicle-IR. $n=6$ in each group

(Fig. 8g, h). These data implicate that EZH2 regulates caspase-3 activation and proinflammatory molecules released by targeting p38 signaling.

Discussion

In light of the steadily increasing AKI epidemic, a better understanding of molecular mechanisms that are affected to develop potential novel therapeutic strategies is extremely required [3]. The histone methyltransferase EZH2 is a member of polycomb repressive complex 2, which is specifically responsible for the trimethylation of H3K27me3, a well-known histone mark involved in transcriptional repression of target genes [26, 27]. Mounting evidences have documented that EZH2 functions as a double-facet molecule in tumorigenesis as an oncogene and tumor suppressor gene, depending on the different cellular contexts [28, 29]. Recently, accumulating data implicate that EZH2 has been involved in the pathogenesis of chronic renal fibrosis. Zhou et al. have observed that unilateral ureteral obstruction induces high expression of EZH2 in fibrotic kidneys of mice and inhibition of EZH2 by DZNeP impedes the progression of renal fibrosis [22, 30]. Moreover, EZH2 inhibition exerts a protective role against acute kidney injury via attenuation of the Raf-1/ERK1/2 pathway [14]. In this work, we demonstrate that pharmacological inhibition of EZH2 by DZNeP reduces kidney dysfunction and diminishes renal tubular injury via the regulation of apoptosis and inflammatory response. Of note, EZH2 inhibition attenuates IR-induced AKI by inactivating p38 signaling.

A large number of studies have demonstrated that apoptosis of tubular epithelial cells during IR stress exacerbates AKI, resulting in kidney dysfunction [31, 32]. In the present study, our findings reveal that IR insult leads to a significant increment of apoptotic cells in the kidneys of mice. Conversely, treatment with DZNeP markedly decreases apoptotic cells in the kidneys. Simultaneously, we show that the activation of caspase-3 is significantly induced in the kidneys of mice with IR injury, whereas DZNeP treatment obviously impairs the activation of caspase-3. Consistent with the results of mice, DZNeP treatment or EZH2 knockdown reduces apoptosis rates and caspase-3 activation in H/R-treated HK-2 cells. These data indicate that EZH2 inhibition suppresses the apoptosis of tubular cells through regulation of caspase-3 activation in IR-induced AKI.

Infiltrating inflammatory cells have been implicated to play critical roles in the initiation and development of kidney dysfunction and tubular injury during IR-induced AKI [33, 34]. In the current study, we show that IR stress substantially promoted infiltration of T cells and macrophages into the kidneys. By contrast, the kidneys of mice treated with DZNeP present a markedly reduction of these inflammatory cells. These data suggest that EZH2 inhibition impairs T cells and macrophage infiltration into the kidney of mice in response to IR injury. IR-induced AKI is also manifested by the release of a series of proinflammatory cytokines and chemokines such as TNF-α, MCP-1, IL-6, and IL-18 [34–36]. These proinflammatory cytokines and chemokines causing tubular injury and apoptosis have been regarded as the major cause of IR-induced AKI [18, 35]. There is evidence that EZH2 mediates proinflammatory gene expression. The absence of EZH2 diminishes the cytokine secretion and reduces inflammatory response in colitis and experimental autoimmune encephalomyelitis [17]. In addition, a study has reported that the inhibition of EZH2 suppresses gene expression of proinflammatory cytokine such as IL-6 and TNF-α in a mouse model of colitis-associated cancer [16]. In this study, our results show the gene expressions of TNF-α, MCP-1, IL-6, and IL-18 are increased considerably in the

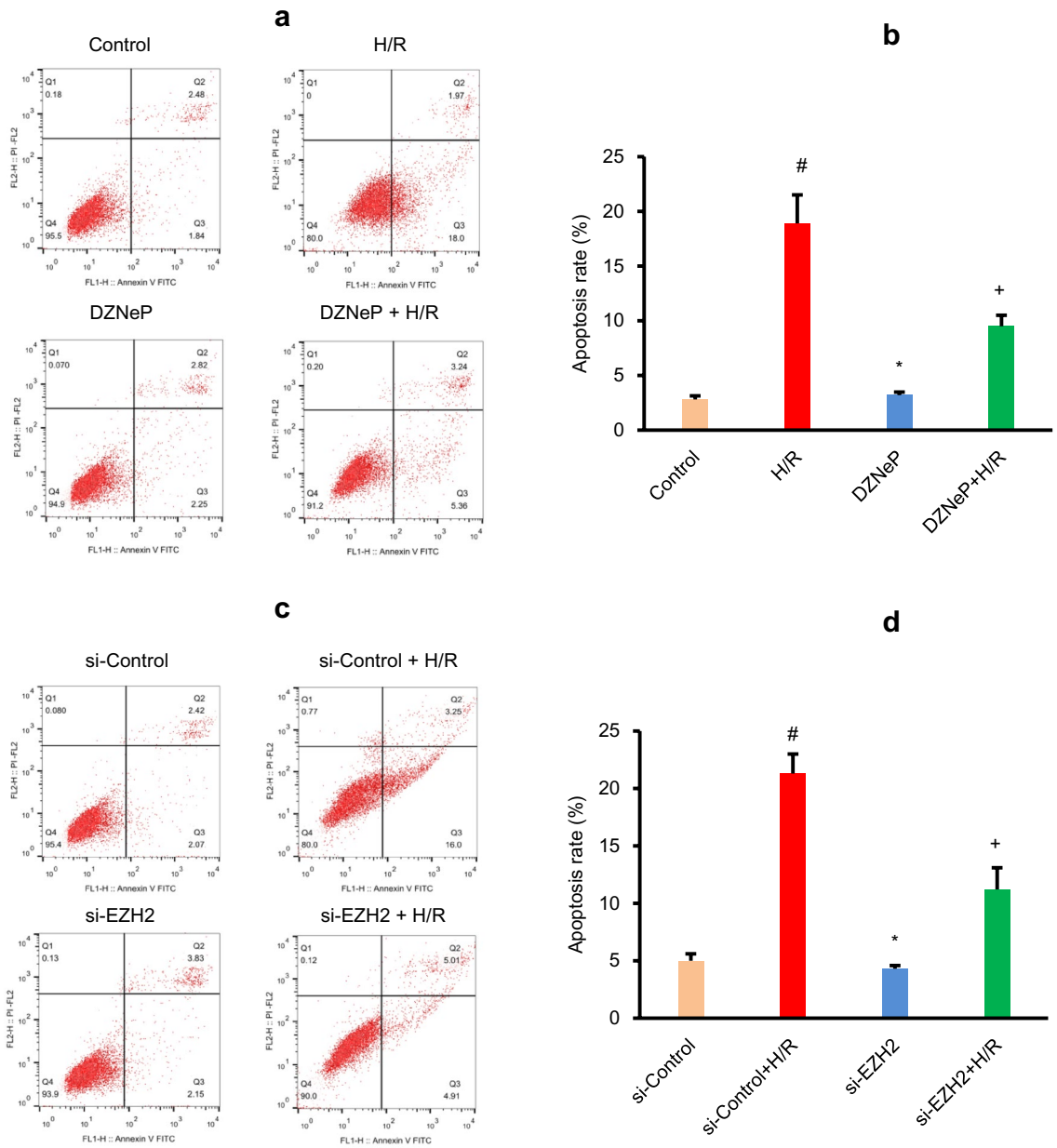

Fig. 6 Inhibition of EZH2 reduces H/R-induced apoptosis of HK-2. **a, b** Representative photomicrographs of flow cytometry show apoptosis and quantitative analysis of apoptosis rates in vehicle-treated or DZNeP-treated HK-2 cells with or without H/R treatment. **c, d** Representative photomicrographs of flow cytometry show apoptosis and quantitative analysis of apoptosis rates in HK-2 cells of EZH2 knockdown with or without H/R treatments. $^{\#}P<0.05$ vs. Control group or si-Control group; $^{*}P<0.05$ vs. DZNeP + H/R group or si-EZH2 + H/R group; $^{+}P<0.05$ vs. or H/R group or si-Control + H/R group

kidneys after IR treatment, whereas application of DZNeP profoundly reduces these gene expressions in the kidneys. In agreement with the results of mouse IR model, DZNeP treatment or EZH2 knockdown decreases proinflammatory production in H/R-treated HK-2 cells. These data implicate that EZH2 inhibition diminishes the levels of proinflammatory molecules during IR-induced AKI.

The p38 MAPK signaling plays a key role in the regulation of apoptosis and inflammatory response [37, 38]. Recent data implicate that EZH2 regulates the activation of p38 signaling pathway in cancer cells [15]. In this study, we reveal that inhibition of EZH2 decreases p38 phosphorylation in IR-treated kidneys and H/R-treated tubular epithelial cells. EZH2

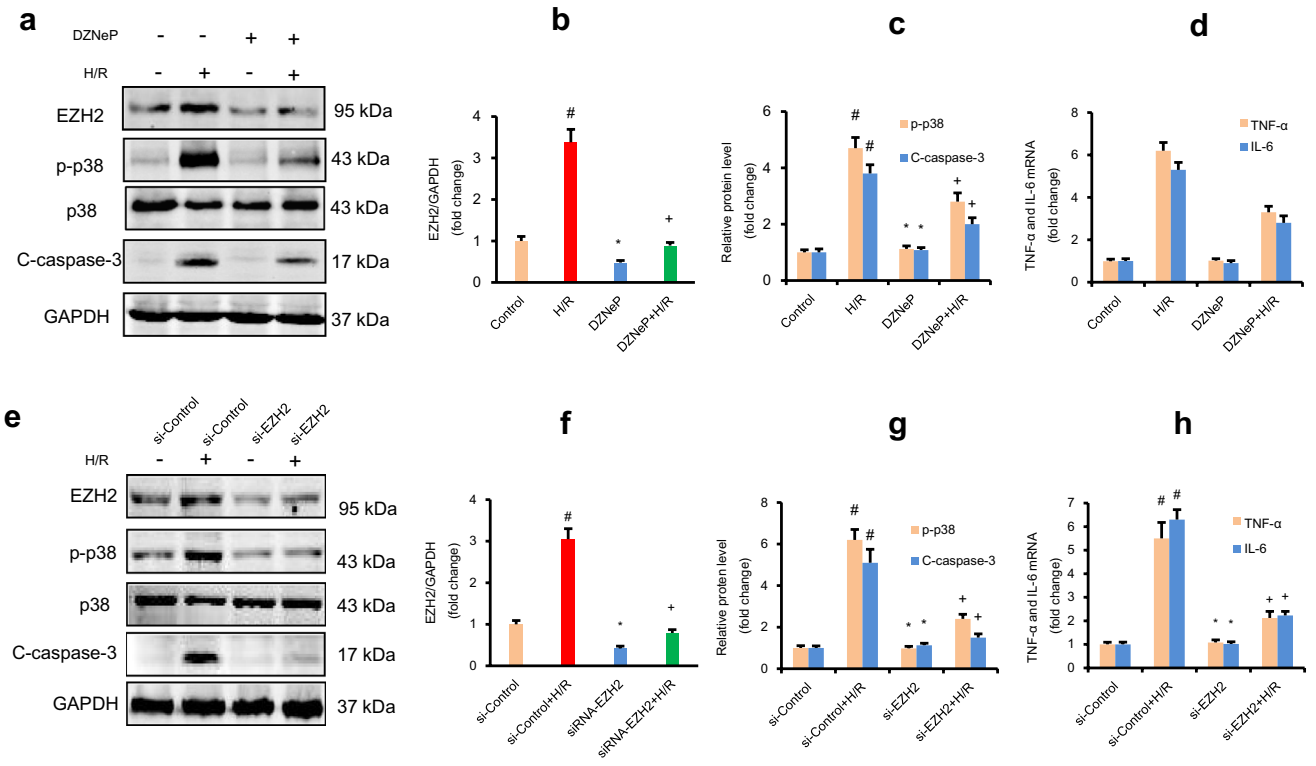

Fig. 7 Inhibition of EZH2 down-regulates p38 phosphorylation, caspase-3 activation, and proinflamamtory molecules release. **a**–**c** Representative Western blots and quantitative analysis of levels of EZH2, p38 phosphorylation, and cleaved caspase-3 in vehicle-treated or DZNeP-treated HK-2 cells with or without H/R treatments. **d** Quantitative analysis of TNF-α and IL-6 mRNA expression in vehicle-treated or DZNeP-treated HK-2 cells with or without H/R treatments. **e**–**g** Representative Western blots and quantitative analysis of levels of EZH2, p38 phosphorylation, and cleaved caspase-3 in si-Control-treated or si-EZH2-treated HK-2 cells with or without H/R treatments. **h** Quantitative analysis of TNF-α and IL-6 mRNA expression in si-Control-treated or si-EZH2-treated HK-2 cells with or without H/R treatments. $^{\#}P<0.05$ vs. Control group or si-Control group; $^{*}P<0.05$ vs. DZNeP + H/R group or si-EZH2 + H/R group; $^{+}P<0.05$ vs. or H/R group or si-Control + H/R group

overexpression up-regulates the levels of p38 phosphorylation in H/R-treated HK-2 cells. Of note, SB203580 treatment reverses H/R-induced caspase-3 activation and proinflammatoryproinflammatory molecule production in the context of EZH2 overexpression. These data suggest that EZH2 regulates apoptosis and inflammation during IR-induced AKI by targeting p38 signaling.

Collectively, we demonstrate that EZH2 plays a crucial role in ischemia–reperfusion-induced acute kidney injury via the regulation of p38 signaling. Targeting EZH2/p38 signaling pathway may offer novel strategies to protect kidneys from acute kidney injury induced by ischemia–reperfusion.

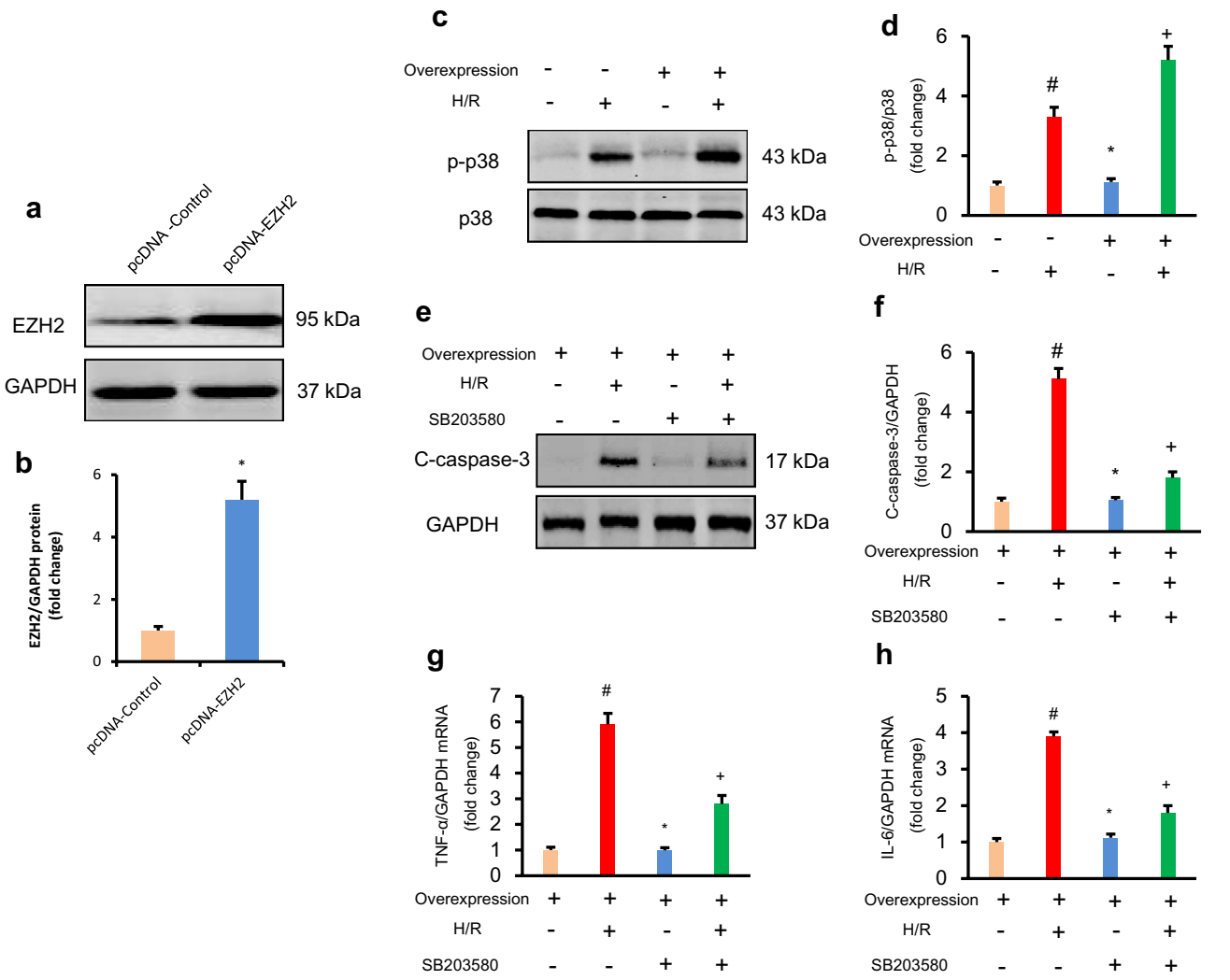

Fig. 8 EZH2 regulates caspase-3 activation and proinflammatorypro-inflammatory molecules release by targeting p38. **a, b** Representative Western blots of EZH2 and quantitative analysis of protein levels in the context of EZH2 overexpression. **c** Representative Western blots show p38 phosphorylation in H/R-treated HK-2 cells in the context of EZH2 overexpression. **d** Quantitative analysis of the levels of p38 phosphorylation in HK-2 cells. **e** Representative Western blots show cleaved caspase-3 in H/R-treated HK-2 cells in the context of EZH2 overexpression. **f** Quantitative analysis of the levels of cleaved caspase-3 in the HK-2 cells. **g, h** Quantitative analysis of TNF-α and IL-6 mRNA expression in H/R-treated HK-2 cells in the context of EZH2 overexpression. $^{\#}P<0.05$ vs. Control group; $^{*}P<0.05$ vs. Overexpression + H/R group or Overexpression + H/R + SB203580 group; $^{+}P<0.05$ vs. H/R group

Acknowledgements This work was supported by Natural Science Foundation of Guangdong Province (2018A030313613) and Guangdong Province Medical Science Technology Investigation Project of China (A2017032 and A2018016).

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

References

1. Hobson C, Ruchi R, Bihorac A. Perioperative acute kidney injury: risk factors and predictive strategies. Crit Care Clin. 2017;33(2):379–96.
2. Meersch M, Schmidt C, Zarbock A. Perioperative Acute kidney injury: an under-recognized problem. Anesth Analg. 2017;125(4):1223–32.
3. Rewa O, Bagshaw SM. Acute kidney injury-epidemiology, outcomes and economics. Nat Rev Nephrol. 2014;10(4):193–207.
4. He L, Wei Q, Liu J, Yi M, Liu Y, Liu H, Sun L, Peng Y, Liu F, Venkatachalam MA, et al. AKI on CKD: heightened injury, suppressed repair, and the underlying mechanisms. Kidney Int. 2017;92(5):1071–83.

5. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest. 2011;121(11):4210–21.
6. Thurman JM. Triggers of inflammation after renal ischemia/reperfusion. Clin Immunol. 2007;123(1):7–13.
7. Ying Y, Kim J, Westphal SN, Long KE, Padanilam BJ. Targeted deletion of p53 in the proximal tubule prevents ischemic renal injury. J Am Soc Nephrol. 2014;25(12):2707–16.
8. Sharfuddin AA, Molitoris BA. Pathophysiology of ischemic acute kidney injury. Nat Rev Nephrol. 2011;7(4):189–200.
9. Eltzschig HK, Eckle T. Ischemia and reperfusion—from mechanism to translation. Nat Med. 2011;17(11):1391–401.
10. Wang Y, Yan L, Zhang Z, Prado E, Fu L, Xu X, Du L. Epigenetic regulation and its therapeutic potential in pulmonary hypertension. Front Pharmacol. 2018;9:241.
11. Stoll S, Wang C, Qiu H. DNA Methylation and Histone modification in hypertension. Int J Mol Sci. 2018; 19(4).
12. Mar D, Gharib SA, Zager RA, Johnson A, Denisenko O, Bomsztyk K. Heterogeneity of epigenetic changes at ischemia/reperfusion- and endotoxin-induced acute kidney injury genes. Kidney Int. 2015;88(4):734–44.
13. Jones BA, Varambally S, Arend RC. Histone methyltransferase EZH2: a therapeutic target for ovarian cancer. Mol Cancer Ther. 2018;17(3):591–602.
14. Zhou X, Zang X, Guan Y, Tolbert T, Zhao TC, Bayliss G, Zhuang S. Targeting enhancer of zeste homolog 2 protects against acute kidney injury. Cell death disease. 2018;9(11):1067.
15. Moore HM, Gonzalez ME, Toy KA, Cimino-Mathews A, Argani P, Kleer CG. EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis. Breast Cancer Res Treat. 2013;138(3):741–52.
16. Cascio S, Faylo JL, Sciurba JC, Xue J, Ranganathan S, Lohmueller JJ, Beatty PL, Finn OJ. Abnormally glycosylated MUC1 establishes a positive feedback circuit of inflammatory cytokines, mediated by NF-kappaB p65 and EzH2, in colitis-associated cancer. Oncotarget. 2017;8(62):105284–98.
17. Zhang X, Wang Y, Yuan J, Li N, Pei S, Xu J, Luo X, Mao C, Liu J, Yu T, et al. Macrophage/microglial Ezh2 facilitates autoimmune inflammation through inhibition of Socs3. J Exp Med. 2018;215(5):1365–82.
18. Liang H, Liao M, Zhao W, Zheng X, Xu F, Wang H, Huang J. CXCL16/ROCK1 signaling pathway exacerbates acute kidney injury induced by ischemia–reperfusion. Biomed Pharmacother. 2018;98:347–56.
19. Jin X, Chen J, Hu Z, Chan L, Wang Y. Genetic deficiency of adiponectin protects against acute kidney injury. Kidney Int. 2013;83(4):604–14.
20. Liang H, Xu F, Wen XJ, Liu HZ, Wang HB, Zhong JY, Yang CX, Zhang B. Interleukin-33 signaling contributes to renal fibrosis following ischemia reperfusion. Eur J Pharmacol. 2017;812:18–27.
21. Liang H, Zhang Z, Yan J, Wang Y, Hu Z, Mitch WE, Wang Y. The IL-4 receptor alpha has a critical role in bone marrow-derived fibroblast activation and renal fibrosis. Kidney Int. 2017;92(6):1433–43.
22. Zhou X, Zang X, Ponnusamy M, Masucci MV, Tolbert E, Gong R, Zhao TC, Liu N, Bayliss G, Dworkin LD, et al. Enhancer of zeste homolog 2 inhibition attenuates renal fibrosis by maintaining Smad7 and phosphatase and tensin homolog expression. J Am Soc Nephrol. 2016;27(7):2092–108.
23. Crea F, Fornaro L, Bocci G, Sun L, Farrar WL, Falcone A, Danesi R. EZH2 inhibition: targeting the crossroad of tumor invasion and angiogenesis. Cancer Metastasis Rev. 2012;31(3–4):753–61.
24. Momparler RL, Cote S. Targeting of cancer stem cells by inhibitors of DNA and histone methylation. Expert Opin Investig Drugs. 2015;24(8):1031–43.
25. Yang Y, Zhang ZX, Lian D, Haig A, Bhattacharjee RN, Jevnikar AM. IL-37 inhibits IL-18-induced tubular epithelial cell expression of pro-inflammatory cytokines and renal ischemia–reperfusion injury. Kidney Int. 2015;87(2):396–408.
26. Yamagishi M, Uchimaru K. Targeting EZH2 in cancer therapy. Curr Opin Oncol. 2017;29(5):375–81.
27. Italiano A. Role of the EZH2 histone methyltransferase as a therapeutic target in cancer. Pharmacol Ther. 2016;165:26–31.
28. Safaei S, Baradaran B, Hagh MF, Alivand MR, Talebi M, Gharibi T, Solali S. Double sword role of EZH2 in leukemia. Biomed Pharmacother. 2018;98:626–35.
29. Yan KS, Lin CY, Liao TW, Peng CM, Lee SC, Liu YJ, Chan WP, Chou RH. EZH2 in cancer progression and potential application in cancer therapy: a friend or foe? Int J Mol Sci. 2017; 18(6).
30. Zhou X, Xiong C, Tolbert E, Zhao TC, Bayliss G, Zhuang S. Targeting histone methyltransferase enhancer of zeste homolog-2 inhibits renal epithelial-mesenchymal transition and attenuates renal fibrosis. FASEB J. 2018:fj201800237R.
31. Ortiz A, Justo P, Sanz A, Lorz C, Egido J. Targeting apoptosis in acute tubular injury. Biochem Pharmacol. 2003;66(8):1589–94.
32. Saikumar P, Venkatachalam MA. Role of apoptosis in hypoxic/ischemic damage in the kidney. Semin Nephrol. 2003;23(6):511–21.
33. Jang HR, Rabb H. Immune cells in experimental acute kidney injury. Nat Rev Nephrol. 2015;11(2):88–101.
34. Fukazawa K, Lee HT. Volatile anesthetics and AKI: risks, mechanisms, and a potential therapeutic window. J Am Soc Nephrol. 2014;25(5):884–92.
35. Nishikawa H, Taniguchi Y, Matsumoto T, Arima N, Masaki M, Shimamura Y, Inoue K, Horino T, Fujimoto S, Ohko K, et al. Knockout of the interleukin-36 receptor protects against renal ischemia–reperfusion injury by reduction of proinflammatory cytokines. Kidney Int. 2018;93(3):599–614.
36. Wu H, Craft ML, Wang P, Wyburn KR, Chen G, Ma J, Hambly B, Chadban SJ. IL-18 contributes to renal damage after ischemia–reperfusion. J Am Soc Nephrol. 2008;19(12):2331–41.
37. Yong HY, Koh MS, Moon A. The p38 MAPK inhibitors for the treatment of inflammatory diseases and cancer. Expert Opin Investig Drugs. 2009;18(12):1893–905.
38. Peti W, Page R. Molecular basis of MAP kinase regulation. Protein Sci. 2013;22(12):1698–710.