ER stress triggers MCP-1 expression through SET7/9-induced histone methylation in the kidneys of db/db mice

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Chen J, Guo Y, Zeng W, Huang L, Pang Q, Nie L, Mu J, Yuan F, Feng B. ER stress triggers MCP-1 expression through SET7/9-induced histone methylation in the kidneys of db/db mice. Am J Physiol Renal Physiol 306: F916–F925, 2014. First published January 22, 2014; doi:10.1152/ajprenal.00697.2012.—Epigenetics plays a key role in the pathogenesis of diabetic nephropathy (DN), although the precise regulatory mechanism is still unclear. Here, we examined the role of endoplasmic reticulum (ER) stress in histone H3 lysine 4 (H3K4) methyltransferase SET7/9-induced monocyte chemoattractant protein-1 (MCP-1) expression in the kidneys of db/db mice. Our results indicate that the expression of MCP-1 significantly increased in the kidneys of db/db mice in a time-dependent manner. An increased chromatin mark associated with an active gene (H3K4me1) at MCP-1 promoters accompanied this change in expression. The expression of SET7/9 and the recruitment to these promoters were also elevated. SET7/9 gene silencing with small interfering (si) RNAs significantly attenuated the expression of H3K4me1 and MCP-1. Furthermore, expression of signaling regulator glucose-regulated protein 78 (GRP78), a monitor of ER stress, significantly increased in the kidneys of db/db mice. The expression of spliced X-box binding protein 1 (XBP1s), an ER stress-inducible transcription factor, and recruitment to the SET7/9 promoters were also increased. XBP1s gene silencing with siRNAs significantly attenuated the expression of SET7/9, H3K4me1, and MCP-1. The chaperone betaine not only effectively downregulated the GRP78 and XBP1s expression levels but also markedly decreased the SET7/9, H3K4me1, and MCP-1 levels. Luciferase reporter assay demonstrated that XBP1s participated in ER stress-induced SET7/9 transcription. Taken together, these results reveal that ER stress can trigger the expression of MCP-1, in part through the XBP1s-mediated induction of SET7/9.

Diabetic nephropathy; ER stress; epigenetics; MCP-1

Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes mellitus (DM) and has become the principal cause of end-stage renal disease (ESRD) in the Western world. Increasing evidence suggests that complex interactions between hemodynamic and metabolic factors may play a major role in the pathogenesis of DN, although the precise mechanisms are unknown. In recent years, inflammation has emerged as being a key pathogenic mechanism of DN. Disordered metabolism and hemodynamics trigger the activation of numerous inflammatory molecules and proinflammatory cytokines that may be critical factors in the development of DN (19, 25). Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C class of the β chemokine family and is one of the key factors involved in the initiation of inflammation. Experimental studies have demonstrated that MCP-1-mediated macrophage accumulation and activation is a critical mechanism in the development of DN (3, 32, 33). Thus, exploring the activation mechanism of MCP-1 in DN is very important for developing new strategies to suppress the development of DN.

Epigenetic modifications have been found to play an important role in altering gene expression patterns. This gene-environment interaction involving epigenetic changes may be particularly relevant to the pathogenesis of diabetes complications (16, 18, 29). Recent research has demonstrated that epigenetic histone modifications play a key role in diabetes and its complications (21, 22, 23, 26). Further studies have indicated that histone modifications lead to sustained activation of pro-inflammatory pathways, which are likely to participate in the progression of diabetic complications (1, 34). Earlier reports have identified SET7/9 as a histone lysine methyltransferase which generates monomethylation at histone 3 lysine 4 (7). Several studies found that SET7/9 and the associated H3K4me are involved in the regulation of inflammatory genes (2, 14). In addition, endoplasmic reticulum (ER) stress is also increasingly recognized as an important mechanism in the pathogenesis of DM and diabetic vascular complications (9, 11, 15, 17). It has been shown that the ER mediates a specific set of intracellular signal pathways. These signal pathways and inflammation are interconnected with each other. All this evidence demonstrates that both ER stress and epigenetics play important roles in the induction of inflammatory response, although it is not clear whether ER stress and epigenetic modifications have a direct molecular link.

In this report, we demonstrate that ER stress leads to the enrichment of H3K4me1 at proinflammatory cytokine MCP-1 promoters in the kidneys of db/db mice through the preferential induction of SET7/9. To elucidate the underlying mechanisms involved, we focused this investigation on the role of the ER stress-inducible transcription factor XBP1s in the ER stress-induced activation of SET7/9. Our data indicate that XBP1s is involved in the activation of SET7/9. Furthermore, XBP1s or SET7/9 gene silencing with siRNAs significantly reverses the ER stress-induced activation of SET7/9, H3K4me1, and MCP-1. These data demonstrate that ER stress triggers MCP-1 expression through SET7/9-induced histone methylation in the kidneys of db/db mice.

Materials and Methods

Animals. All animal experimental protocols were approved by the Animal Experiments Ethics Committee of the Third Military Medical University. All mice were purchased from the Nanjing University Animal Experiment Center in the People’s Republic of China. Male db/db mice (initial weight, 20–26 g; average age, 4 wk) were randomly divided into two groups: the diabetic nephropathy group (DN;
Blood urea nitrogen (BUN) with an automatic biochemistry analyzer for determination of blood glucose (BG), serum creatinine (Scr), and drawn from the heart at the end of containing protease inhibitors, and the cortices were weighed and used. All the kidneys were cut into two portions, with one part fixed in 10% neutral formaldehyde were embedded in paraffin blocks. Histo-logical sections of the kidneys were stained with PAS (the mesangial area stains purple), and the slides were examined using light microscopy. The sequences for XBP1s were 5'-GAGTCCGCAGCAGGTG-3' (sense) and 5'-GTGTCAGAGTCCATGGGA-3' (antisense). The primer sequences for SET7/9 were 5'-AGTTAAACGCCCACTCACC-3' (sense) and 5'-TGGTTCCGATCCAGGGTTT-3' (antisense). The SET7/9 primers were 5'-GTCGGGGAAGTCAATGAAGA-3' (sense) and 5'-GCCACTCTTCATTCGAG-3' (antisense). siRNA knockdown. XBPI and SET7/9 siRNA were synthesized, high-performance purified (Qiagen, Valencia, CA), and dissolved in RNase-free PBS. A nonsilencing siRNA oligonucleotide sequence that does not recognize any known homology to mammalian genes was also generated as a negative control. The target sequence used for XBPI was 5'-ATGCCAATGAACTCTCCAG-3' (antisense). The target sequence for SET7/9 was 5'-AACCUCGGGAGACGUGCA-3'. The control target sequence was 5'-AAUCGCAUAGCGUAUGCCGUU-3'. Male ddY mice (initial weight, 20–26 g; average age, 4 wk) were randomly allocated into groups (6 mice/group) receiving XBPI, SET7/9, or control siRNA (50 μg in 1 ml PBS) or 1 ml normal saline alone once every 10 days for 40 days. After the total urine volume (ml) was measured, six mice from each group were killed at the end of 4, 8, and 12 wk after treatment began, and the kidneys were harvested. All the kidneys were cut into two portions, with one part fixed in 10% formalin for periodic acid-Schiff (PAS) staining. The remaining portions stored in liquid nitrogen were washed thoroughly with PBS containing protease inhibitors, and the cortices were weighed and used for RNA or protein extraction.

Biochemical analysis and urinary metabolites. Blood samples were drawn from the heart at the end of weeks 4, 8, and 12 and were used for determination of blood glucose (BG), serum creatinine (Scr), and blood urea nitrogen (BUN) with an automatic biochemistry analyzer (Toshiba 120FR). In addition, 24-h urine samples were collected in metabolic cages at the end of weeks 4, 8, and 12. After the total urine volume (UV) was determined, the urine samples were stored at −20°C. The urinary protein and urinary MCP-1 concentration were assayed with a quantitative sandwich ELISA using a commercial kit according to the manufacturer’s instructions (Biosource, Camarillo, CA). For the analysis, the 24-h urine protein excretion rate (UPER) was calculated using the following formula: UPER (mg/24 h) = 24-h total volume of urine (ml) × urinary protein concentration (mg/ml).

Light microscopy morphological study. The renal tissues fixed in 10% neutral formaldehyde were embedded in paraffin blocks. Histological sections of the kidneys were stained with PAS (the mesangial area stains purple), and the slides were examined using light microscopy (magnification ×400). A glomerulosclerosis index (GSI) (17) was used to evaluate the glomerulosclerosis score with PAS-stained tissue. Scores between 0 and 4 were given to glomeruli based on the assessment of degrees of sclerosis (0, no lesions; 1, sclerosis <25%; 2, sclerosis 25–50%; 3, sclerosis 50–75%; and 4, sclerosis >75%). A GSI was calculated with the following formula: GSI = Σ Fi (i) = 0 and Fi is the percentage of glomeruli in the mice with a given score (i).

Real-time PCR. Total RNA was extracted from renal cortices with TRizol reagent (Tiangen, Beijing, China). A standard reverse transcriptase reaction kit (Toyobo) was used to synthesize cDNA. The primer pairs were designed based on published cDNA sequences encoding the mice SET7/9, XBPIs and MCP-1 genes (RuiBo, Guangzhou, China). The primer sequences for SET7/9 were 5'-GTGGCCGAATCTGCAT-3' (sense) and 5'-GCGCACTCTCATCTCCAG-3' (antisense). The sequences for XBPIs were 5'-GAGTCCGACAGGTG-3' (sense) and 5'-GTGTCAGAGTCCATGGGA-3' (antisense). The primers for SET7/9 were 5'-AGTTAAACGCCCACTCACC-3' (sense) and 5'-TGGTTCCGATCCAGGGTTT-3' (antisense). A GSI was calculated with the following formula: GSI = Σ Fi (i) = 0 and Fi is the percentage of glomeruli in the mice with a given score (i).

Western blot analysis. Renal cortical homogenate and Western blot analysis were used to quantify SET7/9, GRP78, ATF6α, and XBPIs. The protein concentration of the tissue was determined with a Bio-Rad protein assay kit (Bio-Rad; bovine serum albumin was used as a standard). A total of 50 μg of protein was separated by 15% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membranes (DuPont). The membranes were blocked with 6% nonfat dry milk in PBS (pH 7.2) overnight at 4°C. The membranes were incubated with one of the following antibodies: mouse anti-SET7/9 (1:250; Santa Cruz Biotechnology), rabbit anti-GRP78 (1:250; BoAoSen, Beijing, China), mouse anti-ATF6α (1:250; Santa Cruz Biotechnology), or rabbit anti-XBPIs (1:250; Santa Cruz Biotechnology). All membranes were incubated for 1 h at room temperature with a monoclonal anti-β-actin antibody (1:2,000; Novus) as an internal reference that recognizes the β-actin protein at 42 kDa. The membranes were washed with PBS (pH 7.2) and incubated for 1 h at room temperature with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2,500; Sigma). The proteins were detected with the luminescence method. Band density was quantified by image-scanning analysis software (Labworks) and reported as optical density per square millimeter.

Chromatin immunoprecipitation assay. The renal cortices were homogenized with liquid nitrogen and fixed with 1% formaldehyde at 37°C for 10 min. The cross-linking reaction was stopped by the addition of 0.125 M glycine. After being washed twice with PBS, the cell suspensions were pelleted. The chromatin was then sheared to 200–1,000 bp using an ultrasonic cell disruptor. The supernatant was diluted in chromatin immunoprecipitation (ChIP) dilution buffer and precleared using protein A agarose/salmon sperm DNA. Then, 2 ml of the supernatant solution was incubated with antibodies against SET7/9, XBPI, H3K4me1, and control IgG at 4°C overnight. The protein/DNA complex was incubated with protein A agarose/salmon sperm DNA at room temperature for 1 h and then washed, and the protein-DNA cross-links were reversed. DNA was extracted, and PCR was performed. The QPCR data were analyzed using the (2−ΔΔCt) method. The MIP1 primers were 5'-AGTTAAACGCCCACTCACC-3' (sense) and 5'-TGGTTCCGATCCAGGGTTT-3' (antisense). The SET7/9 primers were 5'-GTCGGGGAAGTCAATGAAGA-3' (sense) and 5'-GCCACTCTTCATTCGAG-3' (antisense). siRNA knockdown. XBPI and SET7/9 siRNA were synthesized, high-performance purified (Qiagen, Valencia, CA), and dissolved in RNase-free PBS. A nonsilencing siRNA oligonucleotide sequence that does not recognize any known homology to mammalian genes was also generated as a negative control. The target sequence used for XBPI was 5'-ATGCCAATGAACTCTCCAG-3' (antisense). The target sequence for SET7/9 was 5'-AACCUCGGGAGACGUGCA-3'. The control target sequence was 5'-AAUCGCAUAGCGUAUGC GUU-3'. Male ddY mice (initial weight, 20–26 g; average age, 4 wk) were randomly allocated into groups (6 mice/group) receiving XBPI, SET7/9, or control siRNA (50 μg in 1 ml PBS) or 1 ml normal saline alone once every 10 days for 2 mo by tail vein injection (within 10 s). To dilate the tail veins, the tail was immersed in warm water (50–55°C) under ether narcosis for 5 s. After 2 mo, all the kidneys were harvested, cut into two portions, one portion was fixed in 10% formalin for pathology examination; the cortices of the remaining portions were stored in liquid nitrogen and used for real-time PCR or Western blotting. Cultured 80% confluent mouse mesangial cells (MCCs) were divided into four cultivation groups: the saline group as a blank control (cultured with RPMI 1640 but without serum media), high-glucose group (HG; cultured with RPMI 1640 and 30 mmol/l glucose media), and high-glucose plus XBPIs knockdown or control siRNA group [cells cultured with 30 mmol/l glucose media were transfected with XBPI siRNAs or control siRNA by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions]. After 24 h, cells were harvested for real-time PCR to detect the expression of MCP-1.

SET7/9 promoter cloning and reporter gene assay. The 3'-untranslated region (UTR) sequences of SET7/9 promoter including the putative XBPIs target site were generated by PCR. The PCR products were cloned into the pGL3-basic vector (Promega) downstream of the luciferase gene. Twenty-four hours before transfection, MMs were plated at 1.5 × 10^5 cells/well in 12-well plates. Then, 240 ng pGL3-SET7/9-3'UTR plus 30 ng pRL-TK (Promega) was transfected with the expression plasmid for XBPIs mimics or XBPIs control using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Luciferase activity was measured 24 h after...
transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. Each experiment was repeated at least three times in triplicate.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 16.0. Data are presented as means ± SE. Statistical significance was determined using either Student’s t-test when two groups were compared or by a one-way ANOVA when multiple groups were compared. We assigned significance at P < 0.05.

RESULTS

Evidence of ER stress and XBP1 expression in the kidneys of db/db mice. To examine whether ER stress is induced in the kidneys of db/db mice, we examined the expression of GRP78, ATF6β, and XBP1s by Western blot analysis. As is well known, GRP78, ATF6β, and XBP1s are important molecular indicators of ER stress. We observed that GRP78, ATF6β, and XBP1s protein levels increased significantly in a time-dependent manner in the DN group compared with the NC group. Treatment with betaine, a molecular chaperone drug with the ability to assist protein folding and raise ER stress threshold, significantly suppressed their expression (Fig. 1, A–C). These results suggest that ER stress is induced in the kidneys of db/db mice, and betaine effectively downregulates the level of ER stress.

Effect of ER stress on renal MCP-1 in db/db mice. To examine whether ER stress has a potential role in inducing activation of MCP-1, we examined the effect of ER stress on renal MCP-1 levels in db/db mice by real-time PCR. The results indicated that the expression of renal MCP-1 was elevated significantly in the DN group, and the attenuation of ER stress by betaine could inhibit this increase (Fig. 2). These results indicate that ER stress might play a key role in the pathogenesis of DN, and downregulating ER stress could markedly prevent the progression of DN.

Effect of ER stress on the renal morphology of db/db mice. PAS and light microscopy revealed typical renal pathological changes in db/db mice at 12 wk, including mesangial cell proliferation, mesangial matrix accumulation and expansion, as well as the focal thickening of the glomerular basement membrane. The GSI was significantly higher in the DN group (0.56 ± 0.13) than in the NC group (0.05 ± 0.02) (P < 0.05). Downregulating ER stress with betaine prevented these changes (Fig. 3). The GSI was also significantly decreased in the DN+B group (0.24 ± 0.08) than in the DN group (P < 0.05).

Effect of ER stress on renal SET7/9 in db/db mice. We next examined the effect of ER stress on renal SET7/9, a H3K4 excretion of MCP-1 in the DN group were elevated progressively with time. The attenuation of ER stress by using betaine could inhibit the increase in BG, BUN, BW, 24-h UPER, and urinary excretion of MCP-1 but had no effect on Scr (Table 1). These results indicate that ER stress might play a key role in the pathogenesis of DN, and downregulating ER stress could markedly prevent the progression of DN.

Fig. 1. Evidence of endoplasmic reticulum (ER) stress in the kidneys of db/db mice. Shown is Western blot analysis of renal GRP78, ATF6β, and spliced X-box binding protein 1 (XBP1s). A: at the end of 4, 8, and 12 wk, renal GRP78 protein expression in diabetic nephropathy group (DN) mice was significantly increased. Expression was inhibited by betaine treatment. B: at the end of 4, 8 and 12 wk, renal ATF6β protein expression in DN mice was significantly increased. The expression was inhibited by betaine treatment. C: at the end of 4, 8 and 12 wk, renal XBP1s protein expression in DN mice was significantly increased. The expression was inhibited by betaine treatment. NC, normal control group; DN, betaine treatment group. Values are means ± SE; n = 6. See the text for more definitions. *P < 0.05 vs. NC. #P < 0.05 vs. DN.

Fig. 2. Effect of ER stress on renal monocyte chemoattractant protein-1 (MCP-1) in db/db mice. At the end of 4, 8, and 12 wk, the results of real-time PCR indicated that the renal MCP-1 mRNA level in the DN group was significantly increased. This effect was inhibited by betaine treatment. Values are means ± SE; n = 6. *P < 0.05 vs. NC. #P < 0.05 vs. DN.
monomethyltransferase, in the kidneys of db/db mice. We first observed that SET7/9 mRNA levels were increased in a time-dependent fashion in the kidneys of db/db mice. In accordance with SET7/9 mRNA expression, the protein expression of SET7/9 was also increased. The attenuation of ER stress with betaine significantly suppressed both renal SET7/9 mRNA and protein expression at the end of 8 and 12 wk (Fig. 4, A and B).

Effect of ER stress on SET7/9 recruitment and H3K4me1 levels to MCP-1 promoters. We next examined whether regulating ER stress could alter SET7/9 recruitment and H3K4me1 levels (an epigenetic “active” mark) at the promoters of MCP-1 using ChIP-QPCR assays. The results indicated that SET7/9 recruitment and H3K4me1 levels at MCP-1 promoters in the DN group were markedly higher compared with the NC group and that the attenuation of ER stress strength by betaine could reverse these changes (Fig. 5, A and B). These results suggest that SET7/9-mediated H3K4me1 may be involved in the induction of MCP-1 in the kidneys of db/db mice and that ER stress may regulate SET7/9-mediated H3K4me1 expression.

Effect of ER stress on XBP1 recruitment to SET7/9 promoters. We next observed whether regulating ER stress alters XBP1s recruitment to SET7/9 promoters using ChIP-QPCR assays. The results indicated that XBP1s recruitment was significantly increased at the SET7/9 promoters in the DN group compared with the NC group. The attenuation of ER stress with betaine significantly suppressed these changes (Fig. 6). These results suggest that XBP1s may play a key role in the induction of SET7/9.

Effect of SET7/9 siRNA on H3K4me1, MCP-1, and the renal morphology of db/db mice. We further studied the functional role of SET7/9 in the expression of MCP-1. SET7/9 was knocked down by siRNA, and the knockdown efficiency was determined by assessing the reduction of SET7/9 mRNA with real-time PCR. Compared with the saline- and control siRNA-treated groups, the mice treated with SET7/9 siRNA presented significantly reduced levels of SET7/9 mRNA (Fig. 7, A and B). We next examined whether SET7/9 siRNA can affect the levels of H3K4me1 and MCP-1. The results indicated that H3K4me1 levels were clearly decreased in SET7/9 knockdown mice compared with saline- and control siRNA-treated mice, as measured by CHIP-QPCR (Fig. 7C). MCP-1 mRNA levels were also significantly attenuated by SET7/9 siRNA in the treatment group compared with saline- and control siRNA-treated mice (Fig. 7D). Furthermore, SET7/9 knockdown
markedly attenuated glomerular constructive damage (Fig. 7E). The GSI was significantly higher in the control siRNA-treated group (0.64 ± 0.13) than in the NC group (0.05 ± 0.02) (P < 0.05) and decreased in SET7/9 knockdown mice (0.34 ± 0.08) vs. the control siRNA-treated group (P < 0.05). These results further support a key role for SET7/9 in the induction of MCP-1.

Fig. 4. Effect of ER stress on renal SET7/9 in db/db mice. A: at the end of 4, 8, and 12 wk, renal SET7/9 mRNA expression in DN mice as analyzed by real-time PCR was significantly increased. The expression was inhibited by betaine treatment. B: at the end of 4, 8, and 12 wk, renal SET7/9 protein expression in DN mice as analyzed by Western blot analysis was significantly increased. The expression was inhibited by betaine treatment. Values are means ± SE; n = 6. *P < 0.05 vs. NC. #P < 0.05 vs. DN.

Effect of XBP1s siRNA on SET7/9, H3K4me1, and MCP-1 expression, SET7/9 recruitment, BG concentration, UPER, MCP-1 excretion, and the renal morphology of db/db mice. Next, we further examined the functional role of XBP1s in SET7/9-induced MCP-1 expression. Real-time PCR and Western blotting were used to evaluate the expression levels of XBP1s after siRNA treatment. XBP1s mRNA and protein levels were significantly reduced in XBP1s knockdown mice compared with saline- and control siRNA-treated mice (Fig. 8, A and B). Furthermore, we determined whether the loss of XBP1s influences the levels of SET7/9, SET7/9 recruitment, H3K4me1, MCP-1 expression, and the renal morphology of db/db mice. The results indicated that SET7/9, SET7/9 recruitment, H3K4me1, and MCP-1 expression were significantly reduced in XBP1s knockdown mice compared with saline- and control siRNA-treated mice (Fig. 8, C–F). XBP1s knockdown markedly attenuated glomerular constructive damage (Fig. 7E).

The GSI was significantly decreased in the XBP1s knockdown group (0.29 ± 0.11) than in the control siRNA-treated group (0.64 ± 0.13) (P < 0.05). In addition, XBP1s knockdown can reduce significantly the UPER and MCP-1 excretion in urine but has no effect on the concentration of BG in the db/db mice (Table 2). Moreover, in an vitro study, we found XBP1s knockdown can obviously inhibit MCP-1 expression of cultured mesangial cells even if cocultured with high glucose (Fig. 8G). These results indicate that there is a direct correlation between XBP1s and SET7/9-induced MCP-1 expression.

Fig. 5. Effect of ER stress on SET7/9 recruitment and H3K4me1 levels at MCP-1 promoters. A: at the end of 4, 8, and 12 wk, SET7/9 recruitment to MCP-1 promoters was significantly increased in the DN group. The recruitment was inhibited by betaine treatment. B: at the end of 4, 8, and 12 wk, the H3K4me1 levels at MCP-1 promoters was significantly increased in the DN group. The level of increase was inhibited by betaine treatment. Values are means ± SE; n = 6. *P < 0.05 vs. NC. #P < 0.05 vs. DN.

Fig. 6. Effect of ER stress on XBP1s recruitment to SET7/9 promoters. At the end of 4, 8, and 12 wk, XBP1s recruitment to SET7/9 promoters was significantly increased in the DN group. The recruitment was inhibited by betaine treatment from 8 wk. Values are means ± SE; n = 6. *P < 0.05 vs. NC. #P < 0.05 vs. DN.
Relative luciferase activity of SET7/9 promoter in MMCs. Finally, we constructed a luciferase reporter vector with the putative SET7/9 3'-UTR target site for XBP1s downstream of the luciferase gene (pGL3-SET7/9-3'-UTR). Luciferase reporter vector together with the XBPIs mimics or the XBPIs mimic control was transfected into MMCs for 24 h. Results showed that a significant increase in relative luciferase activity was noted when pGL3-SET7/9-3'-UTR was cotransfected with the XBPIs mimics but not with the miRNA mimic control (Fig. 9), suggesting that SET7/9 was the target gene of XBPIs.

DISCUSSION

It is widely known that inflammation plays a key role in diabetes and its vascular complications, such as diabetic nephropathy (24). Recent research, including our previous studies, has demonstrated that both ER stress and epigenetic modification may be triggers of chronic inflammation (8, 35, 36). For instance, attenuating ER stress with the chemical molecular chaperone 4-PBA contributes to the deactivation of inflammatory factors (20, 28). Furthermore, SET7/9-mediated...
H3K4me1 promotes NF-κB subunit p65 gene transcription in aortic endothelial cells (2) and regulates the expression of a subset of NF-κB downstream of inflammatory genes in monocytes (14). Our recent studies found that human glomerular mesangial cells under high-glucose conditions exhibited increased levels of ER stress and SET7/9. In addition, we also found that 4-PBA not only significantly inhibits ER stress but also reduces the expression of SET7/9 (5). However, to the best...
of our knowledge, there are no data about the regulation of the relationship between ER stress and SET7/9-mediated downstream signals.

In this report, we confirmed for the first time that ER stress triggers MCP-1 expression through SET7/9-mediated histone methylation in the kidneys of db/db mice. First, our data demonstrated that the expression of SET7/9 and MCP-1 in the kidneys of db/db mice and the total excretion of MCP-1 in urine were increased and could be significantly suppressed through downregulating ER stress with betaine, a recognized molecular chaperone. Second, our results also indicated that SET7/9-mediated H3K4me1 in the kidneys of db/db mice. The knockdown of SET7/9 with siRNAs significantly suppressed the H3K4me1 levels and the expression of MCP-1. Furthermore, we also found that XBPs was responsible for the induction of SET7/9 in the kidneys of db/db mice; this finding is supported by three pieces of evidence: XBPI recruitment was significantly increased at the promoters of SET7/9 and could be inhibited by suppression of ER stress with betaine. SET7/9 could be upregulated by XBPs, and SET7/9 promoter activity was promoted byXBPs in MMCs. The knockdown of XBPs with siRNAs significantly decreased SET7/9 expression. All these results suggest that ER stress induces the activation of MCP-1, in part through XBPs-mediated induction of SET7/9- and SET7/9-mediated H3K4me1 of the MCP-1 promoter.

The ER is mainly recognized as a protein synthesis and folding factory, as well as mediating multicomponent signal transduction pathways in response to the accumulation of unfolded or misfolded proteins. These pathways are known as the unfolded protein response (UPR). The UPR is mediated by three major transmembrane transducers: pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6), and interferon-response element 1 (IRE1)/XBPs. These ER membrane-bound transcription factors can regulate a great majority of human ER stress-inducible genes (27). A previous report indicated that ER stress-induced ATF6 can impair insulin gene expression via upregulation of SHP in INS-1 cells (30). Furthermore, IRE1, PERK, and ATF6 can regulate VEGFA mRNA expression under various stress conditions (4). We first confirmed that the XBPs protein levels were increased significantly in a time-dependent manner in the kidneys of db/db mice, a diabetic nephropathy model. Based on our recent studies, we supposed that there is some type of regulatory relationship between XBPs and SET7/9 in the pathogenesis of diabetic nephropathy. We searched using computational analysis and found that, as shown in the database of XBPI gene (Uniprot accession no. P18850), XBPI can bind to two DNA sequences, termed the TRE-and CRE-like sequences. Data from DNAStar software indicated that the proximal promoter region of the human SET7/9 gene (the 46- and 1979-bp sequence upstream of the transcriptional start site) contains the CRE mode sequence (5'-TGATGCTA-3') and (5'-TGATGCTC-3'). Furthermore, the proximal 402-bp sequence upstream of the transcriptional start site of SET7/9 contains the TRE mode sequence (5'-AGCGTCA-3'). Only one or two bp of these three mode sequences are different from XBPI-binding DNA sequences. These results suggest XBPI may bind to the TRE- and CRE-like sequences in the promoter regions of the SET7/9 gene.

We investigated the role of XBPs in the regulation of SET7/9 transcription in db/db mice. XBPs is a transcriptional regulator of the ER stress response that lies downstream of IRE1 activation and regulates the expression of many adaptive UPR genes (12). Evidence indicates that XBPs can regulate hepatic lipogenesis (13) and activate the transcription of IGF1 and Akt phosphorylation in zebrafish embryonic cell lines (6). First, our study demonstrated that XBPs and SET7/9 expression increased at the same time in the kidneys of DN mice and that it could be suppressed by betaine, a molecular chaperone often used to downregulate the extent of the ER stress response. Furthermore, we found that the recruitment of XBPs at SET7/9 promoters increased significantly in a time-dependent manner in the kidneys of DN mice. Downregulating ER stress with betaine could attenuate the levels of XBPs recruitment to the SET7/9 promoters. More importantly, ChIP assays demonstrated that knockdown of XBPs not only decreased SET7/9 expression but also significantly impaired the recruitment of SET7/9 to the promoters of MCP-1 and reduced the levels of H3K4me1 of MCP-1 promoters. Furthermore, a significant increase in relative luciferase activity was noted when pGL3-SET7/9-3'-UTR was cotransfected with the XBPI mimics but not with the miRNA mimic control. These results suggest that ER stress induces SET7/9 expression via the XBPs transcription factor.

In addition, we demonstrate the functional role of XBPs and SET7/9 in the induction of MCP-1 expression in the kidneys of DN mice. Histone modifications such as H3 lysine-9 methylation (H3K9me) correlate with gene silencing and transcriptional repression, whereas H3 lysine-4 methylation (H3K4me) is usually associated with active chromatin and increased gene expression. A previous report indicated that a dynamic cooperation between decreased levels of H3K9me3 and increased H3K4m1 was associated with an increase in NF-kB-p65 gene expression (1). We used ChIP assays effectively to demonstrate that both H3K4me1 levels and SET7/9 recruitment at MCP-1 promoters were elevated in the kidneys of db/db mice. The knockdown of SET7/9 with siRNA signif-
icantly suppressed the expression of MCP-1 and attenuated glomerular lesions such as accumulation of mesangial matrix, suggesting that SET7/9-mediated H3K4me1 plays a key role in MCP-1 expression. These findings are in line with those reported by El-Osta (2). We found that XBP1s had the same role in the regulation of MCP-1 expression in the kidneys of db/db mice. First of all, attenuating ER stress with betaine not only effectively downregulated XBP1s expression levels but also markedly decreased renal and urinary MCP-1 levels. Meanwhile, the knockdown of XBP1s also significantly suppressed the expression of MCP-1 and attenuated glomerular lesions such as accumulation of mesangial matrix. These results further support our hypothesis that ER stress plays a key role in the pathogenesis of DN through XBP1-induced SET7/9 expression and SET7/9-mediated epigenetic modification (histone H3K4me1 at the MCP-1 promoter).

An interesting discovery in this study is that treatment with betaine not only decreased the expression of inflammatory factors such as MCP-1 and attenuated the glomerular lesions in the kidney of db/db mice but also lowered BG (still higher than in control mice) at the same time. To exclude the effect of lowering BG on the improvement of glomerular lesions, we conducted an in vitro study that on the condition of coculture with high glucose, MCP-1 expression of mesangial cells still could be suppressed by knockdown of XBP1 with siRNA. The result indicated that the reduction of glomerular damage by betaine therapy was not related with the lowering of BG but was mainly associated with modification of the epigenetic code of inflammatory factors. This inference has been confirmed by Sun et al. (31), and this was also supported by recent research which found diabetic vascular complications such as DN can progress despite subsequent glycemic control, suggesting a metabolic memory of previous exposure to hyperglycemia.

In conclusion, our data demonstrate that the ER stress-induced activation of XBP1s leads to a significant increase in the expression of SET7/9, resulting in parallel increases in the expression of MCP-1, all of which are related to the pathogenesis of DN. Thus ER stress is likely to be an important regulator of epigenetic modifications by UPR-inducible transcription factors. These data suggest that ER stress-induced epigenetic modulation plays a key role in the pathogenesis of DN and provides a theoretical basis for further study of a new clinical target and strategies for DN prevention and treatment.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.C., Y.G., W.Z., L.H., Q.P., and L.N. performed experiments; J.C. analyzed data; J.C. prepared figures; J.C. drafted manuscript; B.F. approved final version of manuscript.

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