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HMGB1 Mediates IFN-γ-Induced Cell Proliferation in MMC Cells Through Regulation of Cyclin D1/CDK4/p16 Pathway

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ABSTRACT

Previous studies have revealed the elevated serum levels of High-mobility group box-1(HMGB1) and the interferon-γ (IFN-γ)-induced proliferation of renal mesangial cells in patients or experimental animals with systemic lupus erythematosus (SLE). However, it is still not elucidated whether HMGB1 involves in the pathogenesis of lupus nephritis (LN) and mediates IFN-γ-induced mesangial cell proliferation. Therefore, in the present study we demonstrated HMGB1 mRNA and protein levels were increased in the glomeruli of LN patients and BXSB mice. HMGB1 increased the proliferation index of mouse mesangial cells (MMC) that was accompanied with the up-regulation of cyclin D1, CDK4 and the down-regulation of p16, subsequently promoting the transition from the G0/G1 to S stage. Inhibition of HMGB1 by a specific short hairpin RNA vector prevented cyclin D1/CDK4/p16 up-regulation and attenuated IFN-γ-induced MMC cell proliferation and PCNA (proliferating cell nuclear antigen, PCNA) expression. These findings indicate that HMGB1 mediates IFN-γ-induced cell proliferation in MMC cells through regulation of cyclin D1/CDK4/p16 pathway and promoting the cell cycle transition from G1 to S stage. J. Cell. Biochem. 113: 2009–2019, 2012.

KEY WORDS: LUPUS NEPHRITIS; HMGB1; IFN-γ; CELL PROLIFERATION; CELL CYCLE; CYCLIN D1/CDK4/p16

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease affecting multiple organs; and glomerulonephritis is a significant cause of death and disability [D'Cruz et al., 2007]. Mesangial cell proliferation is the main pathological change and it can be detected in nearly every type, especially in type IV and V lupus nephritis (LN). Recent evidence has revealed that many cytokines are likely involved in LN [Chun et al., 2007; Suh and Kim, 2008] and the unbalance between proinflammatory and anti-inflammatory cytokines plays an important role in the immune response.

Among SLE-related cytokines, interferon-γ (IFN-γ) is a multi-functional cytokine that plays a central role in the pathogenesis of lupus nephritis, which markedly increased at the early stage of the disease [Segal et al., 1997]. Many researches have confirmed that IFN-γ can mediate various renal injuries including the proliferation of renal mesangial cells [Sheu et al., 2005]. However, the exact mechanism involved in IFN-γ-induced proliferation of renal mesangial cells is still not well known.

High-mobility group box-1 (HMGB1) is a novel, later cytokine mediator [Czura et al., 2003] that is proved to activate many biological functions. The extracellular HMGB1 plays an important role in many diseases such as immune disease, malignant tumor and so on [Kuniyasu et al., 2005; Ek et al., 2006; Pisetsky et al., 2008; Winter et al., 2009; Yoshizaki et al., 2009]. Some studies also indicate that HMGB1 levels increase in the skin of patients with cutaneous lupus and the extracellular localization of HMBG1 is an important difference between lesional and non-lesional skin [Barkauskaite et al., 2007; Abdulahad et al., 2010]. In addition, elevated plasma levels of HMGB1 are associated with disease activity and may act as a proinflammatory mediator of antibody-induced kidney damage [Qing et al., 2008; Ma et al., 2012; Pan et al., 2010; Abdulahad et al., 2011]. Further, cytoplasmic HMGB1 related to cancer progression by involving in cell-cycle progression and inducing cell proliferation [Gnanasekar et al., 2009; Lee et al., 2010]. Our previous studies showed that recombinant HMGB1 could induce the proliferation of RSC-364 cells [Guo et al., 2011] and elevated

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serum levels of HMGB1 are associated with renal injury in patients with SLE [Liu et al., 2008]. As renal injury is a major complication of SLE [Bomback and Appel, 2010], we speculated that HMGB1 might be a putative "late" mediator and mediate IFN-γ-induced the proliferation of renal mesangial cells in LN through regulating cell cycle.

Therefore, in order to confirm this hypothesis, we first determined the expression of HMGB1 protein and its localization in the renal tissue of patients with LN and BXSB mice (LN model mice). Furthermore, a mouse mesangial cell line (MMC) was used to determine the effect of HMGB1 on the cell proliferation and cyclin D1/CDK4/p16 pathway. Finally, MMC was transfected with sh-RNA vector aimed to HMGB1 to elucidate whether HMGB1 was involved in IFN-γ-induced MMC proliferation.

MATERIALS AND METHODS

PATIENTS

Twenty patients (3 males and 17 females, aged between 19 and 55 years), diagnosed with SLE and type IV (n = 13) or type V LN (n = 7) (ISN/RPS2003 classification criteria) [Tan et al., 1982] between 2008 and 2009 at the inpatient Department of Nephrology at the Second Hospital of Hebei Medical University were enrolled in this study. Ten cases of control renal tissues were obtained away from the tumor tissue of renal tumor patients without the history of primary glomerulonephritis, hypertension, or diabetic nephropathy. Renal tissue was stored at −80°C for immunofluorescence analysis and fixed with 4% formaldehyde in 0.1% DEPC for in situ hybridization.

ANIMALS

Sixteen 11-week male BXSB mice (weight: 18–22 g) were kindly provided by the Department of Medical Immunology at the Peking University Health Science Center. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University (approval ID: HebMU 20080026). Hereafter, these mice were referred to as the LN group. Fourteen C57BL/6 mice (age and weight matched to the BXSB mice) were chosen as normal control animals. Normal control mice and BXSB mice were sacrificed after 6 weeks. Serum, urine, and renal cortex were collected for relevant detections.

CELL CULTURE AND GROUPS

MMC (ATCC No.CRL-1927) were obtained from the American Type Culture Collection (Manassas, VA). They were cultured in DMEM-F12 medium (Gibco BRL) supplemented with 5% fetal bovine serum. Cells were synchronized by culturing in serum-free medium for 24 h. (1) To determine the effect of HMGB1 on cell proliferation, cells cultured in DMEM-F12 medium were randomly divided into two groups: control group and HMGB1 group (containing 50 $\mu g.L^{-1}$ mouse recombinant HMGB1 protein, R&D). Cells were respectively collected at 4, 8, and 12 h after stimulation. (2) In the RNA interference (RNAi) experiment, the cells were randomly divided into four groups: normal group, untransfected IFN-γ-treated ($5\mu g\,L^{-1}$) group, control vector sh-Scramble-transfected IFN-γ-treated group and sh-HMGB1-transfected IFN-γ-treated group. At 12 h after stimulation, MMC cells were collected and the relevant detections were performed.

EXPRESSION VECTORS AND TRANSIENT TRANSFECTION

The sh-HMGB1 vector and negative control sh-Scramble vector were designed and produced by Santa Cruz. The sh-HMGB1 vector contains a short hairpin RNA (shRNA) sequence directed against the mouse HMGB1 gene (5′-CAC CCG GAU GCU UCU GUC AAC UUC U-3′; 5′- AGA AGU UGA CAG AAG CAU CCG GGU G-3′). Transient transfection of MMC cells was carried out using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instructions. At approximately 80% confluence, the cells were transfected with 2.0 μg of vector DNA with 4 μl of Lipofectamine 2000 in 2 ml of serum-free DMEM-F12 medium. At 12 h after transfection, the medium was replaced with normal DMEM-F12 medium containing 5% fetal bovine serum and cells were incubated for 24 h. Then the cells were cultured for 12 h in medium containing 1% FBS and $5\,\mu g\,L^{-1}$ IFN-γ, subsequently, HMGB1 expression, the proliferation index, cell cycle distribution, cyclin D1, CDK4, p16, and PCNA expression were determined.

IN SITU HYBRIDIZATION

In situ hybridization kit for detecting HMGB1 mRNA was from Tianjin Haoyang Co. Paraffin sections were used for in situ hybridization to detect the mRNA of HMGB1. The sections were filled with 0.1% Triton X-100 solution for 10 min at room temperature. Tissues were incubated for 30 min at 37°C in 1 mg/ml proteinase K followed by blocking with 3% H_2O_2 solution for about 20 min at room temperature. After equilibration in 2 × SSC, the sections were prehybridized for 1 h at 37°C. Hybridization was carried out for 4 h at 37°C, and then the slides were washed with 2 × SSC for 5 min × 3 at 37°C. The sections were stained with DAB and negative controls were performed with PBS. Brownish yellow granules in the cytoplasm were considered as positive region. The sections were imaged with Olympus microscope. Sequences of oligonucleotide probes were respectively:

(1) 5′-GTGCT TCTTC TTATG CTCC TCCCGA CAA-3′
(2) 5′-GTTCT CCTTT GATTT TTGGG CGATA CTC-3′
(3) 5′-ATTCC ACATC TCTCC CAGTT TCTTC GCA-3′

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After deparaffinization, rehydration, and antigen recovery, the paraffin sections were blocked with 10% goat serum in PBS overnight at 4°C. Then, the paraffin sections were incubated with HMGB1 antibody (1:25; diluted by 10%GS/0.01 M PBS; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. At the second day, the slides were incubated with Rhodamine labeled goat anti-rabbit IgG (H + L) (1:50; Kirkegaard & Perry Laboratories, Inc) for 1 h at 37°C. After rinsed with PBS the slides were counterstained with DAPI (100 ng/ml, Solarbio Biotechnology) for 10 min at room temperature. Then the slides were observed with fluorescence microscopy after rinsing with PBS for three times. Antigen recovery was done by the method of anticyclone and the negative control was performed by replacing the primary and secondary antibody with

TABLE I. Primers and Product for HMGB1, 18SrRNA, Cyclin D1, and β-Actin

Gene	Sense	Antisense	Product
Mouse HMGB1	5′-TGC TGC ATA TCG AGC TAA AGG -3′	5′-CCA TAC TGT ACC AGG CAA GGT-3′	399
18SrRNA	5′-ACACGGACAGGATTGACAGA-3′	5′-GGACATCTAAGGGCATCACAG-3′	238
Cyclin D1	5′-AGC TCC TGT GCT GCG AAG TGG AAA C-3′	5′-AGT GTT CAA TGA AAT CGT GCG GGG T-3′	480
β-actin	5′-GTG GGG CGC CCC AGG CAC CA-3′	5′-CTT CCT TAA TGT CAC GCA CGA TTT C-3′	540

PBS buffer. Images were examined with a Olympus BX61 digital microscope.

SEMI-QUANTITATIVE RT-PCR

Mice glomeruli were isolated as previously described [Qing et al., 2006]. Kidneys were forced through 180-μm and 100-μm stainless steel sieves. Glomeruli were then collected on a 71-μm sieve, washed with phosphate buffered saline (PBS). Total RNA was extracted from the glomeruli of mice or cells using TRIzol reagent. Total RNA (2 μg) was reverse transcribed using oligo (dT) at 42°C for 1 h. The cDNA was amplified with specific primers for HMGB1, cyclin D1, 18S rRNA, and β-actin (Table I). The PCR products were subjected to 1.5% agarose gel electrophoresis and analyzed with GDS-8000 Bioimaging system (UVP, CA) and LabWorks 4.5 software. The gene expression was quantified by comparison with internal-control 18S rRNA or β-actin. All experiments were repeated at least three times.

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The renal cortex was immediately fixed in 4% formaldehyde. Antigen recovery was performed using a microwave. The sections were respectively incubated with primary antibodies against HMGB1 (1:200; Abcam, USA) and PCNA (1:100; Santa Cruz) overnight at 4°C. In the following day, the sections were incubated with polymer helper and polyperoxidase-anti-rabbit IgG at 37°C, and finally stained with diaminobenzidine. The sections were imaged with Olympus microscope and characterized quantitatively by digital image analysis using the Image Pro-Plus 5.0 software (Media Cybernetics, Silver Spring, MD) by using the method introduced by Jun et al. [2009].

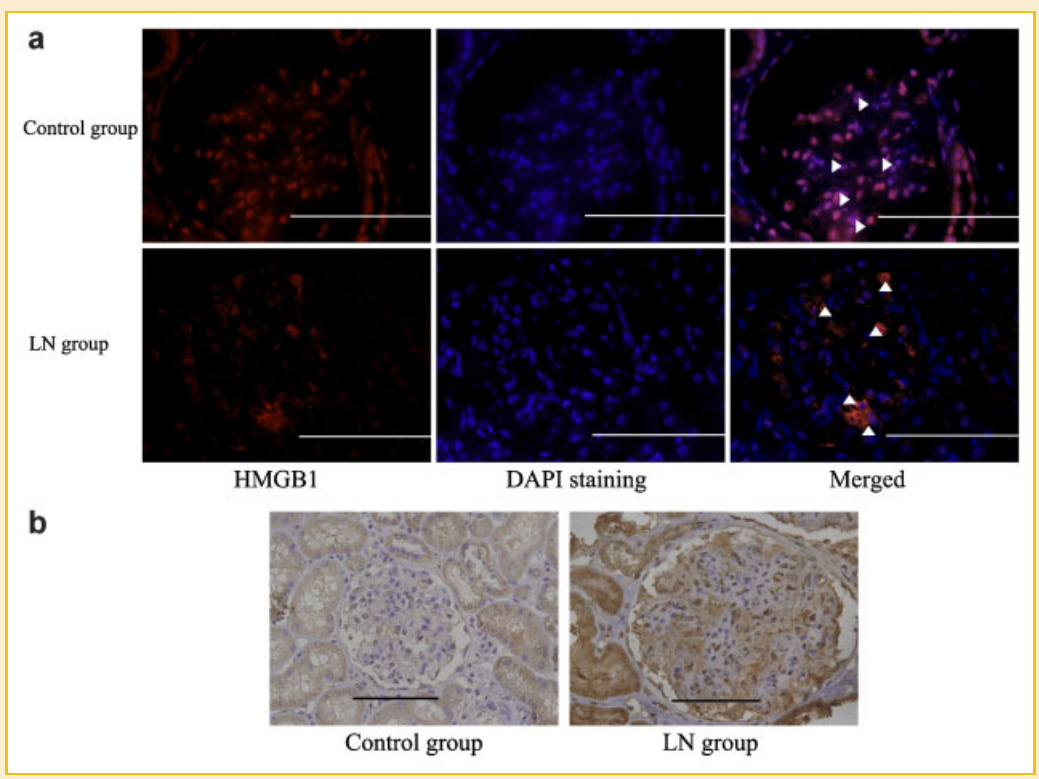

Fig. 1. Expression of HMGB1 mRNA and protein in glomeruli of patients with lupus nephritis. a: Immunofluorescence of HMGB1 protein in kidney of patients with LN and control people ×400. In control group, the expression of HMGB1 protein was located in nuclei of glomeruli (pink), which was located in cytoplasm and extracellular in LN group (red). b: In situ hybridization was used to detect the HMGB1 mRNA expression, increase expression of HMGB1 mRNA was found in the glomeruli of LN group compared with control group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

The cells were fixed in a 4% paraformaldehyde solution and permeabilized with 0.1% Triton X-100. Following incubation with anti-PCNA and anti-cyclin D1 (1:100; Santa Cruz) antibodies, second antibody conjugated with horseradish peroxidase, the slices were stained with diaminobenzidine.

FLOW CYTOMETRY ANALYSIS

After treating cells with HMGB1 or IFN-γ, the cell pellets were fixed in 70% ethanol, and stained with 500 $\mu g \cdot ml^{-1}$ propidium iodide (Sigma) containing 3 Kunitz RNase and 1% fetal bovine serum in PBS. Then, the stained cells were analyzed in an Epics-XLII Flow Cytometer (Beckman Coulter, FL) and the number of the cells in each phase was calculated using the ModFit LT cell cycle analysis program according to the manufacturer's instructions. The proliferation index (PI) represents the number of proliferating cells and was calculated using the following equation:

$$PI = \frac{(S + G2M)}{(S + G2M + G0/G1)} \times 100\%$$

TOTAL PROTEIN EXTRACTION AND WESTERN BLOTTING

The glomeruli of mice were collected according to previous method. Total protein extraction from collected glomeruli and cultured cells was performed as described previously [Hao et al., 2011]. The protein extracts were separated by 10% SDS–PAGE and then transferred to PVDF membranes. The membranes were incubated overnight at 4°C with anti-HMGB1 (1:1000; Abcam), cyclin D1(1:500), CDK4 (1:300), p16 (1:250), and β-actin (1:1000; Santa Cruz) antibodies. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) and then exposed to X-ray film using an enhanced chemiluminescence system (Pierce, IL). The intensity of the bands was measured using LabWorks 4.5.

STATISTICAL ANALYSIS

The quantitative data are presented as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) with the Student–Newman–Keuls test.

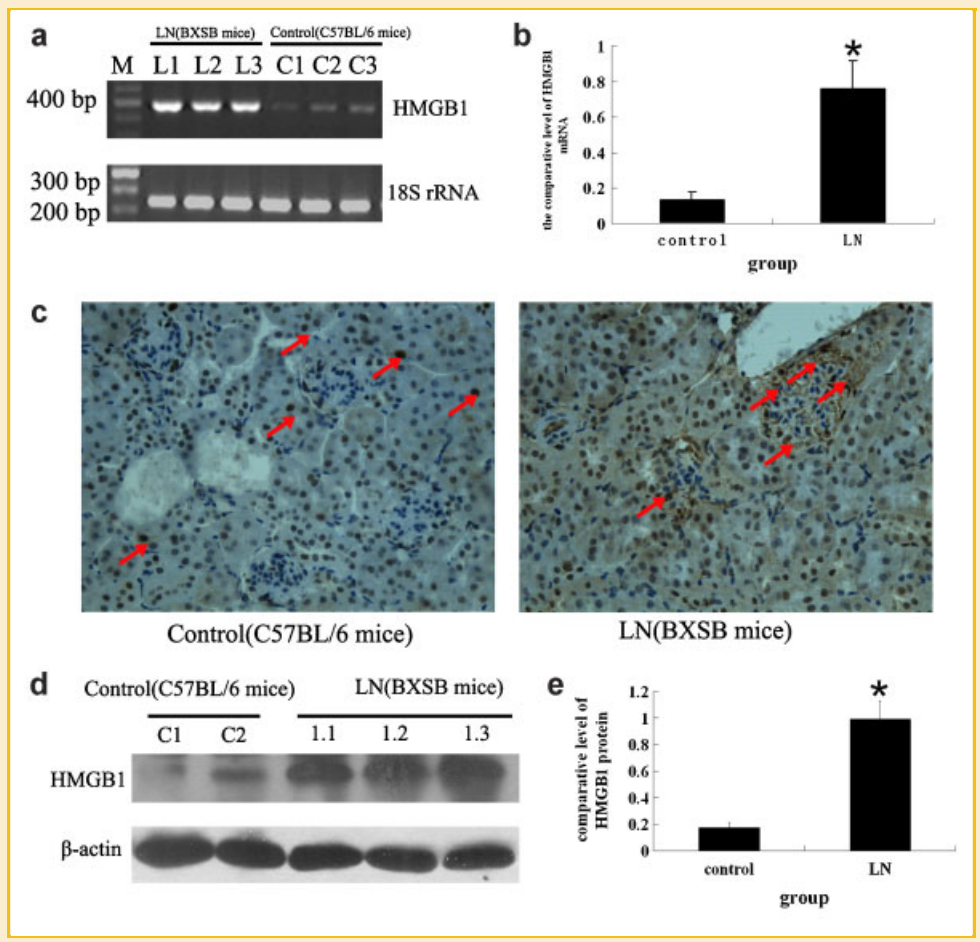

Fig. 2. Increased HMGB1 expression in glomeruli of BXSB mice. a,b: RT-PCR of HMGB1 mRNA in glomeruli of BXSB mice and C57BL/6 mice (mean ± SD, n = 8). As shown in (a and b), there were higher expression of HMGB1 mRNA in glomeruli of BXSB mice compared with in C57BL/6 mice. c: Immunohistochemistry of HMGB1 ×400. There was evident positive expression of HMGB1 protein in glomeruli of BXSB mice, and located in cytoplasm and extracellular, whereas it was located in nuclei of renal tubule epithelial cell in C57BL/6 mice. d,e: Western blot of HMGB1 in glomeruli of BXSB mice and C57BL/6 mice (mean ± SD, n = 8). Western bloting showed increase expression of HMGB1 in glomeruli of BXSB mice compared with C57BL/6 mice. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

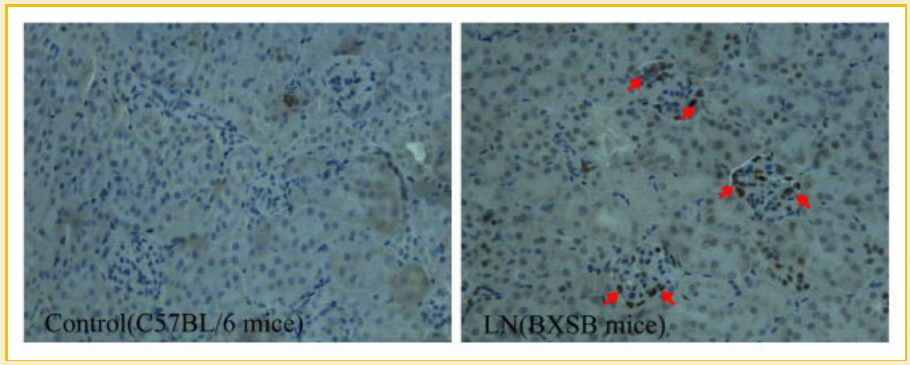

Fig. 3. Increased PCNA protein in glomeruli of BXSB mice (Immunohistochemistry, ×400). Image revealed that the positive expression of PCNA protein was located in nuclei of cells, and there was evident positive expression in glomeruli of BXSB mice compared with control mice by immunohistochemistry (red arrow represented positive expression). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Spreman' test was performed to assess the correlation between two sets of data. A P-value of <0.05 was considered statistically significant.

RESULTS

HMGB1 EXPRESSION WAS UP-REGULATED IN THE GLOMERULI OF PATIENTS WITH LUPUS NEPHRITIS AND BXSB MICE

Indirect immunofluorescence showed strong red fluorescence in the glomeruli of patients with LN that was located mainly in the cytoplasm, in some areas, there was also extra-cellular expression of HMGB1 (Fig. 1a). Moreover, there were positive staining located in nucleus in renal tubule epithelial cell and in individual glomeruli. However, as shown in Figure 1a, the positive expression of HMGB1 protein was confined to nucleus not only in glomeruli but also in renal tubule, there was no clear fluorescent staining in cytoplasm or/and extracellular of glomeruli in the control group.

In situ hybridization was used to detect the mRNA of HMGB1 in renal cortex of LN group and control group. The mRNA of HMGB1 was located in the cytoplasm of glumeruli and renal proximal

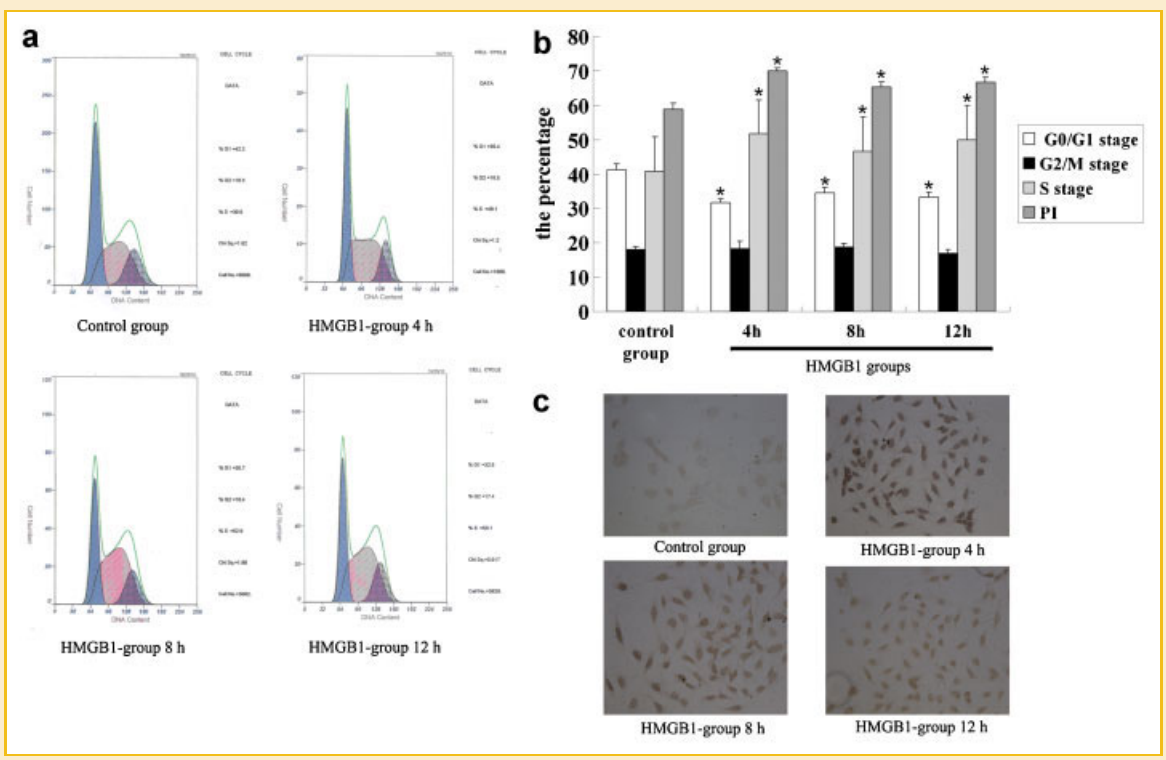

Fig. 4. HMGB1 induces cell proliferation, decreases the percentage of cells in G0/G1 phase and increase the percentage in S phase in MMC cells. a,b: FCM of cell cycle distribution and PI (proliferation index)in MMC cells stimulated by HMGB1. c: Immunocytochemical staining of PCNA protein in MMC cells. The PCNA expression was increased in MMC cells exposed to HMGB1. $^{*}P < 0.01$ versus control group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

tubular cells and was found to increase in the renal cortex of patients with lupus neprtitis (Fig. 1b), especially in glomeruli.

HMGB1 mRNA expression in the glomeruli of BXSB mice was 5.85 times higher than that of C57BL/6 mice (Fig. 2a,b). By immunohistochemistry, there was little HMGB1 expression in the glomeruli of the C57BL/6 mice, and the protein was mostly located in the nuclei of the tubules. However, HMGB1 protein expression in BXSB mice was markedly increased and the expression was primarily restricted to the cytoplasm or the extracellular milieu of the glomeruli (Fig. 2c). The results of Western blotting also

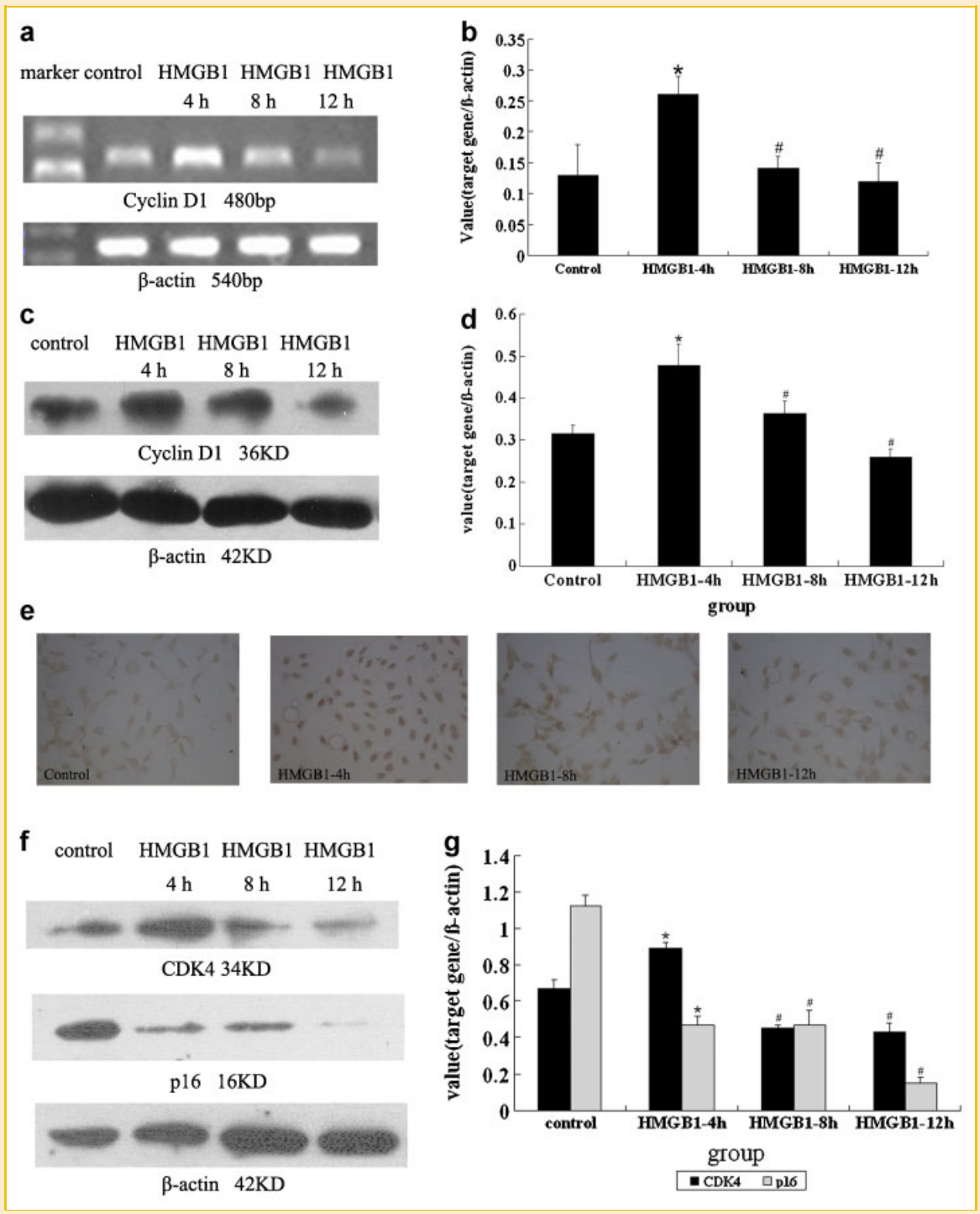

Fig. 5. HMGB1 increases cyclin D1 mRNA and protein expression, CDK4 protein expression, and decreases expression of p16 protein in MMC cells. a,b: Normalized by β-actin, mRNA level of Cyclin D1 was determined by RT-PCR and were quantified by densitometry (mean ± SD, n = 6). c,d: Expression of Cyclin D1 was measured by Western blot analysis and quantified by densitometry. e: Immunohistochemistry of Cyclin D1, ×400. f,g: Western blot of CDK4 and p16 protein (mean ± SD, n = 6). $^{*}P < 0.01$ versus control group. $\#P < 0.01$ versus HMGB1-4h.

confirmed that glomerular expression of HMGB1 in BXSB mice was increased in comparison with C57BL/6 mice (Fig. 2d,e).

As shown in Figure 3, PCNA, the key marker of cell proliferation, presented the evident up-regulation in glomeruli of BXSB mice, coincident with increased HMGB1 expression. The final mean IOD (integrated optical density) of immunohistochemistry images was (1.99 ± 0.14) that is 5.82 times than that of control mice. In addition, the correlation analysis revealed that the HMGB1 protein showed significantly positive correlation with PCNA protein in glomeruli of BXSB mice ($r = 0.771$, $P < 0.05$).

HMGB1-INDUCED MMC CELL PROLIFERATION BY PROMOTING THE CELL CYCLE TRANSITION FROM G0/G1 TO S PHASE

In order to explore the direct role of HMGB1 in cell proliferation, we used recombinant HMGB1 to stimulate MMC. FCM analysis showed that the percentage of cells in G0/G1 phase decreased while the percentage of cells in S phase increased in the HMGB1-stimulated cells compared with the control group, and reached a peak at 4 h (Fig. 4a,b). HMGB1 had no significant effect on the percentage of cells in G2/M phase. Therefore, the PI increased in the HMGB1-treated cells (Fig. 4b). In addition, immunocytochemical staining showed that PCNA expression increased in the HMGB1-stimulated cells compared with the control cells and peaked at 4 h (Fig. 4c).

As shown in Figure 5a,b, cyclin D1 mRNA expression was increased in the HMGB1-treated cells. The highest expression was found at 4 h and then cyclin D1 mRNA returned to baseline at 12 h. It can be seen in Figure 5c–e that the expression of cyclin D1 protein showed the similar trend to cyclin D1 mRNA. As illustrated in Figure 5f,g, incubation with HMGB1 resulted in the significant up-regulation of CDK4 protein and down-regulation of P16 protein in a time-dependent manner in MMC cells ($P < 0.05$) as shown by Western blotting.

THE SH-HMGB1 VECTOR EFFECTIVELY PREVENTED IFN-γ-INDUCED CELL PROLIFERATION, DECREASED CYCLIN D1/CDK4/P16 PATHWAY AND PCNA EXPRESSION, AND PREVENTED CELL PROGRESSION

After confirming the influence of HMGB1 on cell cycle, the sh-HMGB1 vector was transfected into MMC cells in order to determine whether HMGB1 mediates IFN-γ-induced MMC proliferation by regulating cell cycle. As shown in Figure 6, HMGB1 mRNA and protein increased after the stimulation of IFN-γ compared with unstimulated cells. In contrast, MMC cells transfected with the sh-HMGB1 vector showed low HMGB1 mRNA and protein expression after stimulated by IFN-γ for 12 h (inhibited by 78.57% and 98.74%, respectively). On the contrary, no change in HMGB1 protein expression was found between cells transfected with the blank control vector and untransfected MMC cells.

FCM revealed that the number of cells in S phase decreased and the number of cells in G0/G1 phase increased. Therefore, the PI decreased following transfection of IFN-γ-stimulated MMC cells with the sh-HMGB1 vector (Fig. 7). No up-regulation of cyclin D1 mRNA and protein was observed in cells transfected with the

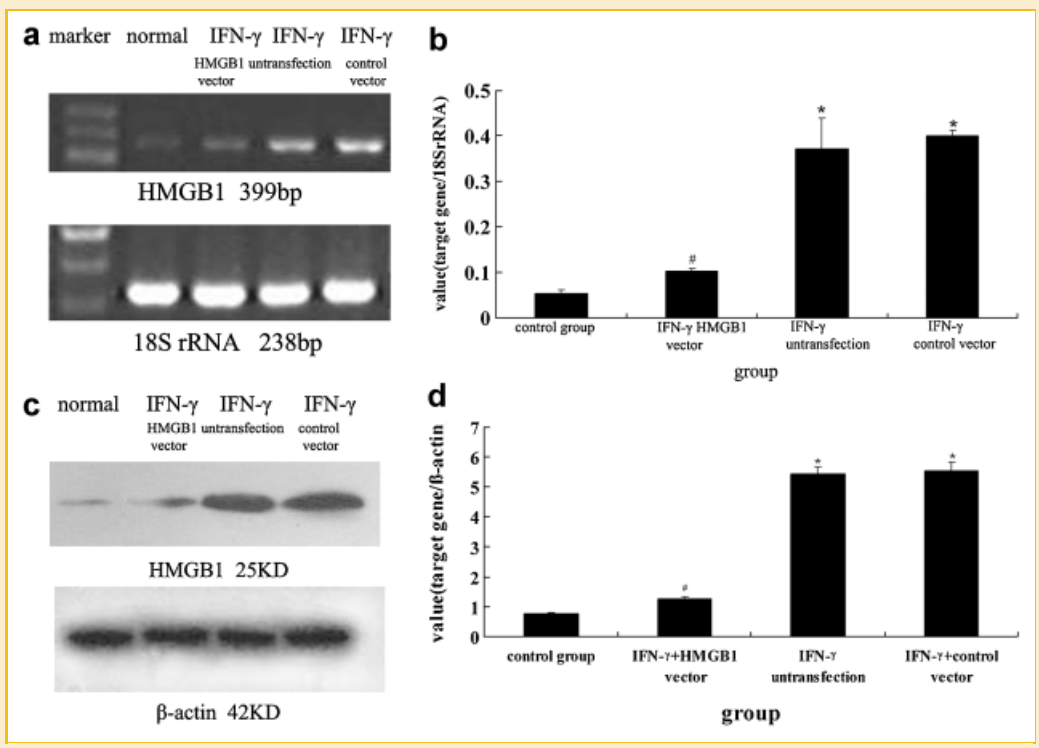

Fig. 6. Specific sh-HMGB1 vector effectively prevents IFN-γ-induced increase of HMGB1 in MMC cells. a,b: HMGB1 mRNA expression was determined by RT-PCR analysis and quantified by densitometry. c,d: Expression of HMGB1 protein was measured by Western blotting analysis and quantified by densitometry. $^{*}P < 0.01$ versus normal group, $\#P < 0.01$ versus control vector group.

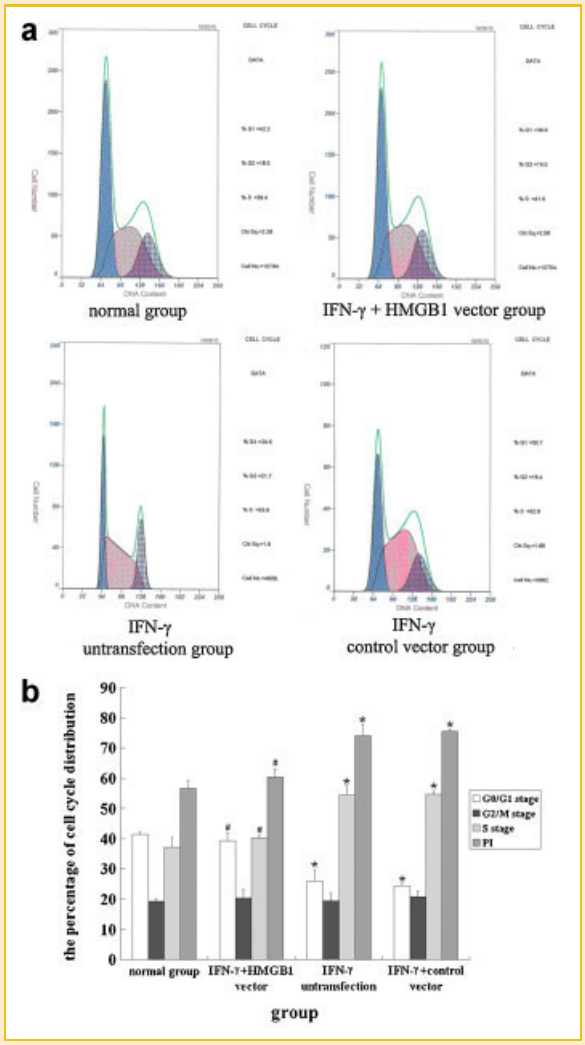

Fig. 7. Specific sh-HMGB1 vector effectively prevents IFN-γ-induced cell proliferation, increases percentage of the cells in G0/G1 phase and decreases the percentage in S phase in MMC cells. a: Histogram of cell cycle distribution by FCM analysis in MMC cells. b: The changes of cell cycle distribution and PI (proliferation index) in MMC cells. $^{*}P < 0.01$ versus normal group, $\#P < 0.01$ versus IFN-γ + control vector group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

sh-HMGB1 vector in medium containing IFN-γ. However, the up-regulation of cyclin D1 mRNA and protein could be easily seen in blank vector-transfected MMC cells and untransfected cells treated with IFN-γ (Fig. 8a,b). Similarly, sh-HMGB1 vector decreased the IFN-γ-induced CyclinD1 protein, CDK4 protein, and PCNA protein overexpression and up-regulated the expression of p16 protein (Fig. 8c–e).

DISCUSSION

Recent evidence has revealed that HMGB1 is a new cytokine that can activate many biological functions [Winter et al., 2009], and extracellular HMGB1 plays an important role in many diseases [Liu et al., 2011]. Moreover, HMGBl, as an endogenous immune adjuvant, is an important factor regulating dendritic cell maturation and Thl polarization, which play a vital role in promoting an effective inflammatory immune response. HMGB1 is either released by activated cells (such as activated macrophages/monocytes, and erythroleukemia cells) or passively released by necrotic or damaged cells into the extracellular milieu [Wang et al., 1999; Scaffidi et al., 2002; Rendon-Mitchell et al., 2003], triggering an inflammatory response. In addition, HMGB1 stimulates macrophages, monocytes, and neutrophils to release proinflammatory cytokines including IL-1β, TNF-α, and IL-8 [Wang et al., 1999; Andersson et al., 2000; Park et al., 2003]. Many studies have shown that HMGB1, as a late inflammatory mediator, is related to autoimmune diseases [Rendon-Mitchell et al., 2003], and it is a novel therapeutic target for arthritis [Scaffidi et al., 2002]. HMGB1 has also been presumed to induce LN through the aforementioned abnormal immune response. Recent studies showed that the HMGB1 protein in serum or in lesional skin increased in patients with LN [Barkauskaite et al., 2007; Abdulahad et al., 2010]. However, the precise role of HMGB1 is unknown in pathogenesis of lupus nephritis, especially the correlation between HMGB1 and the pathological changes in glomeruli is rarely reported.

In this study, HMGB1 protein expression in the glomeruli of patients with LN was higher than that in the control group. The results of animal experiments showed that the HMGB1 protein was primarily restricted to the tubule nuclei in control mice, whereas, HMGB1 expression was increased in BXSB mice, and was largely translocated to the cytoplasm and released into the extracellular space, concurrent with the up-regulation of PCNA protein. From the above results, it is therefore possible that the inherent cells in glomeruli synthesized and excreted HMGB1, thereby contributing to cell proliferation.

In order to further investigate whether HMGB1 is related with the cell proliferation of glomeruli, MMC cells were treated with HMGB1. The results of FCM and immunocytochemistry showed that HMGB1 induced the proliferation of MMC and promoted cell cycle transition from G0/G1 to S phase. The mechanism of cell cycle regulation is the reciprocity of cyclins, cyclin-dependent kinases (CDKs), and CDKIs [Hirama and Koeffler, 1995]. The cyclin D1/CDK4/p16-pRb pathway is one of the key signal transduction pathways at the G1/S checkpoint in the cell cycle [Morgan, 1995; Sato et al., 2011]. Alterations of any component of the pathway, such as deletion or mutation of the p16 gene, amplification or the overexpression of CDKs of cyclin D, and mutations to CDKs that affect p16 binding, will lead to Rb phosphorylation and subsequent progression of G1 into S phase transition [Paggi et al., 1996; Zuo et al., 1996]. Song et al. [2005] reported that the decrease of p16 cooperated with cyclin D1 and caused deregulation of G1/S checkpoint, leading to abnormal cell proliferation in NPC (nasopharyngeal carcinoma). Rho et al.'s study have shown that down-regulation of Cyclin D1, CDK4 and up-regulation of p16, p21,p-CDC25A caused the $G_{(0)}/G_{(1)}$ arrest of ovarian cancer cells exposed to thioridazine [Rho et al., 2011]. Our study confirmed that HMGB1 could promote the switch from G0/G1 to S phase. However, whether this change is correlated with abnormal expression of cyclin D1/CDK4/p16 is still unknown. In order to explore this hypothesis, we determined the expression of cyclin D1/CDK4/p16 in HMGB1-stimulated cells. The expression of

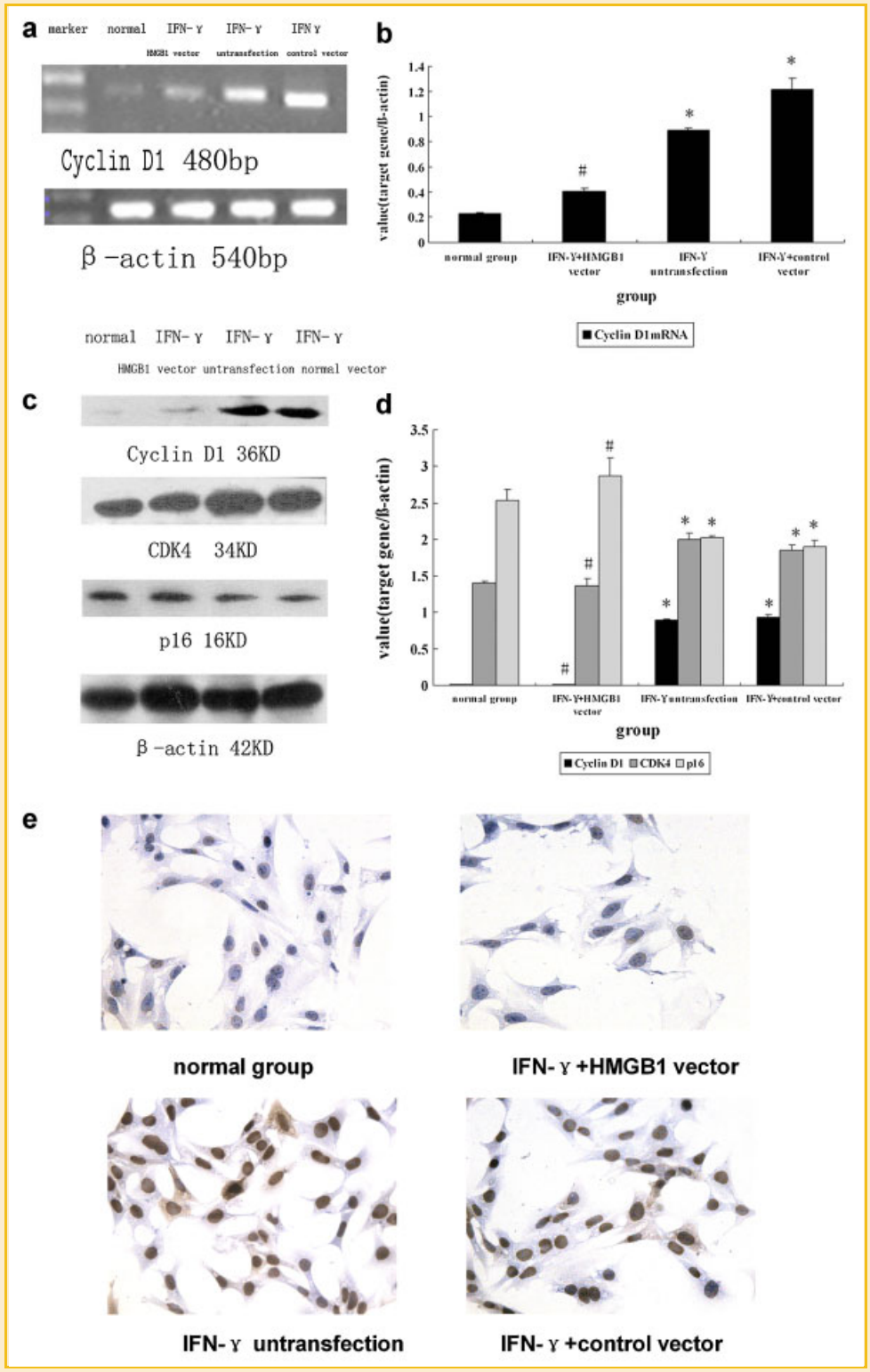

Fig. 8. Specific sh-HMGB1 vector effectively prevents IFN-γ-induced cyclin D1 mRNA and protein, CDK4 protein expression, and increases p16 protein expression in MMC cells. a,b: mRNA levels of cyclin D1 were determined by RT-PCR and quantified by densitometry. c,d: Expressions of CyclinD1, CDK4, and p16 protein were determined by Western blotting analysis and quantified by densitometry (mean ± SD, n = 3), $^{*}P < 0.01$ versus normal group, $\#P < 0.01$ versus IFN-γ + control vector group. e: Expression of PCNA was detected by immunocytochemical staining (400×). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cyclin D1 and CDK4 increased, while the expression of p16 protein decreased in HMGB1-stimulated cells. Therefore, the above data indicate that HMGB1 mediates cyclin D1/CDK4/p16 pathway expression, promotes the transition from G0/G1 to S phase, and drives cell proliferation in MMC cells. How HMGB1 regulates PCNA, cyclin D1, p16, and CDK4 expression in mesangial cell is unknown. Bassi et al. [2008] reported that HMGB1 induced a dose-dependent increase in cell proliferation, which was found to be RAGE-mediated and involved the MAPK/ERK pathway in human malignant glioma cell lines. Our present studies aim to further explore whether HMGB1 regulates cell proliferation and Cyclin D1/CDK4/p16 expression through interacting with its receptors (such as TOLL-like receptors) and activating PI3K/Akt signal pathway in MMC cells.

Some studies have shown that IFN-γ is an important cytokine in SLE [Segal et al., 1997]. Our previous studies have also showed that IFN-γ could induce the expression of the proliferation of mesangial cell in vitro. To further elucidate the role of HMGB1 in mediating IFN-γ-induced cell proliferation, we constructed MMC cell line transfected with a specific shRNA vector aimed at the mouse HMGB1 gene. FCM analysis showed that the PI of MMC treated with IFN-γ decreased after HMGB1 knockdown. The percentage of cells in S phase decreased, while the percentage of cells in G0/G1 phase noticeably increased. Therefore, we thought that HMGB1 mediated the proliferation of MMC treated with IFN-γ and drive the cell cycle transition from G0/G1 to S phase. In addition, blocking HMGB1 prevents cell cycle progression and suppresses cell proliferation through the inhibition of cyclin D1/CDK4/p16 pathway.

In summary, our data demonstrate that HMGB1 expression is increased in the glomeruli of patients and mice with lupus nephritis. HMGB1 induces mice mesangial cell proliferation and promotes the transition from G1 to S stage in the cell cycle by up-regulating the cyclin D1/CDK4/p16 pathway. These results provide evidence for HMGB1 as an important cytokine, which might be a potential molecular target in gene therapy for lupus nephritis. However, further studies are needed to examine the precise role of HMGB1 in the pathogenesis of LN.

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