

# HMGB1 induces lung fibroblast to myofibroblast differentiation through NF- $\kappa$ B-mediated TGF- $\beta$ 1 release

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**Abstract.** The proinflammatory factor high-mobility group box protein 1 (HMGB1) has been implicated in the pathogenesis of lung fibrosis; however, the role of HMGB1 in lung fibrosis remains unclear. It has previously been reported that nuclear factor (NF)- $\kappa$ B and transforming growth factor (TGF)- $\beta$ 1 may be involved in lung fibrosis. Therefore, the present study aimed to examine the potential molecular mechanisms that underlie HMGB1-induced lung fibrosis via the regulation of NF- $\kappa$ B and TGF- $\beta$ 1. The results demonstrated that HMGB1 stimulation increased the activation of NF- $\kappa$ B and the release of TGF- $\beta$ 1, as well as the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I in human lung fibroblasts *in vitro*. In addition, inhibition of NF- $\kappa$ B activation blocked HMGB1-induced TGF- $\beta$ 1 release, as well as  $\alpha$ -SMA and collagen I expression in lung fibroblasts. Preventing the release of TGF- $\beta$ 1 inhibited HMGB1-induced  $\alpha$ -SMA and collagen I expression; however, it had no effect on NF- $\kappa$ B activation. Collectively, these findings indicate that HMGB1 induces fibroblast to myofibroblast differentiation of lung fibroblasts via NF- $\kappa$ B-mediated TGF- $\beta$ 1 release.

## Introduction

Lung fibrosis is one the oldest recorded fibroproliferative disorders, which may ultimately lead to lung failure and mortality (1,2). Several distinct injury-associated or inflammatory causes of interstitial lung disease (ILD) can result in progressive lung scarring (3-7). ILDs that arise with no obvious cause are referred to as idiopathic. Despite the use of anti-inflammatory or immunosuppressive drugs, there is currently no effective treatment for lung fibrosis. Lung transplantation remains the only viable intervention for end-stage lung fibrosis.

Fibrosis is characterized by abnormal fibroblast to myofibroblast differentiation (8-10). Resident pulmonary fibroblasts respond to various stimuli under chronic inflammatory conditions during fibrogenesis. Once activated they differentiate into myofibroblasts, which have a more contractile, proliferative and secretory-active phenotype, and are characterized by the increased expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and extracellular matrix (ECM) components (11,12).

High-mobility group box protein 1 (HMGB1) is a recognized nuclear protein and architectural chromatin-binding factor that binds DNA and promotes protein assembly on specific DNA targets (13,14). However, recent studies have indicated that HMGB1 can be passively released from necrotic cells or actively secreted into the extracellular milieu under appropriate signal stimulation (15,16). Extracellular HMGB1 is a multifunctional cytokine involved in the processes underlying infection, inflammation, apoptosis and immune responses, as well as in tumor development by binding to specific cell-surface receptors (17-20). Notably, numerous studies have indicated that HMGB1 may be involved in fibrotic disorders, including myocardial fibrosis, renal fibrosis and liver fibrosis (21-23).

Pulmonary HMGB1 is markedly upregulated in patients with lung fibrosis and experimental lung fibrosis (24-26). Entezari *et al* (27) demonstrated that inhibition of HMGB1 may enhance bacterial clearance and protect against *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. Li *et al* (28) revealed that HMGB1 mediates the epithelial-to-mesenchymal transition in pulmonary fibrosis, which

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involves the transforming growth factor (TGF)- $\beta$ 1/Smad2/3 signaling pathway. However, the role of HMGB1 in lung fibroblast to myofibroblast differentiation remains unclear.

In the present study, the potential role of HMGB1 in fibroblast to myofibroblast differentiation was explored *in vitro*. Firstly, the effects of HMGB1 on  $\alpha$ -SMA expression and ECM production in human lung fibroblasts were detected. Subsequently, the role of TGF- $\beta$ 1 release in HMGB1-induced  $\alpha$ -SMA expression and ECM production in human lung fibroblasts was investigated. In addition, the present study explored the potential signaling pathway underlying the regulation of HMGB1-induced fibroblast to myofibroblast differentiation.

## Materials and methods

**Reagents.** Monoclonal antibodies against human  $\alpha$ -SMA (catalog no. ab32575) and collagen I (catalog no. ab138492) were purchased from Abcam (Cambridge, UK). Monoclonal antibodies against human  $\beta$ -actin (catalog no. 3700), nuclear factor (NF)- $\kappa$ B p65 (catalog no. 6956) and phosphorylated (p)-NF- $\kappa$ B p65 (catalog no. 13346), and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig) G (catalog no. 7074) and anti-mouse IgG (catalog no. 7076) secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The enhanced chemiluminescence (ECL) detection system was purchased from Tanon Science and Technology Co., Ltd. (Shanghai, China). Polyvinylidene difluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). Ammonium pyrrolidinedithiocarbamate (PDTC) was purchased from Abcam. TRIzol<sup>®</sup> reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A RevertAid<sup>™</sup> First Strand cDNA Synthesis kit was obtained from Fermentas; Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA). TaqMan<sup>®</sup> Fast Advanced Master Mix was purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A human TGF- $\beta$ 1 ELISA kit (catalog no. EK0513) was purchased from Boster (Wuhan, China). Monoclonal antibody against human TGF- $\beta$ 1 (TGF- $\beta$ 1 neutralization antibody; catalog no. MAB240) and recombinant human HMGB1 Protein, were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). RIPA lysis buffer and a BCA kit were supplied by Thermo Fisher Scientific.

**Cell culture and treatment.** The MRC-5 human lung fibroblast cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> and were routinely passaged upon reaching 80% confluence, using 0.25% trypsin and a 1:3 cell dilution for each passage (10,29). MRC-5 cells were incubated with various doses of HMGB1 (0, 0.01, 0.1, 1, 10 and 100  $\mu$ g/ml) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The mRNA expression levels of  $\alpha$ -SMA were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), 48 h following HMGB1 stimulation. Additionally,

MRC-5 cells were incubated with 10  $\mu$ g/ml HMGB1 and the mRNA expression levels of  $\alpha$ -SMA and collagen I were detected using RT-qPCR at 0, 3, 6, 12, 24, 36, 48, 72 and 96 h following HMGB1 stimulation, or the phosphorylation levels of NF- $\kappa$ B p65 were detected by western blotting at 0, 20, 40, 60, 80 min following HMGB1 stimulation. Next, MRC-5 cells were incubated with 10  $\mu$ g/ml HMGB1 or PBS solvent control, and the protein expression levels of  $\alpha$ -SMA and collagen I were detected by western blotting at 72 h following HMGB1 stimulation. In order to inhibit the activation of NF- $\kappa$ B p65, the PDTC (20  $\mu$ M) or DMSO solvent control were added into the medium at 30 min prior to addition of HMGB1. In order to block the function of TGF- $\beta$ 1, TGF- $\beta$ 1 neutralization antibody (10  $\mu$ g/ml) or IgG control (10  $\mu$ g/ml) were added into the medium with HMGB1.

**RNA isolation and RT-qPCR.** Total RNA was isolated from the cells using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.). An equal amount (2  $\mu$ g) of total RNA was synthesized as first-strand cDNA using the RevertAid<sup>™</sup> First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA was amplified using the TaqMan<sup>®</sup> Fast Advanced Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) to detect the expression levels of  $\alpha$ -SMA, collagen I and  $\beta$ -actin, according to the manufacturer's protocol. Each sample was assayed in triplicate. The thermal cycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec. The 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for all experiments. The relative levels of target gene expression were obtained by calculating the ratio of cycle numbers of the initial exponential amplification phase as determined by the sequence detection system for specific target genes and  $\beta$ -actin using the following formula:  $2^{-\Delta\Delta C_q}$  (30,31). The sequences of primers used were as follows:  $\alpha$ -SMA forward, 5'-ACTGCCGCATCC TCATCCTC-3',  $\alpha$ -SMA reverse, 5'-ATGCCAGCAGACTCC ATCCC-3';  $\alpha$ -SMA probe 5'-Fam-CGCTGCCAGAGACC CTGTTCCAGCC-Tamra-3'; collagen I forward, 5'-CCCAGT GTGGCCAGAAGAA-3', collagen I reverse, 5'-AGGAAG GTCAGCTGGATGGC-3'; collagen I probe 5'-Fam-TGGCGG CCAGGGCTCCGACC-Tamra-3';  $\beta$ -actin forward, 5'-GGC ACTCTTCCAGCCTTCCT-3',  $\beta$ -actin reverse, 5'-GCACTG TGTTGGCGTACAGG-3';  $\beta$ -actin probe 5'-Fam-CGTGGA TGCCACAGGACTCCATGCCCA-Tamra-3'.

**Western blot analysis.** Cells were lysed using RIPA lysis buffer at 4°C for 30 min and then protein concentration was quantified with a BCA kit according to the manufacturer's protocol. The proteins (50  $\mu$ g) were subjected to 4-20% ExpressPlus<sup>™</sup> PAGE Gel electrophoresis (Genscript, Nanjing, Jiangsu, China) and transferred onto PVDF membranes using a Mini-Protean<sup>®</sup> system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated for 1 h at room temperature in blocking buffer [5% skim milk in tris-buffered saline plus 0.05% Tween 20 (TBS-T)] prior to overnight incubation with the appropriate antibodies including  $\alpha$ -SMA (1:2,000), collagen I (1:3,000), NF- $\kappa$ B p65 (1:1,000) and p-NF- $\kappa$ B p65 (1:1,000) and anti- $\beta$ -actin (1:1,000) as the internal standard

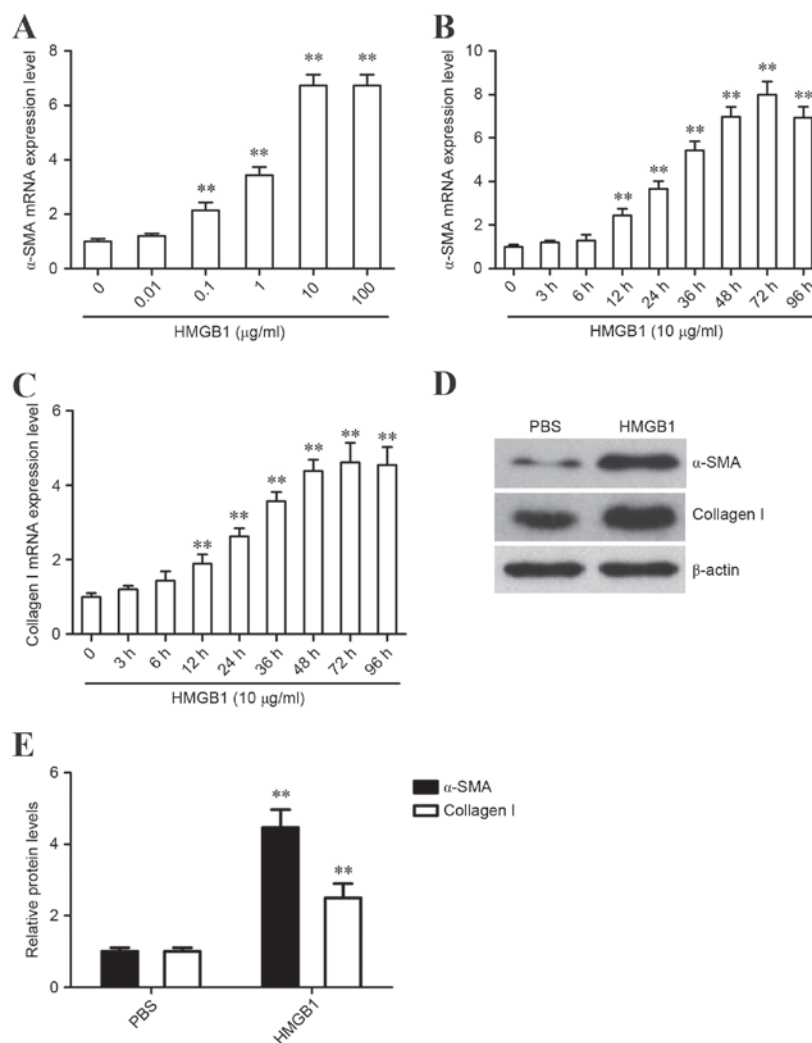


Figure 1. HMGB1 increased  $\alpha$ -SMA and collagen I expression in the MRC-5 cell line. (A) MRC-5 cells were incubated with various doses of HMGB1, and the mRNA expression levels of  $\alpha$ -SMA were detected using RT-qPCR 48 h following HMGB1 stimulation. \*\* $P < 0.01$  vs. 0  $\mu$ g/ml HMGB1 group. MRC-5 cells were incubated with 10  $\mu$ g/ml HMGB1, and the mRNA expression levels of (B)  $\alpha$ -SMA and (C) collagen I were detected using RT-qPCR at various time points following HMGB1 stimulation. \*\* $P < 0.01$  vs. 0 h group. (D) MRC-5 cells were incubated with 10  $\mu$ g/ml HMGB1, and the protein expression levels of  $\alpha$ -SMA and collagen were detected by (D) western blotting at 72 h following HMGB1 stimulation. (E) Quantification of western blot analysis. \*\* $P < 0.01$  vs. PBS group. The data are from one experiment, representative of 3 independent experiments. Results are presented as the mean  $\pm$  standard deviation ( $n=3$ /group). HMGB1, high-mobility group box 1;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

at 4°C. After washing with TBS-T, the membranes were incubated with HRP-conjugated anti-rabbit IgG (1:2,000) and anti-mouse IgG (1:2,000) for 1 h at room temperature. The bands were visualized using the ECL detection system (Tanon Science and Technology Co., Ltd.). The radiographic band density was measured using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc.).

**ELISA.** The levels of TGF- $\beta$ 1 in the cell media were determined using a commercial ELISA kit (Boster Systems, Inc.) according to the manufacturer's protocol. The optical density at a wavelength of 450 nm was detected by a microplate reader. TGF- $\beta$ 1 concentration was calculated by the standard curve.

**Statistical analysis.** All statistical analyses were performed using the SPSS v.11.5 software (SPSS, Inc., Chicago, IL, USA). The statistical significance of the groups was evaluated by one-way analysis of variance; simultaneous multiple comparisons among groups was conducted using the Bonferroni

method.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**HMGB1 induces fibroblast to myofibroblast differentiation in human lung fibroblasts.** Human lung fibroblasts were initially incubated with various doses of HMGB1. After HMGB1 stimulation for 48 h, the expression levels of  $\alpha$ -SMA were detected by RT-qPCR. The results demonstrated that HMGB1 increased  $\alpha$ -SMA expression in human lung fibroblasts in a dose-dependent manner; the maximal effect was detected following treatment with 10  $\mu$ g/ml HMGB1 (Fig. 1A). Therefore, 10  $\mu$ g/ml HMGB1 was chosen for further studies. Human lung fibroblasts were incubated with 10  $\mu$ g/ml HMGB1, and  $\alpha$ -SMA expression was detected at 0, 3, 6, 12, 24, 36, 48, 72 and 96 h using RT-qPCR. The results revealed that  $\alpha$ -SMA mRNA expression increased and peaked at 72 h following HMGB1 stimulation (Fig. 1B). In addition, the expression

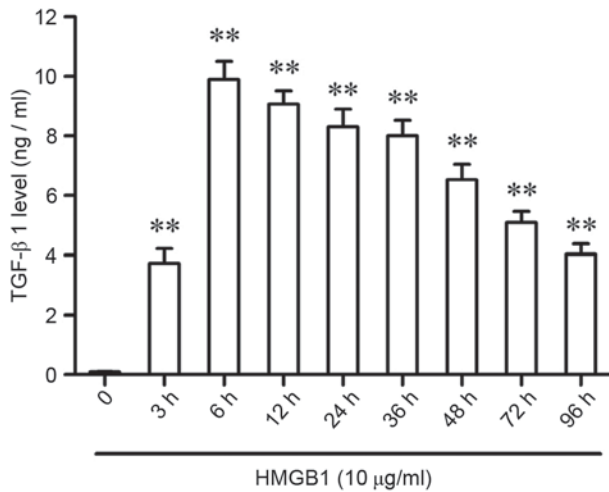


Figure 2. HMGB1 enhanced TGF- $\beta$ 1 release in the MRC-5 cell line. MRC-5 cells were incubated with 10  $\mu$ g/ml HMGB1, and the protein expression levels of TGF- $\beta$ 1 in the media were detected by ELISA at various time points following HMGB1 stimulation. \*\* $P$ <0.01 vs. 0 h group. The data are from one experiment, representative of 3 independent experiments. Results are presented as the mean  $\pm$  standard deviation ( $n$ =3/group). HMGB1, high-mobility group box 1; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1.

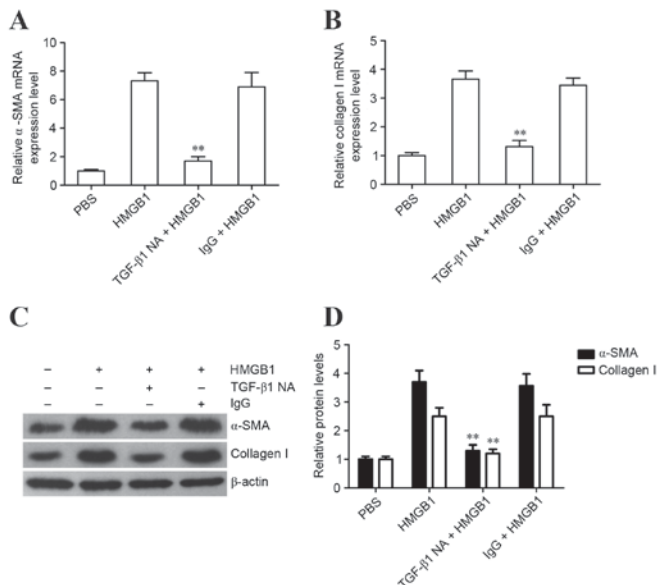


Figure 3. Effects of TGF- $\beta$ 1 NA on HMGB1-induced fibroblast to myofibroblast differentiation in the MRC-5 cell line. TGF- $\beta$ 1 NA (10  $\mu$ g/ml) was used to block the function of TGF- $\beta$ 1 during HMGB1 stimulation (10  $\mu$ g/ml). The expression of  $\alpha$ -SMA and collagen I at (A and B) the mRNA and (C and D) protein level was detected at 72 h following HMGB1 stimulation by (A and B) RT-qPCR and (C and D) western blotting, which was normalized against  $\beta$ -actin. \*\* $P$ <0.01 vs. HMGB1 and IgG + HMGB1 groups. The data are from one experiment, representative of 3 independent experiments. Results are presented as the mean  $\pm$  standard deviation ( $n$ =3/group). TGF- $\beta$ 1 NA, transforming growth factor  $\beta$ 1 neutralization antibody; HMGB1, high-mobility group box 1;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IgG, immunoglobulin G.

of collagen I was examined in human lung fibroblasts at the same range of time points following HMGB1 stimulation. The results demonstrated that collagen I mRNA expression increased and peaked at 72 h following HMGB1 stimulation (Fig. 1C). Further investigations revealed that the protein

expression levels of  $\alpha$ -SMA and collagen I were markedly increased following HMGB1 stimulation for 72 h (Fig. 1D and E). These results indicated that HMGB1 may induce fibroblast to myofibroblast differentiation of human lung fibroblasts.

*HMGB1 increases TGF- $\beta$ 1 release from human lung fibroblasts.* Human lung fibroblasts were incubated with 10  $\mu$ g/ml HMGB1, and the protein expression levels of TGF- $\beta$ 1 in the media were detected by ELISA at various time points (0, 3, 6, 12, 24, 36, 48, 72 and 96 h). The results demonstrated that the level of TGF- $\beta$ 1 secretion increased following HMGB1 stimulation, peaked at 6 h and then gradually decreased up to 96 h (Fig. 2). HMGB1-induced TGF- $\beta$ 1 release peaked earlier than HMGB1-induced expression of  $\alpha$ -SMA and collagen I in human lung fibroblasts (6 vs. 72 h), thus indicating that TGF- $\beta$ 1 release may mediate HMGB1-induced fibroblast to myofibroblast differentiation of human lung fibroblasts.

*TGF- $\beta$ 1 release is essential for HMGB1-induced fibroblast to myofibroblast differentiation of human lung fibroblasts.* In order to investigate whether TGF- $\beta$ 1 is involved in HMGB1-induced  $\alpha$ -SMA expression in human lung fibroblasts, the TGF- $\beta$ 1 neutralization antibody was used to block the function of TGF- $\beta$ 1 during HMGB1 stimulation. Subsequently, the expression levels of  $\alpha$ -SMA and collagen I were detected. The results revealed that the TGF- $\beta$ 1 neutralization antibody effectively inhibited HMGB1-induced  $\alpha$ -SMA and collagen I expression in human lung fibroblasts (Fig. 3A-D). Collectively, these results indicated that HMGB1 may induce fibroblast to myofibroblast differentiation of human lung fibroblasts via TGF- $\beta$ 1 release.

*NF- $\kappa$ B activation is involved in HMGB1-induced TGF- $\beta$ 1 release and fibroblast to myofibroblast differentiation of lung fibroblasts.* It has previously been reported that NF- $\kappa$ B activation may mediate TGF- $\beta$ 1 release (32). Therefore, the present study initially detected whether HMGB1 could induce NF- $\kappa$ B activation in lung fibroblasts. The results revealed that HMGB1 significantly induced NF- $\kappa$ B activation in lung fibroblasts following HMGB1 stimulation for 60 min (Fig. 4A). Subsequently, the NF- $\kappa$ B inhibitor, PDTC, was used to inhibit NF- $\kappa$ B activation to determine the role of NF- $\kappa$ B activation in HMGB1-induced TGF- $\beta$ 1 release, as well as  $\alpha$ -SMA and collagen I expression, in lung fibroblasts. The results demonstrated that the NF- $\kappa$ B inhibitor markedly reduced HMGB1-induced TGF- $\beta$ 1 release (Fig. 4B), as well as  $\alpha$ -SMA and collagen I mRNA and protein expression in lung fibroblasts (Fig. 4C-F). Finally, TGF- $\beta$ 1 suppression did not affect HMGB1-induced NF- $\kappa$ B activation in lung fibroblasts (Fig. 4G), suggesting that NF- $\kappa$ B is an upstream regulator of TGF- $\beta$ 1 release in HMGB1-stimulated lung fibroblasts. Collectively, these results indicated that HMGB1 may induce fibroblast to myofibroblast differentiation via NF- $\kappa$ B-mediated TGF- $\beta$ 1 release.

## Discussion

Lung fibrosis is a common fibroproliferative disorder, which can lead to lung failure and mortality (1,2,33). Despite the use of anti-inflammatory or immunosuppressive drugs,



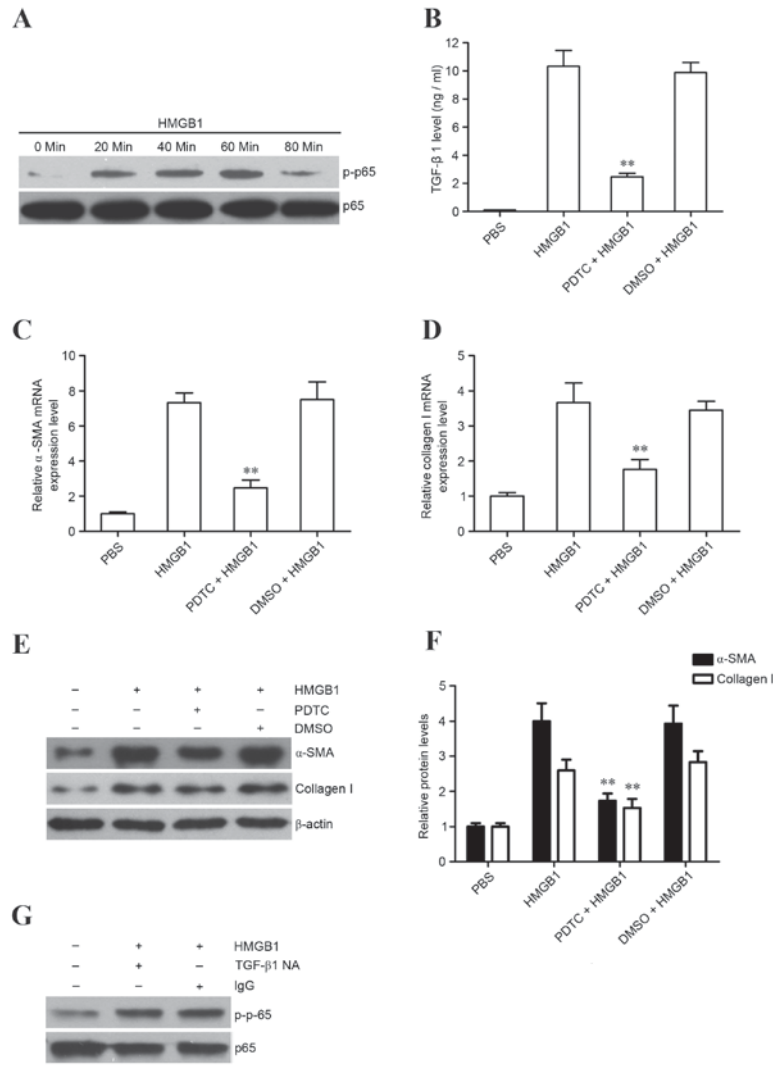


Figure 4. Role of NF-κB activation in HMGB1-induced TGF-β1 release and fibroblast to myfibroblast differentiation in the MRC-5 cell line. (A) Phosphorylation of NF-κB p65 was detected in MRC-5 cells stimulated with HMGB1 (10 μg/ml) at various time points by western blotting. MRC-5 cells were treated with PDTC followed by HMGB1 stimulation (10 μg/ml); subsequently, (B) TGF-β1 release at 6 h was detected by ELISA, and (C) α-SMA and (D) collagen I expression levels at 72 h were detected in MRC-5 cells by reverse transcription-quantitative polymerase chain reaction and (E and F) western blotting. (G) TGF-β1 NA (10 μg/ml) was used to block the function of TGF-β1 during HMGB1 stimulation, and the phosphorylation of NF-κB p65 was detected at 60 min following HMGB1 stimulation by western blot analysis. \*\*P<0.01 vs. HMGB1 and DMSO + HMGB1 groups. The data are from one experiment, representative of 3 independent experiments. Results are presented as the mean ± standard deviation (n=3/group). NF-κB, nuclear factor κB; p, phosphorylated; HMGB1, high-mobility group box 1; PDTC, ammonium pyrrolidinedithiocarbamate; TGF-β1, transforming growth factor β1; α-SMA, α-smooth muscle actin; TGF-β1 NA, TGF-β1 neutralization antibody; DMSO, dimethyl sulfoxide.

there is currently no effective treatment for lung fibrosis. Previous studies have revealed that fibroblast to myfibroblast differentiation is pathologically altered during lung fibrosis. Furthermore, it has been suggested that HMGB1 may be associated with lung fibrosis (24-28); however, the role of HMGB1 in lung fibroblast activation and differentiation is unclear. The present study revealed that HMGB1 increased α-SMA and collagen I expression in human lung fibroblasts, indicating that HMGB1 may induce fibroblast to myfibroblast differentiation of lung fibroblasts.

Since TGF-β1 is a key factor that mediates fibroblast to myfibroblast differentiation (34,35), the present study further investigated HMGB1-induced TGF-β1 release from human lung fibroblasts. The results demonstrated that TGF-β1 secretion was significantly increased following HMGB1 stimulation. Notably, HMGB1-induced TGF-β1 release occurred earlier

than HMGB1-induced expression of α-SMA and collagen I in human lung fibroblasts (6 vs. 72 h), indicating that TGF-β1 release may mediate HMGB1-induced fibroblast to myfibroblast differentiation of human lung fibroblasts. Further experiments demonstrated that treatment with the TGF-β1 neutralization antibody effectively inhibited HMGB1-induced α-SMA and collagen I expression in human lung fibroblasts. Collectively, these results indicated that HMGB1 may induce fibroblast to myfibroblast differentiation of human lung fibroblasts via TGF-β1 release.

Previous studies have reported that NF-κB activation may mediate TGF-β1 release (36,37). Therefore, the present study determined whether HMGB1 could induce NF-κB activation in lung fibroblasts. The results revealed that HMGB1 significantly induced NF-κB activation in lung fibroblasts. The NF-κB inhibitor, PDTC, was applied to inhibit NF-κB activation in order to

investigate the role of NF- $\kappa$ B activation in HMGB1-induced TGF- $\beta$ 1 release and fibroblast to myofibroblast differentiation of lung fibroblasts. The results demonstrated that the NF- $\kappa$ B inhibitor markedly reduced HMGB1-induced TGF- $\beta$ 1 release, and  $\alpha$ -SMA and collagen I expression in lung fibroblasts. Collectively, the results indicated that HMGB1 may induce fibroblast to myofibroblast differentiation of lung fibroblasts via NF- $\kappa$ B-mediated TGF- $\beta$ 1 release.

It has been reported that HMGB1 accelerates lipopolysaccharide-induced lung fibroblast proliferation *in vitro* via the NF- $\kappa$ B signaling pathway (38). Conversely, the present study demonstrated that NF- $\kappa$ B activation in lung fibroblasts, induced by HMGB1, may promote TGF- $\beta$ 1 release and induce fibroblast to myofibroblast differentiation. Signal transducer and activator of transcription-3 (STAT-3) has been reported to contribute to lung fibrosis through epithelial injury and fibroblast to myofibroblast differentiation (39). In addition, TGF- $\beta$  stimulation *in vitro* leads to Smad2/Smad3-dependent phosphorylation of STAT-3 in lung fibroblasts (39). NF- $\kappa$ B and STAT-3 are involved in fibroblast to myofibroblast differentiation, however, the associations between these two molecules is unclear. Therefore, further investigations are required to determine the association between NF- $\kappa$ B and STAT-3 in the regulation of HMGB1-induced TGF- $\beta$ 1 release and fibroblast to myofibroblast differentiation.

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