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High-mobility group box1 mediates epithelial-to-mesenchymal transition in pulmonary fibrosis involving transforming growth factor-β1/Smad2/3 signaling

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Running title: HMGB1 mediates EMT via TGF-β1/Smad2/3 signaling in PF

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Abbreviations: α-SMA, α-smooth muscle actin; AECs, alveolar epithelial cells; BLM, bleomycin; Col-I, type I collagen; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; HMGB1, high-mobility group box1; HYP, hydroxyproline; MAPK, mitogen-activated protein kinase; PF, pulmonary fibrosis; p-Smad2/3, phosphorylated-Smad2/3; RAGE, the receptor for advanced glycation end products; TGF-β1, transforming growth factor-β1.

Abstract

Epithelial-to-mesenchymal transition (EMT) is a crucial event in the cellular origin of myofibroblasts that secrete extracellular matrix (ECM) in the progression of pulmonary fibrosis (PF). High-mobility group box1 (HMGB1) is a novel mediator of EMT. However, whether this process involves the recognized transforming growth factor-β1 (TGF-β1)/Smad signaling that also contributes to EMT in PF, has not been elucidated yet. Here, we developed a PF model induced by bleomycin (BLM) in rats and conducted several simulation experiments in A549 and RLE-6TN (human and rat) alveolar epithelial cells (AECs) lines to unravel the role of TGF-β1/Smad2/3 signaling in HMGB1-mediated EMT. We found that the levels of serum HMGB1 and lung hydroxyproline (HYP) were severely elevated after BLM administration. Moreover, the protein expression of HMGB1, TGF-β1, phosphorylated-Smad2/3 (p-Smad2/3), and the mesenchymal markers including α-smooth muscle actin (α-SMA), vimentin, and type I collagen (Col-I) were significantly increased with the reduced protein expression of epithelial marker (E-cadherin) in the rat model by Western blot or immunohistochemical analysis. Besides, the uptake of both exogenous TGF-β1 and HMGB1 by above AECs could induce EMT; meanwhile, HMGB1 dramatically enhanced TGF-β1 expression and triggered Smad2/3 phosphorylation. In contrast, TGF-β1 deficiency evidently ameliorated HMGB1-mediated EMT with reduced p-Smad2/3 in A549 cells. It provides new insights that the HMGB1 release from injured lungs promotes AECs damage through inducing EMT process, in which TGF-β1/Smad2/3 signaling is activated and contributes to PF. These results suggest

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that HMGB1 may constitute a therapeutic target for developing anti-fibrotic agents in the abnormal lung remodeling.

Introduction

Pulmonary fibrosis (PF), a progressive fatal disorder with unclear etiology, predominantly occurs in middle-aged and elderly adults with a median survival time of less than three years from the time of diagnosis (King et al., 2011; Raghu et al., 2011). It is well known that PF is characterized by the injury of alveolar epithelial cells (AECs), abnormal activation and proliferation of extracellular matrix (ECM)-producing cells (especially myofibroblasts), and excessive deposition of ECM with subsequent scar formation, distorted lung architecture as well as impaired respiratory function (Li et al., 2014a, 2014b; Loomis-King et al., 2013). Despite lots of recent studies are to clarify the molecular mechanisms and treatment strategies for PF, limited substantial therapeutic interventions have been developed to reverse established PF or even halt the chronic progression to respiratory failure. Thus, extensive efforts are still needed to elucidate the mechanisms of PF and dig the novel targets.

Initial studies on abnormal lung remodeling are focused on the role of inflammation in inciting fibroblast activation and fibrosis, while the recent paradigm suggests that AECs undergoing epithelial-to-mesenchymal transition (EMT) also plays a crucial role in the progression of PF (Kalayarasan et al., 2013; Selman and Pardo, 2003). EMT is characterized by the loss of epithelial proteins such as E-cadherin and the acquisition of new mesenchymal markers including α-smooth muscle actin (α-SMA), vimentin and type I collagen (Col-I). It is a reversible process in which epithelial cells transform into cells with mesenchymal characteristics that contribute to ECM

secretion in the progression of PF (Mateen et al., 2013; Kalluri and Weinberg, 2009; Vittal et al., 2007). Accumulated data support that injured AECs have a great contribution to the local activation of fibroblasts and myofibroblasts, and may also directly serve as a source of these cells via EMT process (Kim et al., 2006; Liu et al., 2014; Vyas-Read et al., 2014).

Among the reported inducers, transforming growth factor-β1 (TGF-β1) is a main mediator of EMT and is the key cytokine in the abnormal tissue remodeling, particularly in PF (Chen et al., 2013; Willis et al., 2005). TGF-β latency binding protein 1, which facilitates the release and activation of the biologically latent form TGF-β1, is detected primarily in alveolar macrophages and epithelial cells lining honeycomb cysts in areas of advanced PF (Khalil et al., 2001). The TGF-β1 signaling is partly dependent on combining and activating type I TGF-β receptor, and then the post-receptor signal transducers, Smad2 and Smad3, are phosphorylated, and result in the formation of a stable complex with Smad4, which transfers into the nuclei to act as a transcriptional regulator and then induces AECs injury via promoting the changes of EMT markers (Chen et al., 2014; Sullivan et al., 2011). Therefore, strategies disrupting TGF-β1/Smad signaling have undoubtedly therapeutic potential in the clinical treatment of PF. High-mobility group box1 (HMGB1) is a transcription factor-like protein that acts as a danger signal in inflammatory diseases, tissue injury as well as fibrotic diseases (Ebina et al., 2011; Griffin et al., 2014; Lee et al., 2013; Li et al., 2014a; Ogiku et al., 2011; Rowe et al., 2008). In lung tissues from patients with PF, HMGB1 is predominantly expressed in alveolar macrophages, infiltrating

inflammatory cells and epithelial cells (Hamada et al., 2008). Recent evidence also shows that HMGB1 is a novel mediator of EMT in mice type II AECs and human proximal tubular epithelial cells (He et al., 2007; Lynch et al., 2010). However, whether the canonical TGF-β1/Smad signaling is involved in HMGB1-mediated EMT in the abnormal lung remodeling has not been clarified yet.

This study was conducted to investigate the role of TGF-β1/Smad2/3 signaling in HMGB1-mediated EMT during the development of PF. Here we determined the serum profile of HMGB1 and the lung level of hydroxyproline (HYP), as well as the protein levels of HMGB1, EMT markers, TGF-β1 and phosphorylated-Smad2/3 (p-Smad2/3) in the lung tissues in a rat model of bleomycin (BLM)-induced PF. Meanwhile, A549 and RLE-6TN (human and rat) AECs lines were used to explore whether the uptake of exogenous HMGB1 triggered EMT in both kinds of AECs, and whether it was involved in the regulation of TGF-β1/Smad2/3 signaling. In addition, TGF-β1 deficiency was performed to determine its role in HMGB1-induced EMT in A549 cells.

Materials and methods

Animals

Sprague-Dawley rats weighing 180-220 g were purchased from the Experimental Animal Center of Anhui Medical University, Hefei, Anhui, China. The animal experimental protocol was approved by the University Animal Care and Use

Committee. In compliance with the relevant guidelines, all of the animals received humane care and had free access to food and water during the study.

Reagents and antibodies

Recombinant human TGF-β1 (100-21C, PeproTech) and HMGB1 (pro-581, ProSpec) were respectively purchased from USA and Israel. The primary antibodies described in this paper included GAPDH (AP0063, Bioworld), β-actin (ab52614, abcam), TGF-β1 (ab25121, abcam), p-Smad2/3 (sc-11769, Santa Cruz), HMGB1 (ab79823, abcam), E-cadherin (ab76055, abcam), α-SMA (ab5694, abcam), vimentin (ab92547, abcam) and Col-I (ab34710, abcam), and were all from USA. In addition, the Masson's trichrome kit (MST-8003/8004, Maixin-Bio, China), hydroxyproline (HYP) Assay Kit (A030-2, Nanjing Jiancheng Bioengineering Institute, China) and HMGB1 ELISA Kit (YY42027, Shanghai yuanye Bio-Technology Co., Ltd, China) were also used in this study.

BLM-induced pulmonary fibrosis

Ninety rats were randomly divided into the normal saline (NS) group (instilled with saline) and BLM group (instilled with BLM). To establish the PF model, each vial of BLM A5 hydrochloride (8 mg/vial, Laiboten Pharmaceutical CO., LTD, Harbin, China) was dissolved in saline with a volume of 1.6 ml just before using. The PF model was induced by intratracheal instillation of BLM (5 mg/kg) in rats. On days 7, 14 and 28, the rats were first anesthetized with 10% chloral hydrate (5 ml/kg)

intraperitoneally, and then the lungs and blood were harvested. The lungs were used to stain with H&E and Masson's trichrome, while the content of HYP in lung tissues and the level of HMGB1 in serum were also measured. Furthermore, the protein expression of HMGB1, TGF-β1, p-Smad2/3, E-cadherin, α-SMA, vimentin and Col-I were observed by Western blot or immunohistochemical analysis.

Histological analysis

The left rat lung tissues were fixed in 10% formaldehyde for 48 h, dehydrated in a graded ethanol series and subsequently embedded in paraffin. Sequential 5 μm lung sections were placed on slides and stained with H&E and Masson's trichrome, for morphological analysis and locating collagen expression by using the standard protocols, respectively. The slides were investigated under a light microscope (Olympus Opticals, Tokyo, Japan) with the same magnification times (×200).

HYP and HMGB1 levels determination

The HYP level was determined in concordance with the instruction manual of the kit. The deputy lobes (80-100 mg) of each group were took out to detect the content of HYP which was expressed in microgramme of HYP per milligram of wet weight (μg/mg). The serum level of HMGB1 (ng/ml) was measured by ELISA. The absorbance of HYP and HMGB1 was measured using an automated Multiskan MK3 microplate reader (Thermo Labsystems, USA) at the wavelength of 550 nm and 450 nm.

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Immunohistochemical analysis

This method had been performed in our previous study (Xu et al., 2014a). Briefly, the lung sections were prepared and then the endogenous peroxide activity was blocked using 0.3% H_2O_2 while the non-specific protein staining was blocked with 1.5% normal goat serum in TBS with 0.5% bovine serum albumin. Subsequently, the sections were incubated with primary anti-α-SMA and anti-Col-I antibodies at 1:500 dilution at 37 °C for 30 min, and then stayed overnight at 4 °C. On the next day, the slides were incubated in goat anti-rabbit secondary antibodies for 1 h. Visualization was performed with diaminobenzidine followed by washing with water, and then counterstained with hematoxylin, dehydrated and transparented, sections were cover-slipped with oil of cypress and dried. The images were photographed with the same microscope and magnification times (×400).

Cell culture and isolation

Human pulmonary epithelial cell line A549 and rat epithelial cell line RLE-6TN were obtained from ATCC (Manassas, USA) and cultured in 1640 medium (Gibco, USA) that supplemented with containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C in a humidified atmosphere with 5% CO_2. A549 and RLE-6TN cells were seeded in 6-wellplates at 80% confluence and then cultured in 1640 medium without FBS for 6 h before stimulated with TGF-β1 and HMGB1 in 1640 medium that supplemented with FBS. After stimulated for 24 h and 48 h, the cells were collected

and cracked for Western blot analysis.

Small interference RNA (siRNA)

The TGF-β1 small interfering RNA (siTGF-β1) was synthesized according to human-specific sequences. A549 cells were divided into four groups as follows: control group, A549 cells without treatment; siTGF-β1 group, A549 cells with siTGF-β1 treatment; HMGB1 group, A549 cells with 4 ng/ml HMGB1 stimulation; (HMGB1+siTGF-β1) group, A549 cells with siTGF-β1 treatment and 4 ng/ml HMGB1 stimulation. The siTGF-β1 treatment groups were transfected with the mix of TGF-β1 siRNA and Lipofectamine 2000 (Invitrogen, USA) in Opti-MEM serum free medium (Gibco, USA), while other groups were only treated with Opti-MEM serum free medium for 6 h in 6-well plates according to the instructions of the manufacturer. After 6 h of transfection, the A549 cells were treated with or without HMGB1 (4 ng/ml) for another 24 h in 1640 medium that supplemented with FBS. The protein levels of TGF-β1, p-Smad2/3 and EMT markers were analyzed by Western blot. GAPDH was used as an internal reference for relative quantification. The primer sequences of TGF-β1 siRNA were as follows: TGF-β1 forward, 5'-GACACCAACUAUUGCUUCATT-3', TGF-β1 reverse, 5'-UGAAGCAAUAGUUGGUGUCTT-3', which were synthesized by GenePharma (Shanghai, China).

Western blot analysis

The right lung lobes weighing 80-100 mg were homogenized in ice-cold radio-immunoprecipitation lysis buffer (P0013C, Beyotime Institute of Biotechnology, China) and proteinase inhibitor PMSF (Amresco 0754, Biosharp, USA) cocktail. All the lytic process was performed as the instruction manual described. To observe the protein expression levels of epithelial and mesenchymal markers as well as the role of HMGB1 on TGF-β1/Smad2/3 signaling, cultured A549 cells or RLE-6TN cells were washed three times with ice-cold PBS and lysed in 100 μl of lysis buffer. The lung and cell lysates were centrifuged at 12,000 *g* for 10 min at 4°C. After centrifugation, the supernatant was collected. Before using, the loading buffer was mixed with the supernatant in a ratio of 1:4 and heated at 100°C for 10 min before loading. Equal protein amounts were separated by SDS-PAGE on a 12% gel and then transferred to PVDF membranes (IPVH00010; Millipore, USA). Nonspecific binding to the membrane was blocked for 2 h at room temperature with 5% non-fat dry milk (w/v) (Guangming, China) in TBST (AR0031, BOSTER, China) and incubated at 4 ˚C overnight with the primary anti-HMGB1 (1:8,000 diluted, 25 kDa), anti-TGF-β1 (1:1000 diluted, 44 kDa), anti-p-Smad2/3 (1:500 diluted, 55-60 kDa), anti-E-cadherin (1:500 diluted, 97 kDa), anti-α-SMA (1:400 diluted, 42 kDa), anti-vimentin (1:5000 diluted, 57 kDa), anti-β-actin (1:5,000 diluted, 43 kDa) and anti-GAPDH (1:5,000 diluted, 36 kDa) antibodies. On the next day, the blots were incubated with HRP-conjugated anti-rabbit or anti-goat IgG antibodies (ZSGB-BIO, Beijing, China) for 1 h at room temperature. Immunodetection was developed with enhanced chemiluminescence reagent (ECL, Beyotime, China). All experiments were

performed independently at least three times. The densitometry was performed on protein bands using Image J analysis software (ChemiQ 4600, Bioshine, China). The IOD value was performed by Image-Pro Plus 6.0, while β-actin or GAPDH was used as the internal reference for relative quantification.

Statistical analysis

The data are presented as mean ± SD for each group in the *in vivo* and *in vitro* experiments. Statistical significance was determined by either the Student's t-test for comparison between means or one-way analysis of variance with a post hoc Dunnett's test. Statistical analyses were performed by SPSS 13.0 software. $P<0.05$ was considered to be significant.

Results

Increased release of HMGB1 and activation of TGF-β1/Smad2/3 signaling in BLM-induced pulmonary fibrosis in rats

In the *in vivo* PF model, we first observed the abnormal pathologic changes after BLM administration. As stained by H&E and Masson's trichrome in the lung sections, the intratracheal injection of BLM led to the destruction of normal lung architecture, the infiltration of inflammatory cells in alveolar and the extensive distribution of collagen in cell interval (Fig. 1A and B). Though TGF-β1 and HMGB1 have been reported affecting the pathological process of fibrotic diseases (Hamada et al., 2008;

Hu et al., 2014; Xu et al., 2014a, 2014b), the inner link between them has not been clearly clarified yet in PF.

Previous studies have shown that TGF-β1/Smad signaling and HMGB1 take part in the process of EMT (Chen et al., 2013, 2014; He et al., 2007). However, whether HMGB1 is involved in the activation of TGF-β1/Smad signaling in EMT process? In order to confirm the link between them in the process of EMT in the abnormal lung remodeling, we here examined the HYP level in the lungs as it is the major constituent of collagen (Han et al., 2006), and the protein levels of HMGB1, TGF-β1, p-Smad2/3 and EMT markers (E-cadherin, vimentin and α-SMA) in BLM-induced PF in rats. We found that the level of HYP (Fig. 1C) in lung tissues and the content of HMGB1 (Fig. 1D) in serum were markedly increased after BLM treatment. It showed that the level of lung HYP was gradually increased from day 7 to day 28, while the serum HMGB1 level was the highest on day 7. Furthermore, compared with the NS group, the protein expression of α-SMA (Fig. 2A) and Col-I (Fig. 2B) was significantly increased by immunohistochemical analysis after BLM administration. Meanwhile, the protein levels of HMGB1, TGF-β1 and p-Smad2/3 were sharply elevated in response to BLM treatment, correlating with down-regulated expression of epithelial proteins such as E-cadherin and increased protein expression of mesenchymal markers including α-SMA and vimentin by Western blot analysis (Fig. 2C). However, these changes in the lungs were more obvious on day 28. These results indicated that the increased release of HMGB1, the up-regulated activity of TGF-β1/Smad2/3 signaling as well as the changes of EMT markers may have a

certain internal relations during the development of PF.

TGF-β1- and HMGB1-induced EMT in A549 and RLE-6TN cells

During the pathogenesis of PF, excessive ECM is mainly secreted by abnormal activation of mesenchymal cells including fibroblasts and myofibroblasts, which can arise from EMT. The above findings shown in Fig. 1 and Fig. 2 prompted us to confirm the role of TGF-β1 and HMGB1 in EMT in AECs. First, A549 and RLE-6TN cells were cultured and stimulated with TGF-β1 and HMGB1 for 24 h and 48 h, respectively. In Fig. 3, we found that TGF-β1 significantly decreased the protein expression of E-cadherin (epithelial marker) and increased the protein expression of α-SMA (mesenchymal marker) in A549 and RLE-6TN cells in a certain concentration range. It was consistent with previous researches that TGF-β1 is a crucial mediator of EMT (Chen et al., 2014).

To investigate the potential role of HMGB1 in both kinds of AECs, the effects of direct application of recombinant human HMGB1 to A549 and RLE-6TN cells as well as its role in TGF-β1/Samd2/3 signaling were subsequently examined. In the present study, the uptake of exogenous HMGB1 could induce EMT in both A549 and RLE-6TN cells, as shown by the changed expression profiles of EMT markers including the down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal marker α-SMA (Fig. 4). Nevertheless, we surprisingly found that the stimulation with HMGB1 could not only induce EMT but also elevate the protein expression of TGF-β1 and p-Smad2/3 in both kinds of AECs in a certain

concentration range (Fig. 4). These results strongly suggest that HMGB1 indeed acts as an important mediator of EMT in AECs. In addition, our data show that HMGB1 participates in activating TGF-β1/p-Smad2/3 signaling, may be through which HMGB1 promotes EMT in the process of PF.

TGF-β1 silence inhibited HMGB1-induced EMT in A549 and RLE-6TN cells

The above findings provide *in vitro* evidence that HMGB1-induced EMT may be involved in the activation of TGF-β1/Smad signaling. Thus, we further explored whether TGF-β1/Smad2/3 acted the downstream signaling pathway regulated by HMGB1. Consistent with the above studies, HMGB1 triggered Smad2/3 phosphorylation, increased the protein expression of mesenchymal markers (vimentin and α-SMA), and decreased the expression of epithelial marker (E-cadherin) (Fig. 5). On the contrary, knockdown of TGF-β1 by siRNA significantly prevented EMT with the reduction of the increased mesenchymal markers and the elevation of the decreased epithelial marker (E-cadherin) in response to the uptake of exogenous HMGB1 by A549 cells (Fig. 5). Furthermore, phosphorylation of Smad2/3 in response to HMGB1 stimulation was strongly attenuated with the deficiency of TGF-β1 (Fig. 5), suggesting that HMGB1-mediated EMT requires the activation of TGF-β1/Smad2/3 signaling.

Discussion

PF is a complex disease with a poor prognosis and currently few effective therapies,

which reflects our limited understanding of its basic mechanisms. To our current knowledge, TGF-β1/Smad signaling and HMGB1 release play a crucial role in promoting EMT process and triggering ECM secretion and deposition (Chen et al., 2014; Hamada et al., 2008; Li et al., 2014a; Lynch et al., 2010). Nevertheless, it has not been elucidated whether HMGB1 is involved in the recognized TGF-β1/Smad signaling that contributes to EMT in PF. In this study, we provide the advanced evidence that TGF-β1/Smad2/3 signaling is required for HMGB1-induced EMT, suggesting that HMGB1 may be a potential effective target for PF treatment.

In the present study, the *in vivo* data showed that treating the rats with BLM resulted in typical PF including the excessive ECM deposition and lung structure destruction. Moreover, the levels of the lung HYP (the major constituent of collagen) and serum HMGB1, as well as the positive protein expression of Col-I (one of the ingredients of collagen) and α-SMA (the marker of myofibroblasts) in the lungs, were dramatically elevated after BLM administration. Meanwhile, the protein expression of TGF-β1, p-Smad2/3 and HMGB1 was greatly up-regulated during the development of PF in rats. Previous strong evidence has shown that EMT is widely considered to be a crucial process in the generation of myofibroblasts that secrete ECM and contribute to PF (Selman and Pardo, 2003). In this study, we also demonstrated that BLM treatment could induce the loss of epithelial marker E-cadherin and acquisition of mesenchymal markers including α-SMA and vimentin. These data showed that the increased HMGB1 release and activated TGF-β1/Smad2/3 signaling were accompanied by the induction of EMT in BLM-induced PF in rats.

It is widely recognized that the regulatory mechanisms involving EMT would help to determine the effective therapies to halt the progression of PF. Besides, TGF-β1 silence inhibits the pro-fibrotic role of HMGB1 in renal fibrosis (Lynch et al., 2010). We then designed the *in vitro* study to investigate the role of TGF-β1 and HMGB1 on EMT in A549 and RLE-6TN cells, as well as the interplay between HMGB1 and the canonical TGF-β1/Smad2/3 signaling in the process of EMT. Surprisingly, we observed that both TGF-β1 and HMGB1 can respectively induce EMT in these AECs. In addition, our study also showed that HMGB1 increased TGF-β1 expression and triggered Smad2/3 phosphorylation. Moreover, the deficiency of TGF-β1 blocked HMGB1-induced EMT with down-regulated level of p-Smad2/3 in AECs. These results support that TGF-β1/Smad2/3 signaling is required for HMGB1-mediated EMT in AECs. However, we found that siTGF-β1 could not completely block HMGB1-mediated EMT in AECs, indicating that the canonical TGF-β1/Smad signaling may be not the only signaling pathway involved in this process. Losartan is effective in attenuating PF and cigarette smoke-induced lung injury by antagonizing TGF-β1 signaling (Podowski et al., 2012; Yao et al., 2006). Recent study also shows that losartan reduces lung damage by inhibiting the induction of HMGB1 (Hagiwara et al., 2009). Thus, it may be an interesting and meaningful exploration to investigate whether losartan would inhibit TGF-β1 signaling by targeting HMGB1 in lung injury, or whether it is effective in clinic for treating PF.

Recent studies have demonstrated that HMGB1 up-regulates Smad7 expression on cardiac fibroblasts and triggers Smad2 phosphorylation in hepatic stellate cells (He et

al., 2013; Kao et al., 2014). Combined with our present study, HMGB1 may play a significant role in Smad signaling during the development of organ fibrosis. Thus, further experiments are required to understand whether the activation of HMGB1 takes part in the non-Smad signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway in tissue fibrosis (Mishra et al., 2014). Besides, despite current data supports the pro-fibrotic role of HMGB1 in PF, and the recent studies show that reducing the abnormal expression of HMGB1 attenuates PF in rats (Li et al., 2015; Zhang et al., 2015), the protective role of HMGB1 inhibitors in PF remain to be further identified. Additionally, more detailed sets of TGF-β1/Smad signaling inhibitors should be performed to ensure the interplay between the alarm signal HMGB1 and TGF-β1/Smad signaling pathway. It was found that the receptor for advanced glycation end products (RAGE), one of the receptors of HMGB1, is involved in the process of fibrotic changes in several organs (Englert et al., 2011; He et al., 2007; Rowe et al., 2008; Zhang et al., 2015). Further studies should turn to investigate whether HMGB1/RAGE signaling is involved in elevating the canonical TGF-β1/Smad signaling in the development of PF and which factor is the effective therapeutic target in this process. Besides, the application of transgenic animals would be easier to evaluate the involvement of HMGB1 signaling during PF and clarify whether HMGB1 would be a biomarker or potential therapeutic target for PF.

Our findings in the present study first demonstrated that the increased HMGB1 release was accompanied by the promotion of EMT and the activation of TGF-β1/Smad2/3 signaling in BLM-induced PF. Besides, we provided the first

evidence that HMGB1 could not only induce EMT in both human A549 and rat RLE-6TN AECs, but also activate TGF-β1/Smad2/3 signaling during above process. In addition, the inhibition of TGF-β1 could evidently ameliorate HMGB1-mediated EMT and reverse the elevation of phosphorylated Smad2/3 in AECs. In conclusion, we provide the first evidence that TGF-β1/Smad2/3 signaling involves HMGB1-mediated EMT of AECs, may be due to which the ECM-producing cells are activated and increased, leading to the abnormal accumulation of ECM in the development of PF. It provides novel evidence for further clarifying the mechanism of PF and HMGB1 may represent a superior therapeutic target for PF treatment.

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Authorship Contributions

Participated in research design: L.C.Li, D.L.Li, W.C.Zhou.

Conducted experiments: L.C.Li, D.L.Li, L.Xu.

Contributed new reagents or analytic tools: X.T.Mo, W.C.Zhou, P.Zhao.

Performed data analysis: L.C.Li, W.H.Cui, X.T.Mo.

Wrote or contributed to writing of the manuscript: L.C.Li, J.Li, J.Gao.

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Footnotes

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Figure legends

Fig.1. The lung pathologic abnormalities and the levels of lung HYP and serum HMGB1 after BLM administration in rats. (A) The lung sections from each rat were stained with H&E, ×200. The black and red arrows respectively indicated the inflammatory cells infiltration and ECM deposition. (B) The collagen accumulation was stained by Masson's trichrome, ×200. The black arrows indicated the collagen expression. (C) The content of HYP was evaluated in the lung tissue. (D) The level of HMGB1 was assayed in serum by ELISA analysis. Data are shown as means ± SD, (n = 4–6). ***P*< 0.01, compared to NS group.

Fig.2. The protein expression of HMGB1, TGF-β1, p-Smad2/3, E-cadherin, α-SMA and Col-I after BLM administration. (A) Representative images showing the protein expression of α-SMA by immunohistochemical analysis in BLM rat model, ×400. (B) Representative images showing the protein expression of Col-I with immunohistochemical analysis in BLM rat model, ×400. (C) The protein expression of HMGB1, TGF-β1, p-Smad2/3, E-cadherin, vimentin and α-SMA by Western blot analysis after BLM administration. The black arrows indicated the proteins expression of α-SMA and Col-I respectively. The densitometry values were normalized to β-actin respectively for protein. Data are shown as means ± SD and representative of at least three separate experiments. ***P*< 0.01, compared to NS group.

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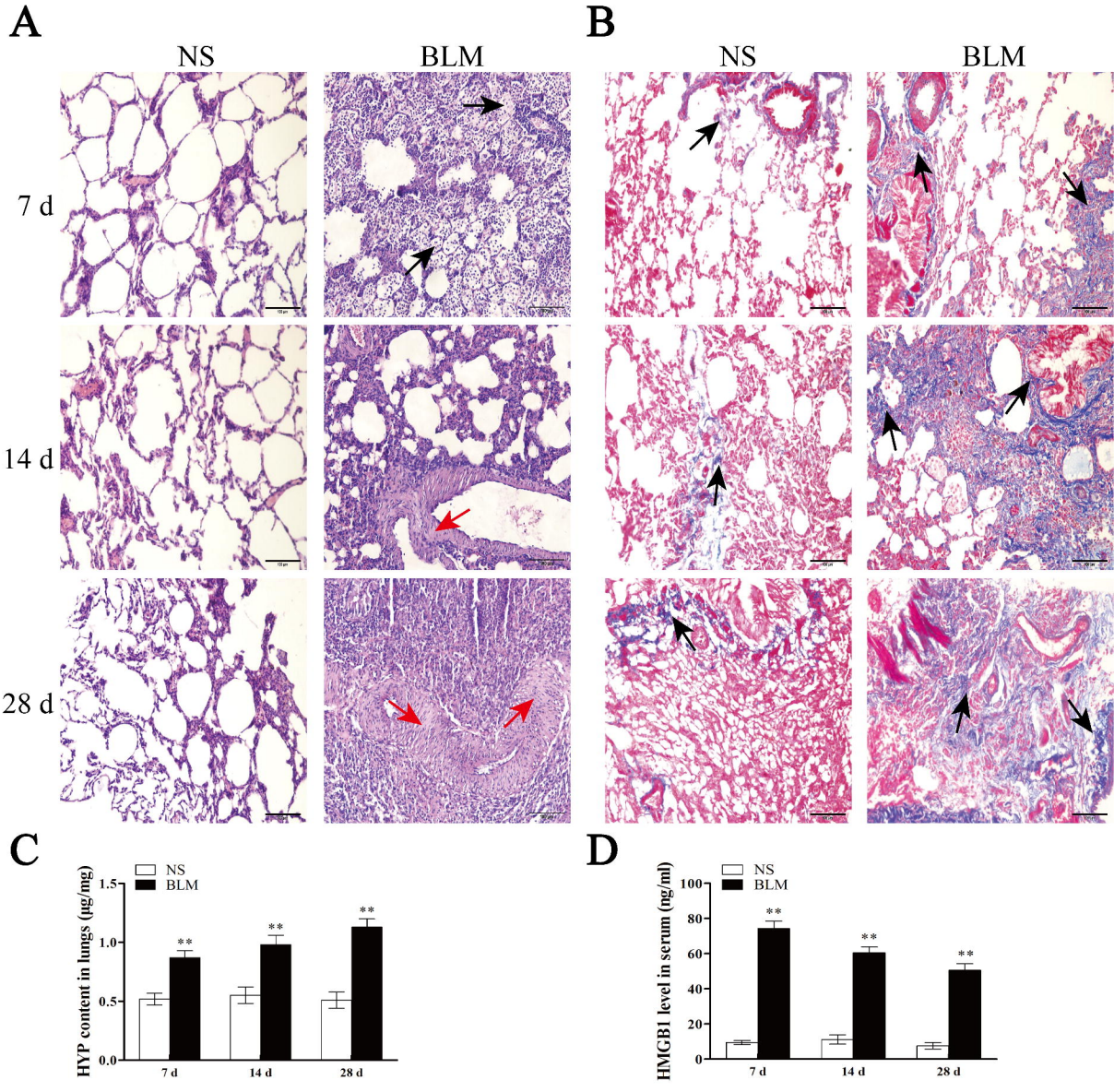
Fig.3. The uptake of exogenous TGF-β1 induced EMT in AECs in a certain concentration range. (A) The protein expression of E-cadherin and α-SMA was observed by Western blot analysis at 24 h and 48 h after TGF-β1 stimulation in human A549 cells. (B) The protein expression of E-cadherin and α-SMA was observed by Western blot analysis at 24 h and 48 h after TGF-β1 stimulation in rat RLE-6TN cells. The densitometry values were normalized to β-actin or GAPDH respectively for protein. Data are shown as means ± SD and representative of at least three separate experiments. $^{**}P < 0.01$, compared to control group (without TGF-β1 stimulation).

Fig.4. The uptake of exogenous HMGB1 induced EMT in AECs in a certain concentration range. (A) The protein expression of E-cadherin, α-SMA, p-Smad2/3 and TGF-β1 was observed by Western blot analysis at 24 h and 48 h after HMGB1 stimulation in human A549 cells. (B) The protein expression of E-cadherin, α-SMA, p-Smad2/3 and TGF-β1 was observed by Western blot analysis at 24 h and 48 h after HMGB1 stimulation in rat RLE-6TN cells. The densitometry values were normalized to β-actin or GAPDH respectively for protein. Data are shown as means ± SD and representative of at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to control group (without HMGB1 stimulation).

Fig.5. Gene silence of TGF-β1 significantly inhibited HMGB1-mediated EMT and Smad2/3 phosphorylation in AECs. Human A549 cells were first treated with

siTGF-β1 for 6 h, and then treated with HMGB1 (4 ng/ml) for another 24 h. The protein expression of TGF-β1, p-Smad2/3, E-cadherin, α-SMA and vimentin was observed by Western blot analysis. The densitometry values were normalized to GAPDH respectively for protein. Data are shown as means ± SD and representative of at least three separate experiments. $^{**}P< 0.01$, compared to control group; $^{\#\#}P< 0.01$, compared to HMGB1 group.

Fig. 1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NS
BLM
7 d
14 d
28 d
B
NS
BLM
C
HYP content in lungs (μg/mg)
NS
BLM
1.5
1.0
0.5
0.0
**
7 d
14 d
28 d
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BLM
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40
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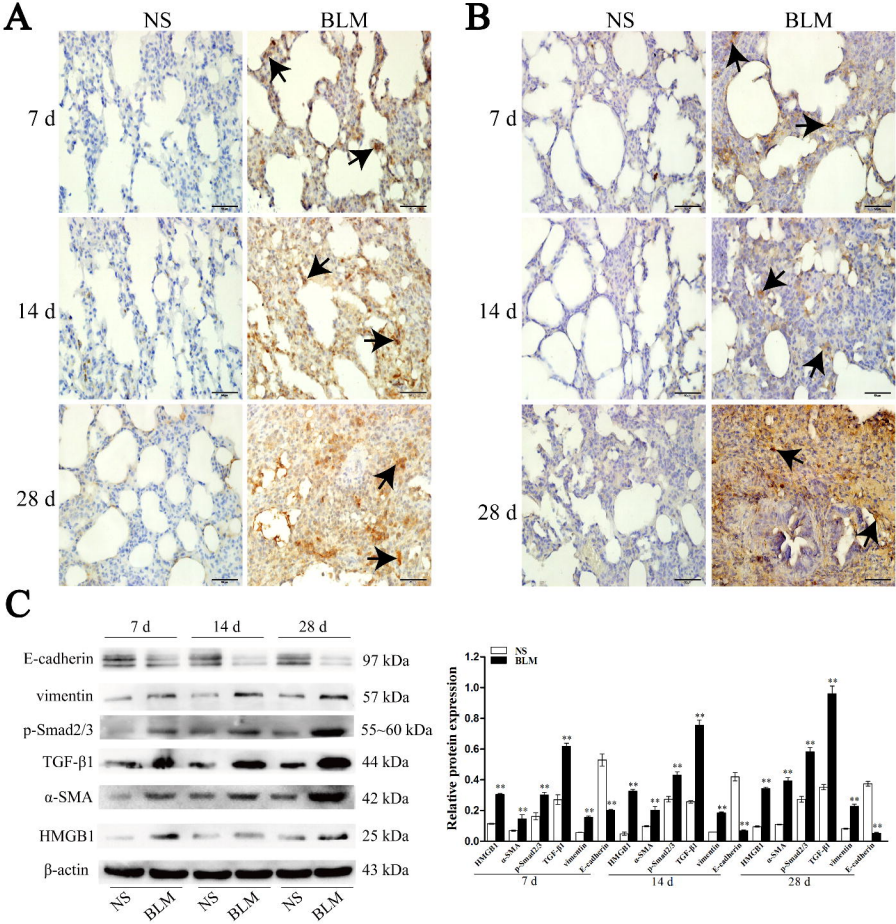
0
**
7 d
14 d
28 d

Fig. 2


A
NS
BLM
7 d
14 d
28 d
B
NS
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7 d
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28 d
E-cadherin
vimentin
p-Smad2/3
TGF-β1
α-SMA
HMGB1
β-actin
97 kDa
57 kDa
55~60 kDa
44 kDa
42 kDa
25 kDa
43 kDa
NS
BLM
NS
BLM
NS
BLM
NS
BLM
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TGF-β1
vimentin
E-cadherin
7 d
14 d
28 d

Fig. 3

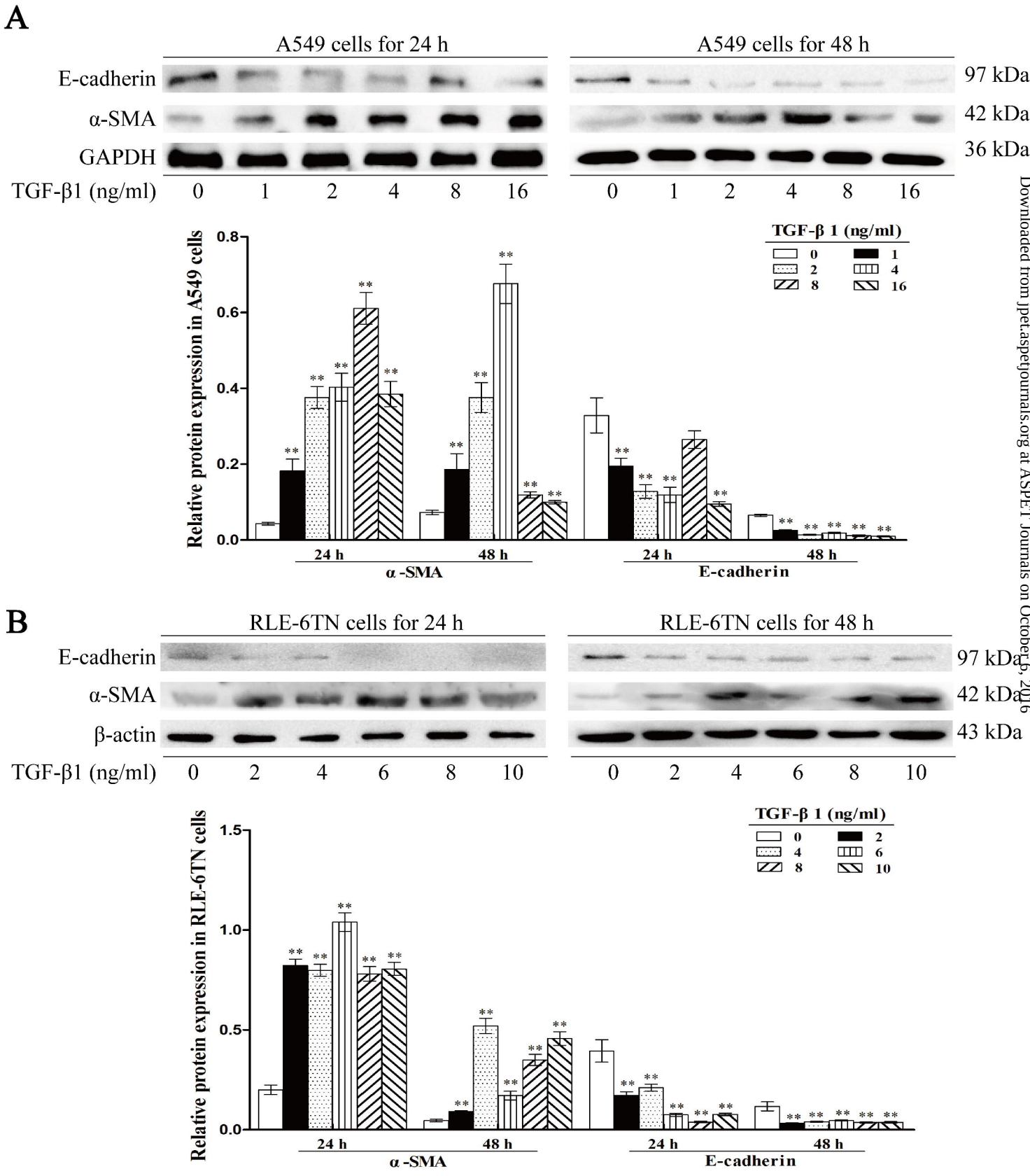


Fig. 4

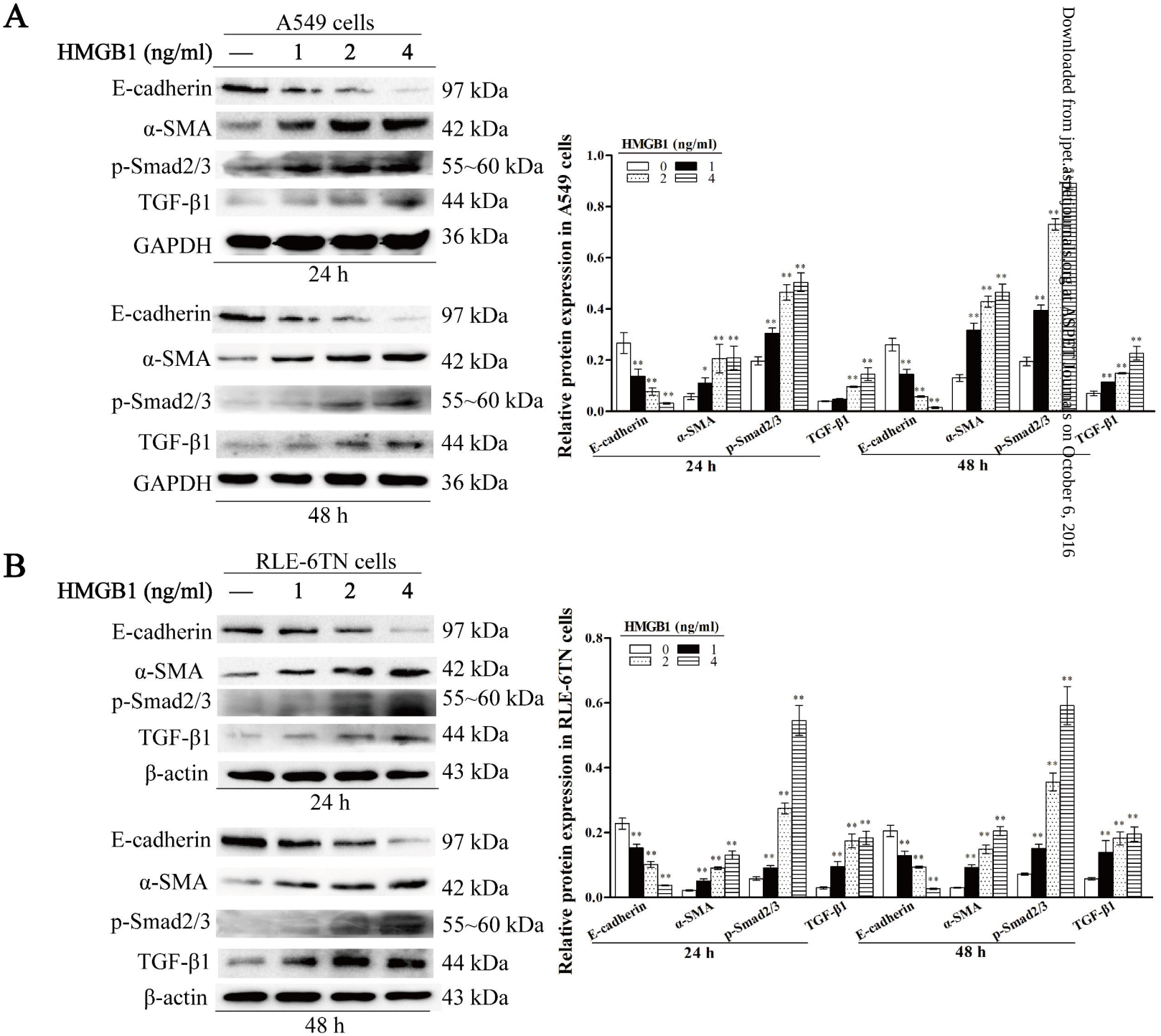
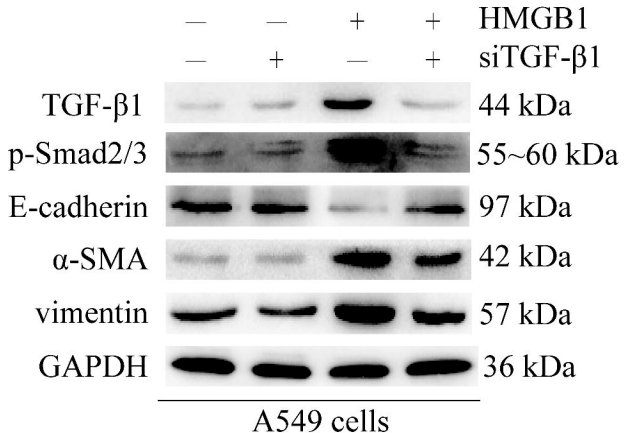
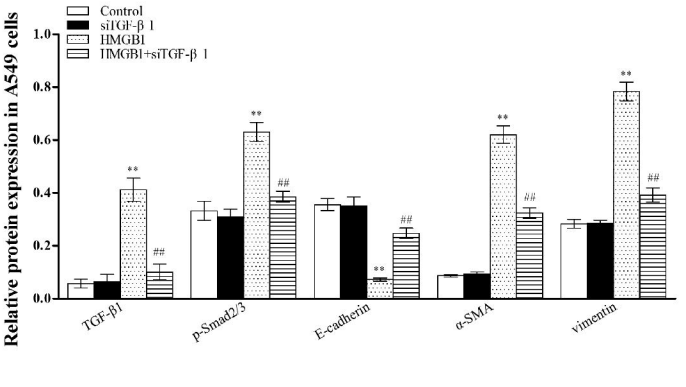


Fig. 5


— — + + HMGB1
— + — + siTGF-β1
TGF-β1
p-Smad2/3
E-cadherin
α-SMA
vimentin
GAPDH
44 kDa
55~60 kDa
97 kDa
42 kDa
57 kDa
36 kDa
A549 cells


Control
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HMGB1
HMGB1+siTGF-β 1
Relative protein expression in A549 cells
1.0
0.8
0.6
0.4
0.2
0.0
**
##
TGF-β1
p-Smad2/3
E-cadherin
α-SMA
vimentin