HMGB1/TLR4 promotes hypoxic pulmonary hypertension via suppressing BMPR2 signaling

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ABSTRACT

High mobility group box 1 (HMGB1), a critical nonclassical inflammatory cytokine, has been found up-regulated in patients with idiopathic pulmonary arterial hypertension (IPAH), but its role in vascular remodeling of pulmonary hypertension (PH) is still unknown. In present study, we demonstrated that the plasma level of inflammatory cytokine including HMGB1, interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) were elevated in hypoxia-induced pulmonary hypertension rats model. Moreover, expressions of HMGB1 and Toll like receptor-4 (TLR4) in pulmonary arteries were obviously up-regulated accompanied with down-regulation of bone morphogenetic protein receptor 2 (BMPR2) signaling, characterized by decline of phosphorylated Smad1/5/8 (p-Smsd1/5/8) and inhibitor of differention 1 (Id1) expression. In cultured primary pulmonary arterial smooth muscle cells (PASMCs), we found that HMGB1 incubation significantly promoted proliferation and migration of PASMCs, down-regulated p-Smsd1/5/8 and Id1 expression, which can be abrogated by HMGB1 inhibitors saquinavir, glycyrrhizn and TLR4 inhibitors TAK-242. Furthermore, saquinavir, glycyrrhizn and TAK-242 treatment significantly attenuated the development of PH in rats by recovering homodynamic parameters, pulmonary vascular remodeling and BMPR2 signaling pathway. In summary, our results suggest that HMGB1/TLR4 signaling promotes hypoxia-induced pulmonary hypertension via suppressing BMPR2 signaling.

1. Introduction

Pulmonary hypertension (PH) is an extremely rare cardiovascular disease characterized by increased pulmonary vascular resistance, abnormal vasoconstriction and pulmonary vascular remodeling, finally leading to progressive right ventricular failure and death. In recent years, inflammation has been emerging as a key disease-related factor in PH [1,2]. (1) A large number of inflammatory cells infiltrated in the vascular damaged area of patients with PAH [3]. (2) Clinical studies in our and other laboratories have found that plasma levels of inflammatory factors including IL-1β, IL-6 and TNF-α are significantly elevated, closely related to reduced survival [4]. (3) Animal experiments have confirmed that anti-inflammatory treatment can alleviate

the pathogenesis of experimental PH [5]. Therapy targeting inflammation is thought as the next effective stratagem in PH treatment.

High mobility group box 1 (HMGB1), a highly conserved nuclear protein with extracellular pro-inflammatory activity, has emerged as a novel regulator in PH. Bauer and coworker found that HMGB1 contributed to the pathogenesis of chronic hypoxia-induced PH [6]. Anti-HMGB1 neutralizing antibody significantly improved the survival in MCT-induced PAH rats [7]. However, the underlying mechanism remains unknown. Toll-like receptor 4 (TLR4) is a major receptor of HMGB1, mediating the pro-inflammation effect of HMGB1. It is demonstrated that HMGB1 exhibits inflammatory cytokine activity by binding to the TLR4/MD2 receptor complex on macrophages and stimulating release of TNF-α and other cytokines [8]. Moreover, it has
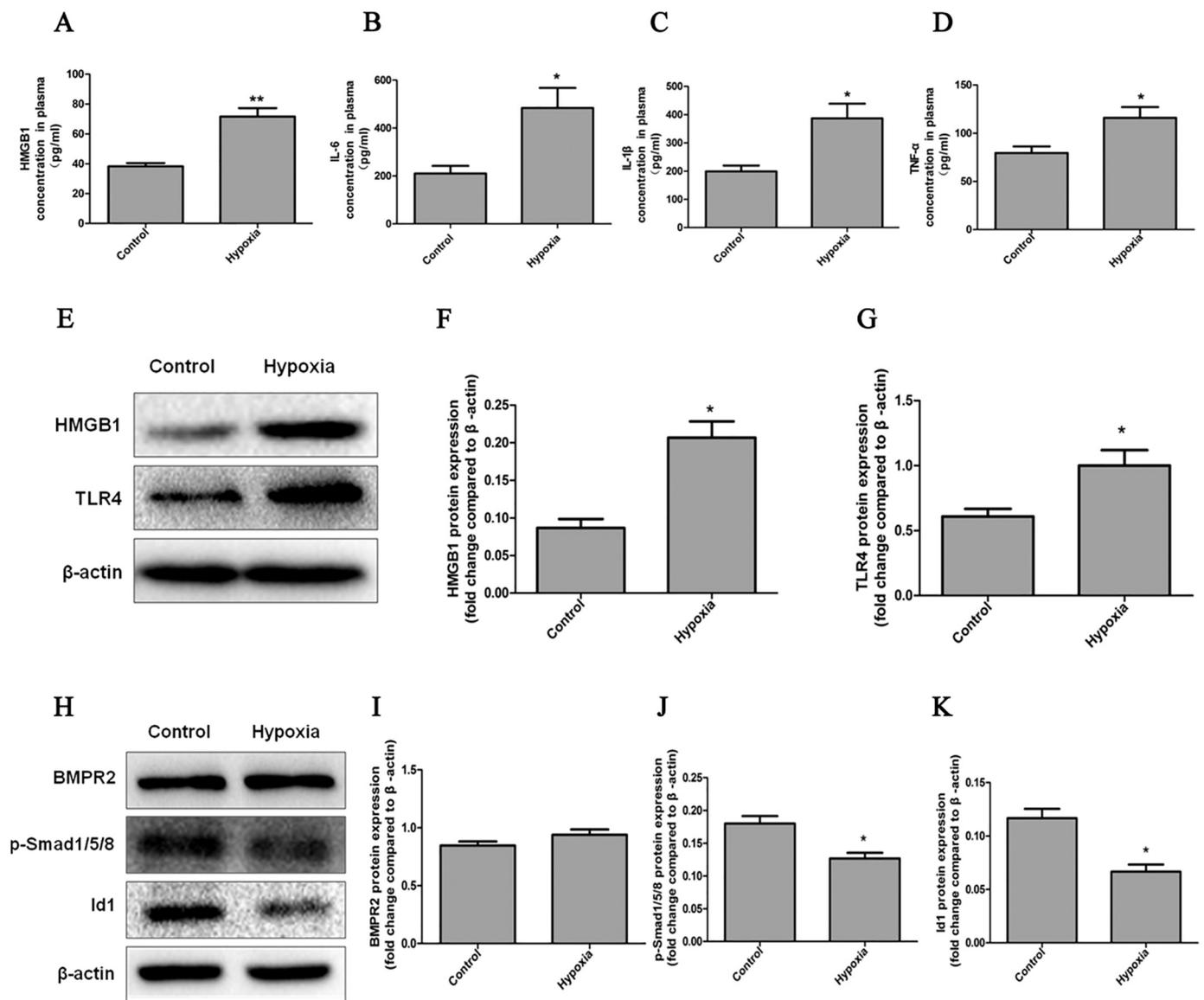

Fig. 1. Levels of inflammatory factors, expression of HMGB1/TLR4 pathway and BMPR2 signaling pathway in hypoxia-induced PH rats. A-D: HMGB1, IL-6, IL-1β and TNF-α plasma level respectively. Inflammatory factors level was measured by ELISA. Data are mean ± standard error. n = 6. *P < 0.05, **P < 0.01 vs. Control group. E: The protein expression of HMGB1 and TLR4 in hypoxia-induced in pulmonary vascular was determined by Western blot; F-G: Quantitative analysis results of graph E. Data expressed as mean ± standard error, n = 6. *P < 0.05 vs. Control group. H: The protein expression of BMPR2, p-Smad1/5/8 and Id1 in hypoxia-induced in pulmonary arterial were determined by Western blot. I-K: Quantitative analysis results of graph H. Data expressed as mean ± standard error, n = 3. *P < 0.05 vs. Control group.

been reported that TLR4 deficiency mice may turn down the susceptibility to developing pulmonary hypertension by attenuating the pulmonary vascular inflammatory response to chronic hypoxia [9]. Considering the significance of inflammatory response in PH, we speculated that HMGB1/TLR4 signaling plays a critical role in the development of PH.

It is well recognized that bone morphogenetic protein receptor 2 (BMPR2) signaling pathway, including BMPR2, p-Smads and Id1, are of vital importance in regulating pulmonary vascular homeostasis. BMPR2 gene loss-of-function mutations is responsible to the majority of hereditary PAH and a proportion of idiopathic PAH. The dysfunction of BMPR2 signaling plays a critical role during the initiation and progress of PH. Animal studies have indicated that recovering BMPR2 signaling protected against experimental PH. Previously studies have indicated that multifarious risk factors including genetics (mutation), epigenetic modification (including microRNAs, histone deacetylases, abnormal methylation), environmental factors (hypoxia), autophagy and

ubiquitin proteasome degration were likely contribute the process [10–12]. Recently, Soon et al. found that BMPR2 deficiency promoted an exaggerated inflammatory response induced by LPS to instigate development of pulmonary hypertension, suggesting the potential link between BMPR2 pathway and inflammation [13]. These finding inspired us to investigate whether BMPR2 signaling pathway is involved in HMGB1/TLR4-regulated inflammation in PH.

Therefore, by utilizing hypoxia model in rats and PASMCs, the present study firstly tested the expression of HMGB1/TLR4 and BMPR2 pathway. Furthermore, HMGB1 inhibitors saquinavir, glycyrrhizn and TLR4 inhibitor TAK-242 were used to explore whether HMGB1/TLR4 regulated the BMPR2 pathway during hypoxic PH in vivo and in vitro.
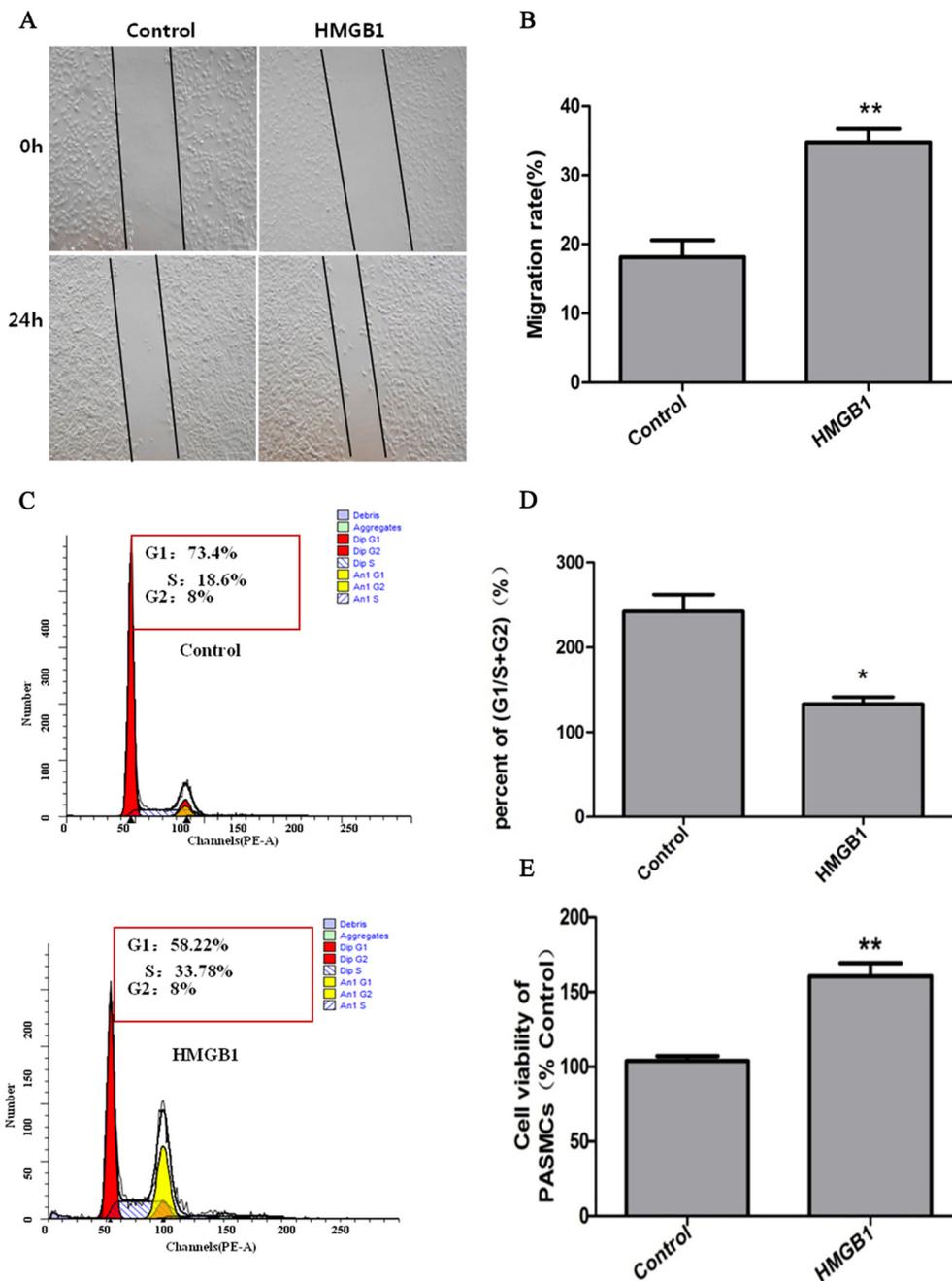
Fig. 2. Effects of HMGB1 on cell migration, proliferation in cultured PASMCs. A: Scratch test results, ($\times 40$); B: Quantitative analysis results of graph A. Data expressed as mean $\pm$ standard error, n = 3. **P $<$ 0.01 vs. Control group. C: Flow cytometry results of the cell cycle; D: Quantitative analysis results of graph c; E: The MTS measures the statistical results of cell viability. Data expressed as mean $\pm$ standard error, n = 3. *P $<$ 0.05, **P $<$ 0.01 vs. Control group.

2. Materials and methods

2.1. Animal experiments

Male Sprague–Dawley (SD) rats (weighing 180–200 g) were obtained from the Laboratory Animal Center, Xiangya School of Medicine, Central South University (Changsha, China). The whole experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. And the experimental protocol was approved by the medicine animal welfare committee of Xiangya Medical School, Central South University (Changsha, China).

All animals were housed in a room acclimated for 1 week, with a controlled temperature (18 °C–25 °C) and humidity (50%–60%), and

was kept on an alternating 12 h light-dark cycle. Food and water were available ad libitum. Then SD rats were randomly allocated to 7 groups as follows (n = 10 per group): Control group, rats were placed in a chamber with normobaric normoxia (21% O_2); Hypoxia group, rats were placed in a chamber and exposed to 10% O_2 continuously for 4 weeks; Hypoxia plus vehicle group (1% DMSO, ~1 mL/100 g per day, i.p.) and subjected to hypoxia treatment; Hypoxia plus saquinavir (cell experiments have identified its inhibitory effect on HMGB1-induced inflammation) low-dosage group (SQL) or saquinavir high-dosage group (SQH) (3 or 15 mg/kg per day, i.g., Genentech, USA) and subjected to hypoxia treatment; Hypoxia plus glycyrrhizn (an HMGB1 inhibitor) group (GLY) (100 mg/kg per day, i.g, Sigma-Aldrich, USA) and subjected to hypoxia treatment; Hypoxia plus TAK-242 (a TLR4 specific inhibitor) (0.3 mg/kg per day, i.p, MedChem Express, USA) group and
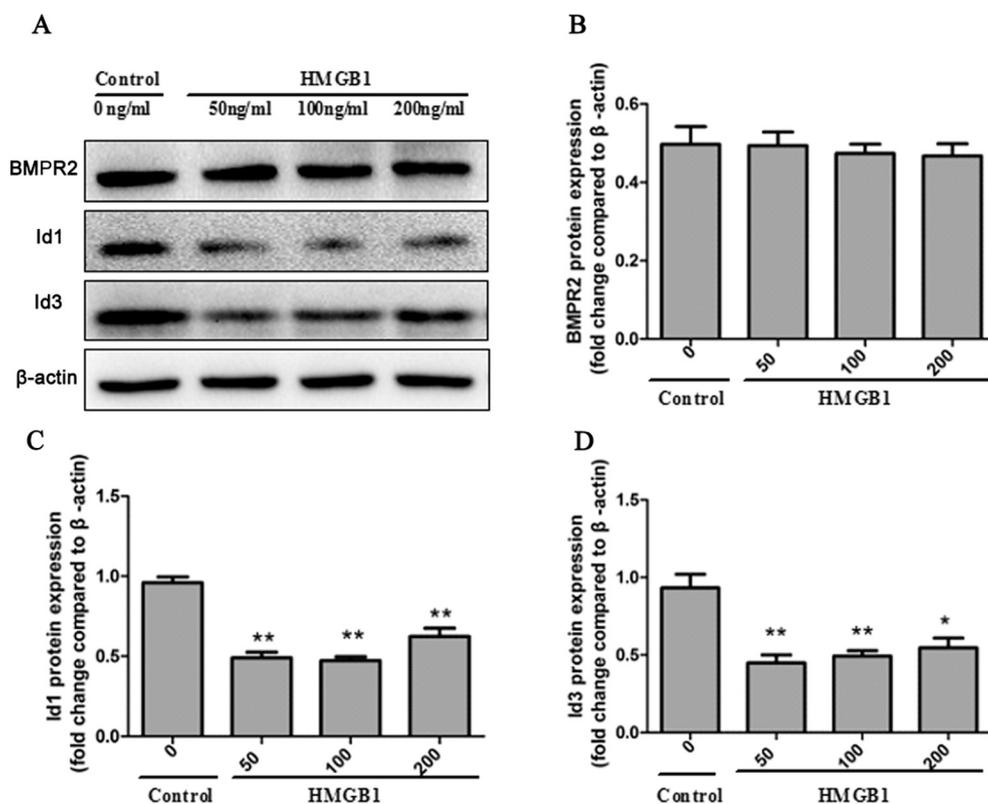
subjected to hypoxia treatment. At the end of hypoxia, rats were anesthetized with sodium pentobarbital and were subjected to measure right ventricle systolic pressure (RVSP). Then blood samples from all animals were collected immediately. After sacrificing the animals, the left ventricle (LV), right ventricle (RV) and the interventricular septum (S) were dissected from the heart and were weighed for calculating the ratio of RV to (LV + S) and the ratio of RV weight to the length of the tibia, which are crucial parameters for evaluating RV hypertrophy. The freshly isolated pulmonary arterial samples were used for protein expression analysis. Excised lungs were fixed in 4% paraformaldehyde for hematoxylin–eosin (HE) staining as well as immune- histochemical analysis. All samples operation were keeping on ice during experiments.

2.2. Elisa

The measurement of plasma concentration of inflammatory factors including HMGB1, IL-1β, IL-6 as well as TNF-α were measured by ELISA kits (R&D Systems, USA) according to the manufacturer's instructions as previously described [14].

2.3. Histology and immunohistochemistry

HE and immunohistochemistry staining were performed to evaluate the morphological changes of pulmonary arteries in lung as previously described [14]. Briefly, pulmonary arteries were fixed with 4% paraformaldehyde and embedded in paraffin and then cut into 5 mm sections. Then staining the slices with HE before observation under a microscope (Olym-pus Corporation, Japan). Each slide were randomly selected at least 6 microscopic fields. For immunohistochemistry staining, sections were stained with anti-HMGB1 antibody (1:100, CalBioreagents, USA) and anti-TLR4 antibody (1:200, Abcam, UK), Diaminobenzidine was used for color reaction to detect a positive signal according to routine procedure. Slides were examined microscopically at 400 magnification and photographed by a high-resolution digital camera (Olym-pus Corporation, Japan).

2.4. Cell experiments

Primary pulmonary arterial smooth cells (PASMCs) were prepared from the pulmonary artery of male 10-week-old SD rats using the explant method as described previously [15]. The cells were cultured at 37 °C under 5% CO_2 in DMEM containing 20% fetal bovine serum (Hyclone, USA). PASMCs were identified by immunofluorescence staining with the antibody of smooth muscle α-actin (1:50, Abcam). The cells between passages 3 and 8 were used for the experiments.

2.5. Cell proliferation assay

Cell proliferation was measured by 2 methods as follows:

2.5.1. Flow cytometry

For the cell cycle analysis using flow cytometry according to the manufacturer's instructions (GENVIEW, China). Briefly, cells were counted and seeded into 6-well culture plates (1×10^5 cells per well). After 24 h, the medium was changed to DMEM containing 0.1% FBS to make them quiescent for 24 h. The cells were then treated with 0.25% Triton X-100 for 2 min in an ice bath, and then resuspended in 1.5 mL Eppendorf with 1 mL cold Wash Buffer. Then the cells were fixed gently with 70% cold alcohol at 4 °C overnight, and then resuspended in 200 μL Propidium Staining Solution. Cells were incubated in a dark room for 0.5 h at 37 °C and then subjected to cell-cycle analysis using a FACScan flow cytometer (BD Biosciences, USA) and FACS Di Va software. At least 10,000 cells were counted for each measurement. Data presented are the percentage of cells in a given subpopulation.

2.5.2. MTS assay

Cell proliferation assay was performed using CellTiter 96 AQ ueous One Solution cell Proliferation Assay (a) (Promega, USA) according to the manufacturer's instructions as previously described [16].
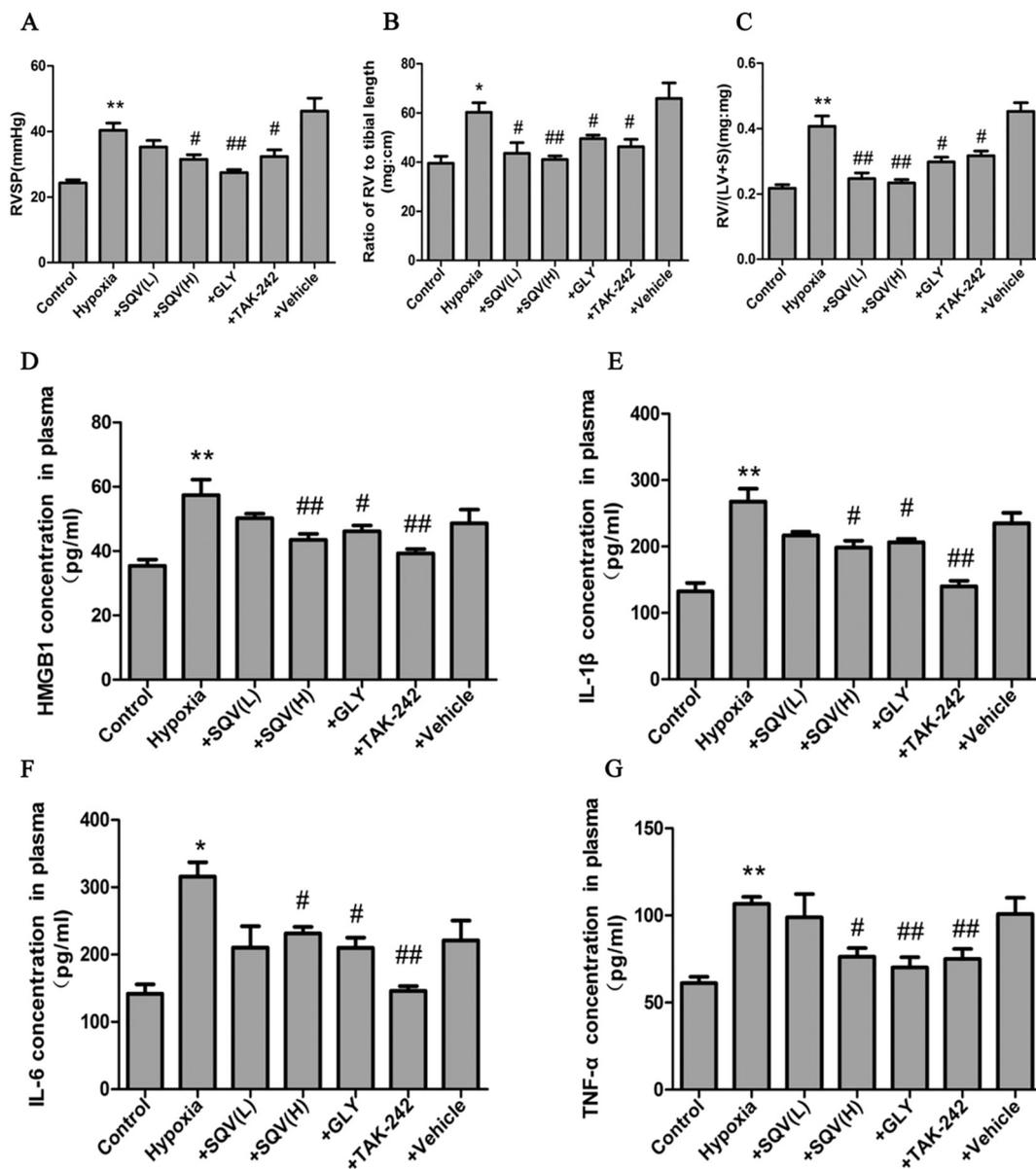
Fig. 4. Effects of HMGB1/TLR4 inhibitors on cardiovascular remodeling and plasma inflammatory factors in hypoxia-induced PH rats. A: Right ventricle systolic pressure (RVSP). SQVL: Saquinavir low-dosage group (3 mg/kg per day); SQVH: Saquinavir high-dosage group (15 mg/kg per day); GLY: Glycyrrhizin (100 mg/kg per day); TAK-242 (TLR4 inhibitor, 0.3 mg/kg per day); Vehicle: 1% DMSO. B: The ratio of right ventricle (RV) weight to tibial length. C: The ratio of right ventricle (RV) weight to that of left ventricle (LV) plus interventricular septum (S). D-G: HMGB1, IL-1β, IL-6 and TNF-α plasma level respectively. Inflammatory factors level was measured by ELISA. Data are mean ± standard error. n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Hypoxia group.

2.6. Cell migration assay

Cell scratch test was used to detect PASMCs migration. Briefly, a 6-well plate was marked on the back with evenly distributed horizontal straight lines at 1 cm intervals. Then each well was seeded with 5×10^5 cells, and cells were serum starved overnight after reaching 70–80% confluency. Then, 200 μL pipette tips were used to randomly scratch three parallel lines. The cells were washed three times with PBS and cultured with starvation medium containing HMGB1 (50 ng/mL based on the preliminary experiment). Then, the cells were cultured at 37 °C and 5% CO_2 under normoxic conditions and photographed at 0 and 24 h of culture. Migration rate was calculated by the following formula: Migration rate (%) = (distance$_{before}$ − distance$_{after}$)/distance$_{before}$ × 100, where distance$_{before}$ are the scratch distance before migraton and distance$_{after}$ are the scratch distance after migration.

2.7. siRNA transfection

The siRNA was diluted to a storage concentration of 20 uM with RNase-free Water. Add 250 μL of RNase-free Water to 5 nmol siRNA, mix gently by pipetting, and store at −20 °C to avoid repeated freezing and thawing. Preparation of siRNA mixture: At a final concentration of 50 nM, first dilute 5 μL of siRNA (20 μM) with 120 μL of transfected medium (Opti-MEM) and mix gently. Then add 12 μL of Hiperfect Transfection Reagent (QIAGEN, GER), gently pipette and mix, incubate for 15 min at room temperature to prepare transfection complex; also set control group (only medium) and negative control. Take out the 6-well plate that has been planted overnight, discard the cell culture medium, and wash it three times with PBS; make the prepared transfection complex to make up the medium to 2 mL, mix gently, add to the six-well plate, and incubate for 72 h.
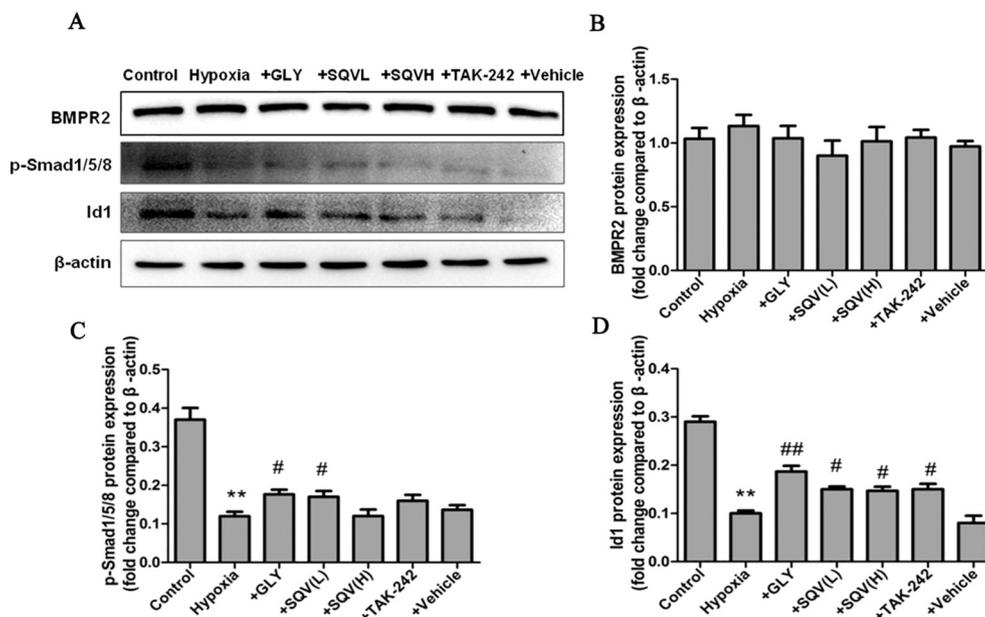
Fig. 5. Effects of HMGB1/TLR4 inhibitors on BMPR2 signaling in hypoxia-induced PH rats. A: The protein expression of BMPR2, p-Smad1/5/8 and Id1 in pulmonary arteries of rats determined by Western blot. GLY: Glycyrrhizin (100 mg/kg per day); SQVL: Saquinavir low-dosage group (3 mg/kg per day); SQVH: Saquinavir high-dosage group (15 mg/kg per day); TAK-242 (TLR4 inhibitor, 0.3 mg/kg per day); Vehicle: 1% DMSO. B-D: Quantitative analysis results of graph A. Data expressed as mean ± standard error, n = 3. **$P < 0.01$ vs. Control group; #$P < 0.05$, ##$P < 0.01$ vs. HMGB1 group.

2.8. Western blot analysis

Protein was extracted from pulmonary arteries with RIPA buffer (containing 0.1% PMSF), and equal amounts of protein from each sample (30 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. And incubated with 1% BSA for 1 h at room temperature. The membranes were then incubated with primary antibodies against HMGB1 (CalBioreagents, USA), TLR4 (Abcam, UK), BMPR2 (CalBioreagents, USA), p-Smad1/5/8 (Cell Signaling Technology, USA), Id1 (CalBioreagents, USA), Id3 (Abcam, UK), β-actin (Beyotime, China) overnight at 4 °C, followed by horseradish peroxidase–conjugated secondary anti-bodies. The signals of bands were measured by Luminata Creseendo Western HRP Substrate (Millipore) through Molecular Imager ChemiDoc XRS System (Bio-Rad, PA). The densitometric quantification was conducted with Image J 1.43 (National Institutes of Health).

2.9. Statistical analysis

Statistical analysis was performed by SPSS 18.0 software. And the results were presented as mean ± SD. All data was analyzed using one way ANOVA analysis followed by Newman–Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Hypoxia affects inflammatory factors, HMGB1/TLR4 protein expression and BMPR2 signaling pathway

More and more evidences showed that inflammation plays an important role in PH. Thus, the inflammatory factor levels in hypoxia-induced pulmonary hypertension rats were examined. The levels of HMGB1, IL-6, IL-1β, and TNF-α were measured by ELISA kits. As shown in Fig. 1A–D, hypoxia markedly increased inflammatory factors including HMGB1, IL-6, IL-1β as well as TNF-α level. HMGB1 has been reported to promote the pathogenesis of chronic hypoxia-induced pulmonary hypertension via activation of TLR4 [6]. Therefore, we further detected the protein expression of HMGB1/TLR4. The results showed that the expression of HMGB1/TLR4 protein was significantly increased in the pulmonary arteries of hypoxia-induced PH rats (Fig. 1E–G). Moreover, the expression of BMPR2 signaling pathway including BMPR2, Smad1/5/8 phosphorylation, Id1/Id3 in the pulmonary

arterial was determined by Western Blot. The results showed that there was no significant difference in the expression of BMPR2 in the pulmonary artery of hypoxia rats (Fig. 1H–I), but the downstream Smad1/5/8 phosphorylation and Id1 expression were significantly reduced (Fig. 1H,J,K).

3.2. HMGB1 promotes PASMCs migration and proliferation

The migration and proliferation of PASMCs play an important role in the process of vascular remodeling in PAH. In order to confirm the effect of HMGB1 on PASMCs proliferation and migration, exogenous HMGB1 recombinant proteins was used to directly treat PASMCs for 24 h, and then the migration of PASMCs were determined by Scratch test. (Fig. 2A–B). The results showed that exogenous HMGB1 can significantly promote the migration of PASMCs. And we also examined the effect of HMGB1 on the proliferation of PASMCs (Fig. 2C–E). The results of MTS assay and flow cytometry test showed that HMGB1 could promote the proliferation and G1/(S + G2) ratio of PASMCs.

3.3. HMGB1 regulates BMPR2 signaling pathway in PASMCs

Our previous results showed that hypoxia treatment can up-regulate the expression of HMGB1/TLR4, down-regulate the BMPR2 signaling pathway and promotes PASMCs migration and proliferation. To further explore whether HMGB1 promotes the migration and proliferation of PASMC via regulating BMPR2 signaling pathway, Different concentrations (0, 50, 100, 200 ng/mL) of HMGB1 recombinant protein were used to directly stimulate PASMCs for 24 h, and measure the expression of BMPR2 signaling pathway-related protein. The results showed that HMGB1 can significantly inhibit the BMPR2 downstream Id1 and Id3 protein expression (Fig. 3A–D).

3.4. HMGB1/TLR4 inhibitors improves pulmonary vascular remodeling and reduce plasma inflammatory factors level in hypoxia-induced PH rats

To confirm the role of HMGB1/TLR4 signaling in PH, HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242 were used to treat hypoxia-induced pulmonary hypertension in rats. In consistent with previous study [17], 4 weeks of exposure to hypoxia-induced PH in rats, as indicated by a significant elevation in RVSP (Fig. 4A). Hypoxia also significantly induced hypertrophy of right ventricle and pulmonary arteries, showing an increase in ratio of RV weight to the length of the
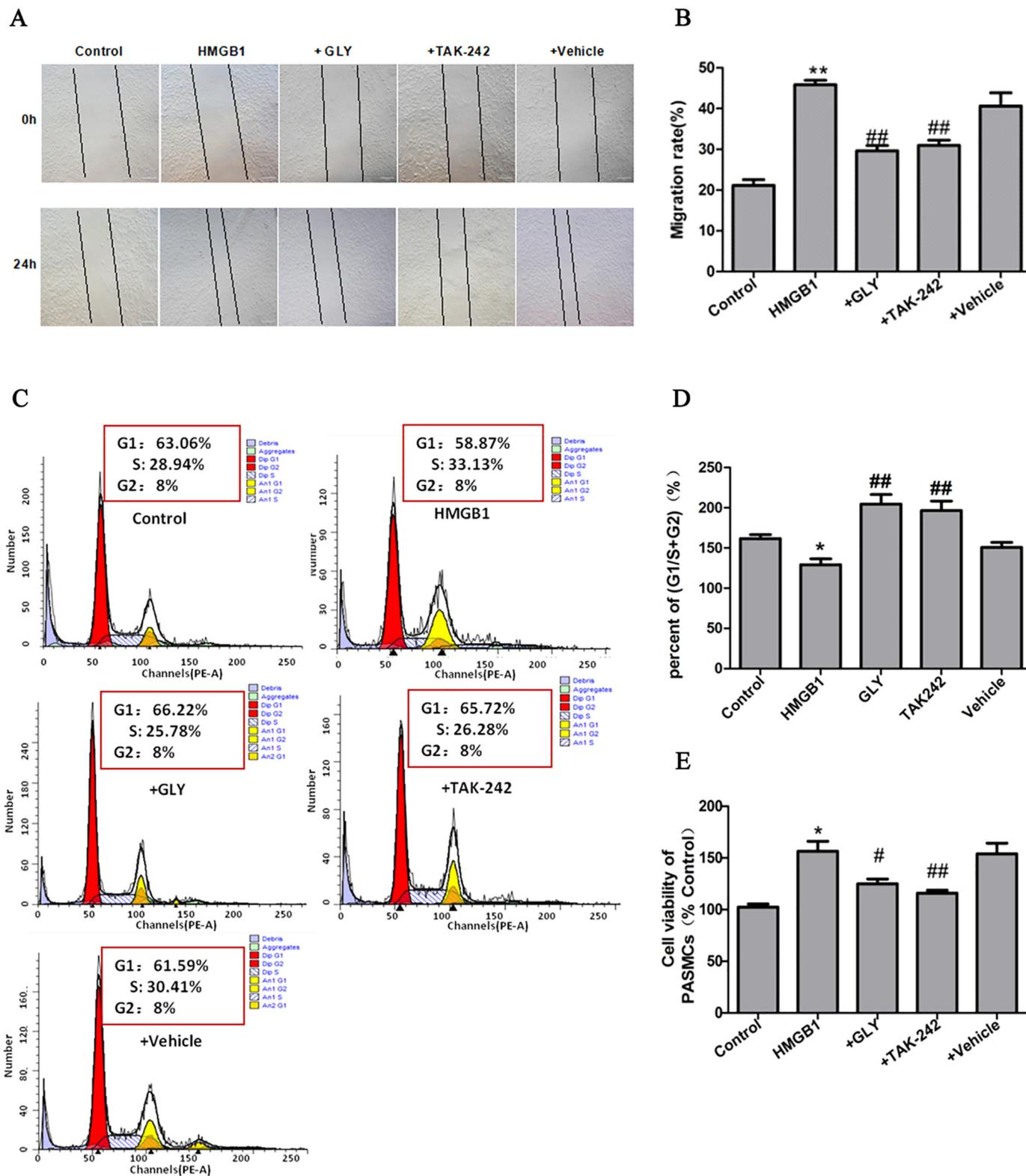
Fig. 6. Effects of HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242 on cellular migration, proliferation in cultured PASMCs. A: Migration of PASMCs under a microscope ($\times$40) after incubating with HMGB1 (50 ng/ml), Vehicle (1% DMSO) and HMGB1/TLR4 inhibitors glycyrrhizin (GLY) and TAK-242 (TLR4 inhibitor) for 24 h. Migration rate was calculated by the following formula: Migration rate (%) = (distance$_{before}$ − distance$_{after}$)/distance$_{before}$ $\times$ 100, where distance$_{before}$ are the scratch distance before migraton and distance$_{after}$ are the scratch distance after migration. B: Quantitative analysis results of graph A. Data expressed as mean $\pm$ standard error, n = 3. **$P < 0.01$ vs. Control group; ##$P < 0.01$ vs. HMGB1 group. C-D: Cell cycle analysis by flow cytometry. E: Cell viability analysis by MTS assay. Data expressed as mean $\pm$ standard error, n = 3. *$P < 0.05$ vs. Control group; #$P < 0.05$, ##$P < 0.01$ vs. HMGB1 group.
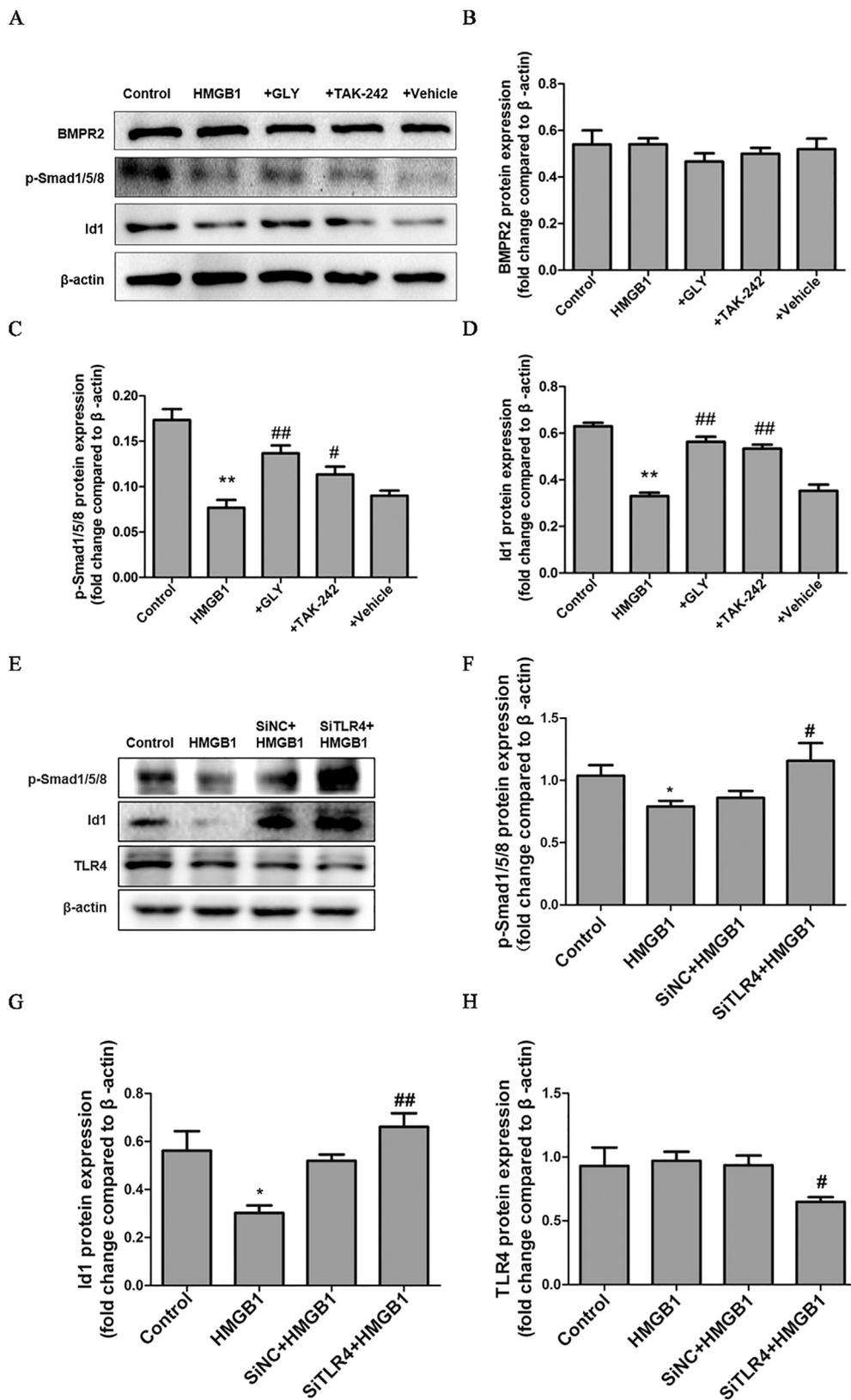
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B

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D

E

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H

Fig. 7. Effects of HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin, TAK-242 and siTLR4 on BMP signaling pathway in HMGB1-treated PASMCs. A: The protein expression of BMPR2, p-Smad1/5/8 and Id1 in HMGB1 and HMGB1/TLR4 inhibitors glycyrrhizin(GLY) and TAK-242 (TLR4 inhibitor) stimulated PASMCs was determined by Western blot. B-D: Quantitative analysis results of graph A. Data are mean ± standard error. n = 3. $^{**}P < 0.01$ vs. Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. HMGB1 group. E: The protein expression of p-Smad1/5/8, Id1, and TLR4 in PASMCs was determined by Western blot. F-H: Quantitative analysis results of graph A. Data are mean ± standard error. n = 3. $^{*}P < 0.05$ vs. Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. siNC + HMGB1.

tibia, RV/(LV + S) and proliferation of smooth muscle cells in the vascular media of small pulmonary arteries (Fig. 4B–C). All of these effects induced by hypoxia were dramatically alleviated by treating with HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242. Hypoxia obviously increased plasma inflammatory factors including HMGB1, IL-1β, IL-6 as well as TNF-α level. These effects were significantly inhibited by treating the rats with HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242 (Fig. 4D–G).

3.5. Inhibition of HMGB1/TLR4 restores BMPR2, Smad1/5/8 phosphorylation and Id1 expression in hypoxia-induced PH rats and HMGB1-treated PASMCs

To further determine the relationship between HMGB1/TLR4 signaling and BMPR2 signaling pathway in pulmonary hypertension. The expression of BMPR2 signaling pathway was measured in PH rats treated with HMGB1/TLR4 inhibitors. And HMGB1/TLR4 inhibitors and siRNA of TLR4 were used to incubate PASMCs before treated with HMGB1. The results showed that hypoxia and HMGB1 decreased the expression of Id1 and phosphorylation level of Smad1/5/8 (Figs. 5A, C and D; 7A, C, D and E–G), but exerted no effect on BMPR2 expression (Figs. 5B; 7B). However, all of these effects induced by hypoxia or HMGB1 were reversed by treating the rats and PASMCs with HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242 or siRNA of TLR4 (Figs. 5A; 7A and E).

3.6. HMGB1/TLR4 inhibitors regulate cell migration, proliferation in cultured PASMCs

In view of the key roles of PASMCs migration and proliferation in PH, we further examined the effect of HMGB1 inhibitors on HMGB1-induced migration and proliferation of PASMCs. In consistent with previous studies [18], HMGB1 stimulated migration and proliferation of PASMCs, as shown by a significant higher migration rate (Fig. 6A–B) and an increase in the percentage of cells in the S + G2 phase (Fig. 6C–E), while HMGB1-induced migration and proliferation of PASMCs were significantly reduced under treatment with HMGB1/TLR4 inhibitors saquinavir, glycyrrhizin and TAK-242. The results of Scratch test showed that glycyrrhizin and TAK-242 significantly inhibited HMGB1-induced cell migration. The results of flow cytometry and MTS showed that glycyrrhizic and TAK-242 could significantly inhibit the HMGB1-induced proliferation and increased cell cycle G1/(S + G2) ratio in PASMCs.

4. Discussion

In summary, this study suggested an important role of BMPR2 signaling in inflammation, provided new proof supporting anti-inflammation therapy in PH. Especially the results revealed that HMGB1, a novel inflammatory factor and regulator, promoted vascular remodeling in hypoxic PH by suppressing BMPR2 signaling pathway. In 1994, Tuder et al. firstly detected the infiltration of inflammatory cells such as T lymphocytes, B lymphocytes, and macrophages around the arterial wall of plexiform lesions in patients with PH [19]. In recent years, an increasing number of studies have shown that inflammation play an important role in the progression of PH. The levels of inflammatory cytokines or chemokines such were significantly elevated in peripheral plasma, injured blood vessels and lung tissues in PAH [4,20]. The follow-up study further found that the level of inflammatory factors was closely related to the degree of pulmonary vascular injury and prognosis. Anti-inflammatory therapy has been shown to be effective in improving endothelial function and delaying pulmonary vascular remodeling in animal studies. This study also found that inflammatory response in hypoxia-induced PH rats was significantly higher than those under normoxia. And hypoxia treatment markedly increased the level of inflammatory factors including HMGB1, IL-1β, IL-6 as well as TNF-α in animal and cell models.

Previous studies have shown that HMGB1 and the key downstream receptor TLR4 play a critical role in regulating inflammatory responses. Activated HMGB1 is released to outside of cells from nucleus, binds to TLR4 receptor on the cell membrane surface, which promotes the production of inflammatory factors such as IL-6, TNF-α, etc., finally results in the inflammatory response. The inflammatory responses mediated by HMGB1 participates in many disease, such as cardiovascular diseases, cancer, lung disease [21]. Studies have shown that inflammatory processes are associated with the occurrence and development of PH and pulmonary vascular remodeling, sustained and excessive release of HMGB1 may lead to remodeling of pulmonary blood vessel walls and increased resistance of pulmonary blood vessels. We found that both of HMGB1 and TLR4 were up-regulated in hypoxic PH rats and hypoxia-treating PASMCs. Exogenous HMGB1 could promote the proliferation and increase the G1/(S + G2) ratio of PASMCs, which can be abrogated by HMGB1 inhibitors and TLR4 inhibitor TAK-242. These reults support a role of HMGB1/TLR4 in vascular remolding of PH.

The dysfunction of BMPR2 signaling pathway has been generally recognized as an important pathological basis of PAH. The inhibition of BMPR2 signaling leads to a loss of the growth-suppressive effects of BMPs in PASMCs via a reduction in downstream BMPR2, Smad1/5 phosphorylation and Id1, which is a major target gene in response to BMP signaling pathway [22,23]. Recently, Soon and coworkers found that BMPR2 deficiency promotes an exaggerated inflammatory response in vitro and in vivo induced by LPS. LPS elicits strong inflammatory response through TLR4 signaling, which can instigate development of pulmonary hypertension. Their work established a direct link between BMPR2 deficiency and excessive pro-inflammatory cytokine production for the first time [24]. Association between BMPR2 and inflammation are bringing new concepts into the pulmonary arterial hypertension world.

Furthermore, we observed a significantly increased expression of HMGB1/TLR4 signaling and a dramatically reduced expression of BMPR2 pathway downstream Smad1/5/8 phosphorylation and Id1 in pulmonary artery or lung tissue in hypoxia-induced PH rats, although BMPR2 expression in pulmonary artery has no significant changes. Notably, all of these effects induced by hypoxia were significantly abrogated by pre-treating the rats with HMGB1/TLR4 inhibitors. Similarly, PASMCs incubated with HMGB1 performed an abnormal proliferation and migration, concomitantly with an obvious suppression of BMPR2 signaling. These results suggested that HMGB1/TLR4 inhibited BMPR2 signaling pathway in hypoxia-induced PH. Actually the relationship between inflammation and BMPR2 signaling pathway has been revealed by more and more studies recently. It has been demonstrated that BMPR2 loss always means up-regulated inflammation. IL-1β induces an exaggerated inflammatory response in pulmonary arteries when BMPR2 signaling is reduced [25]. On the other hand, ligands as well as activators to BMPR2 such as BMP7 and BMP9 can protect against inflammation via BMPR2/Smads signaling pathway [26,27]. Thus the benefit from anti-inflammation therapy may partly attribute to restoration of BMPR2 signaling pathway. Considering that BMPR2 signaling recovery are involved in other therapeutical medications such as sildenafil and treprostinil [28,29]. It is justified that BMPR2 signaling pathway has very potential to be new therapeutical target in PH.

Furthermore, we believe the relationship between inflammation and BMPR2 signaling doesn't only exit in PASMCs but also in PAECs, fibroblast and other cell lines, further study using BMPR2 and HMGB1 knockout animal would be helpful to evaluate the effect of anti-inflammatory therapy and its mechanism in PH. By utilizing BMPR2 activators (BMP4/9), our further experiments would reveal whether activated BMPR2 signaling can restrict the damage effects of HMGB1/TLR4 in PH. Therapy integrating genetic and inflammatory factors would have great potential in maintaining vascular homeostasis in PH.

5. Conclusion

Present study found that HMGB1/TLR4 promoted the proliferation and migration of PASMCs and participates in the vascular remodeling of hypoxic PH, the underlying mechanism may involve down-regulation of BMPR2 signaling. This study creatively investigated the direct relationship between HMGB1/TLR4 signaling and BMPR2 signaling in hypoxic PH, which may provide a novel insight into pathophysiology of

PH.

References

[1] L.C. Price, S.J. Wort, F. Perros, P. Dorfmuller, A. Huertas, D. Montani, S. Cohen-Kaminsky, M. Humbert, Inflammation in pulmonary arterial hypertension, Chest 141 (2012) 210–221.

[2] S. Cohen-Kaminsky, A. Hautefort, L. Price, M. Humbert, F. Perros, Inflammation in pulmonary hypertension: what we know and what we could logically and safely target first, Drug Discov. Today 19 (2014) 1251–1256.

[3] J. Saleby, H. Bouzina, J. Lundgren, G. Radegran, Angiogenic and inflammatory biomarkers in the differentiation of pulmonary hypertension, Scand. Cardiovasc. J. 51 (2017) 261–270.

[4] M. Rabinovitch, C. Guignabert, M. Humbert, M.R. Nicolls, Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension, Circ. Res. 115 (2014) 165–175.

[5] L.A. Hurst, B.J. Dunmore, L. Long, A. Crosby, R. Al-Lamki, J. Deighton, M. Southwood, X. Yang, M.Z. Nikolic, B. Herrera, G.J. Inman, J.R. Bradley, A.A. Rana, P.D. Upton, N.W. Morrell, TNFalpha drives pulmonary arterial hypertension by suppressing the BMP type-II receptor and altering NOTCH signalling, Nat. Commun. 8 (2017) 14079.

[6] E.M. Bauer, R. Shapiro, H. Zheng, F. Ahmad, D. Ishizawar, S.A. Comhair, S.C. Erzurum, T.R. Billiar, P.M. Bauer, High mobility group box 1 contributes to the pathogenesis of experimental pulmonary hypertension via activation of Toll-like receptor 4, Mol. Med. 18 (2013) 1509–1518.

[7] Y. Sadamura-Takenaka, T. Ito, S. Noma, Y. Oyama, S. Yamada, K. Kawahara, H. Inoue, I. Maruyama, HMGB1 promotes the development of pulmonary arterial hypertension in rats, PLoS ONE 9 (2014) e102482.

[8] H. Yang, H.S. Hreggvidsdottir, K. Palmblad, H. Wang, M. Ochani, J. Li, B. Lu, S. Chavan, M. Rosas-Ballina, Y. Al-Abed, S. Akira, A. Bierhaus, H. Erlandsson-Harris, U. Andersson, K.J. Tracey, A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 11942–11947.

[9] K.C. Young, S.M. Hussein, R. Dadiz, D. DeMello, C. Devia, C. Hehre, C. Suguihara, Toll-like receptor 4-deficient mice are resistant to chronic hypoxia-induced pulmonary hypertension, Exp. Lung Res. 36 (2010) 111–119.

[10] M. Brock, V.J. Samillan, M. Trenkmann, C. Schwarzwald, S. Ulrich, R.E. Gay, M. Gassmann, L. Ostergaard, S. Gay, R. Speich, L.C. Huber, AntagomiR directed against miR-20a restores functional BMPR2 signalling and prevents vascular remodelling in hypoxia-induced pulmonary hypertension, Eur. Heart J. 35 (2014) 3203–3211.

[11] D.R. Fraidenburg, J.X. Yuan, Hungry for more: autophagy in the pathogenesis of pulmonary arterial hypertension, Circ. Res. 112 (2013) 1091–1093.

[12] C. Hwangbo, H.W. Lee, H. Kang, H. Ju, D.S. Wiley, I. Papangeli, J. Han, J.D. Kim, W.P. Dunworth, X. Hu, S. Lee, O. El-Hely, A. Sofer, B. Pak, L. Peterson, S. Comhair, E.M. Hwang, J.Y. Park, J.L. Thomas, V.L. Bautch, S.C. Erzurum, H.J. Chun, S.W. Jin, Modulation of endothelial bone morphogenetic protein receptor type 2 activity by vascular endothelial growth factor receptor 3 in pulmonary arterial hypertension, Circulation 135 (2017) 2288–2298.

[13] E. Soon, A. Crosby, M. Southwood, P. Yang, T. Tajsic, M. Toshner, S. Appleby, C.M. Shanahan, K.D. Bloch, J. Pepke-Zaba, P. Upton, N.W. Morrell, Bone

[14] X.W. Li, J. Du, G.Y. Hu, C.P. Hu, D. Li, Y.J. Li, X.H. Li, Fluorofenidone attenuates vascular remodeling in hypoxia-induced pulmonary hypertension of rats, Can. J. Physiol. Pharmacol. 92 (2014) 58–69.

[15] X.H. Li, J. Peng, N. Tan, W.H. Wu, T.T. Li, R.Z. Shi, Y.J. Li, Involvement of asymmetric dimethylarginine and Rho kinase in the vascular remodeling in monocrotaline-induced pulmonary hypertension, Vasc. Pharmacol. 53 (2010) 223–229.

[16] A.P. Wang, X.H. Li, S.X. Gong, W.Q. Li, C.P. Hu, Z. Zhang, Y.J. Li, miR-100 suppresses mTOR signaling in hypoxia-induced pulmonary hypertension in rats, Eur. J. Pharmacol. 765 (2015) 565–573.

[17] B. Liu, X.J. Luo, Z.B. Yang, J.J. Zhang, T.B. Li, X.J. Zhang, Q.L. Ma, G.G. Zhang, C.P. Hu, J. Peng, Inhibition of NOX/VPO1 pathway and inflammatory reaction by trimethoxystilbene in prevention of cardiovascular remodeling in hypoxia-induced pulmonary hypertensive rats, J. Cardiovasc. Pharmacol. 63 (2014) 567–576.

[18] D. Zabini, S. Crnkovic, H. Xu, M. Tscherner, B. Ghanim, W. Klepetko, A. Olschewski, G. Kwapiszewska, L.M. Marsh, High-mobility group box-1 induces vascular remodelling processes via c-Jun activation, J. Cell. Mol. Med. 19 (2015) 1151–1161.

[19] N.A. Vonk, B.E. Westerhof, N. Westerhof, The relationship between the right ventricle and its load in pulmonary hypertension, J. Am. Coll. Cardiol. 69 (2017) 236–243.

[20] S. Isobe, M. Kataoka, Y. Aimi, S. Gamou, T. Satoh, K. Fukuda, Improved survival of patients with pulmonary arterial hypertension with BMPR2 mutations in the last decade, Am. J. Respir. Crit. Care Med. 193 (2016) 1310–1314.

[21] R. Kang, R. Chen, Q. Zhang, W. Hou, S. Wu, L. Cao, J. Huang, Y. Yu, X.G. Fan, Z. Yan, X. Sun, H. Wang, Q. Wang, A. Tsung, T.R. Billiar, H.R. Zeh, M.T. Lotze, D. Tang, HMGB1 in health and disease, Mol. Asp. Med. 40 (2014) 1–116.

[22] K. Miyazono, S. Maeda, T. Imamura, BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk, Cytokine Growth Factor Rev. 16 (2005) 251–263.

[23] J. Yang, R.J. Davies, M. Southwood, L. Long, X. Yang, A. Sobolewski, P.D. Upton, R.C. Trembath, N.W. Morrell, Mutations in bone morphogenetic protein type II receptor cause dysregulation of id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension, Circ. Res. 102 (2008) 1212–1221.

[24] F. Perros, S. Bonnet, Bone morphogenetic protein receptor type II and inflammation are bringing old concepts into the new pulmonary arterial hypertension world, Am. J. Respir. Crit. Care Med. 192 (2015) 777–779.

[25] J. Pickworth, A. Rothman, J. Iremonger, H. Casbolt, K. Hopkinson, P.M. Hickey, S. Gladson, S. Shay, N.W. Morrell, S.E. Francis, J.D. West, A. Lawrie, Differential IL-1 signaling induced by BMPR2 deficiency drives pulmonary vascular remodeling, PULM CIRC 7 (2017) 768–776.

[26] X. Chen, M. Orriols, F.J. Walther, E.H. Laghmani, A.M. Hoogeboom, A. Hogen-Esch, P.S. Hiemstra, G. Folkerts, M. Goumans, D.P. Ten, N.W. Morrell, G. Wagenaar, Bone morphogenetic protein 9 protects against neonatal hyperoxia-induced impairment of alveolarization and pulmonary inflammation, Front. Physiol. 8 (2017) 486.

[27] M. Chemel, R. Brion, A.I. Segaliny, A. Lamora, C. Charrier, B. Brulin, Y. Maugars, B. Le Goff, D. Heymann, F. Verrecchia, Bone morphogenetic protein 2 and transforming growth factor beta1 inhibit the expression of the proinflammatory cytokine IL-34 in rheumatoid arthritis synovial fibroblasts, Am. J. Pathol. 187 (2017) 156–162.

[28] J. Yang, X. Li, R.S. Al-Lamki, C. Wu, A. Weiss, J. Berk, R.T. Schermuly, N.W. Morrell, Sildenafil potentiates bone morphogenetic protein signaling in pulmonary arterial smooth muscle cells and in experimental pulmonary hypertension, Arterioscler. Thromb. Vasc. Biol. 33 (2013) 34–42.

[29] J. Yang, X. Li, R.S. Al-Lamki, M. Southwood, J. Zhao, A.M. Lever, F. Grimminger, R.T. Schermuly, N.W. Morrell, Smad-dependent and smad-independent induction of id1 by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo, Circ. Res. 107 (2010) 252–262.