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Inhibition of Siah2 ubiquitin ligase ameliorates monocrotaline-induced pulmonary arterial remodeling through inactivation of YAP

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Abstract

Aims: It has been shown that up-regulation of E3 ubiquitin ligase seven-in-absentia-homolog 2 (Siah2) and activation of Hippo signaling pathway effector yes-associated protein (YAP) are involved in the development of pulmonary arterial hypertension (PAH). However, it is still unclear whether Siah2 activates YAP in MCT-induced PAH rat model.

Main methods: Intraperitoneal injection of monocrotaline (MCT) was used to induce PAH rat models. The right ventricular systolic pressure (RVSP), right ventricle hypertrophy index (RVHI), percentage of medial wall thickness (%MT), α-SMA, Ki-67 and TUNEL staining were performed to evaluate the development of PAH. Protein levels of Siah2, Lats1/2, YAP phosphorylation and total YAP, and the subcellular localization of YAP were examined using immunoblotting. Proteasome activity was measured by an assay kit.

Key findings: The protein level of Siah2 was significantly increased in MCT-induced PAH rats, this was accompanied with the proteasome-dependent degradation of Lats1/2 and subsequent up-regulation and dephosphorylation of YAP and its nuclear localization. Administration of PAH rats with Siah2 inhibitor Vitamin K3 or proteasome inhibitor MG-132 dramatically suppressed MCT-induced down-regulation of Lats1/2 and activation of YAP, finally reduced RVSP, RVHI, %MT, pulmonary arterial muscularization, pulmonary arterial smooth muscle cells (PASMCs) proliferation and enhanced PASMCs apoptosis in PAH rats.

Significance: Siah2 contributes to the development of MCT-induced PAH by destabilizing Lats1/2 and subsequently stimulating YAP activation. Inhibition of Siah2 or proteasome alleviates pulmonary arterial remodeling through inactivation of YAP, indicating Siah2 ubiquitin ligase as a novel target might have potential value in the management of PAH.

Keywords

Siah2, YAP, Vitamin K3, Pulmonary arterial remodeling

1. Introduction

Pulmonary arterial hypertension (PAH) is a multifactorial disorder characterized by sustained elevation of pulmonary vascular resistance and pulmonary artery pressure, eventually resulting in right heart dysfunction and death [1, 2]. Different types of PAH share a similar pathogenesis including pulmonary vasoconstriction, vascular remodeling and thrombosis in situ [2]. However, it is now widely recognized that pulmonary vascular remodeling, major caused by excessive proliferation of pulmonary arterial smooth muscle cells (PASMCs), is the prominent hallmark of PAH pathologic mechanism [3]. Therefore, it is critical to elucidate the molecular mechanisms responsible for PASMCs proliferation to supply novel targets for attenuating pulmonary arterial remodeling and preventing PAH.

Seven-in-absentia-homolog 2 (Siah2) is the mammalian homolog of Drosophila seven-in-absentia (Sina), it is an evolutionarily conserved really interesting new gene (RING) finger E3 ubiquitin ligase that mediates substrate protein degradation by functioning as scaffold to transfer ubiquitin from E2 ubiquitin conjugating enzyme to substrate protein [4, 5]. It has been demonstrated that Siah2 ubiquitin ligase recognizes and targets many proteins for proteasomal degradation by poly-ubiquitination, such as deleted in colorectal cancer (DCC), prolyl hydroxylase 3 (PHD3) and β-catenin [6-8]. In addition, up-regulation of Siah2 has been found to participate in various aspects of pathological processes, including hypoxic response, tumorigenesis, cancer growth, metastasis and angiogenesis [9-13]. A recent study has shown that elevated expression of Siah2 involves in the occurrence of hypoxic pulmonary hypertension by initiating the ubiquitination and proteasomal degradation of PHD3 [14]. However, the role and detailed molecular mechanisms of Siah2 in monocrotaline (MCT)-induced PAH rats remains indefinite.

The Hippo signaling pathway is a highly conserved pathway that controls organ size by regulating cell proliferation and apoptosis [15, 16]. It has been revealed that

down-regulation of large tumor suppressor 1/2 (Lats1/2) suppresses yes-associated protein (YAP) phosphorylation and promotes YAP nuclear translocation [17], which allows YAP as a transcriptional coactivator to induce the transcription of growth-promoting genes, such as CCND1, forkhead box M1 (FOXM1), CYR61 and connective tissue growth factor (CTGF), therefore contributes to cells proliferation [18-20]. Furthermore, inactivation of Lats1 and up-regulation of its effector YAP have been shown to promote PASMCs proliferation and small pulmonary arterial remodeling in idiopathic PAH [21]. Recently, it has been demonstrated that Siah2 ubiquitin ligase interacts with Lats1/2 and negatively regulates its stability through proteasome pathway [17]. Therefore, it is attractive to explore whether Siah2 induces the proteasomal degradation of Lats1/2 and actives its effector YAP, leading to the proliferation of PASMCs and occurrence of PAH.

Vitamin K3 (also known as menadione), a Vitamin K metabolite which is required to synthesize coagulation factors, has been reported to show anti-cancer and anti-angiogenic effects in tumor [22, 23]. Meanwhile, Vitamin K3 has been used as a novel Siah2 inhibitor to ameliorate hypoxia and MAPK signaling and consequently to suppress melanoma carcinogenesis [10]. MG-132, a proteasome inhibitor, plays an important role in reducing PASMCs proliferation in vitro and decreasing the thickness of pulmonary vascular walls in chronic hypoxia-induced PAH rats [24, 25]. However, it is still unclear whether Vitamin K3 or MG-132 benefits PAH by suppression of Siah2 mediates Lats1/2 degradation and YAP activation.

2. Materials and methods

2.1. Animals

56 male Sprague-Dawley (SD) rats (180-210g) were purchased from the Experimental Animal Center of the Medical College of Xi'an Jiaotong University and used in the current study. All rats were kept in the same room of the specific pathogen free (SPF) animal laboratory under standard conditions (18-22°C, 40%-60% humidity and 12h

light/dark cycle). The animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of Xi'an Jiaotong University Animal Experiment Centre and approved by the Laboratory Animal Care Committee of Xi'an Jiaotong University.

2.2. Drug and chemical reagents

MCT (Sigma-Aldrich, USA) was dissolved in 1 mol/L HCL and then titrated with 1 mol/L NaOH to pH 7.4 with a final concentration of 20 mg/mL. Siah2 inhibitor Vitamin K3 (Selleck, USA) and proteasome inhibitor MG-132 (Selleck, USA) were dissolved in dimethyl sulfoxide (DMSO) and then diluted with normal saline to a final concentration of 0.1 mg/mL, while the ultimate concentration of DMSO was 0.5%. DMSO was diluted with normal saline to a final concentration of 0.5% as a vehicle for Vitamin K3 and MG-132.

2.3. Experimental design

56 rats were divided into 7 groups randomly (n=8 per group): Group 1 (Control group), Group 2 (Vitamin K3 group), Group 3 (MG-132 group), Group 4 (MCT group), Group 5 (MCT + Vehicle group), Group 6 (MCT + Vitamin K3 group) and Group 7 (MCT + MG-132 group). Control group received a single intraperitoneal injection of 60mg/kg vehicle solution (1 mol/L HCL titrated with 1 mol/L NaOH to pH 7.4) at day 1, then were administered with DMSO vehicle by daily intraperitoneal injection for 4 weeks. Vitamin K3 (3.5mg/kg) was used by intraperitoneal injection twice a week for 4 weeks. MG-132 (0.1 mg/kg) was administered to rats by daily intraperitoneal injection for 28 days. Experimental PAH was induced by a single intraperitoneal injection of MCT (60 mg/kg) at day 1. MCT+Vehicle group received a single intraperitoneal injection of MCT (60mg/kg) at day 1 and then the rats were regulated by daily intraperitoneal injection of DMSO vehicle for 28 days until the rats were sacrificed. The dose selected in the present study for MCT, Vitamin K3 [10] and MG-132 [26] were shown to be effective and safe in previous experimental studies.

2.4. Measurement of the RVSP and the RVHI

After 4 weeks of MCT injection, all survived rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3-5mL/kg). After stable anesthesia, we put the rats in supine position and isolated its right external jugular vein, from which a polyvinyl catheter filled with heparin saline solution were inserted into the right ventricle to measure the right ventricle systolic pressure (RVSP) monitored by a Grass polygraph (Power Lab, Australia). After hemodynamic measurement, the thorax was opened and the lung and heart were collected, then the heart was separated along the interventricular septum and divided into two parts: the right ventricle (RV) and the left ventricle (LV) plus the interventricular septum (S). Each chamber was weighed separately to calculate the right ventricular hypertrophy index (RVHI), which is the ratio of RV and LV + S (RVHI = RV/ LV + S).

2.5. Hematoxylin and eosin staining

The obtained right upper pulmonary lobes were completely immersed in 4% paraformaldehyde for 24h at room temperature and then the fixed tissues were embedded in paraffin wax. The paraffin embedded tissue blocks were sectioned at 5μm thickness and then stained with hematoxylin and eosin (HE) for morphological analysis. To evaluate the degree of pulmonary arterial remodeling by the percentage of medial wall thickness (%MT) of vessels (diameter 50-200μm, 30 arteries each rat), the distance between the inner and outer elastic fiber layers (medial wall thickness, MT) and the average diameter of the outer elastic fiber layer (external diameter, ED) were measured by two observers blinded to the treatments of the rats using a light microscope (×400 magnification). The %MT was calculated as follows: %MT = (2 x MT/ ED) x100%.

2.6. α-SMA, Ki-67 and TUNEL staining

To assess the degree of pulmonary arterial muscularization, the paraffin embedded

tissue blocks were sectioned at 5μm thickness and then performed an immunostaining with anti α-smooth muscle actin (α-SMA) antibody (Boster Biological Technology, China, #BM0002, 1:100 dilution). Under light microscopy (×400 magnification), counted arteries (diameter 20-100μm, 30 arteries each rat) were categorized as muscular, partially muscular or non-muscular. The artery number and the degree of muscularization were quantified by a method described previously [27]. Similarly, Ki-67 (ImmunoWay Biotechnology, USA, YM3064, 1:200 dilution) immunostaining was conducted to evaluate the cell proliferation. 30 arteries (diameter 20-100μm) in each rat were randomly selected to detect the proliferation by microscopy at ×400 magnification. Cells with brown-stained nuclei in the medial layer of pulmonary artery were Ki-67 positive PASMCs. In addition, cell apoptosis was evaluated by the terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay. 30 arteries (diameter 20-100μm) in each rat were randomly selected for detection of apoptosis and the ratios of apoptosis cells to total PASMCs in individual arterioles were calculated. All experiments were evaluated by two independent examiners in blinded.

2.7. PASMCs culture

Primary rat PASMCs were obtained from pulmonary arteries of male SD rats (100-120g). All animal experiments were approved and performed in accordance with the Laboratory Animal Care Committee of Xi'an Jiaotong University. Pulmonary arteries were rapidly isolated from euthanized rats, and scraped off the adventitia and endothelium gently and cut the remaining smooth muscle layer into 1 mm^3 tissue blocks and transferred them into a culture flask with Dulbecco's modified Eagle medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Sijiqing, China) and 1% antibiotics (100 U/mL penicillin and 100μg/mL streptomycin). Then tissues blocks were incubated at 37 °C in a humidified atmosphere of 5% CO2. Cells were subcultured by trypsinization using 0.25% trypsin (Invitrogen, USA) until reaching 80% confluence. Cells between passages 3-6 were used for further

experiments. α-SMA immunostaining were used to confirm the purity and identity of PASMCs.

2.8. siRNA transfection

To silence the expression of Siah2, PASMCs were transfected with Siah2 sequence-specific (sense 5'-GCAUCAGGAACCUGGCUAUTT-3', antisense 5'-AUAGCCAGGUUCCUGAUGCTT-3') or negative control siRNAs (GenePharm, China) using Lipofectamine^{TM} 2000 reagent (Invitrogen, USA). Briefly, PASMCs were seeded in 6-well plates and cultured until reaching 30-50% confluence, then 100nM siRNA and 5μL Lipofectamine were separately diluted in 250μL serum-free DMEM. Then the complex was added to cells and cells were incubated in serum-free DMEM for 6 h, then replaced with complete DMEM for further 48 h incubation in a 37 °C, 5% CO2 humidified incubator. The effect of siRNA transfection was analyzed using immunoblotting.

2.9. Immunoblotting

The left lung tissues saved in the -80 °C refrigerator were homogenized in RIPA lysis buffer (HEART, China) containing protease inhibitors, phosphatase inhibitors and PMSF. Tissue lysates were centrifuged at 12,000 rpm at 4 °C for 20 min to extract the total proteins. Cytoplasmic and nuclear proteins were collected using the Cytoplasmic and Nuclear Protein Extraction Kit (Beyotime Biotechnology, China) according to the sequential extraction procedure recommended by the manufacturer. The protein concentration was measured by BCA protein assay kit (Pierce, USA). Equal amounts of protein extracts were separated on SDS-PAGE gel (10%) and transferred to nitrocellulose membrane (Merck Millipore, Germany) by a Bio-Rad Trans-Blot system. After being blocked with 5% skim milk or 5% BSA at room temperature for 1 h, the membranes were incubated at 4 °C overnight with the following primary antibodies: Siah2 (Proteintech, USA, 12651-1-AP, 1:500 dilution), Lats1/2 (ImmunoWay Biotechnology, USA, YT6125, 1:500 dilution), YAP (ABclonal, USA,

A11430, 1:1000 dilution), phosphor YAP (ABclonal, USA, AP0489, 1:1000 dilution), β-actin (ImmunoWay Biotechnology, USA, YM3028, 1:5000 dilution) and lamin B (Proteintech, USA, 12987-1-AP, 1:2000 dilution). Then the membranes were washed by PBST solution for 3 times and incubated with a horseradish peroxidase-conjugated goat anti-mouse (Sigma, USA, 1:5000 dilution) or anti-rabbit (Sigma, USA, 1:5000 dilution) IgG at room temperature for 1 h. After fully washed by PBST solution, the membranes were visualized by the enhanced chemiluminescence kit (Millipore, USA) and analyzed by Quantity One software (Bio-Rad, USA).

2.10. Proteasome activity assay

The proteasome activity was detected strictly in accordance with the instruction of the Proteasome Activity Assay Kit (Merck, USA, APT280). Briefly, the left lung tissues saved in the -80 °C refrigerator were homogenized in RIPA lysis buffer and then the tissue lysates were centrifuged at 12,000 rpm at 4 °C for 20 min to extract the total proteins. After measuring the protein concentration by BCA protein assay kit, 20μg protein in each group was used to prepare an assay mixture in a 96-well fluorometer plate according to the instruction of the Kit. And incubated the sample for 2 h at 37°C, read the fluorescence using a 380/460 nm filter set in a fluorometer before and after the incubation, the difference of fluorescence between the incubation is the value of the sample.

2.11. Statistical analysis

All data were presented as mean ± standard deviation (SD). Data were analyzed using one- way analysis of variance (ANOVA) with Tukey's post hoc test by SPSS 24.0 software (SPSS Inc., USA). P value < 0.05 was considered to represent a significance difference between groups.

3. Results

3.1. Inhibition of Siah2 or proteasome function prevents the increase of RVSP and

RVHI in MCT-induced PAH rats

As shown in Fig. 1A, after 28 days of MCT injection, the RVSP was significantly increased compared with the control group (52.85 ± 2.24mmHg vs. 19.24 ± 0.95mmHg, $P < 0.05$), suggesting that MCT successfully induced PAH in rats. However, treatment with Siah2 inhibitor Vitamin K3 or proteasome inhibitor MG-132 after MCT injection dramatically reduced RVSP to 34.69 ± 1.42 mmHg or 29.47 ± 2.12 mmHg, respectively (both $P < 0.05$ vs. MCT + Vehicle group). Meanwhile, Fig. 1B showed that the RVHI was elevated from 0.28 ± 0.03 in the control rats to 0.57 ± 0.13 in MCT-induced PAH rats ($P < 0.05$). However, after administration of Vitamin K3, the RVHI was decreased compared with the DMSO vehicle solution treated rats (0.35 ± 0.08 vs. 0.55 ± 0.11, $P < 0.05$). MG-132 treatment also reduced the RVHI in PAH rats (0.33 ± 0.09, $P < 0.05$ vs. MCT + Vehicle group). These results suggest that inhibition of Siah2 or proteasome function suppresses the development of pulmonary arterial hypertension.

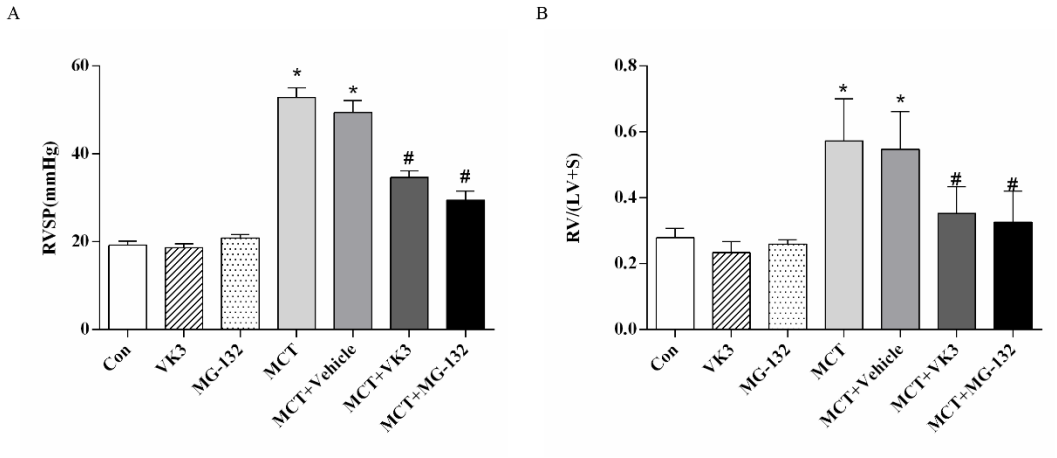

Fig. 1. Vitamin K3 or MG-132 prevents MCT-induced PAH in rats. (A) Changes of the right ventricular systolic pressure (RVSP) in each group (n = 6-8). (B) Changes of right ventricular hypertrophy index (RVHI) in each group, RVHI = RV/ (LV + S) (n = 6-8). *$P < 0.05$ vs. Control group, #$P < 0.05$ vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.2 Inhibition of Siah2 or proteasome function suppresses pulmonary arterial remodeling in MCT-induced PAH rats

Pulmonary arterial remodeling plays an important role in the pathogenesis of PAH. As the HE-staining results shown in Fig. 2A, MCT-induced PAH rats showed a significantly increased medial wall thickness of small pulmonary arteries compared with the control rats. Administration of Vitamin K3 or MG-132 markedly reversed the pulmonary arterial remodeling induced by MCT. The quantitative morphometric analysis indicated that %MT was increased from 24.34 ± 6.26% in control rats to 69.09 ± 3.60% in MCT-treated rats ($P < 0.05$, Fig. 2B). After Vitamin K3 or MG-132 treatment, %MT decreased from 64.50 ± 2.05% (MCT + Vehicle group) to 33.61 ± 4.86% or 31.16 ± 2.12%, respectively (both $P < 0.05$, Fig. 2B). Meanwhile, we classified the small pulmonary arteries observed in lung sections as muscular, partially muscular and non-muscular. After 4 weeks of MCT injection, the percentage of muscularization of pulmonary arteries was significantly higher than that in the control group (48.89% vs. 8.75%, $P < 0.05$, Fig. 2C, D). Treatment with Vitamin K3 or MG-132 markedly reduced the percentage of MCT-induced pulmonary arterial muscularization to 28.57% or 30.95%, respectively (both $P < 0.05$ vs. MCT + Vehicle group, Fig. 2C, D). These results suggest that inhibition of Siah2 or proteasome function alleviates pulmonary arterial remodeling.

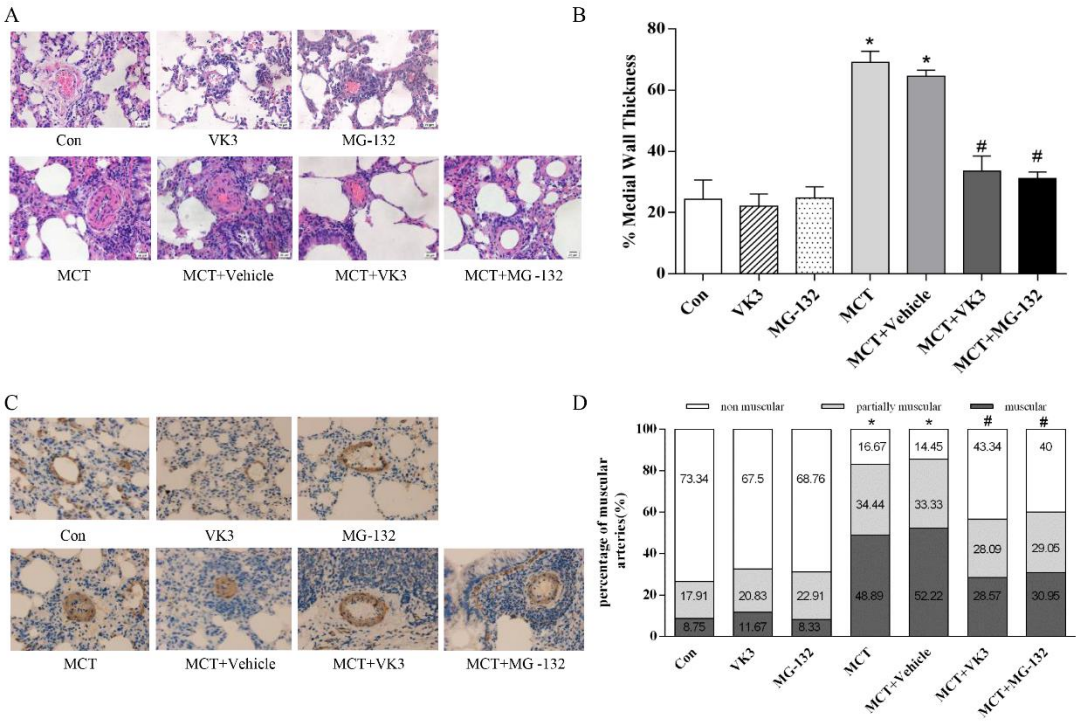

Fig. 2. Vitamin K3 or MG-132 suppresses the pulmonary arterial remodeling in MCT-induced PAH rats. (A) Hematoxylin and eosin staining of small pulmonary arteries (×400 magnification). (B) Quantitative morphometric analysis of the medial wall thickness of small pulmonary arteries. (C) The degree of muscularization of pulmonary arteries was evaluated by immunostaining of α-SMA (×400 magnification). (D) Percentage of muscular, partially muscular and non-muscular arteries in each group. *P < 0.05 vs. Control group, #P < 0.05 vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.3 Inhibition of Siah2 or proteasome function reduces proliferation and enhances apoptosis of PASMCs in MCT-induced PAH rats

Increased proliferation and reduced apoptosis of PASMCs are major pathological changes of pulmonary arterial remodeling in PAH. Therefore, we examined the degree of cells proliferation in the medial layer of pulmonary artery. Compared with the control rats, the percentage of Ki-67 positive PASMCs was markedly increased in MCT-induced PAH rats (21.81 ± 1.99% vs. 5.97 ± 0.86%, P < 0.05, Fig. 3A, B).

However, the presence of Vitamin K3 or MG-132 reduced the percentage of Ki-67 positive PASMCs from 20.67 ± 1.08% (MCT + Vehicle group) to 14.86 ± 2.41% or 14.66 ± 0.75%, respectively (both $P < 0.05$, Fig. 3A, B). In addition, we also examined cells apoptosis in the medial layer of pulmonary artery. As shown in Fig. 3C, D, MCT group showed a decreased percentage of apoptotic PASMCs compared with control group (1.29 ± 0.36% vs. 3.54 ± 0.32%, $P < 0.05$). However, treatment of MCT-induced PAH rats with Vitamin K3 or MG-132 markedly increased the percentage of apoptotic PASMCs to 2.42 ± 0.23% or 2.54 ± 0.32%, respectively (both $P < 0.05$ vs. MCT + Vehicle group, Fig. 3C, D). These results suggest that inhibition of Siah2 or proteasome function reduces proliferation and enhances apoptosis of PASMCs in MCT-induced PAH rats.

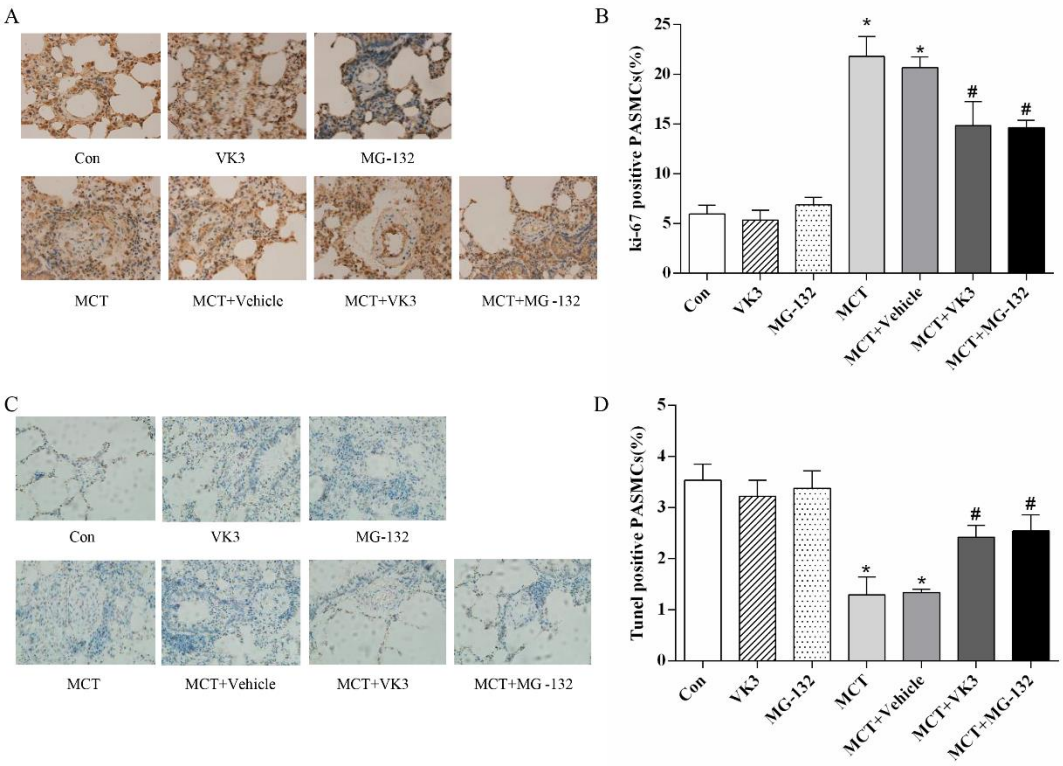

Fig. 3. Vitamin K3 or MG-132 reduces proliferation and enhances apoptosis of PASMCs in MCT-induced PAH rats. (A) Ki-67 immunostaining was conducted to assess the cell proliferation, cells with brown-stained nuclei in the medial layer of pulmonary artery are Ki-67 positive PASMCs (×400 magnification). (B) The percentage of Ki-67 positive PASMCs in each group. (C) TUNEL was performed to

determine the apoptosis of PASMCs, cells with brown-stained nuclei in the medial layer of pulmonary artery are apoptotic cells (×400 magnification). (D) The percentage of Tunel positive PASMCs in different groups. *P < 0.05 vs. Control group, #P < 0.05 vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.4 Siah2 is increased in MCT-induced PAH rats

It has been demonstrated that elevation of Siah2 ubiquitin ligase involved in the development of various tumor and hypoxic pulmonary hypertension [4, 10, 12, 14]. To determine the potential role of Siah2 in MCT-induced PAH rats, we detected the protein level of Siah2. As shown in Fig. 4, Siah2 protein level in MCT treated rats showed a 3.01-fold increase over control rats (P < 0.05 vs. Control group), which was declined to 1.58-fold over control after Vitamin K3 treatment (P < 0.05 vs. MCT + Vehicle group). These results suggest that Siah2 shows a significantly increased expression in MCT-induced PAH rats.

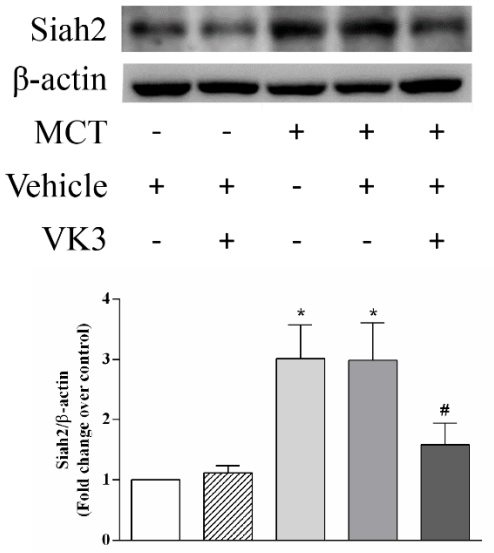

Fig. 4. Siah2 is increased in MCT-induced PAH rats. Siah2 protein level in lung tissues from different groups was determined using immunoblotting, β-actin served as a loading control (n = 4 each group). *P < 0.05 vs. Control group, #P < 0.05 vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.5 Siah2 promotes proteasome-dependent degradation of Lats1/2 in MCT-induced PAH rats

It has been shown that Siah2 diminishes Lats1/2 stability by promoting its ubiquitination and proteasomal degradation in cancer cells [17]. To determine whether Siah2 down-regulates Lats1/2 through the ubiquitin-proteasome pathway in MCT-induced PAH rats, we examined the expression of Lats1/2 and the activity of proteasome. As shown in Fig. 5A, MCT significantly reduced the protein level of Lats1/2 to 0.41-fold compared with control group ($P < 0.05$ vs. Control group), while treatment of PAH rats with Vitamin K3 or MG-132 restored the Lats1/2 protein levels to 0.75-fold or 0.77-fold over control, respectively (both $P < 0.05$ vs. MCT + Vehicle group). Furthermore, the proteasome activity in MCT treated rats showed a 1.54-fold increase over control rats ($P < 0.05$ vs. Control group, Fig. 5B), while administration of MCT-induced PAH rats with proteasome inhibitor MG-132 reduced proteasome activity to 1.19-fold over control ($P < 0.05$ vs. MCT + Vehicle group, Fig. 5B). These results suggest that Siah2 decreases Lats1/2 by promoting its proteasome-dependent degradation in MCT-induced PAH rats.

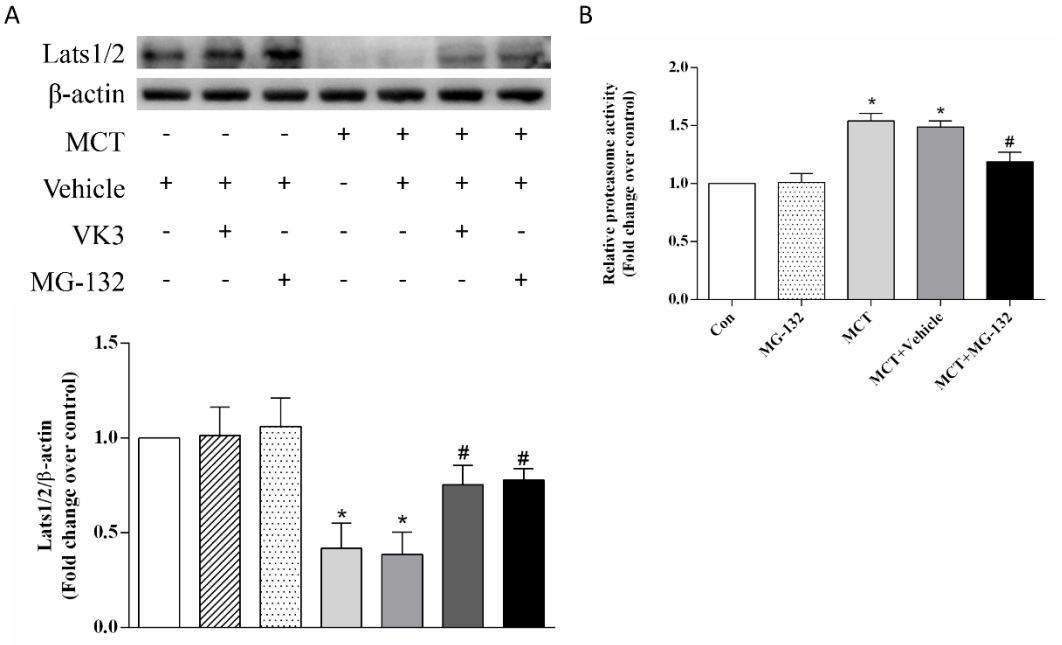

Fig. 5. Siah2 promotes proteasome-dependent degradation of Lats1/2 in MCT-induced PAH rats. (A) Lats1/2 protein level in lung tissues from different groups was

determined using immunoblotting, β-actin served as a loading control (n = 4 each group). (B) The relative proteasome activity in different groups. *P < 0.05 vs. Control group, #P < 0.05 vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.6 Siah2 promotes the overexpression and activation of YAP in MCT-induced PAH rats

Since the expression, phosphorylation and subcellular localization of Hippo signaling pathway effector YAP is associated with Lats1/2, we next determined whether Siah2 affects the expression and phosphorylation of YAP and its subcellular localization in MCT-induced PAH rats. As shown in Fig. 6A, YAP phosphorylation declined to 0.51-fold over control in MCT-induced PAH rats (P < 0.05 vs. Control group), while treatment of MCT-induced PAH rats with Vitamin K3 or MG-132 increased YAP phosphorylation to 0.85-fold or 0.87-fold over control, respectively (both P < 0.05 vs. MCT + Vehicle group). Meanwhile, YAP protein level was increased to 2.70-fold over control in MCT-induced PAH rats (P < 0.05 vs. Control group, Fig. 6A), after administration of MCT-induced PAH rats with Vitamin K3 or MG-132, YAP expression declined to 1.32-fold or 1.23-fold over control, respectively (both P < 0.05 vs. MCT + Vehicle group, Fig. 6A). In addition, the protein level of YAP in the nuclear fraction increased to 2.19-fold over control in MCT-induced PAH rats compared with the control rats (P < 0.05, Fig. 6B), while treatment of MCT-induced PAH rats with Vitamin K3 or MG-132 suppressed YAP nuclear translocation, which declined to 1.11-fold or 1.25-fold over control, respectively (both P < 0.05 vs. MCT + Vehicle group, Fig. 6B). Meanwhile, YAP protein level in cytoplasmic extracts was increased from 0.38-fold over control in MCT treated rats (MCT group vs. Control group, P < 0.05) to 0.62-fold over control in Vitamin K3 treated MCT rats and 0.72-fold over control in MG-132 treated MCT rats (both P < 0.05 vs. MCT + Vehicle group, Fig. 6B). These results suggest that Siah2 leads to the development of MCT-induced PAH by increasing total YAP expression, reducing its phosphorylation and promoting its nuclear translocation.

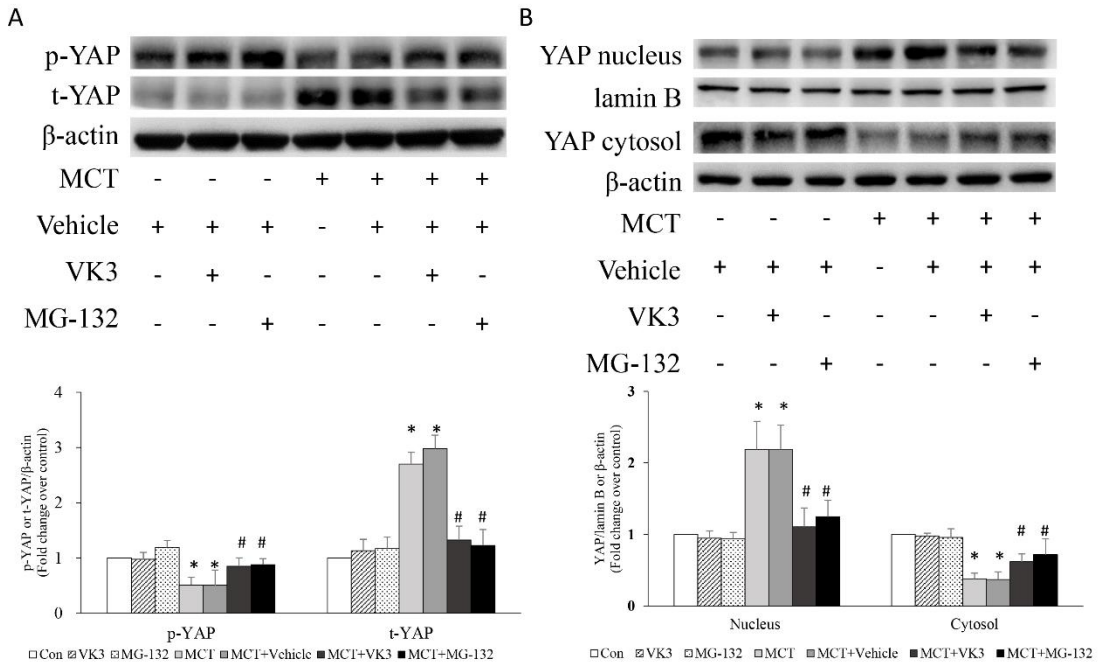

Fig. 6. Siah2 promotes the expression and activation of YAP in MCT-induced PAH rats. (A) p-YAP, t-YAP protein level in lung tissues from different groups was determined using immunoblotting, β-actin served as a loading control (n = 4 each group). (B) The protein level of YAP in the nuclear and cytoplasmic fraction, lamin B and β-actin served as loading control for the nuclear and cytoplasmic protein, respectively (n = 4 each group). *P < 0.05 vs. Control group, #P < 0.05 vs. MCT + Vehicle group. MCT, monocrotaline; VK3, Vitamin K3.

3.7 Siah2 increases YAP function in PASMCs

To clarify the specific role of Siah2 in regulation of YAP in PAH, we used specific siRNA targeting Siah2 in rat primary cultured PASMCs. As shown in Fig. 7, transfection of rat PASMCs with sequence-specific Siah2 siRNA for 48 h significantly reduced Siah2 protein level to 36% of the control (P < 0.05), whereas non-targeting siRNA did not change the expression of Siah2. And lack of Siah2 significantly increased the expression of Lats1/2 to 1.84-fold over control, decreased the expression of total YAP to 0.44-fold over control and increased YAP phosphorylation to 1.77-fold over control, respectively (P < 0.05 vs. Control group, Fig. 7A). Meanwhile, loss of Siah2 modulated YAP distribution in cytosolic versus nuclear

fraction. YAP in nuclear fraction decreased to 0.26-fold over control and in cytoplasmic fraction increased to 1.77-fold over control, respectively ($P < 0.05$ vs. Control group, Fig. 7B). These results suggest that Siah2 increases YAP function in PASMCs by increasing its expression, decreasing its phosphorylation and promoting its nuclear translocation.

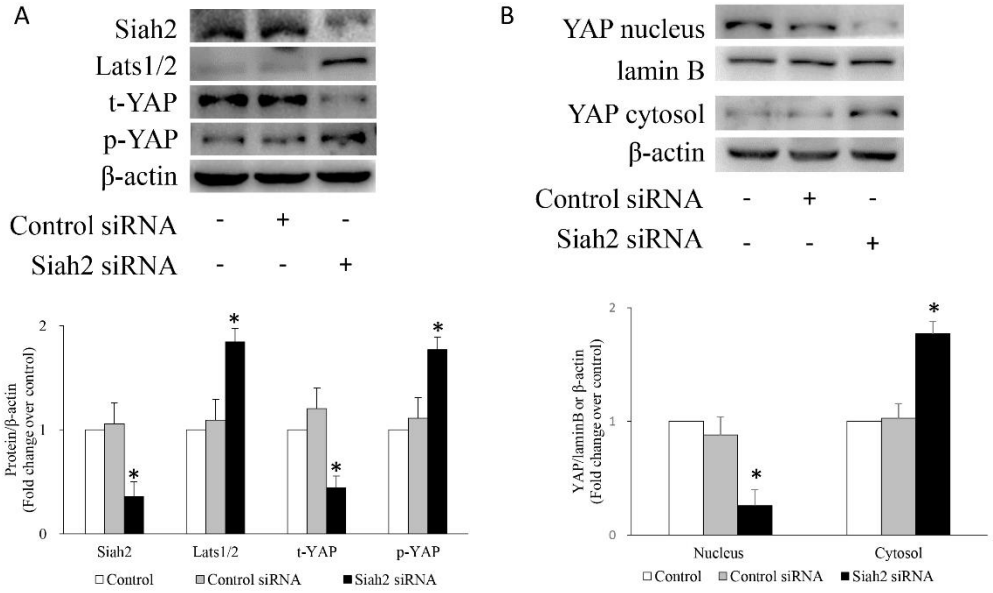

Fig. 7. Siah2 increases YAP function in PASMCs. PASMCs were transiently transfected with the control siRNA or the Siah2-specific siRNA for 48 h. Protein was extracted and subjected to immunoblotting for Siah2, Lats1/2, YAP and p-YAP. (n = 4 each group). *$P < 0.05$ vs. Control group, PASMCs, pulmonary arterial smooth muscle cells; siRNA, small (or short) interfering RNA.

4. Discussion

In the present study, we have demonstrated that Siah2 ubiquitin ligase shows elevated expression in the lung tissues of MCT-induced PAH rat models, which subsequently regulates Hippo signaling pathway by promoting Lats1/2 proteasome-dependent degradation and YAP upregulation and activation. In addition, inhibition of Siah2 by Vitamin K3 or suppression of proteasome by MG-132 effectively ameliorates pulmonary arterial remodeling and prevents the development of MCT-induced PAH.

Our results suggest that Siah2 as a novel target might have potential value in the management of PAH.

Ubiquitin proteasome system (UPS) is a sophisticated protein quality control system that is responsible for the degradation of misfolded, damaged, redundant or unneeded intracellular proteins [28, 29], which is a multi-enzyme process including the covalent conjugation of ubiquitin to substrate protein and its degradation by a large multicomponent proteolytic complex, the proteasome [29, 30]. The E3 ubiquitin ligase plays a critical role in determining substrate specificity, recognizing substrate degradation signals and catalyzing the transfer of activated ubiquitin to substrate [31-33], and its aberrant regulations are related with various cellular processes and human diseases. Previous studies have suggested that elevated expression of Siah2 ubiquitin ligase is a common event in numerous tumors such as epithelial ovarian carcinoma, lung cancer, leukemia and breast cancer [34-37]. And it has been showed that Siah2 is highly expressed in the hypoxic-induced pulmonary hypertension [14]. In this study, we found that the protein level of Siah2 was significantly enhanced in the lung tissues of MCT-induced PAH rat models, along with the excessive proliferation of PASMCs and pulmonary arterial remodeling. And administration of Siah2 inhibitor Vitamin K3 suppressed pulmonary arterial remodeling and prevented MCT-induced PAH, indicating the important role of Siah2 in the occurrence and development of PAH.

In the Hippo signaling pathway, downregulation and destabilization of Lats1/2 results in increased nuclear translocation and activation of YAP, along with the activation of pro-proliferative and anti-apoptotic genes, thus promoting tumor initiation and growth, such as liver, lung, breast and pancreatic cancers [17, 38, 39]. Further study has demonstrated that the deficiency of Lats1 and up-regulation of YAP leads to small pulmonary arterial remodeling and PASMCs proliferation in idiopathic PAH [21]. Consistent with these studies, our research showed that Lats1/2 was down-regulated

and YAP was highly expressed and activated in the MCT-induced PAH rats, accompanied with enhanced PASMCs proliferation and pulmonary arterial remodeling. A set of E3 ubiquitin ligases, such as Itch [40], WWP1[41], Siah2[17], have been revealed to negatively regulate the stability of Lats1 or Lats2 leading to increased YAP activity. The present study found that elevation of Siah2 ubiquitin ligase reduced Lats1/2 protein level by promoting its proteasomal degradation, and then up-regulated YAP expression, decreased its phosphorylation and promoted its nuclear translocation, while inhibition of Siah2 by Vitamin K3 or suppression of proteasome by MG-132 ameliorated MCT-induced Lats1/2 down-regulation and YAP up-regulation and activation, thus prevented the development of PAH in rats.

Vitamin K3 is able to produce potent antitumor effects on hepatocellular carcinoma by promoting G1 arrest of the cell cycle [42]. It has been further reported that Vitamin K3 also inhibits triple-negative breast cancer growth by phosphorylation of extracellular signal-regulated kinase (ERK) [43]. In addition, Vitamin K3 has been used as a novel Siah2 inhibitor to suppress melanoma carcinogenesis [10]. In the present study, we showed that Vitamin K3 reduced the proliferation of PASMCs and pulmonary arterial remodeling by inhibiting the function of Siah2, indicating that Vitamin K3 might have a potential therapeutic effect on PAH. Enhanced proteasome function has been observed in many types of cancers due to proteasome degrades several cancer suppressors and cell cycle checkpoint inhibitors necessary for rapid cell division [29, 44, 45]. Therefore, inhibition of proteasome function has been shown to be an important strategy in the treatment of many cancers. Previous studies have shown that MG-132 inhibits PASMCs proliferation by promoting the accumulation of negative cell cycle regulatory p21(WAF1) [24] and suppressing proteasome-dependent degradation of phosphatase and tensin homologue deleted on chromosome ten (PTEN) [26]. Our results have further suggested that treatment of MG-132 also suppressed PASMCs proliferation by blocking Siah2-induced Lats1/2 degradation and subsequent YAP activation, indicating the potential therapeutic value

of MG-132 for PAH.

5. Conclusion

Siah2 ubiquitin ligase plays a significant role in the occurrence and development of PAH by promoting PASMCs proliferation and pulmonary arterial remodeling. And elevation of Siah2 contributes to PASMCs proliferation by destabilizing Lats1/2 and up-regulating YAP and stimulating its activation. Furthermore, Siah2 inhibitor Vitamin K3 and proteasome inhibitor MG-132 suppress the development of MCT-induced PAH by inhibiting Siah2/Lats1/2/YAP axis-mediated pulmonary arterial remodeling. These results indicate that inhibition of Siah2 or proteasome function might be a promising therapeutic strategy in the prevention and treatment of PAH.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Conflict of Interest Policy Form and Author Contribution to Study Form

Conflict of interest

The authors declare that there is no conflict of interest.

Author Contribution

Qingting Wang, Wenhua Shi, Jian Wang, Manxiang li conceived and designed research;
Qingting Wang, Wenhua Shi, Qianqian Zhang performed experiments;
Qingting Wang, Qianqian Zhang, Wei Feng, Jian Wang analyzed data;
Qingting Wang, Wenhua Shi, Cui Zhai, Manxiang li interpreted results of experiments;
Qianqian Zhang, Qianqian Zhang, Wei Feng, Jian Wang prepared figures;
Qingting Wang, Wei feng, Cui Zhai, Xin Yan drafted manuscript;
Qingting Wang, Wenhua Shi, Xin Yan, Manxiang li approved final version of manuscript.