

Original Article

TGF- β 1/Smad3 signaling promotes collagen synthesis in pulmonary artery smooth muscle by down-regulating miR-29b

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Abstract: Deposition of fibronectin and collagen in the extracellular matrix (ECM) and the proliferation, migration, and hypertrophy of vascular smooth muscle cells (VSMCs) result in pulmonary arterial (PA) hypertrophy and muscularization, leading to increased pulmonary vascular resistance in pulmonary arterial hypertension (PAH). MicroRNA-29 (miR-29) is reported to be associated with diseases such as liver fibrosis, renal fibrosis, pulmonary fibrosis, and cardiac fibrosis in which collagen synthesis plays an important role. Due to the possible link between PAH and collagen, in this study, we examined the role and therapeutic potential of miR-29b in vitro and in a rat model of pulmonary hypertension induced by monocrotaline (MCT). Results revealed that miR-29b treatment PAH rats showed a lower level of collagen synthesis. Furthermore, in pulmonary arterial smooth muscle cells (PASMCs), TGF- β 1/Smad3 signaling negatively regulated the expression of miR-29b, and miR-29b suppressed collagen synthesis by directly targeting collagen I and blocking PI3K/AKT signaling. In addition, TGF- β 1/Smad3 signaling promoted collagen synthesis in PASMCs by down-regulating miR-29b. Interestingly, Smad3 decreased the expression of miR-29b by interacting with its promotor. In conclusion, our results revealed that miR-29b plays an important role in collagen synthesis and may be a therapeutic target for PAH when regulated by the TGF- β 1/Smad3 pathway.

Keywords: miR-29b, collagen synthesis, TGF- β 1, Smad3, PASMCs

Introduction

Pulmonary arterial hypertension (PAH) is a common and multifactorial disease characterized by the progressive remodeling of the small pulmonary arteries, which, with the progression of the disease, can ultimately lead to an increased pulmonary vascular resistance, right ventricular dysfunction, and even death [1]. Increased pulmonary arterial smooth muscle cells (PASMCs) proliferation is a key pathophysiologic component of pulmonary vascular remodeling in pulmonary arterial hypertension (PAH) [2]. Recent studies reported that pulmonary arterial collagen accumulation plays an important role in hypoxic pulmonary hypertension (HPH)-induced pulmonary vascular remodeling in distal arteries and large proximal arteries [3]. In addition, studies discovered that hypoxia increases the expression of type I collagen in cultured fibroblasts in hypoxia-induced pulmonary vascular adventitial remodeling [4].

Changes in the levels of extracellular matrix (ECM) proteins, particularly collagen, have been proven to contribute to arterial stiffening, and the content of collagen in mice remains at a higher level even after being removed from the hypoxic environment due to impaired type I collagen degradation [5]. Thus, treatment with inhibitors of collagen synthesis may be an effective method for lowering pulmonary artery pressure and preventing pulmonary artery remodeling [6].

ECM is a dynamic microenvironment that contributes to idiopathic pulmonary fibrosis (IPF), by activation of profibrotic pathways and matrix metalloproteinases that enhance collagenase activity [7, 8]. Fibroblasts and myofibroblasts play essential roles in the pathological process by secreting growth factors, as well as excessive collagens, and other matrix proteins [9]. The synthesis and accumulation of collagen play an important role in the formation and pro-

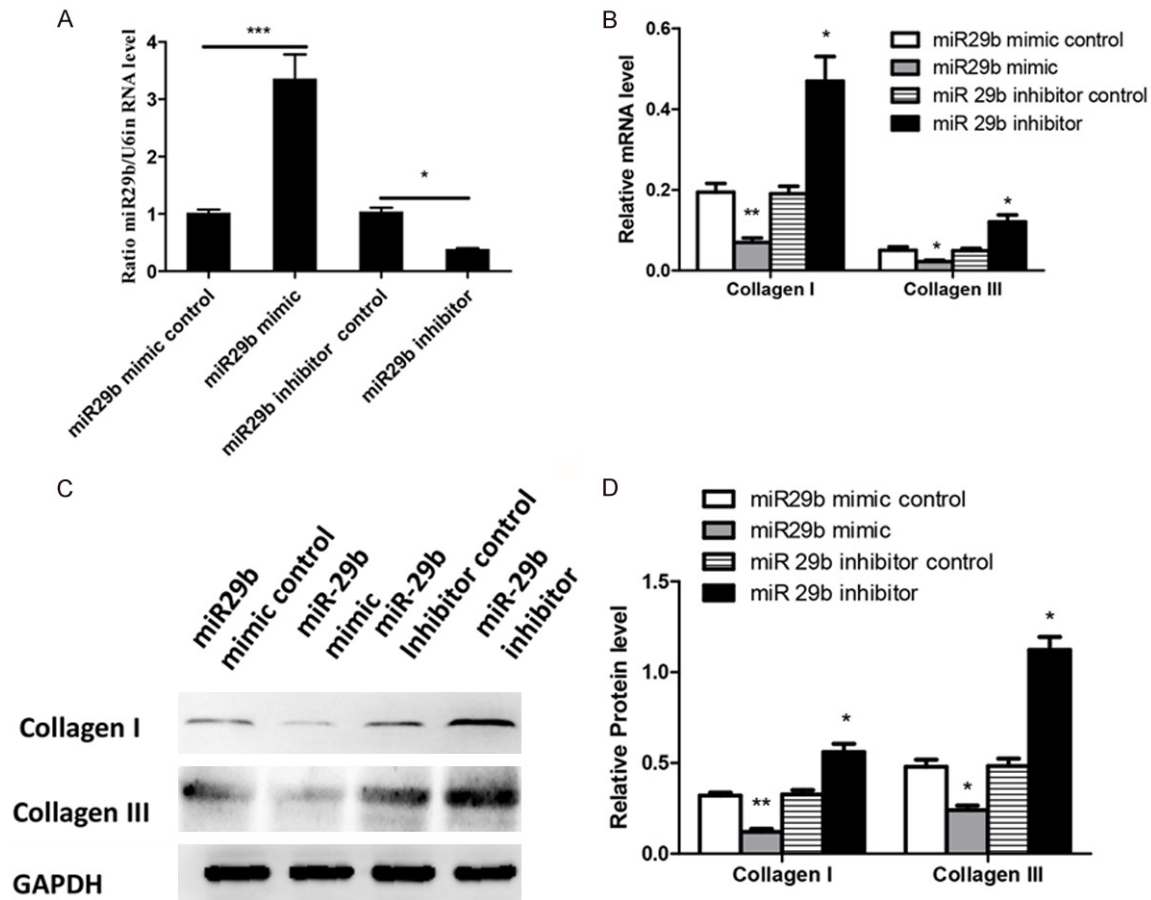


Figure 1. miR-29b suppresses collagen synthesis in PSMC. A. The relative mRNA level of miR-29b in hypoxia-cultured PSMC transfected with miR-29b mimic, miR-29b inhibitor or their controls. $n = 3$. B. The relative mRNA level of collagen I and collagen III in hypoxia-cultured PSMC transfected with miR-29b mimic, miR-29b inhibitor or negative control. $n = 3$. C, D. The representative image and the statistical graph of collagen I and collagen III expression in hypoxia-cultured PSMC transfected with miR-29b mimic, miR-29b inhibitor, or their controls analyzed by western blot. $n = 3$. * $P < 0.05$, ** $P < 0.01$.

gression of hypoxic pulmonary hypertension [9]. Recently, studies also showed vascular stiffness in PAH driving miRNA function and pathogenesis [10, 11].

The microRNA-29 (miR-29) family consists of three members (miR-29a, miR-29b, and miR-29c) [12]. There is overwhelming evidence that aberrant miR-29 family expression was involved in the development of tissue fibrosis, for instance liver fibrosis [13, 14], renal fibrosis [15], pulmonary fibrosis [16], and cardiac fibrosis [17]. In addition, overwhelming evidence has suggested that aberrant expression of the members of the miR-29 family is involved in multiple cancers including lung cancer, acute myeloid leukemia, and hepatocellular carcinoma [18-20]. 16 α OHE promotes the develop-

ment of HPAH via upregulation of miR-29, and antagonism of miR-29 improves in vivo and in vitro features of HPAH [21]. In this research, we speculated that miR-29 plays a key role in PAH and could exhibit as a potential therapeutic target for this disease.

The transforming growth factor TGF- β 1 is well known to induce the phenotypic transformation of fibroblasts to myofibroblasts in the lung and is thought to play a significant role in fibrosis [22]. It is also an important mediator of lung fibrosis and can induce differentiation of pulmonary fibroblasts into myofibroblasts characterized by α -smooth muscle actin expression and active synthesis of ECM proteins [23].

In the present study, based on the possible links among PAH, collagen and TGF- β 1/

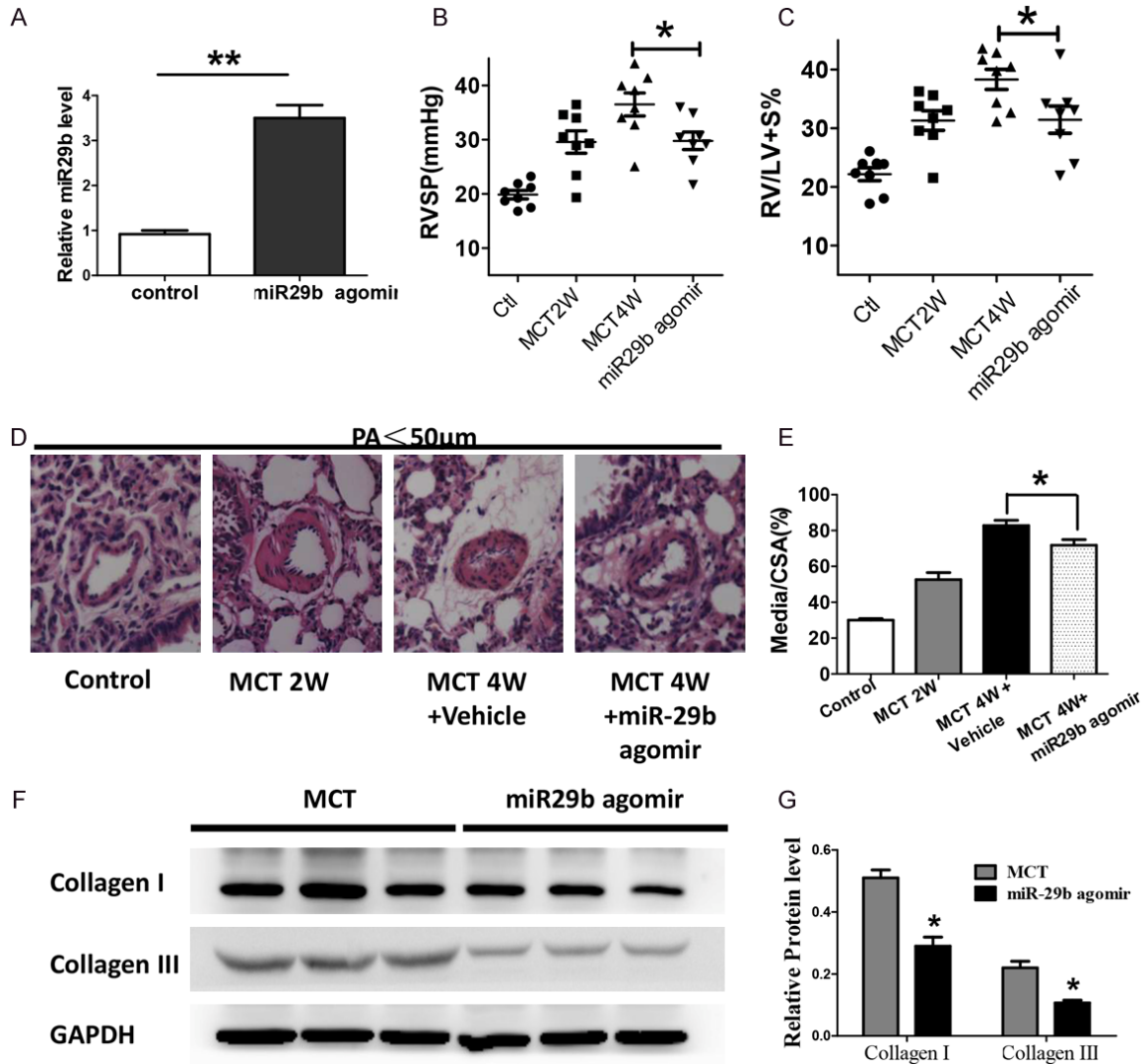


Figure 2. miR-29b mimic down-regulates collagen synthesis in MCT-induced PAH rats. **A.** The expression of miR-29b in the pulmonary arteries after miR-29b agomir injection. **B, C.** Significant differences were detected among the right ventricular systolic pressure (RVSP) and right ventricular hypertrophy [RV/(LV + S)] of rats treated as indicated. $n = 6$. **D, E.** The wall thickness of the small and medium-sized pulmonary arteries was assessed from HE-stained lung sections. Bar graphs showing the percentage of the wall thickness to the external diameter of the vessels in each rat group. $n = 6$. **F, G.** The expression level of collagen I and III in the MCT-induced PAH rats interfered with miR-29b agomir or negative control by tracheal administration. $n = 6$. * $P < 0.05$, ** $P < 0.01$.

Smad3 signaling, we established a rat model of PAH, and measured the expression levels of collagen and miR-29b in order to determine the effects of miR-29b on the synthesis of collagen in rats with PAH.

Materials and methods

Animals

Eight weeks old Sprague-Dawley male rats weighing 350-400 g were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). Ani-

mals were fed standard chow and administered a subcutaneous injection of monocrotaline and maintained in a 12-hr-light-dark cycle under controlled temperature conditions. All animal experiments were approved by The Institutional Animal Care and Use Committee of Shanghai Jiaotong University (Shanghai, China) and were in accordance with the Guide for the Care and Use of Laboratory Animals. Rats were treated with an intraperitoneal injection with MCT (60 mg/kg) once, four weeks later, the following research on this model was done. We randomized the rats and performed the study blinded.

miR-29b in PSMCs

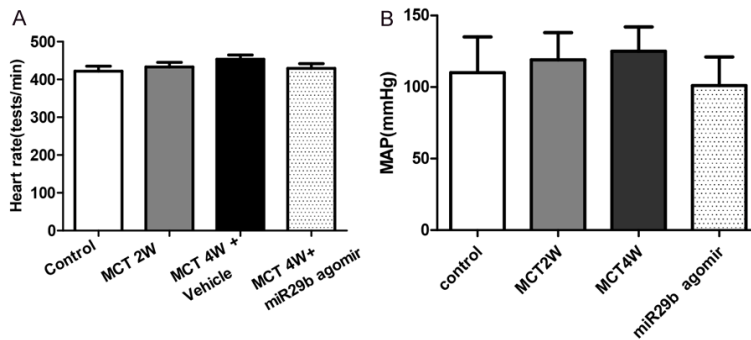


Figure 3. The effects of miR-29b on heart rate and systemic blood pressure (MAP). A, B. No differences were detected among the heart rate and MAP in MCT induced PAH rat transfected with agomir. n = 6.

Green PCR system (Applied Biosystems) with the following cycling settings: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. The threshold cycle (Ct) of each target gene was automatically defined and was located in the linear amplification phase of the PCR and normalized to the U6 (DCt value) of each group. Mature miR-29b expression was normalized to U6.

Tail vein injection

For further study, all PAH rats were divided randomly into two groups: (i) The control group was treated with physiological saline (10 ml/rat, tail-vein injection) at 24 h before the tests; (ii) The miR-29b agomir (Ribobio, Guangzhou, China) group was treated with miR-29b agomir (80 mg/kg, tail-vein injection). 21 days later, animals were used to do the tests.

Histomorphometric analysis

The pulmonary arterial vascular was fixed by 4% paraformaldehyde at 4°C in the dark, then embedded in paraffin, and sectioned into 5 mm thick sections. Finally, sections were stained with picric acid and hematoxylin-eosin for histology and histomorphometric analysis.

Cell culture and treatment

Human PSMCs were purchased from ATCC (Manassas, VA, USA). Cells were directly plated on regular 60-mm cell culture dishes, 12-well plates, or 24-well plates kept under hypoxia (1% O₂) in incubators that maintained a constant environment (5% CO₂) for indicated periods of time. Then, cells were transfected with miR-29b mimic, miR-29b inhibitor, or negative control (Biotend, Shanghai, China). For drug treatment, 5 μM of LY294002 (MCE, USA,) or LY364947 (MCE, USA) were applied.

Quantitative real-time PCR

miRNAs were isolated from cells using the miRNA isolation Kit. Taqman probes were used for measuring mature miR-29b, and U6 snRNA expression. The reaction was performed by miRNA assay kit (Ribobio, Guangzhou, China) in accordance with the manufacturer's instructions. The PCR was conducted using the SYBR

For mRNA expression analysis, total RNA was isolated with Trizol (Invitrogen, USA) and 1 μg RNA was used to synthesize cDNA through a reverse transcription reaction with oligo (dT) primers (Invitrogen). The cDNA was then used for real-time PCR with SYBR chemistry (Applied Biosystems, CA, USA). mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Primers used were as follows (5'→3'): collagen I (forward: GT-GCGATGACGTGATCTGTGA; reverse: CGGTGGT-TTCTTGGTCGGT), collagen III (forward: ACAG-TGACGAGGTGGAGATCA; reverse: GATAGCGCA-GTCGGTGTAGG).

Western blotting

Proteins from total cell lysates were separated by 10% SDS-PAGE and probed with different primary antibodies against Collagen I/III (Proteintec, 1:1000), phospho-PI3K/PI3K/phospho-AKT/AKT/GAPDH (Cell Signaling Technology, MA, USA, 1:1000).

Luciferase assay

PSMCs were plated into 24 well plates. Luciferase reporter constructs containing the putative miR-29b binding sites from Col1A1, Col3A1. PGL3-luciferase plasmid (Biotend) and Renilla luciferase control plasmid were cotransfected into the cells using Lipofectamine 2,000 (Invitrogen, Waltham, MA) as well as miR-29b mimic, inhibitor or negative control as indicated. After incubation for 24 hours, luciferase activity was measured by performing dual-luciferase assay using a Glomax96 Microplate Luminometer (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

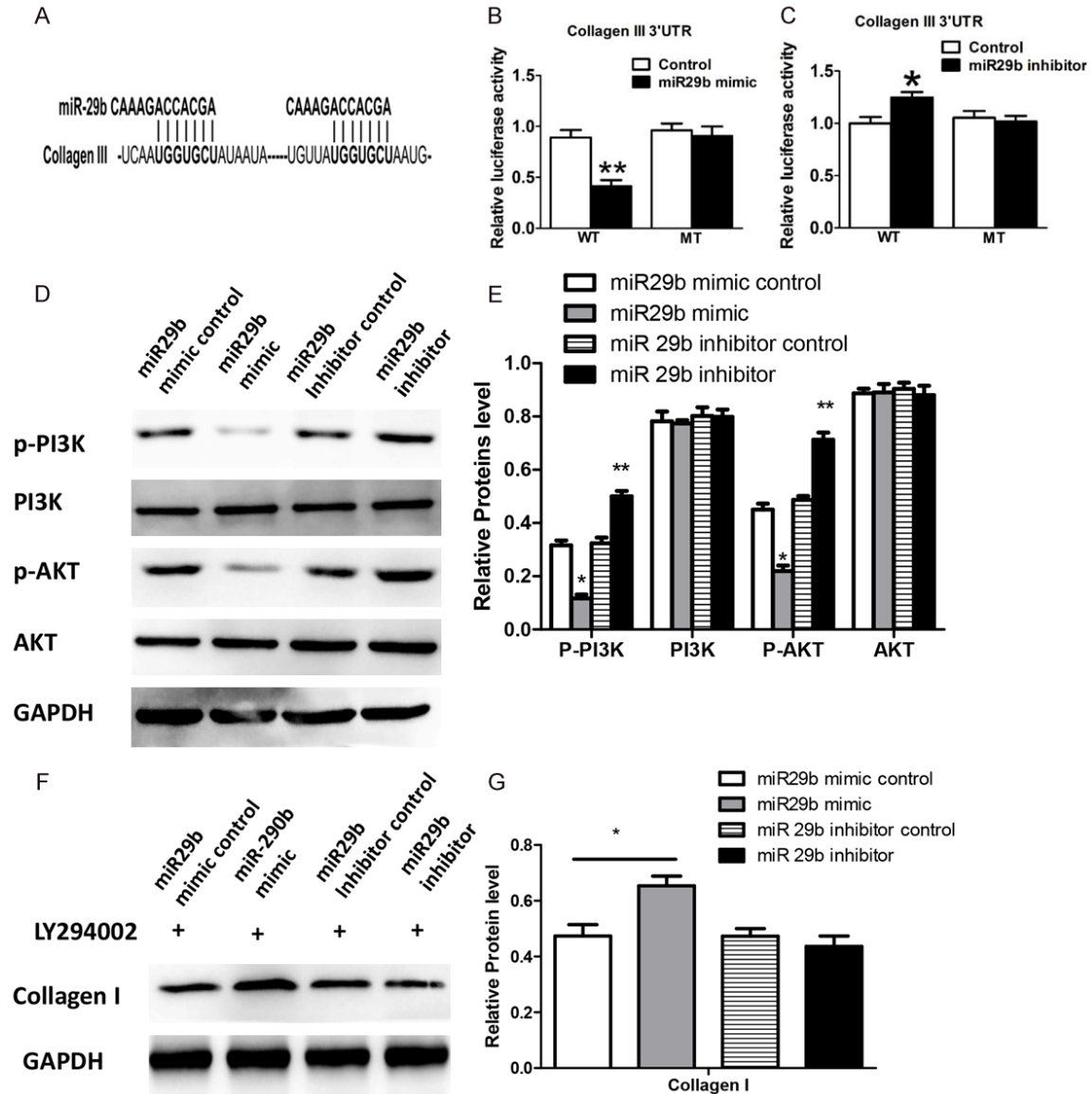


Figure 4. miR-29b suppresses collagen synthesis by directly targeting collagen III and blocks PI3K/AKT signaling. A. The potential binding sites of miR-29b on the 3'-UTR of the collagen III predicted by miRDB. B, C. The effect of miR-29b mimic and inhibitor on the wildtype and mutant 3'UTR of collagen III analyzed by luciferase assay. n = 4. D, E. The effect of miR-29b mimic and inhibitor on the phosphorylation of PI3K and AKT detected by western blot. F, G. Representative image and the statistical graph of the effect of miR-29b mimic and inhibitor on the collagen I expression in the present of LY294002 (PI3K inhibitor) in hypoxia-cultured PSMC by western blot analysis. n = 3. * $P < 0.05$, ** $P < 0.01$.

Chip

We used a ChIP assay kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol. Briefly, cells were incubated with 1% formaldehyde for 10 min at 37°C and each ChIP reaction was performed using 2.0×10^6 cells. For DNA precipitation, we added 2 µg monoclonal anti-Flag M2 (Clone No.9A3, Cat No.8146, Cell Signaling Technology, Beverly,

MA, USA). The precipitated DNA samples were analyzed by PCR.

Statistical analysis

The data are expressed as mean \pm SEM. Data more than three groups were analyzed by one-way ANOVA, and Student's t-test was used for all other statistical analyses (GraphPad Prism 5.0 software). $P < 0.05$ was considered significant.

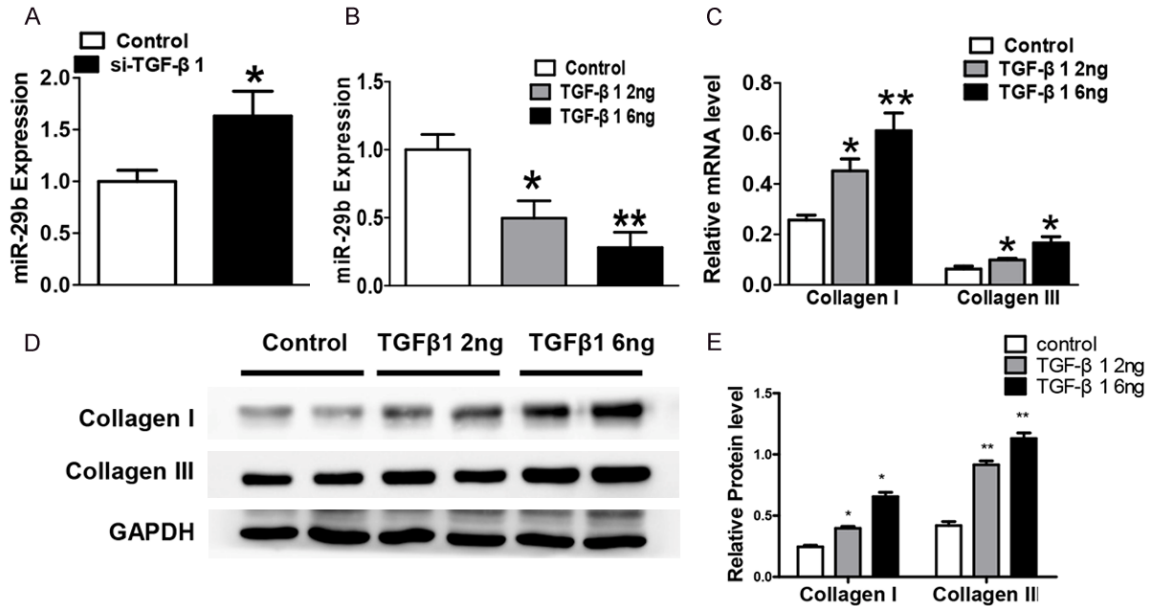


Figure 5. TGF-β1 down-regulates the expression of miR-29b and up-regulates collagen synthesis in PSMC. A. The expression level of miR-29b in PSMC cultured in hypoxia (1% O₂) treated with si-TGFβ1 or negative control. n = 4. B. The level of miR-29b expression in PSMC cultured in hypoxia (1% O₂) treated with TGFβ1 or vehicle. n = 4. C. The relative mRNA level of collagen I and collagen III in hypoxia-cultured PSMC treated as indicated, analyzed by quantitative Real-time PCR. n = 4. D, E. The representative image and the statistical graph of collagen I and collagen III expression in hypoxia-cultured PSMC treated as indicated, analyzed by western blot. n = 3. *P < 0.05, **P < 0.01.

Results

miR-29b suppresses collagen synthesis in PSMCs

In order to clarify the role of miR-29b in collagen synthesis of PSMCs, we transfected PSMCs with miR-29b mimic and inhibitor and total RNA and proteins were isolated for analysis of collagen I and III expression. Interestingly, we found miR-29b mimic inhibited collagen synthesis at the mRNA level (**Figure 1A, 1B**) and the protein level (**Figure 1C, 1D**) in PSMCs. The opposite trend was observed after transfection with miR-29b inhibitor (**Figure 1B-D**). We also detected the role of miR-29a and miR-29c in collagen synthesis of PSMCs. Our results also revealed that miR-29a and miR-29c did not affect collagen synthesis (**Figures S1 and S2**). These results together indicated that miR-29b negatively regulates collagen synthesis in PSMCs.

miR-29b mimic down-regulates collagen synthesis in MCT-induced PAH rats

Further, we evaluated the role of miR-29b in collagen synthesis of MCT-induced PAH rats.

Firstly, we observed the effect of the miR-29b agomir or control on RVSP, RVHI (RV/LV + S) and pulmonary vascular muscularization in rats exposed to MCT. Data revealed that miR-29b agomir could significantly increase the expression of miR-29b in the pulmonary arteries (**Figure 2A**). Following two weeks of MCT-induced PAH, the RVSP in rats increased from 18 mmHg to 30 mmHg. Rats treated with the miR-29b agomir exhibited a significantly attenuated increase in miR-29b mimic (32 mmHg) compared with 38 mmHg RVSP in MCT at four weeks (P < 0.05) (**Figure 2B**). Coincident with the effects on RVSP, the RVHI in rats treated with the miR-29b agomir also exhibited an attenuated increase compared to the four weeks MCT group (**Figure 2C**). As pulmonary vascular remodeling is a characteristic feature of PAH and always occurs in the media of the vessels, we noted that the delivery of the synthetic miR-29b agomir markedly reduced the MCT-induced media wall thickness of the small-sized pulmonary arteries (**Figure 2D, 2E**). Further, the collagen synthesis was assessed by western blotting in the MCT-induced PAH model rats. Western blotting results showed that miR-29b decreased the synthesis of collagen I and III in pulmonary artery of MCT-induced

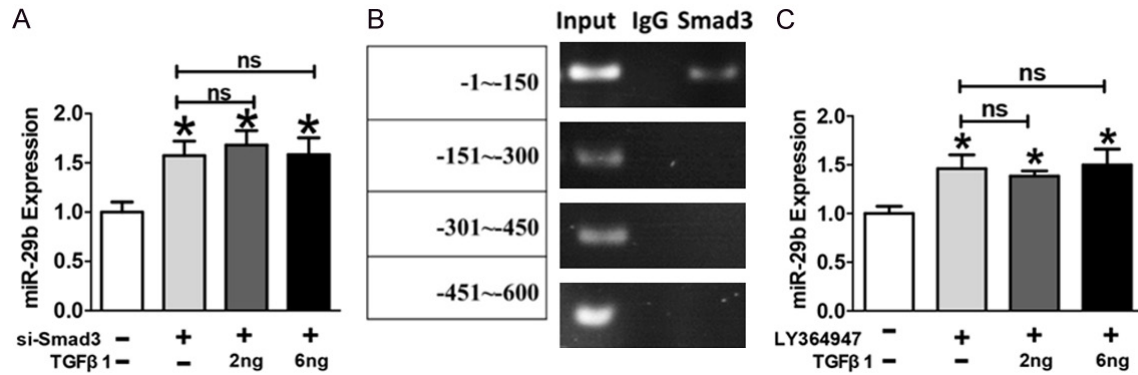


Figure 6. TGF-β1 inhibits miR-29b expression by Smad3 signaling. **A.** The level of miR-29b expression in PSMC cultured in hypoxia (1% O₂) treated with TGFβ1 or vehicle in the presence of Smad3 siRNA. n = 4. **B.** The interaction of Smad3 and miR-29b promoter detected by chromatin immunoprecipitation assay. **C.** The level of miR-29b expression in PSMC cultured in hypoxia (1% O₂) treated with TGFβ1 or vehicle in the presence of LY364947 (TGFβR-I inhibitor). n = 4. *P < 0.05.

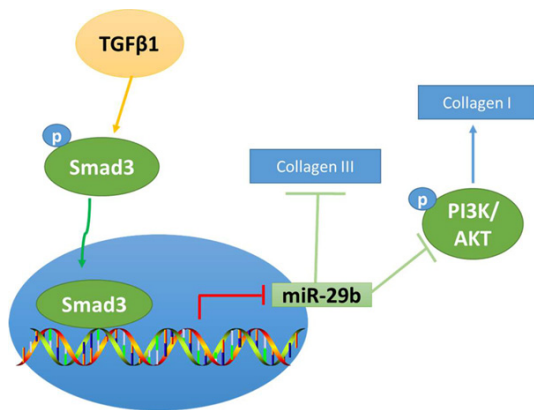


Figure 7. The underlying mechanism of TGF-β1/Smad3 signaling promotes collagen synthesis in PSMC.

PAH rats compared to the PAH control animals (**Figure 2F, 2G**). We also calculated heart rate, systemic blood pressure (MAP), which could in turn affect pulmonary vascular remodeling and RVSP, results indicated no change in heart rate and MAP (**Figure 3A, 3B**).

MiR-29b suppresses collagen III synthesis by directly targeting its 3'-UTR and blocking PI3K/AKT signaling

To further address the mechanism, using miRDB, we found that collagen III 3'-UTR, rather than collagen I 3'-UTR, had a seed sequence for miR-29b and was predicted as its potential target (**Figure 4A**). Luciferase assay showed that miR-29b mimic transfection into PSMCs significantly decreased the luciferase activity

when wildtype collagen III 3'-UTR was cloned into pGL3 plasmid. In the meantime, miR-29b mimic had no effect on the luciferase activity when mutant collagen III 3'-UTR was cloned into pGL3 plasmid. Opposite results were observed when miR-29b inhibitor was transfected into PSMCs (**Figure 4B, 4C**). These results indicated that collagen III 3'-UTR was a direct target of miR-29b.

Additionally, we assessed the effect of miR-29b on the PI3K/AKT pathway. Results displayed that miR-29b significantly reduced the level of PI3K and AKT phosphorylations (**Figure 4D, 4E**). Furthermore, inhibition of PI3K/AKT signaling efficiently abolished the effect of miR-29b on the expression of collagen I (**Figure 4F, 4G**). Thus, these findings suggested that miR-29b suppresses collagen synthesis by directly targeting collagen I and blocking PI3K/AKT signaling.

TGF-β1 down-regulates the expression of miR-29b and up-regulates collagen synthesis in PSMCs

TGF-β1 was reported to modulate the expression of miR-29b in different types of cells [24]. In order to detect the effect of TGF-β1 on the miR-29b expression in PSMCs, cells were transfected with TGF-β1 siRNA or treated with TGF-β1. The results showed that silencing of TGF-β1 was followed by the upregulation of miR-29b (**Figure 5A**). On the contrary, miR-29b was down-regulated in PSMCs treated with TGF-β1 in a dose-dependent manner (**Figure**

5B). In addition, both qRT-PCR and western blot analyses indicated that treatment with TGF- β 1 significantly increased the expression levels of collagen I and collagen III (**Figure 5C-E**), dose-dependently.

TGF- β 1 inhibits miR-29b expression by Smad3 signaling

As it was evidenced above that TGF- β 1 down-regulated the expression of miR-29b, we sought to elucidate the potential mechanism involved in this regulatory process. Smad3 signaling is an important downstream signaling pathway of TGF- β 1 [25]. Therefore, we investigated whether TGF- β 1 down-regulated the expression of miR-29b through Smad3. Silencing of Smad3 by siRNA led to the abrogation of the effect of TGF- β 1 on miR-29b expression (**Figure 6A**). By blocking TGF- β 1/Smad3 with LY364947, we equally found that the effect of TGF- β 1 treatment on miR-29b expression was abolished (**Figure 6B**). Interestingly, the interaction of Smad3 and miR-29b promotor was assessed by chromatin immunoprecipitation assay, and Smad3 was found to interact with the promotor of miR-29b (**Figure 6C**). All these observations led to the conclusion that TGF- β 1 inhibits miR-29b expression by Smad3 signaling. The underlying mechanism of TGF- β 1/Smad3/miR-29b signaling promotes collagen synthesis in PSMC was shown in **Figure 7**.

Discussion

In recent years, increasing attention has been paid to the association between PAH and collagen accumulation. Studies have discovered that collagen accumulation plays a key role in the stiffening of the proximal pulmonary artery, which leads to sustained increase in pulmonary artery pressure to finally result in the failure of the right ventricle [25]. Research on newborn Wistar rats exposed to hypoxia has also revealed increased mPAP, right ventricular hypertrophy, collagen deposition in the ECM, and pulmonary vascular remodeling [26]. In a previous study, it was suggested that total collagen content was critical for extralobar pulmonary artery stiffening during PAH [27]. Type I collagen is a fibrillar collagen subtype that plays a dominant role in the composition and strength of the arteries [28]. The expression of collagen, including collagen I in rats exposed to

hypoxia has been shown to markedly increase pulmonary vascular remodeling and this increase may be alleviated by inhibiting the collagen accumulation in pulmonary arteries [29].

The miR-29 family is composed of miR-29a, miR-29b, and miR-29c, differing only in two or three bases [26]. miRs of the miR-29 family are encoded and transcribed in tandem by two genes located on chromosome 7 or chromosome 1, respectively [27]. The members of the miR-29 family are of special interest, because miR-29 family has been shown to be involved in inhibiting the synthesis of extracellular matrix ECM proteins indicating an antifibrotic function [28]. Although deregulation of miR-29b has been reported in fibrosis of different type of tissues, experimental evidence of its involvement in PSMC is currently lacking. Here we report for the first time that miR-29b negatively regulated the expression of collagen directly or indirectly in PSMC.

TGF- β 1 has been shown as a key modulator in the synthesis and degradation of ECM, and also mediates the proliferation of human lung fibroblasts [21]. Likewise, miR-29 has been shown to be an important player in this process by down-regulating several extracellular proteins, including collagens [22]. Also, miR-29 plays an important role in integrating functionally connected pathways involved in pulmonary fibrotic disease through its ability to regulate multiple important signaling events involved in fibrogenesis [23]. Thus, it may represent an attractive therapeutic target for pulmonary fibrosis. TGF- β 1 is a central pathologic mediator of lung fibrosis by inhibiting the expression of miR-29, but whether TGF- β 1 regulates miR-29 expression directly or indirectly is unknown since pathways such as PI3K/AKT pathway, Smad, MAPK, and Wnt are also related to this process [24].

Activation of pulmonary adventitial fibroblasts plays a key role in pulmonary vascular remodeling in PAH [29]. The increased activation of TGF- β 1 signaling in PAs was reported in idiopathic PAH patients and in different PAH animal models, and inhibition of the TGF- β 1 receptor activin receptor-like kinase 1 prevents the development of PAH [30]. In conclusion, miR-29b plays an important role in collagen synthesis and may be a therapeutic target for PAH

under the regulation of the TGF- β 1/Smad3 pathway.

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Disclosure of conflict of interest

None.

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miR-29b in PSMCs

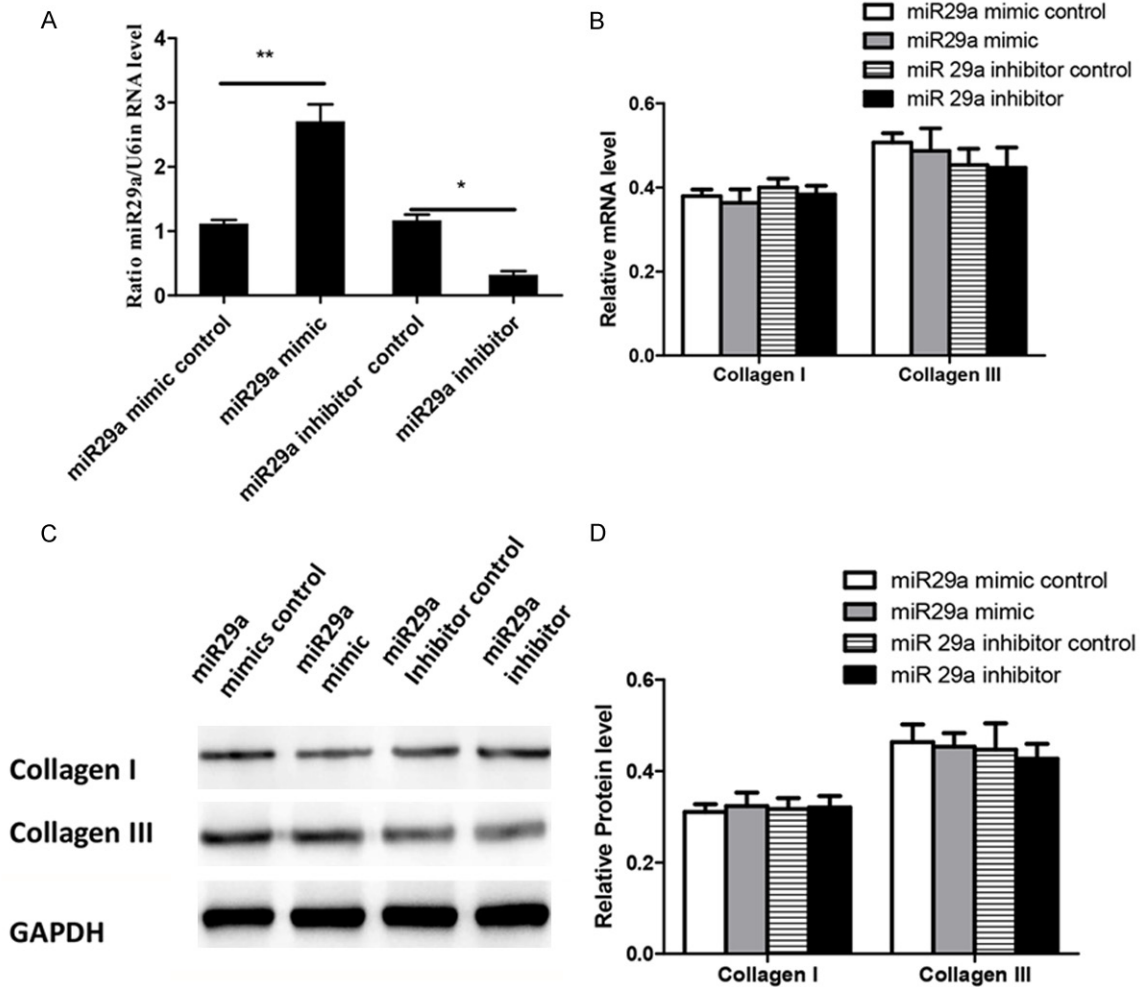


Figure S1. miR-29a does not affect collagen synthesis in PSMC. A. The relative mRNA level of miR-29a in hypoxia-cultured PSMC transfected with miR-29a mimic, miR-29a inhibitor, or their controls. n = 3. B. The relative mRNA level of collagen I and collagen III in hypoxia-cultured PSMC transfected with miR-29a mimic, miR-29a inhibitor, or negative control. n = 3. C, D. The representative image and the statistical graph of collagen I and collagen III expression in hypoxia-cultured PSMC transfected with miR-29a mimic, miR-29a inhibitor, or their controls analyzed by western blot. n = 3. * $P < 0.05$, ** $P < 0.01$.

miR-29b in PSMCs

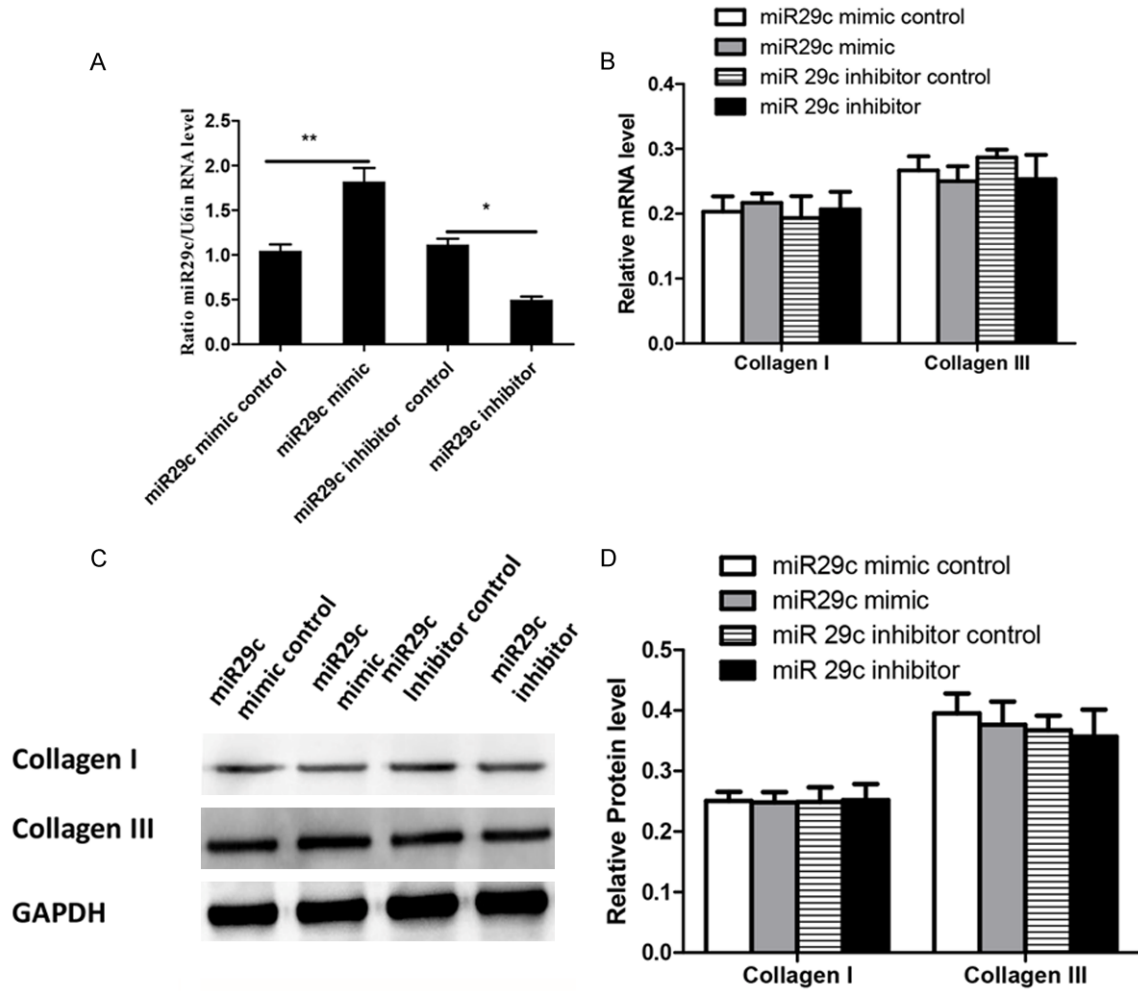


Figure S2. miR-29c does not affect the collagen synthesis in PSMC. A. The relative mRNA level of miR-29c in hypoxia-cultured PSMC transfected with miR-29c mimic, miR-29c inhibitor or their controls. n = 3. B. Relative mRNA levels of collagen I and collagen III in hypoxia-cultured PSMC transfected with miR-29c mimic, miR-29c inhibitor, or negative control. n = 3. C, D. The representative image and the statistical graph of collagen I and collagen III expression in hypoxia-cultured PSMC transfected with miR-29c mimic, miR-29c inhibitor, or their controls analyzed by western blot. n = 3. * $P < 0.05$, ** $P < 0.01$.