miR-29a-3p Attenuates Hypoxic Pulmonary Hypertension by Inhibiting Pulmonary Adventitial Fibroblast Activation

Ying Luo, Hai-Ying Dong, Bo Zhang, Zhao Feng, Yi Liu, Yu-Qi Gao, Ming-Qing Dong, Zhi-Chao Li

Abstract—Activation of pulmonary adventitial fibroblasts plays a key role in the pulmonary vascular remodeling in hypoxic pulmonary hypertension. Previous studies showed that miRNAs participated in the regulation of fibroblast activation. This study explored the role of miR-29 in the activation of pulmonary adventitial fibroblasts and the therapeutic potential in hypoxic pulmonary hypertension. We found that hypoxia-induced pulmonary adventitial fibroblasts activation was accompanied with a drastic decrease of miR-29a-3p expression. Knockdown of hypoxia-inducible factor-1 α or Smad3 reversed the hypoxia-induced decrease of miR-29a-3p in cultured pulmonary adventitial fibroblasts. In vitro, miR-29a-3p mimic inhibited the hypoxia-induced proliferation, migration, and secretion of pulmonary adventitial fibroblasts, suppressed the hypoxia-induced expression of α-smooth muscle actin and extracellular matrix collagen in pulmonary adventitial fibroblasts; however, miR-29a-3p inhibitor mimicked the effect of hypoxia on the activation of pulmonary adventitial fibroblasts. Further studies revealed that preventative or therapeutic administration of miR-29a-3p significantly decreased pulmonary artery pressure and right ventricle hypertrophy index and ameliorated pulmonary vascular remodeling in hypoxic pulmonary hypertension rats. These findings suggest that miR-29a-3p regulates the activation and phenotype of pulmonary adventitial fibroblasts in hypoxia and has preventative and therapeutic potential in hypoxic pulmonary hypertension. (Hypertension. 2015;65:414-420. DOI: 10.1161/HYPERTENSIONAHA.114.04600) • Online Data Supplement

Key Words: hypoxia ■ pulmonary hypertension

Hypoxic pulmonary hypertension (HPH) is still a life-threatening disorder lack of effective agents. It is highly prevalent in advanced chronic obstructive pulmonary disease and high altitude hypoxia. With the increase of chronic obstructive pulmonary disease patients, the morbidity of HPH is rising. Although most of HPH is mild to moderate, a subpopulation (1%–4%) with grim prognosis present with severe pulmonary hypertension.1 Pharmacological agents moderately improve the symptoms and hemodynamic parameters of severe pulmonary hypertension, but none significantly reduces mortality.23 There is a pressing need to identify novel target to treat HPH.

One of the characteristics of HPH is the dramatic remodeling of pulmonary vascular adventitia.46 During the remodeling, pulmonary adventitial fibroblasts (PAFs) are highly activated and undergo phenotype switch characterized by excessive proliferation, migratory, and inflammatory activity.67 Therefore, inhibition of the aberrant activation of PAFs may reverse the remodeling process of pulmonary vascular adventitia and have therapeutic potential for HPH.8 Increasing evidence shows that miRNAs, especially miR-29 family members, participate in the development of organ fibrosis via regulating the activation of fibroblasts.9-11 Downregulation of miR-29 underlies transforming growth factor-β–mediated pulmonary fibrosis, and overexpression of miR-29 inhibits bleomycin-induced pulmonary fibrosis.12 Although PAFs in HPH undergo the similar activated phenotype to that in lung fibrosis,6 the role of miR-29 family member in the hypoxia-induced activation of PAFs and the development of HPH is not known. On the other hand, hypoxia-inducible factor-1α (HIF-1α), a key transcription factor mediating cellular response to hypoxia, and Smad3, which can inhibit miR-29 expression in organ fibrosis, are interdependent in hypoxia.13-16 Therefore, HIF-1α and Smad3 may be jointly involved in the regulation of miR-29 in the activation of PAFs in hypoxia.

The present study explored the effects of miR-29a in the activation of PAFs in hypoxia and the therapeutic potential in HPH. The results show that miR-29a-3p plays a key role in hypoxia-induced activation of PAFs and has protective effects in HPH in rats.
Materials and Methods

For additional details, please see the online-only Data Supplement.

Vector Construction and Virus Production

The rat pre-miR-29a was synthesized, annealed, cloned to construct adeno-associated virus (AAV) vector. The constructed AAV-miR-29a or control vector was transfected into 293AAV cells to package AAV serotypes 5 (AVV5-miR-29a and AVV5-control).

Animal Experiments

Protocols were approved by the Animal Care and Use Committee of the Fourth Military Medical University. Rats were randomly divided into 4 groups (normoxia+AAV5-control, normoxia+AVV5-miR-29a, hypoxia+AAV5-control, and hypoxia+AVV5-miR-29a) and maintained in normoxia (21% O2) or hypobaric hypoxia (10% O2) for 4 (preventive experiment) or 6 (therapeutic experiment) weeks. A double (at day-0 and day-14 after hypoxia, preventive experiment) or a single (at day-28 after hypoxia, therapeutic experiment) intratracheal instillation of AAV5-miR-29a or AAV5-control (1×1012 vg/kg/each instillation) were given. The right ventricle systolic pressure (RVSP) was measured as the indicator of pulmonary artery pressure.

Statistical Analysis

All values were presented as means±SD. The statistical differences between groups were evaluated by 1-way analysis of variance, followed by Bonferroni’s test for multiple comparisons.

Results

Hypoxia Downregulated the Expression of miR-29a-3p in HIF-1α/Smad3-Associated Pathway

We first examined the expression profiles of miR-29a/b family members in the pulmonary adventitia isolated from HPH rats and in PAFs cultured in hypoxia via real-time polymerase chain reaction. Figure 1A and 1B shows the profiles and the normalized levels of the miR-29a/b in pulmonary adventitia from HPH rats. Significant downregulation of miR-29a-3p, miR-29a-5p, and miR-29b-5p was seen; moreover, miR-29a-3p decreased most by >30-folds. Similar results were seen in PAFs cultured in hypoxia (Figure 1C and 1D). So we chose miR-29a-3p as the representative of miR-29a/b family members in the following studies.

Next, we determined whether HIF-1α and Smad3 jointly mediated the hypoxia-induced reduction of miR-29a-3p. The expression of HIF-1α protein, the total protein of Smad3, and the phosphorylation of Smad3 significantly increased in PAFs in hypoxia (Figure S1A–S1D in the online-only Data Supplement). Knockdown of HIF-1α or Smad3 with specific siRNA markedly reversed the hypoxia-induced reduction of miR-29a-3p in PAFs; however, the control sequence had no such effect (Figure S1E and S1F). Knockdown of HIF-1α also significantly decreased the mRNA (Figure S1G) and the total and phosphorylated protein (Figure S1H–S1J) of Smad3 in PAFs in hypoxia. These results suggest that miR-29a-3p is downregulated in HIF-1α/Smad3–associated pathway in PAFs in hypoxia.

Role of miR-29a-3p in the Hypoxia-Promoted Proliferation and Migration of PAFs

Then we explored the role of miR-29a-3p in hypoxia-promoted proliferation and migration of PAFs. Hypoxia significantly accelerated the proliferation of PAFs by ≈25%. The mimic of miR-29a-3p significantly inhibited the hypoxia-induced proliferation (Figure 2A). The inhibitor did not further increase the proliferation of PAFs in hypoxia, but it significantly accelerated the proliferation of PAFs in normoxia by ≈30% (Figure 2B), suggesting that miR-29a-3p inhibitor mimicked
the promotion effect of hypoxia on the proliferation of PAFs in normoxia.

The representative migration results were shown in Figure 2C and 2D. Hypoxia promoted the migration of PAFs; miR-29a-3p mimic significantly inhibited the migration of PAFs both in normoxia and in hypoxia (Figure 2C). However, miR-29a-3p inhibitor only promoted the migration of PAFs in normoxia (Figure 2D), suggesting it mimicked the effect of hypoxia on the migration of PAFs in normoxia. These results showed that the downregulation of miR-29a-3p underlay the promotion effects of hypoxia on the proliferation and migration of PAFs.

**Role of miR-29a-3p in the Secretion of PAFs in Hypoxia**

We explored the role of miR-29a-3p in the secretion of PAFs in hypoxia. Hypoxia significantly increased the secretion of transforming growth factor-β, endothelin-1, and platelet-derived growth factor in PAFs; miR-29a-3p mimic significantly inhibited the hypoxia-enhanced secretion of these cytokines (Figure 3A–3C). However, miR-29a-3p inhibitor significantly promoted the secretion of transforming growth factor-β, endothelin-1, and platelet-derived growth factor in PAFs in normoxia (Figure 3D–3F), mimicking the effects of hypoxia on the secretion of PAFs. These results showed that
loss of miR-29a-3p mediated the increased secretion of PAFs in hypoxia.

Role of miR-29a-3p in the Activation Phenotype–Associated Proteins Expression of PAFs in Hypoxia

The expression of activation phenotype–associated proteins, such as α-SMA and Col1A1, is one of the important characters of activated PAFs; therefore, we explored the role of miR-29a-3p in the expression of α-SMA and Col1A1 in PAFs in hypoxia. Hypoxia significantly increased the expression of α-SMA and Col1A1 in PAFs at both the mRNA and the protein levels, whereas miR-29a-3p mimic significantly inhibited the hypoxia-induced expression of α-SMA and Col1A1 in PAFs (Figure S2A–S2E). Moreover, miR-29a-3p inhibitor mimicked the effects of hypoxia: significantly increased expression of α-SMA and Col1A1 in PAFs in normoxia (Figure S2F–S2J). These data indicated that the reduction of miR-29a-3p played a key role in the increased expression of α-SMA and Col1A1 in PAFs in hypoxia.

Preventive and Therapeutic Effects of miR-29a on HPH In Vivo

We further investigated the preventive effects of miR-29a, which can be processed into miR-29a-3p and miR-29a-5p in the transduced cells, on HPH in rats via AAV5 gene transfer. AAV5-miR-29a was preventively administrated at day-0 and day-14 after hypoxia, and the rats were kept in hypoxia for total 4 weeks. Figure 4A and 4B showed that hypoxia for 4 weeks significantly increased the RVSP from 22.9±4.2 to 42.8±6.6 mm Hg and right ventricle hypertrophy index from 0.22±0.04 to 0.39±0.09, respectively, whereas AAV5-miR-29a treatment significantly reduced the RVSP from 42.8±6.6 to 35.0±4.2 mm Hg and RVHI from 0.39±0.09 to 0.30±0.05, respectively (all P<0.05; n=6). Figure 4C–4E shows the representative hematoxylin and eosin staining of lung tissue and the summarized data of MT% (percent medial wall thickness) and MA% (percent medial wall area) of pulmonary artery, where hypoxia significantly increased MT% from 16.6%±3.6% to 55.0%±8.0% and MA% from 27.6%±3.2% to 79.7%±8.6%, whereas AAV5-miR-29a significantly prevented the hypoxia-induced increase of MT% from 55.0%±8.0% to 26.6%±5.0% and MA% from 79.7%±8.6% to 45.6%±6.2% (all P<0.05; n=60).

To evaluate the therapeutic effects of miR-29a on the HPH, AAV5-miR-29a was administrated 4 weeks after hypoxia and then the rats were kept in hypoxia for another 2 weeks. Figure 5A and 5B shows that hypoxia for 6 weeks significantly increased the RVSP from 22.9±4.2 to 42.8±6.6 mm Hg and right ventricle hypertrophy index from 0.22±0.04 to 0.39±0.09, respectively, whereas AAV5-miR-29a treatment significantly reduced the increased RVSP from 42.8±6.6 to 35.0±4.2 mm Hg and RVHI from 0.39±0.09 to 0.30±0.05, respectively (all P<0.05; n=6). Figure 5C–5E shows that hypoxia for 6 weeks significantly increased the RVSP from 22.9±4.2 to 42.8±6.6 mm Hg and right ventricle hypertrophy index from 0.22±0.04 to 0.39±0.09, respectively, whereas AAV5-miR-29a treatment significantly reduced the hypoxia-induced increase of MT% from 55.0%±8.0% to 34.0%±5.0% and MA% from 79.7%±8.6% to 55.6%±6.0% (all P<0.05; n=60). However, preventively or therapeutic administration of AAV5 control had no such effects (Figures 4 and 5A–5E). In addition, preventively or therapeutic administration of AAV5-miR29a had

Figure 4. Preventive effects of miR-29a on hypoxic pulmonary hypertension (HPH) in rats. A and B, Adeno-associated virus (AAV5)-miR-29a significantly decreased the right ventricle systolic pressure (RVSP) and right ventricle hypertrophy index (RVHI) (n=6). C, Representative hematoxylin and eosin staining of pulmonary vessels. D and E, Quantification of MT% and MA% of pulmonary arteries/arterioles. Total 60 vessels from 6 animals (10 vessels/animal) were analyzed in panel D and E. F, Expression of miR-29a-3p in pulmonary adventitia from HPH rats with preventive administration of AAV5-miR-29a (n=3). Data are expressed as mean±SD. *P<0.05, **P<0.01 vs normoxia+C; #P<0.05, ##P<0.01 vs hypoxia+C. C indicates AAV5-control; M, AAV5-miR-29a; MA%, percent medial wall area; and MT%, percent medial wall thickness.
no significant effect on the mean carotid artery pressure (data not shown). These results demonstrated that miR-29a-3p had preventive and therapeutic effects on HPH in rats.

Furthermore, we measured the expression of miR-29a-3p in the pulmonary adventitia isolated from the rats administrated with AA V5-miR-29a or AA V5-control. Figures 4 and 5F show that hypoxia significantly decreased the expression of miR-29a-3p by >31-folds in pulmonary adventitia, similar to the result in Figure 1. Both preventive and therapeutic administration of AAV5-miR-29a significantly increased the expression of miR29a-3p in pulmonary adventitia in HPH and normal rats. However, administration of AAV5-control had no effect on the expression of miR-29-3p.

Discussion

In this study, we showed that hypoxia downregulated the expression of miR-29a-3p in PAFs via HIF-1α/Smad3-associated pathway. Loss of miR-29a-3p mediated the hypoxia-induced PAFs activation and phenotype. In vitro, miR-29a-3p mimic reversed, but its inhibitor mimicked, the effects of hypoxia on PAFs. Moreover, preventive or therapeutic transduction of miR-29a-3p markedly ameliorated HPH in rats. The present study first demonstrates that miR29a-3p is a protective factor in PAFs. The present study added novel evidence that miR-29a-3p had a protective role in HPH. This was supported by the data that HPH was associated with a marked reduction of miR-29a-3p in pulmonary artery adventitia, and overexpression of miR-29a-3p ameliorated HPH. The result from in vivo experiments is somewhat striking, just as previous study showed that overexpressing miR-29b prevented Angiotensin II–mediated cardiac fibrosis, restoration of miR-29b in hypertensive heart could block progressive cardiac fibrosis and improve cardiac dysfunction. However, considering that AA V5 can consistently express target gene miR29a for >2 weeks and the broad biological effects of miR-29a, the results are believable.

Moreover, in vitro, the mimic of miR-29a-3p significantly inhibited the hypoxia-induced aberrant proliferation, migration, secretion of transforming growth factor-β, endothelin-1, and platelet-derived growth factor, and expression of α-SMA and CollA1, all of which are the characteristic phenotypes of activated PAFs. Conversely, the inhibitor of miR-29a-3p mimicked the effects of hypoxia on PAFs (Figures 2 and 3; Figure S1). Therefore, the in vitro and in vivo data suggest that inhibition of PAFs activation underlies the protective effect of miR-29a-3p on HPH.

Recently, Li et al18 reported that the natural agent, isoflavone, could demethylate the methylation sites in the promoter of miR-29a, leading to the upregulation of miR-29a. Interestingly, isoflavone (genistein) is previously found to attenuate/reverse pulmonary hypertension in rats. Whether isoflavone exerted the benefit effect on pulmonary hypertension partly via upregulation of miR-29a is not known.

This study revealed that hypoxia reduced miR-29a-3p in PAFs via HIF-1α/Smad3–associated pathway. It is supported...
by the findings that HIF-1α and Smad3 were significantly increased in PAFs in hypoxia, concomitant with the reduction of miR-29a-3p, and that knockdown of HIF-1α or Smad3 inhibited hypoxia-induced reduction of miR-29a-3p. Moreover, knockdown of HIF-1α also decreased the expression of Smad3 in PAFs in hypoxia, suggesting that Smad3 may be the downstream effector of HIF-1α. These results are consistent with those of previous studies that showed that there is interdependence of HIF-α and Smad3 signaling in hypoxia.\textsuperscript{15,16} However, the detailed mechanism for the regulation of HIF-1α and Smad3 on miR-29a-3p in PAFs in hypoxia is still to be explored.

In the development of HPH, pulmonary artery smooth muscle cells and pulmonary endothelial cells also participate in the remodeling of pulmonary artery.\textsuperscript{13,22} Previous studies showed that miRNAs regulated the phenotype of pulmonary artery smooth muscle cells in hypoxia and mediated the hypoxia-promoted proliferation of pulmonary endothelial cells.\textsuperscript{23–25} In addition, decrease of miR-124 contributes to the activation of PAFs from severe pulmonary hypertension patient.\textsuperscript{6} In this study, we only focused on the role of miR-29a-3p in the activation of PAFs in hypoxia and the hypoxic remodeling of pulmonary artery and found its protection in HPH. It must be in mind the species difference and the limitations of chronically hypoxic rats as models of human pulmonary hypertension.\textsuperscript{2} Future works are needed to examine the role of miR-29a-3p in the phenotype regulation of pulmonary artery smooth muscle cells and pulmonary endothelial cells in hypoxic pulmonary vascular remodeling, especially in human PAFs and HPH patients, before it is translated to clinic.

**Perspectives**

HPH is still a life-threatening disorder characterized by the dramatic remodeling of pulmonary vascular adventitia caused by hypoxia-induced aberrant activation of PAFs. This study showed that hypoxia-induced activation of PAFs was accompanied with the drastic decrease of miR-29a-3p. In vitro, miR-29a-3p mimic inhibited the hypoxia-induced proliferation, migration, and secretion of PAFs, suppressed hypoxia-induced expression of activation-associated proteins in PAFs; moreover, miR-29a-3p inhibitor mimicked the effects of hypoxia on PAFs. Preventative or therapeutic administration of miR-29a-3p significantly ameliorated pulmonary vascular remodeling and pulmonary hypertension in HPH rats. These results demonstrate that miR-29a-3p regulates the hypoxia-induced activation and phenotype of PAFs and may be a treatment target for HPH.

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**Disclosures**

None.

**References**


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### What Is New?

- Hypoxia-induced activation of pulmonary adventitial fibroblasts (PAFs) was accompanied by the drastic decrease of miR-29a-3p. miR-29a-3p inhibited hypoxia-induced activation of PAFs in vitro and ameliorated hypoxic pulmonary hypertension in vivo.

### What Is Relevant?

- Hypoxic pulmonary hypertension is one type of pulmonary hypertension characterized by pulmonary vascular remodeling and increased pulmonary artery pressure. Hypoxia-induced PAF activation is key in pulmonary vascular remodeling. Inhibition of aberrant PAF activation may reverse pulmonary vascular remodeling. The role of miR-29a-3p in hypoxia-induced activation of PAFs and hypoxic pulmonary hypertension is not known.

### Summary

miR-29a-3p regulated hypoxia-induced activation of PAFs and had preventative and therapeutic potential on hypoxic pulmonary hypertension.

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### Novelty and Significance

- Hypoxia-induced activation of pulmonary adventitial fibroblasts (PAFs) was accompanied by the drastic decrease of miR-29a-3p. miR-29a-3p inhibited hypoxia-induced activation of PAFs in vitro and ameliorated hypoxic pulmonary hypertension in vivo.

- Hypoxic pulmonary hypertension is one type of pulmonary hypertension characterized by pulmonary vascular remodeling and increased pulmonary artery pressure. Hypoxia-induced PAF activation is key in pulmonary vascular remodeling. Inhibition of aberrant PAF activation may reverse pulmonary vascular remodeling. The role of miR-29a-3p in hypoxia-induced activation of PAFs and hypoxic pulmonary hypertension is not known.

- miR-29a-3p regulated hypoxia-induced activation of PAFs and had preventative and therapeutic potential on hypoxic pulmonary hypertension.
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miR-29a-3p attenuates hypoxic pulmonary hypertension by inhibiting pulmonary adventitial fibroblast activation

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Running title: miR-29a-3p attenuates pulmonary hypertension

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Supplemental Materials and Methods

Vector construction and virus production
The rat pre-miR-29a complementary DNA was synthesized (Sangon, Shanghai, China) (forward:
5'-GATCCACCCCTAGAGGATGACTGATTTCTTTTGGTGTTCA
GAGTCAATAGAATTTTCTAGCACCATCTGAAATCGGTATAATGATTGAGGAG-3’, reverse:
5'-AATTCTCCCCAATCATTATAACCAGATTTTCAATGGTCTAG
AAAATTCTTATGACTGAAACAAACAAAATCGATCTCTCTAAGGGGTG-3’), annealed and cloned into pAAV-ZsGreen-miRNA vector (Biowit Technologies, Shenzhen, China) to construct the target vector (referred as AA V5-miR-29a) according to the manufacturer’s instruction. The constructed AA V-miR-29a or the control vector (referred as AA V5-control) and the packaging vectors (pHelper and pAAV-RC) were transfected into 293AAV cells with HET kit (Biowit Technologies, Shenzhen, China). The adeno-associated virus serotypes 5 (AAV5) was produced and concentrated. The ultimate virus titer (vector genomes/ml) for AA V5-miR-29a and AA V5-control is $7.5 \times 10^{12}$ and $1.0 \times 10^{13}$ respectively.

Animal experiments
All the experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals. All animals were maintained in a 12 to 12 hours light-dark cycle in an air-conditioned room (25 °C) and had free access to food and water. Male Sprague-Dawley rats (200-220 g) were randomly divided into four groups (Nomoxia+AA V5-control, Normoxia+AA V5-miR-29a, Hypoxia+AA V5-control, Hypoxia+AA V5-miR-29a, n = 6), and accordingly maintained in normoxia (21% O2) or hypobaric (depressurized to 380 mmHg corresponding to 10% O2) hypoxia environment for four weeks (for preventive experiment) or for six weeks (for therapeutic experiment). Rats were anesthetized with pentobarbital (30 mg / kg, i.p.), and given a double intratracheal instillation of AA V5-miR-29a or AA V5-control at day-0 and day-14 (for preventive experiment) or a single intratracheal instillation at day-28 after hypoxia (for therapeutic experiment) ($1 \times 10^{12}$ v.g / kg / each instillation) according to the grouping.

Pulmonary hemodynamics
Rats were anesthetized with pentobarbital (30 mg kg$^{-1}$, i.p.). The right ventricle systolic pressure (RVSP) was measured according to a right cardiac catheterization procedure and used as an indicator of pulmonary artery pressure. A polyethylene catheter was inserted into the right ventricle via the right external jugular vein. The other end of the catheter was connected to a transducer and the pressure tracings were simultaneously recorded on a physiologic recorder (PowerLab system, AD Instruments, Castle Hill, NSW, Australia). Meanwhile, the mean carotid artery pressure was recorded via a special catheter inserted into the carotid artery. The data were analyzed by using the Chart program supplied by the system. After measurement, the anesthetized animal was euthanized by exsanguination, and the heart and lung were harvested for subsequent experiments.

Morphological investigation
The right ventricle and the left ventricle with septum were isolated and individually weighted to calculate the right ventricle hypertrophy index (RVHI, the ratio of right ventricle weight to left ventricle plus septum weight). The left lung was fixed in 4% (w/v) paraform and processed into 5 μm paraffin sections used for morphometric analysis with hematoxylin & eosin staining. Pulmonary vascular remodeling was assessed by the percent medial wall thickness (MT%) and percent medial wall area (MA%)$^{1-3}$, which are calculated as the following respectively: $MT\% = 100 \times ($medial wall thickness)/(vessel...
semi-diameter) and $MA% = 100 \times (\text{cross-sectional medial wall area})/(\text{total cross-sectional vessel area})$. Pulmonary arteries / arterioles, with external diameter from 50 to 100 $\mu m$, were measured using an image processing program (Image-Pro Plus, Version 5.1, Media Cybernetics, USA) to calculate $MT%$ and $MA%$. Total sixty vessels from six rats were analyzed for each group (ten vessels for each rat). All the morphological analysis was conducted in a double-blind method.

Cell culture
Pulmonary artery fibroblasts (PAFs) were obtained by tissue explant culturing method. Pulmonary arteries were isolated from adult male Sprague-Dawley rats as previous described. Briefly, muscular tissue and endothelial cell layers were removed by gentle abrasion of the vessel. The remaining tissue (adventitia) was then dissected into 1 $mm^3$ portions. The explanted tissue was cultured in RPMI 1640 medium supplemented with 20% (v/v) fetal bovine serum (Gibco, Melbourne, VIC, Australia) at 37°C in 95% $O_2$, 5%$CO_2$. Fibroblasts cell grew from the plants about 5 days. They were passaged and cultured in in RPMI 1640 medium with 10% FBS. The three to six passages were used for the following experiments.

Cell proliferation assay
PAFs were cultured in 24-well plate to 40% confluence, then transfected with 10 nM miR-29a-3p mimic, 50 nM miR-29a-3p inhibitor or corresponding amounts of negative controls (all from Ribobio, Guangzhou, China, the catalog number were shown in Table S1). After 6 hours, the medium was changed and the cells were cultured for another 24h. Then PAFs were passaged and used for cell proliferation assay and cell cycle analysis.

PAFs proliferation was measured by 3 - (4, 5 - dimethylthiazol - 2 - yl) - 2, 5 - diphenyltetrazoliubromide (MTT) assay and cell counting as previously described. For MTT assay, the transfected PAFs were seeded in 96-well plates at a density of 5,000 cells each well. After serum starved for 24h in serum-free medium, the infected cells were cultured in RPMI 1640 containing 5% FBS for 24h in normoxia (21%) or hypoxia (3%). Then MTT (5 mg / mL, 10$\mu$L / well) was added to the plates, and incubated for another 4h at 37°C. The supernatant was then carefully removed, and dimethyl sulfoxide (DMSO, 75$\mu$L / well) was added to dissolve the formazan crystals. The absorbance of the solubilized product at 490 nm (A490) was measured with microplate spectrophotometer (PowerWave XS, BioTek Inc, Winooski, VT, USA). For cell counting, the transfected PAFs were seeded in 24-well plates at a density of $5 \times 10^4$ cells / well, and then were treated as the above procedure. At the end of treatment, they were washed with phosphate buffered solution, harvested by mild trypsinization, and counted with a hematocytometer (Qiujin, Shanghai, China).

Migration assay
Migration was performed using scrape assay with modification. The cells were seeded in 6-well plate, and transfected with miR-29a-3p mimics, inhibitors or negative controls. After 6 hours, culture medium was removed and the cell-coated surface was scraped with a 200-$\mu$l pipette tip in a single stripe. Then the cells was washed twice with the medium and cultured in the medium with FBS for 48h for the wound healing. The images were acquired using microscope (Olympus, Tokyo, Japan) immediately and 48h after scrape respectively.

Mimic and inhibitor transfection
1x10$^5$ cells/well were seeded in 6-well plates and transfected with 10 nM of each miR-29a-3p mimics or with 50 nM miR-29a-3p inhibitor or corresponding amounts of negative controls using the DharmafectDuo transfection reagent (all from Dharmacon, CO, USA) according to the supplied protocol; After 6 hours, culture medium was replaced to remove the transfection reagent and the cells were cultured for another 48h. Then total RNA/miRNA and protein were extracted.
ELISA assay of TGF-β, ET-1 and PDGF

To collect conditioned medium, cells were plated in 6-well plate in RPMI 1640 medium supplemented with 10% FBS and grown until confluent. Next, cells were rinsed with PBS, and growth medium was replaced with serum-deprived RPMI 1640. In 48h, conditioned medium was collected for ELISA analysis.

RNA extraction and quantitative real-time PCR

Extraction of total RNA was performed using the miRNeasy Kit (Qiagen, Germany) and quantified using the NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA) according to the manufacturer’s instructions.

For miR-29a/b, the reverse transcription and quantitative real-time PCR were performed using Bulge-LoopTM qPCR kit (RiboBio, Guangzhou, China) according to the manual. For α-SMA and Col1A1, total RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent Kit Perfect Real Time, and Real-time PCR was performed using PrimeScript™ RT Master Mix (Takara, Dalian, China) with the following cycling conditions: 95 °C for 5 minutes followed by 40 cycles of amplification (95 °C denaturation for 30 seconds, annealing for 30 seconds (60 °C for Col1A1, 59°C for α-SMA), 72 °C extension for 30 seconds). The amplicon size for α-SMA and Col1A1 is 84 bp and 74 bp respectively. The catalog numbers of the commercial miR-29a/b primer sets (from RiboBio, Guangzhou, China) and the primers for α-SMA and Col1A1 were shown in Table S1. The ratio of the mRNA of interest was normalized with U6 (for miR-29a-b) or β-actin (for Col1A1, α-SMA).

Western blot analysis

The total lysate was obtained from the harvested cells or lung arteries, and used for the extraction of protein according to the instructions of Total Protein Extraction Kit (Millipore, Bedford, MA, USA). The lung arteries (the first- to the third-division) were harvested under a light microscope with a forceps. The adventitia is carefully isolated from arteries under a high-magnification microscope (Leica, Narishige Micromanipulator, Wetzlar, Germany).

The protein concentration was determined with BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were separated on denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with Tris buffered saline containing 5% non-fat dry milk at room temperature for 2h, followed by incubation with primary antibody against β-actin (1:10000, Sigma-Aldrich, St Louis, MO), α-SMA (1:1000, Cell Signaling Technology, MA, USA), HIF-1α (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA), Smad3 (1:1000,Cell Signaling Technology, MA, USA), phosphorylated Smad3 (1:1000, Cell Signaling Technology, MA, USA) or Col1A1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Then secondary antibody (anti-mouse IgG peroxidase conjugated, 1 : 5000) was incubated. The signal detection was performed by using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA) of a commercial ECL kit.

Statistical analysis

All values were presented as means ± SD. The statistical differences between groups were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni’s test for multiple comparisons. P value <0.05 was considered statistically significant.

References:


## Table S1. The catalog number or sequence for mimic, inhibitor and primers

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Figure S1. HIF-1α and Smad3 mediated the hypoxia-induced reduction of miR29a-3p in PAFs
(A) Representative western blot of HIF-1α, phosphorylated and total Smad3 in cultured PAFs in normoxia and hypoxia. (B-D) Summarized data for the western blot. (E,F) Knockdown of HIF-1α or Smad3 significantly reversed the hypoxia-induced reduction of miR-29a-3p in PAFs. (G) Normalized data of the mRNA level of Smad3 in PAFs transfected with the specific siRNA for HIF-1α. The mRNA level of Smad3 was normalized by dividing the value for Normoxia+CH group. (H-J) Representative western blot (H) and the summarized data of the phosphorylated (I) and the total protein (J) of Smad3 in PAFs transfected with specific siRNA for HIF-1α. β-actin was used as control. CH, scramble control sequence for HIF-1α; SH, specific siRNA for HIF-1α; CS, scramble control sequence for Smad3; and SS, specific siRNA for Smad3. Each bar represents mean±SD from three independent experiments. * P<0.05, ** P<0.01 vs Normoxia. # P<0.01 vs Hypoxia.
Figure S2. Effects of miR-29a-3p mimic and inhibitor on the expression of activation phenotype-associated protein in PAFs in hypoxia and/or normoxia

(A-E) Effects of miR-29a-3p mimic on the mRNA (A,B) and protein (C-E) expression of α-SMA and Col1A1 in PAFs in normoxia and hypoxia. The mRNA data of α-SMA and Col1A1 were normalized by dividing the value of Normoxia+SM. Representative western blot was shown in panel E. (F-J) Effects of miR-29a-3p inhibitor on the mRNA (F,G) and protein (H-J) expression of α-SMA and Col1A1 in PAFs in normoxia. The mRNA data of α-SMA and Col1A1 were normalized by dividing the value of Normoxia. Representative western blot was shown in panel J. SM, scramble sequence of mimic; M, miR-29a-3p mimic; SI, scramble sequence of inhibitor; and I, miR-29a-3p inhibitor. Each bar represents mean±SD for three independent experiments. * P<0.05 vs Normoxia+SM or Normoxia; # P<0.05 vs Hypoxia+SM.