QKI Promotes Hypoxia Induced Smooth Muscle Reprogramming in Pulmonary Hypertension

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Research

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Abstract

Background: Pulmonary hypertension (PH) is a complex and progressive cardiopulmonary disorder with poor prognosis and limited therapeutic treatments. Recent evidence suggests that RNA binding proteins (RBPs) participate in the pathogenesis of human and experimental pulmonary arterial hypertension. Quaking (QKI) as an important RBPs is involved in mRNA biogenesis, export, decay and translation. However, the biological significance of QKI in phenotypic transformation of PASMCs in PH as well as in abnormal pulmonary vascular remodeling remain elusive.

Methods: We assessed the expression pattern, phenotypic transformation effect, and mechanism of QKI in rodent Su/Hx-induced PH model, Human PAH samples and in HPASMCs.

Results: Elevated protein expression level of QKI was found in animal PH and human PAH samples, thus in hypoxic HPASMCs. Inhibition of QKI attenuated proliferation and phenotype switching in HPASMCs. Mechanistically, QKI was found to mediate STAT3 mRNA stabilization by binding to its 3'Untranslated Region (3'-UTR). Downregulation of QKI attenuated STAT3 expression in PASMCs, while overexpression of STAT3 in PASMCs was widely regarded to be involved in the progression of PH. In addition, as a transcription factor, STAT3 was identified to bound to miR-146b promoter to induce its expression, while miR-146b was proved to promote smooth muscle reprogramming through inhibiting STAT1 and TET2 expression during pulmonary vascular remodeling.

Conclusions: Our study demonstrates the QKI-STAT3-miR-146b pathway as a novel mechanistic insights into hypoxic reprogramming that permits vascular remodeling, and thus provides proof of concept for anti-remodeling therapy through the direct modulation this axis in PH.

Background

Pulmonary hypertension (PH) is a complex and progressive cardiopulmonary disorder with poor prognosis and limited therapeutic treatments[1, 2]. The hallmark features of PH are pulmonary vascular remodeling and increased pulmonary vascular resistance, ultimately resulting in right ventricular failure and death[3]. Pulmonary vascular remodeling owes to uncontrolled proliferation of pulmonary artery smooth muscle cells (PASMCs) and fibroblasts as well as endothelial dysfunction[4, 5]. Multiple factors including the unique genetic background are involved in disease susceptibility[6–8]. The PASMCs undergo dedifferentiated phenotypic transformation in PH, exhibiting a high rate of proliferation, migration and protein synthesis as well as a low rate of contractile protein expression[9–11]. Although there are significant advances in the intervention and treatment of PH[12], the prognosis of PH is still poor and the underlying molecular mechanisms remain unclear.

Recently, substantial advances suggest that RNA binding proteins (RBPs) participate in the pathogenesis of cardiovascular diseases, including ischemic injury in diabetic heart and doxorubicin-mediated cardiotoxicity [13, 14]. As an important RBP, the quaking (QKI) gene is proceeded into three major alternatively spliced isoforms with length of 5, 6, 7 kb, named QKI-5, QKI-6 and QKI-7, and their different

3’-UTR determines their respective subcellular localization. QKI-5 is the most abundant isoform which localizes predominantly in nucleolus, while QKI-6 is distributed both in nuclear and cytoplasmic compartments, and QKI-7 is mostly in nucleolus[15]. QKI can post-conscription modulate the expression level and function of target RNAs by specifically interacting with the QKI response element (QRE; ACUAAY[N1-20]UAAY)[16–18]. Studies have shown that QKI is essential for embryonic blood vessel development and visceral endoderm function[19], and is also implicated in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) differentiation [19–21]. A recent study implicated that extracellular matrix stiffening increases QKI expression leading to pathogenic pulmonary arterial endothelial function through QKI-miR-7 signaling pathway in PH [22]. However, the biological significance of QKI in phenotypic transformation of PASMCs in PH as well as in abnormal pulmonary vascular remodeling remain elusive.

STAT3 has been extensively described as a key signaling molecule that controls cell growth, apoptosis, and inflammation in response to growth factors and cytokines stimulation or stress[23–25]. Many studies have showed that dysregulation of STAT3 signaling plays a critical role in the pathogenesis of PH, and specific inhibitors of STAT3 can attenuate or even reverse abnormal proliferation and apoptosis resistance of PASMCs in PH[26–28]. The IL-6/JAK2/STAT3 axis of STAT3 signaling pathway is related to vascular diseases[26, 29]. The mechanism that regulates the expression of STAT3 is largely focused on protein phosphorylation and miRNA biosynthesis[30–32], whether STAT3 can be regulated by QKI is largely unknown.

In this study, we demonstrate that QKI is significantly upregulated in vivo and in vitro in PH, participates in cell proliferation, phenotype switch of PASMCs and contributes to the pathogenesis of PH. The up-regulation of QKI expression contributes to the enhanced expression of STAT3 in PASMCs. STAT3 participates in the abnormal proliferation and migration of PASMCs through increasing the expression of miR-146b in PH. Our study demonstrates a novel potential therapeutic strategy for the treatment of PH through inhibiting the QKI-STAT3-miR-146b pathway in PASMCs.

Materials And Methods

Detailed materials and methods are provided in the online data supplement.

Human lung samples and patient characteristics

Human lung specimens were derived from healthy donor lungs or explanted lungs from subjects with idiopathic pulmoanry arterial hypertension (iPAH) undergoing lung transplantation at Wuxi Lung Transplantation Center. Detailed clinical information can be found in supplemental materials.

Animal models and assessment of PAH

Su/Hx PH mice model: male mice aged 8 weeks received a weekly single subcutaneous injection of SUGEN5416 (20 mg/kg). Experimental animals were exposed to chronic hypoxia (10% O_2) in a ventilated

chamber for 4 weeks. Su/Hx PH rat model: male Sprague-Dawley rats received a single subcutaneous injection of SU5416 (20mg/kg) and were exposed to hypoxia for 3 weeks before returning to room air for 2 weeks.

Cell culture and experiments

Primary murine PASMCs were isolated from PH and Non-PH mice using a modified elastase/collagenase digestion protocol[33].

Western blot

Western blot was used to detect the protein levels of QKI,QKI-5,QKI-6,STAT3,STAT1,TET2, α-SMA, PCNA.

Immunofluorescence staining and microscopy analysis

Lung slides of human subjects and animal models were stained for determining the expression and location of QKI, STAT3, STAT1 and TET2 as previously described and were examined by Olympus microscope.

Statistical analyses

Statistical analyses were performed in the GraphPad Prism Version 6.0 environment (GraphPad Software, San Diego, CA, USA). For comparing, data were analyzed by Student's *t*-test and ANOVA. All pairs of columns were compared with bars denoted as mean ± SD obtained from at least three independent experiments.

Results

QKI is significantly up-regulated in vivo **and** in vitro **in PH**

To investigate the role of QKI in PH, the protein expression of QKI in experimental rodent PH models and human PAH patients was firstly determined. The mRNA and protein expression levels of QKI (QKI-pan, total QKI), QKI-5 and QKI-6 were up-regulated in hypertensive lung tissues, but QKI- 7 is maintained at much lower expression levels and is not detectable (Fig. 1a and suFig. 1). Immunofluorescence staining showed the elevated expression of QKI, QKI-5 and QKI-6 in PAs from rodent PH models and human PAH patients (Fig. 1b and suFig. 2), especially in pulmonary artery smooth muscle cells (PASMCs). Furthermore, exposing HPASMCs to hypoxia and treating with DMOG or $CoCl_2$ increased QKI, QKI-5 and QKI-6 protein levels (Fig. 1c) and mRNA levels except QKI-7(suFig. 3a,b). Elevated of QKI, QKI-5 and QKI-6 expression levels were depicted in PASMCs from mice with PH (Fig. 1d). In addition, the immunofluorescence signal intensities of QKI, QKI-5 and QKI-6 also increased in hypoxia or $CoCl_2$ treated HPASMCs (suFig. 3c).

QKI is participated in cell proliferation and phenotype switch of PASMCs

Phenotype modulation of vascular smooth muscle cells was crucial in pathogenesis of PAH. To determine whether QKI directly regulates phenotype modulation, we used siRNA QKI to knockdown QKI expression. Cell proliferation assay results showed that the depletion of QKI significantly inhibited HPASMCs proliferation (Fig. 2a). Immunofluorescence staining showed that QKI silencing increased the expression of contractile marker α-SMA (Fig. 2b). Furthermore, QKI silencing potentiated α-SMA and Calponin1 and repressed PCNA expression (Fig. 2c and d).

QKI stabilizes STAT3 through directly binding to its 3’-UTR

To determine the downstream target of QKI, we focused on cell cycle- and cell proliferation-associated genes which contain potential QRE. STAT3 is identified as one of the candidates possessing the above-mentioned characteristics which contains one putative QKI response elements, QRE-1 (AUUAAC), at 530 bp in the 3’-UTR. The protein expression levels of STAT3 were much higher in the lungs of rodent PH models and human PAH patients compared to respective controls (Fig. 3a). Immunofluorescence staining also revealed elevated expression of STAT3 in PAs from rodent PH models and human PAH patients (Fig. 3b). STAT3 protein levels were also significantly higher in hypoxia, DMOG or $CoCl_2$ treated HPASMCs (Fig. 3c and suFig. 4a). Loss of function assay was performed by transfecting HPASMCs with STAT3 siRNA (suFig. 4b), which showed a significant inhibition on cell proliferation (suFig. 4c). Furthermore, STAT3 silencing repressed PCNA expression and potentiated α-SMA expression (Fig. 3d and suFig. 4d).. These data revealed that STAT3 was up-regulated in PH and promoted HPASMCs proliferation and phenotypic transformation.

To further confirm the molecular interaction of QKI and STAT3, we tested the cellular localization of QKI and STAT3 in PH lung tissues. QKI and STAT3 were co-located in vessel walls of rodent PH models and human PAH patients (Fig. 4a). Knockdown QKI expression showed significantly decreased STAT3 protein and mRNA expression (Fig. 4b). Overexpression of QKI could increase the protein expression level of STAT3 in Hek293T cells (suFig. 5). To verify whether QKI post-transcriptionally regulated the mRNA stability of STAT3, the mRNA expression level of STAT3 was destabilized when QKI was silencing in HPASMCs (Fig. 4c). In contrast, QKI overexpression increased STAT3 stability in Hek293T cells (Fig. 4c). Furthermore,luciferase assay showed that overexpression of QKI increased the luciferase activity of STAT3-WT, but not the STAT3-MUT sequence (Fig. 4d). These data demonstrated that QKI stabilized STAT3 through directly binding to its 3’-UTR region.

The transcriptional regulation of miR-146b by STAT3 is important to promote PASMCs reprogramming

We next determined the molecular mechanism of STAT3 in promoting cell proliferation and phenotype switching of PASMCs. MiR-146b, a cell proliferation-associated miRNA, contained putative STAT3 binding sites. To understand the regulatory mechanism of STAT3 and miR-146b, we found that the expression level of miR-146b was much higher in lung homogenates of Su/Hx PH mice and Su/Hx PH rats models, thus in hypoxic HPASMCs (Fig. 5a). HPASMCs transfected with STAT3 siRNA significantly reduced the

expression level of miR-146b (Fig. 5b). Furthermore, overexpression of STAT3 significantly up-regulated the expression of miR-146b in Hek293T cells (suFig. 6a,b). Moreover, luciferase reporter assay showed that overexpression of STAT3 significantly up-regulated the expression of luciferase with miRNA-146b-WT promoter, but not with the mutant promoter (Fig. 5c). To further demonstrate whether STAT3 promoted PAMSCs proliferation and phenotype switch through up-regulating of miR-146b, HPASMCs were transfected with miR-146b mimics or control and then STAT3 was silenced with siRNA. Overexpression of miR-146b could attenuated STAT3 silencing induced PASMCs differentiation and inhibited PASMCs proliferation(Fig. 5d). Together, these data demonstrated that the ability of STAT3 to increase miR-146b expression, in some capacity, contributes to PASMCs hyperproliferation and pulmonary vascular remodeling.

MiR-146b promotes PASMCs reprogramming by inhibiting STAT1 and TET2

To better understand the underlying mechanisms of miR-146b in PASMCs phenotypic transformation, we used several available miRNA target prediction websites to predict miR-146b targets, among the possible targets of miR-146b, we focused on STAT1 and TET2, which have been reported to play important roles in PASMCs phenotypic transformation. We firstly tested the expression pattern and cellular localization of STAT1 and TET2 in PH tissues. STAT1 and TET2 protein levels were significantly reduced in the lung tissues of PH mice and rats as well as human PAH patients compared to respective controls (Fig. 6a and suFig.7a). Furthermore, exposing HPASMCs to hypoxia and treating with DMOC or $CoCl_2$ decreased STAT1 and TET2 protein levels (Fig. 6b and suFig. 7b). To investigate the function of miR-146b, we transiently overexpressed miR-146b mimic in HPASMCs. In response to overexpression of miR-146b, the proliferative ability and phenotypic transformation of HPASMCs were significantly increased.(Fig. 6c and sFig. 8a). Given that the 3'-UTRs of STAT1 and TET2 transcripts contained conserved sequences complementary to the miR-146b seed sequence (suFig. 8b). We found that overexpressing miR-146b significantly reduced the protein expressions of STAT1 and TET2 in HPASMCs (Fig. 6d)

Discussion

In this study, we demonstrated that QKI was significantly up-regulated in rodent PH models,clinical PAH samples and hypoxic PASMCs. We confirmed that QKI aberrant overexpression contributed to PASMCs proliferation and phenotype switch during pulmonary vascular remodeling by increasing the expression of STAT3. We also found that STAT3 directly bound to the promoter region of miR-146b and induced miR-146b expression, thereby participated in PASMCs hyperproliferation and phenotypic conversion. Furthermore, mechanistic studies revealed that miR-146b promoted PASMCs proliferation by inhibiting the expression of STAT1 and TET2. Our study provides a novel insight that QKI could promote pulmonary vascular remodeling while the inhibition of QKI might be a therapeutic approach in patients with PH.

QKI as an important post-transcriptional regulatory factors that participates in many diseases, but little is known about its role in regulating vascular remodeling ,especially the effect on PASMCs. QKI-5 is function

in human embryonic stem cells (hESCs) differentiation, the early cardiogenic process, and cardiac disease pathogenesis[34]. QKI-6 provide positive angiogenesis and neovascularization via VSMCs dedifferentiation[35]. QKI-7 negatively regulates endothelial function in diabetic mice ischemic hindlimb[36].Our study provided novel insights on the expression profile of QKI isoforms in both rodent PH animal models and human PAH patients samples. QKI was widely distributed in all layers of small PAs in rodent hypertensive lungs with most abundantly concentrated in the medial layer of human PAs. We further observed that QKI protein level increased in hypoxia-induced HPASMCs and primary PASMCs from PH mice. We also showed that functional significance of QKI in regulating PASMCs proliferation and phenotype switch during pulmonary vascular remodeling.

As a type of RBP, QKI exhibits multiple functions in regulating mRNA metabolism[37]. In our study, QKI improved the stability of STAT3 mRNA through directly binding to the QRE motif of STAT3 mRNA. Further study showed that STAT3 protein levels were increased in rodent PH models and clinical PAH samples. QKI and STAT3 were co-located in the medial layer of small PAs. In addition, knockdown the expression of QKI showed a decreased level of STAT3 in HPASMCs and reduced the half-life of STAT3 mRNA.

STAT3 is a well-known transcriptional activator for many genes[38, 39], but it has been reported to inhibit miR-146b expression by promoting methylation of its promoter through increasing DNMT1 expression[40]. MiR-146b plays important role in controlling cell growth, apoptosis and inflammation[39, 41]. MiR-146b was up-regulated and promoted VSMC proliferation upon PDGF-BB stimulation[42]. The present work indicated that miR-146b was highly expressed in rodent models of PH and PASMCs exposed to hypoxia. Furthermore, luciferase assay and loss/gain function assays revealed that STAT3 induced the expression of miR-146b through directly binding to its promoter. Together, STAT3 promotes HPASMCs proliferation and phenotype switch through up-regulation of miR-146b.

MiRNAs as post-transcriptional regulatory factors, bind to the 3’-UTR of target genes to inhibit gene expression. STAT1 and TET2 as candidates targets of miR-146b which played important role in PH. STAT1 could inhibit the proliferation of PASMCs and was a negative regulator of VEGF expression by inhibiting HIF-1α transcription[43]. TET2 as a master epigenetic regulator plays an important role in PH development, which is decreased in PAH-SMCs. TET2 deficiency and somatic TET2 mutation in hematopoietic cells is associated with overproduction of pro-inflammatory cytokines and SMC dedifferentiation [44–46]. We found that miR-146b inhibited the expression of target genes STAT1 and TET2 which leads to PASMCs hyperproliferation and phenotype switch.

We did acknowledge several limitations of our work. First, we did not test the functional consequence and cell type specific effect of QKI or its downstream targets in animal models of PH due to the lack of tissue-specific and cell type-specific gene editing animal models. Second, the precise mechanism of QKI in regulating STAT3 mRNA stability remains to be understood by biochemistry and molecular biology approaches. Third, we showed the beneficial effects of QKI and STAT3 knockdown in controlling PASMCs hyperproliferation and phenotype switch. It would be clinically and pharmaceutically beneficial to determine which is the best target to achieve the therapeutic effects as well as to avoid side effect.

Conclusions

In summary, our data strongly support the notion that QKI could increase STAT3 mRNA stability through directly binding to QRE motif in 3'-UTR of STAT3 mRNA. The up-regulated STAT3 promoted miR-146b transcription by binding to miR-146b promoter region. Elevated miR-146b inhibited its targets STAT1 and TET2 expression and participated in pulmonary vascular remodeling. Our results provide a novel insight that QKI is a key regulator of the functional plasticity which is required for reprogramming of PASMCs under vascular remodeling.

Declarations

Ethics approval and consent to participate

The protocols for human research were approved by the Ethics committee of Nanjing Medical University (Permit Number: 2019-452). Patient's consented to research use of tissue removed as part of medical care. All animal experiments were performed in compliance with the guidelines for the care and use of laboratory animals and were approved by the Institute for Laboratory Animal Research of Nanjing Medical University. The protocols were approved by the committee of the Ethics of Animal Experiments of Nanjing Medical University (Permit Number: 1705029, 2001008).

Consent for publication

All of the authors have consented to publication of this research.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its additional information files).

Competing interests

The authors declare that there is no conflict of interests.

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Authors' contributions

All authors contributed to the study conception and design. Experiments, data collection and analysis were performed by Huijie Huang and Donghai Lin. Animal model and critical reading/editing of the

manuscript were done by Li Hu and Jie Wang. The draft of the manuscript was written by Huijie Huang. Feng Chen supervised the study. All authors participated in data interpretation.

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Figures

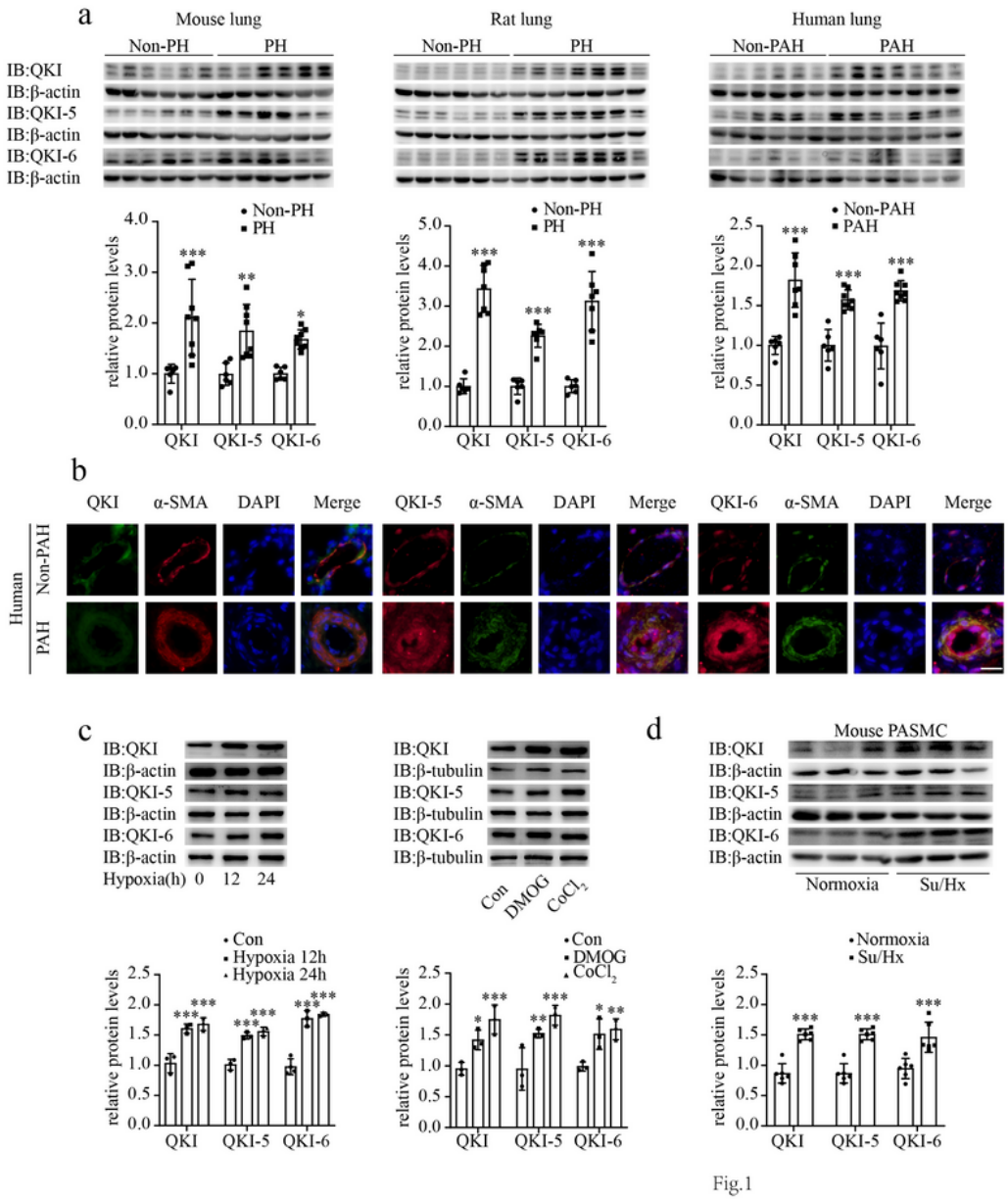

Fig.1

Figure 1

QKI is significantly upregulated in vivo and in vitro in PH. (a) Representative immunoblots and relative densitometric analysis of QKI, QKI-5 and QKI-6 expression in lung tissues (mouse: Non-PH, n =6, PH, n =6, rat: Non-PH, n =6, PH, n =7, human: Non-PAH, n =6, PAH, n =7,) normalized to β-actin. (b) Representative immunofluorescence staining demonstrates the increased QKI-pan, QKI-5 and QKI-6 expression in PAs from human PAH (scale bars = 50μm). (c) Immunoblot and densitometric analyses of QKI, QKI-5 and QKI-

6 in HPASMCs exposed to 1% O2 for indicated times and treated with DMOG (100 μM) or CoCl2 (150 μM) for 24 hours. (d) QKI, QKI-5 and QKI-6 expression in PASMCs from PH mice and control mice (n=3, 3). n=3 independent experiments each, results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P< 0.0001.

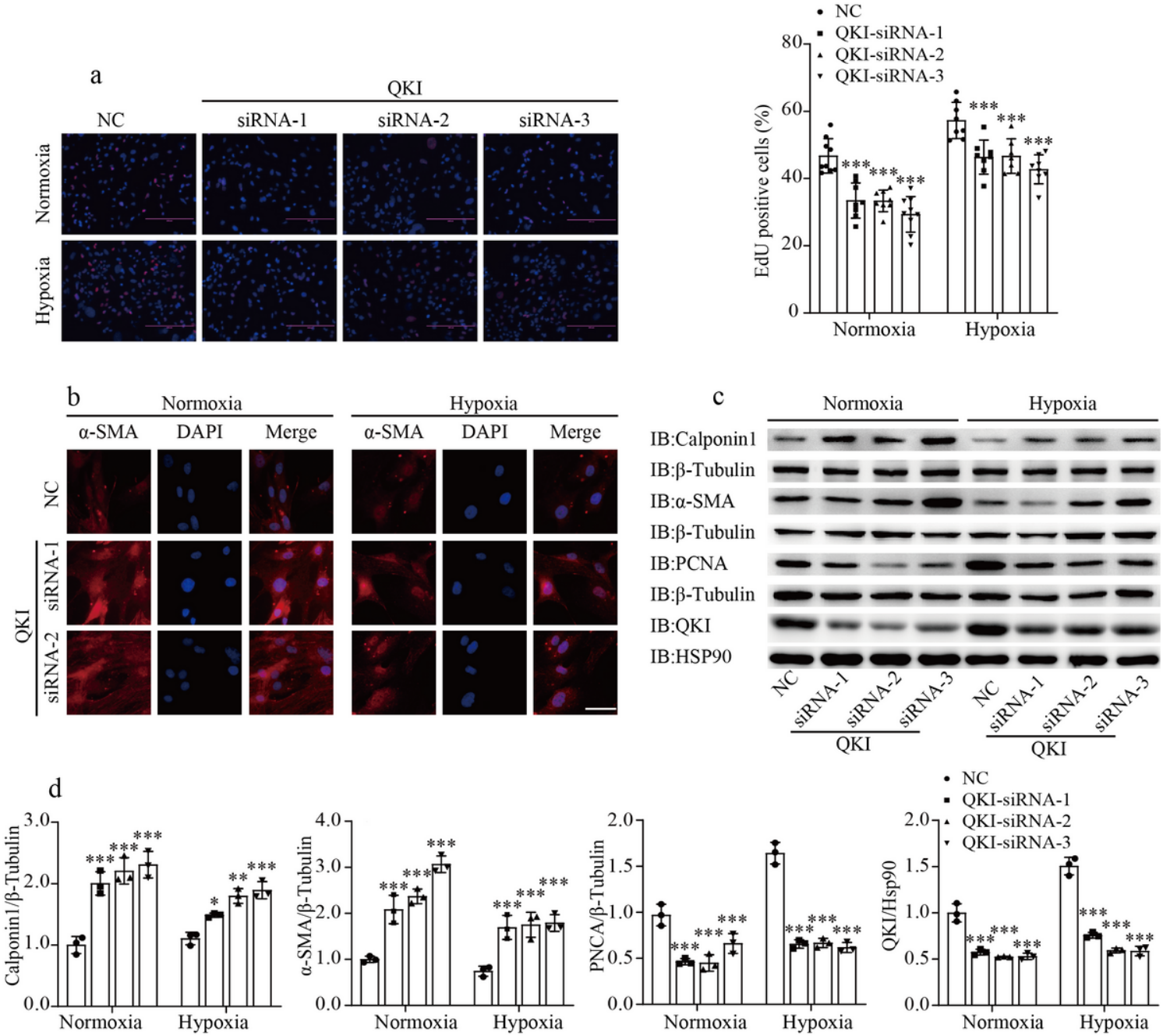

Fig.2

Figure 2

QKI deficiency inhibits hypoxia-induced proliferation and phenotype switch of HPASMCs. (a)Fluorescence images and the quantification of EdU incorporation assays : EdU positive cells (red) and nucleus was

counterstained with DAPI (blue). (b) Representative immunofluorescence staining of α-SMA (red) in HPASMCs, nucleus was counterstained with DAPI (blue). (c) Calponin1, α-SMA, PCNA and QKI protein levels were measured by Western blot. (d) Corresponding densitometric quantification shows Calponin1, α-SMA, PCNA and QKI expression levels of c. Results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.

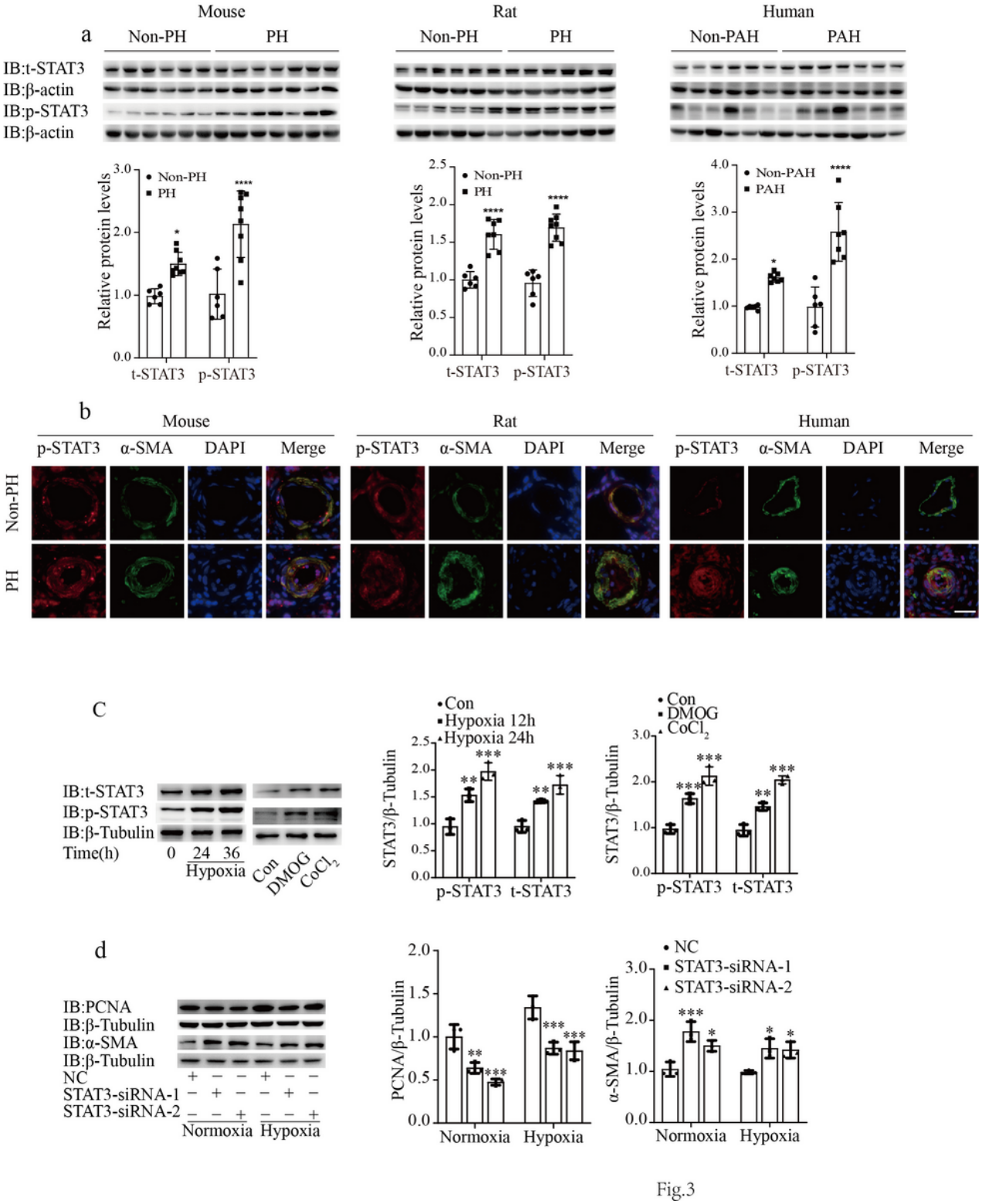

Fig.3

Figure 3

STAT3 expression is increased in PH and contributes to PASMCs reprogramming. (a) Representative immunoblots and relative densitometric analysis of STAT3 protein expression in lung tissues (mouse: Non-PH, n =6, PH, n =7, rat: Non-PH, n =6, PH, n =7, human: Non-PAH, n =6, PAH, n =7,) normalized to β-actin. (b) Representative immunohistochemical staining of STAT3 in lungs from mouse, rat and human with or without PH, scale bars = 50 μm. (c) Immunoblot and densitometric analyses of STAT3 protein expression in HPASMCs exposed to 1% O2 for indicated times and treated with DMOG or CoCl2 for 24 hours. (d) Immunoblot analysis and quantification of PCNA and α-SMA. n= 3 independent experiments each. Results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.

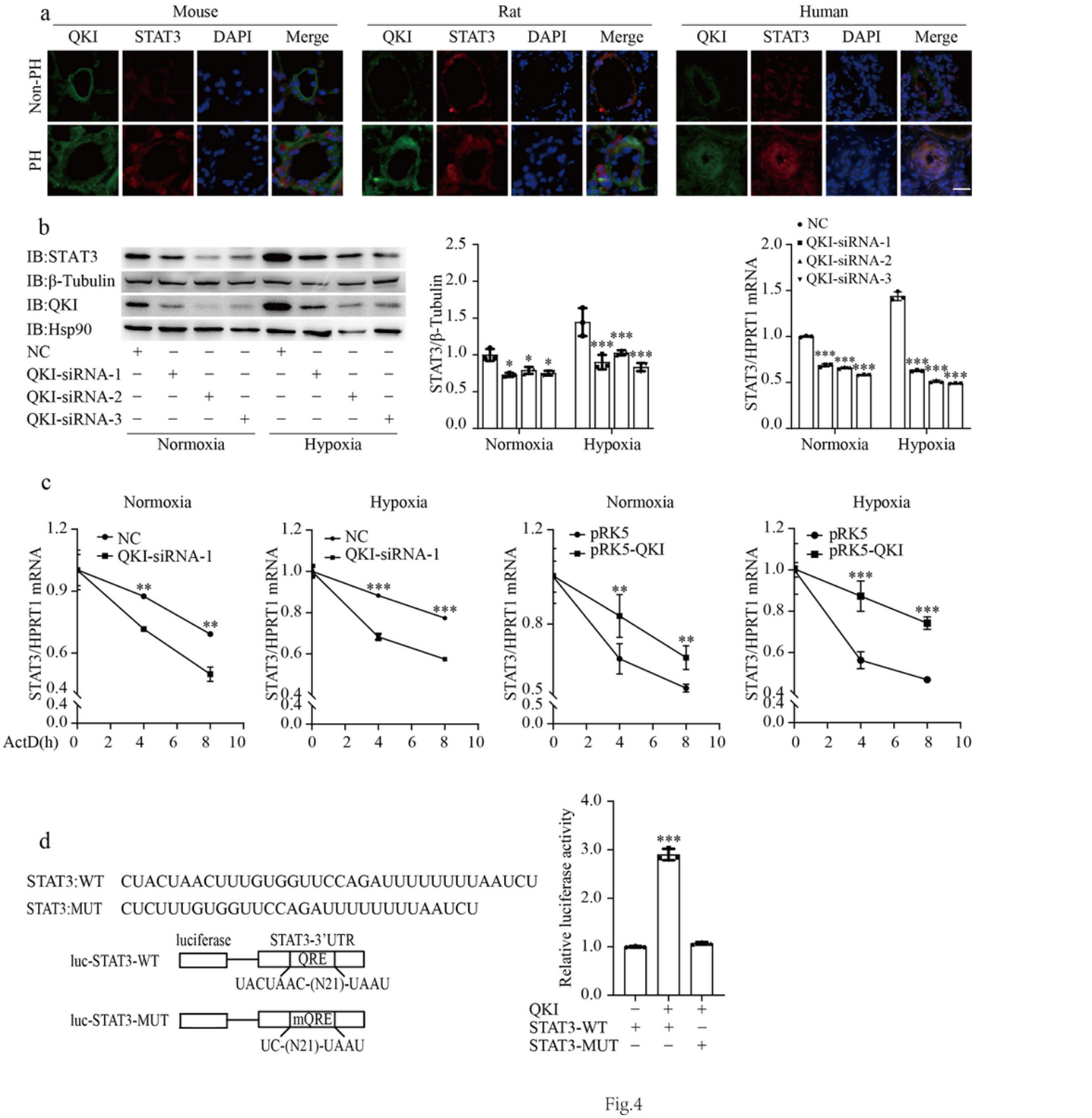

Fig.4

Figure 4

QKI regulates STAT3 expression via binding to QRE of its 3'-UTR in HPASMCs. (a) Representative immunofluorescence staining of QKI and STAT3 in lungs from mouse, rat and human with or without PH, scale bars = 50 μm. (b) Immunoblot and densitometric analyses of STAT3 protein levels and RT-PCR analysis of STAT3 mRNA levels in HPASMCs transiently transfected with QKI siRNA and control for 48 hours. (c) RT-PCR analysis of STAT3 mRNA stability in HPASMCs transfected with QKI siRNA and in

Hek293T cells overexpressing QKI treated with ActD at indicated times. (d) Predicted QKI recognition sites in the 3'-UTR of human STAT3 and schematic representation of human STAT3 3'-UTR reporter constructs and luciferase reporter assays analysis of overexpression of QKI inducing the relative luciferase activity, but not the mutant QRE in STAT3 3'-UTR. n = 3 independent experiments each. Results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.

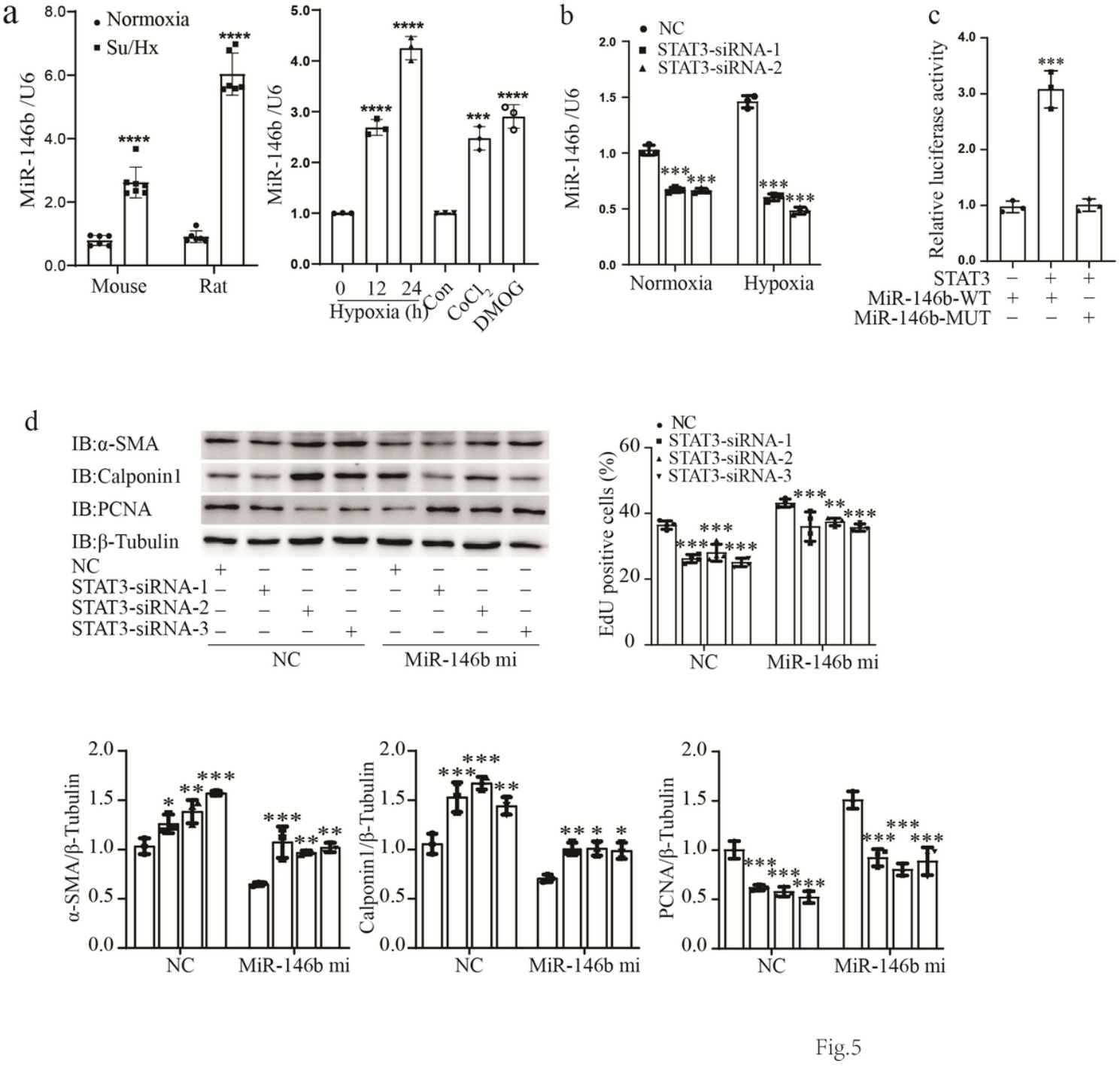

Fig.5

Figure 5

STAT3 promotes hypoxia-induced proliferation and phenotype switching of HPASMCs through upregulating miR-146b.(a) miR-146b is significantly upregulated in the lungs of rodent PH models and PASMCs exposed to hypoxia..(b)Representative RT-PCR analysis of miR-146b in HPASMCs transfected

with STAT3 siRNA. (c) MiR-146b promoter and mutant luciferase-reporter constructs were co-transfected with STAT3 in 293T cells and luciferase activity levels were measured. (d) miR-146b promotes PASMCs proliferation and phenotype switching. Quantitative measurement of EdU incorporation assay of HPASMCs and immunoblot and densitometric analyses of α-SMA, Calponin1 and PCNA protein levels. n = 3 independent experiments each. Results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.

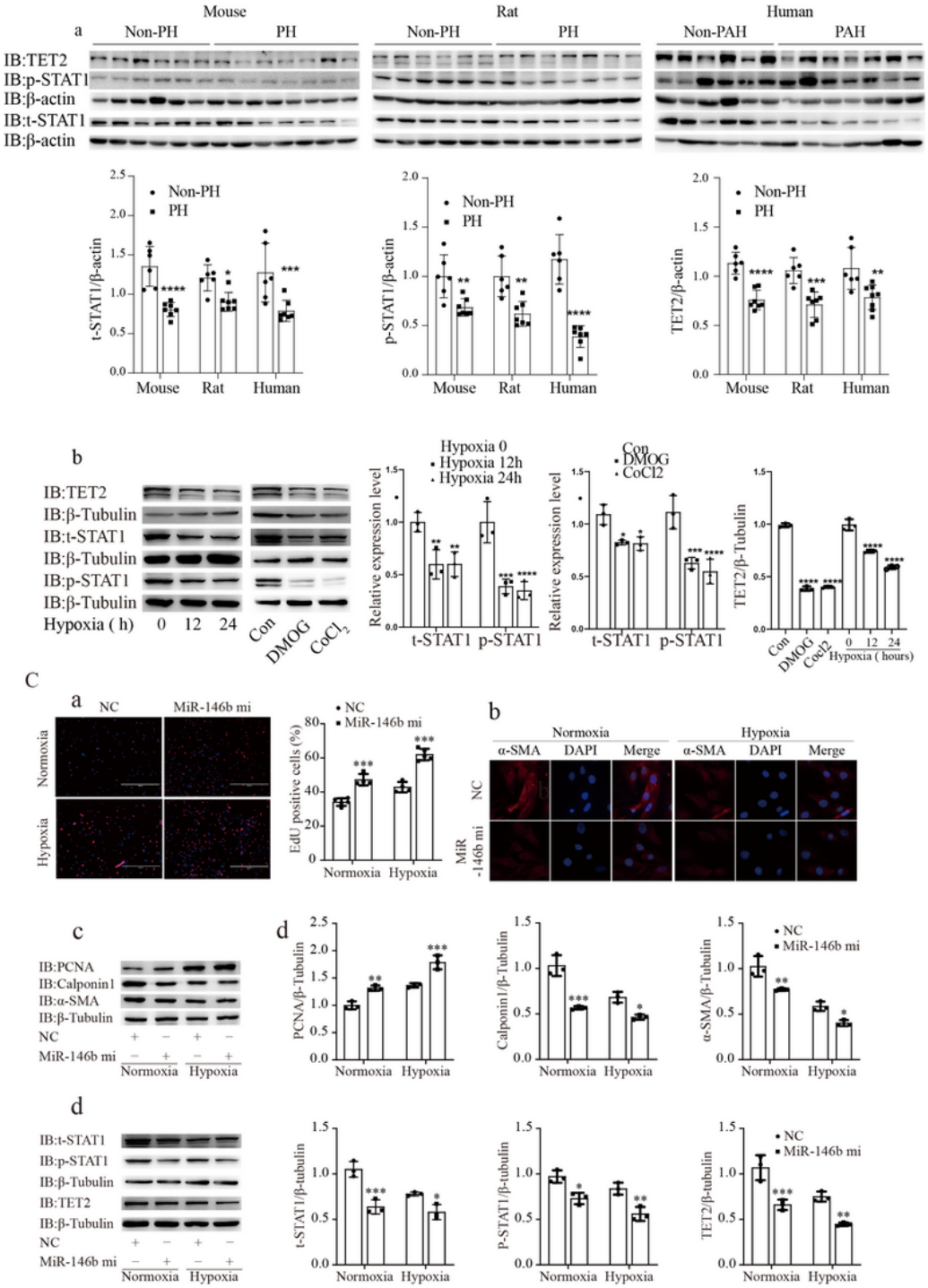

Fig.6

Figure 6

Overexpression of miR-146b promotes hypoxia-induced proliferation and phenotype switch of HPASMCs. (a) Representative immunoblots and relative densitometric analysis of STAT1 and TET2 protein expression in lung tissues (mouse: Non-PH, n=6, PH, n=7; rat: Non-PH, n=6, PH, n =7; human: Non-PAH, n=6, PAH, n=7) normalized to β-actin. (b) Representative immunoblots and relative densitometric analysis of STAT1 and TET2 protein expression in HPAMSCs exposed to hypoxia and treated with DMOG and Cocl2.(c) miR-146b promotes the proliferation and phenotype switch of HPASMCs. Fluorescence images and percentage quantification of EdU incorporation assay and representative immunofluorescence of α-SMA (red) in HPASMCs, nucleus was counterstained with DAPI (blue). (d) Western blot and quantification of STAT1 and TET2 in HPASMCs transfected with miR-146b mimics. n = 3 independent experiments each. Results are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

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