Received: 21 September 2023 | Revised: 5 February 2024 | Accepted: 20 February 2024

DOI: 10.1111/cts.13767

ARTICLE

FAK mediates hypoxia-induced pulmonary artery smooth muscle cell proliferation by modulating mitochondrial transcription termination factor 1/cyclin D1

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Abstract

This study aimed to investigate the mechanism of FAK-dependent hypoxia-induced proliferation on human pulmonary artery smooth muscle cells (HPASMCs). Primary HPASMCs were isolated and cultured in vitro under normal and hypoxia conditions to assess cell proliferation with cell counting kit-8. FAK and mitochondrial transcription termination factor 1 (mTERF1) were silenced with siRNA, mRNA, and protein levels of FAK, mTERF1, and cyclin D1 were determined. HPASMC proliferation increased under hypoxia compared to normal conditions. Knocking down FAK or mTERF1 with siRNA led to decreased cell proliferation under both normal and hypoxia conditions. FAK knockdown led to the reduction of both mTERF1 and cyclin D1 expressions under the hypoxia conditions, whereas mTERF1 knockdown led to the downregulation of cyclin D1 expression but not FAK expression under the same condition. However, under normal conditions, knocking down either FAK or mTERF1 had no impact on cyclin D1 expression. These results suggested that FAK may regulate the mTERF1/cyclin D1 signaling pathway to modulate cell proliferation in hypoxia.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Pulmonary arterial hypertension (PAH) is a form of PAH in which obstruction of small arteries in the lungs results in a sustained increase in pulmonary artery blood pressure. Chronic hypoxic exposure can lead to PAH, which causes excessive muscularization of the pulmonary arteries. Currently, there is no cure for PAH.

WHAT QUESTION DID THIS STUDY ADDRESS?

The study addresses the question of what the mechanism of abnormal proliferation and migration of pulmonary artery smooth muscle cells under hypoxia is, in the hope of identifying new preventive or therapeutic targets for PAH.

Chunlong Lin and Hui Yang contributed equally to this manuscript.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The present study demonstrated that during hypoxia increased human pulmonary artery smooth muscle cells proliferation may be attributed to the activation of FAK. The mechanism of FAK in promoting cell proliferation may be associated with the mTERF1/cyclinD1 signaling pathway.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This may provide new ideas and targets for preventing the progression of PAH and treating PAH.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is one form of pulmonary hypertension characterized as a continuous increase in pulmonary arterial blood pressure due to obstruction in the small arteries in the lungs. It is characterized by dysregulation of pulmonary hemodynamics and vascular growth. Progression of PAH could lead to structural alterations in pulmonary arteries and veins, recognized as pulmonary vascular structure remodeling (PVSR), which could further worsen PAH by increasing the pulmonary vascular resistance.[1] PVSR is a process that involves changes in intima, media, and adventitia, whereas each vascular cell type, including endothelial, smooth muscle, and adventitial fibroblast, undergoes cellular and molecular changes that often also involve inflammatory response.[1,2] Chronic exposure to hypoxia can lead to PAH. Hypoxic pulmonary vasoconstriction as well as the release of growth factors can lead to excessive muscularization of pulmonary arteries, and a remodeling predominate in media, which is composed primarily of smooth muscle cells.[3] Thus, understanding the mechanism of abnormal proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) under hypoxia may provide insights into preventing PAH progression and new targets for PAH treatment.

Focal adhesion kinase (FAK) also known as PTK2, is a protein kinase involved in the regulation of cell adhesion and motility. FAK is originally identified as a target for the protein kinase v-Src and is often found in multiple protein structures connected with extracellular matrix and cytoplasmic cytoskeleton. FAK can be activated by integrin signaling or growth factor stimulation.[4] It plays roles in several cellular processes, including cell migration, mitogen response, and survival.

Studies have shown that FAK activation in response to integrin signaling can promote cell cycle progression through cyclin D1, a key regulator for the G1-S phase, by modulating its gene expression; and this activity requires a FAK/Src complex.[5,6]

One study indicated that hypoxia condition could increase FAK and Src phosphorylation in the embryonic stem cells that are integrin signaling dependent.[7] The study by Jia et al.[8] demonstrated that osteoprotegerin contributes to the hypoxia plus SU5416-induced PAH in animals via the signal transduction pathway through integrin and FAK. Recent studies showed that the FAK/Akt pathway is activated under hypoxia conditions both in vivo assessed with mouse lung tissue and in vitro studied by human pulmonary artery smooth muscle cell (HPASMC).[9] However, another study demonstrated that FAK phosphorylation decreased in the presence of hypoxia-inducible factor 1 (HIF-1) and cobalt chloride in smooth muscle cells.[10]

The role of FAK in PASMC proliferation and migration is not well understood. Studies showed that FAK inhibition in vitro can induce a fivefold increase in apoptosis, a 2.5-fold decrease in proliferation, and an 18% decrease in cell migration of PASMCs from patients with PAH.[11,12] This observation is likely because of FAK on the downstream signaling. It is shown that inhibition of p21-activated kinases, LIM kinases, c-Jun N-terminal kinases, and p38 mitogen-activated protein kinases reduce the dysregulated and the platelet-derived growth factor stimulated migration of PASMCs from patients with PAH.[13] Therefore, the study of the role of FAK in modulating PASMC during hypoxic conditions could provide new insights for PAH treatment.

Metabolic abnormalities are present in human PAH. Mitochondria in pulmonary vascular cells of patients with PAH showed increased glycolysis and glutamine utilization and decreased fatty acid oxidation. Reactive oxygen species generation is seen to increase, and together with changes in the tricarboxylic acid cycle may lead to HIF accumulation, which drives the changes in metabolism observed.[14–18] The metabolic phenotype of PAH has a character that resembles the proliferative cells. Metabolic dysregulation found in PAH also includes changes in the pentose phosphate pathway, fatty acid synthesis, and profound alteration in iron metabolism. The biogenesis of mitochondria is also affected in which increased fission and decreased fusion are observed.[19] Moreover, PHA is found associated with mitochondrial-dependent apoptotic pathways and disrupted oxygen sensing which can create a pseudo-hypoxic condition.[12,16–18,20–22]

The mitochondrial transcription termination factor (mTERF1) is a family of proteins that are present in metazoans and plants. This family has four members, mTERF1 to mTERF4 with similar leucine zipper structures. The reports about mTERF1 have mainly focused on its structure and location rather than its influence on mitochondrial gene transcription, physiological effects, and impact on cell growth. It was shown in HeLa cells that mTERF1 can increase cyclin D1 expression and promote cell proliferation by regulating oxidative phosphorylation.[23] Interestingly, Kasahara et al.[24] showed that overexpression of FAK could prevent mitochondria-dependent ionizing radiation-induced apoptosis.

However, the mechanism of interplay among FAK, mTERF1, and cyclin D1 in the HPASMCs proliferation has not been investigated. In this study, we use small interference RNA (siRNA) technology to explore whether FAK promotes HPASMC proliferation via mTERF1/cyclin D1 signaling under hypoxia conditions.

MATERIALS AND METHODS

Ethics approval and consent to participate

The study was approved by Yueyang Municipal Hospital of Hunan Normal University Ethics Committee (2015030206). Written informed consent was obtained from individual participants. In total, 21 patient samples were collected, in which three primary pulmonary artery smooth muscle cell cultures were established. Experiments were performed with all three cultures.

Reagents

High glucose Dulbecco's modified Eagle's medium (DMEM; essential medium), heat-inactivated fetus bovine serum (FBS), and streptomycin were purchased from Hyclone Laboratories Inc. Rabbit anti-mouse cyclin D1 antibody was purchased from Abgent. Goat anti-rabbit FAK antibody (FAK Polyclonal Antibody) was purchased from Proteintech Group. Anti-calponin-1 antibody, anti-mTERF1 antibody, and electrochemiluminescence (ECL) detection reagents were purchased from Abcam (Cambridge, UK). β-actin monoclonal antibody was purchased from Wuhan Biodiscover (Hubei, China). FAK siRNA and mTERF1 siRNA were synthesized by Guangzhou Ribobio Ltd. (Guangdong, China). TRIzol assay kit, Taka reverse transcription kit, and the real-time polymerase chain reaction (RT-PCR) kit were purchased from Takara (Japan). Human FAK and mTERF1 primers were purchased from Shanghai Sangon Ltd. (China). Human β-actin primers were purchased from Guangzhou Ribobio Ltd. (China). Lipofectamine 2000 was purchased from Invitrogen.

Isolation and primary cell culture

Tissue collection

Primary HPASMCs were isolated from the lung tissues obtained from patients admitted from July 2013 to March 2014 at our Hospital. Lung tissues were obtained from patients who underwent pulmonary segmentectomy, lobectomy, or pneumonectomy due to radiograph abnormality of pulmonary shadowing. Tissue collection was approved by patients who signed written consent, and the study was approved by our hospital.

During the surgical procedure, the normal lung tissues at the edge of tumor tissues that were sectioned together were separated from the tumor tissues and sent immediately for pathological examination. If the edge of the lesion was negative for tumor, the pulmonary artery was then separated from the lung tissue.

Cell culture

HPASMC primary culture was performed according to previously published protocols.[25] Briefly, the isolated pulmonary artery media membrane was placed in a sterile vessel and cut into 0.5 mm × 1 mm × 1 mm size. FBS was added to the vessel to cover the tissue. Pulmonary artery tissue blocks were then transferred into the 50 cm^2 culture flask to allow for tissue attachment to the flask surface. The distance between tissue blocks was about 0.5 cm. 1 mL DMEM supplemented with 20% FBS was added to make sure the tissues were completely submerged in the culture medium and the tissues were incubated in a normal oxygen incubator (37°C, 21% O_2, and 5% CO_2). Cells with long spindle shape emerging from the edge of tissue block became visible gradually. After 2 weeks, the cells grew into a "peak and valley"-like dense cell layer, indicating that they could be subcultured.

Experimental treatments

After subculture, cells with passage numbers 3–10 were seeded in a sterile six-well plate at 1×10^5 cells/mL with 2 mL culture medium per well. When the cells reached 40%–60% of confluence, the cells were starved in serum-free DMEM medium for 24 h before treatment. Cells were divided into knockdown FAK, mTERF1, or negative control (NC) control groups, cultured in normal (37°C, 5% CO_2, and 21% O_2) or hypoxia (37°C, 5% CO_2, and 5% O_2) conditions.

FAK siRNA and mTERF1 siRNA sequences design and cell transfection

The FAK and mTEFR1 siRNA sequences were designed and synthesized by Suzhou Jingweizhi Ltd., China. The sequences are listed in Table 1.

The 2×10^5 primary cultured HPASMCs were inoculated into a sterile six-well plate and cultured in DMEM medium supplemented with 10% FBS. When cells reached 40%–60% of confluence, they were starved in the serum-free medium for 24 h before 50 μM siRNA was transfected by Lipofectamine 2000 (Life Technology, USA) according to manufacturer's instructions. After 6 h, the medium was replaced with DMEM medium containing 2% FBS for continued culture. Changes in target gene expression were assessed by RT-PCR and Western blot 24 h after transfection.

Cell counting kit-8 detection

Cell proliferation was assessed using the Cell counting kit-8 (CCK8). In brief, 1×10^5 cells were seeded in a 96-well plate. After 24 h of transfection, the cells were cultured based on the experimental design. Then, 10 μL of CCK8 solution was added to each well. The cells were incubated for 2 h in the cell incubator and the absorbance was measured at 450 nm (Bio-Red, USA).

Western blot

The cells were harvested 2 h after stimulation and the total protein was extracted by adding the modified RIPA lysis buffer. The protein concentration was measured using the Bradford method. Then, 30 μg total protein for each sample was loaded onto the 10%–15% SDS-PAGE for electrophoresis. Proteins were then transferred to polyvinylidene fluoride transfer membranes followed by blocking nonspecific binding with skim milk for 2 h at room temperature. The membranes were then incubated with the appropriate primary antibodies: FAK (1:1000), cyclin D1 (1:1000), mTERF1 (1:2000), and β-actin (1:3000) overnight at 4°C. The next day, the membranes were washed with phosphate-buffered saline (PBS) three times (10 min each time). Subsequently, the membrane was incubated with an appropriate secondary antibody for 3 h at room temperature. The bound antibody was detected using the ECL substrate assay kit and the protein bands were analyzed with Image J software (Rawak Software, Germany) for grayscale value. The protein level was expressed as the ratio of the densitometry value of the respective protein band to the densitometry value of the β-actin protein band.

Real-time polymerase chain reaction

Total RNA was extracted by the TRIzol method, and the purity and concentration of RNA were determined by UV spectrophotometer. The reverse transcription reaction was carried out with the RT-PCR kit from Takara Bio (Japan) according to the manufacturer's instructions. The internal reference β-actin human PCR primers were provided by Guangzhou Ribobio Ltd., China. The human FAK, mTERF1, and cyclin D1 primers were designed and synthesized by Sangon Biotech (Shanghai) Ltd., China, and the primer sequences are listed in Table 2.

Real-time RT-PCR reaction conditions were as follows: pre-denaturation at 95°C for 10 s, denaturation at 95°C for 5 s, annealing/extension at 60°C for 20 s, and a total of 45 cycles. The relative gene expression was expressed by the $2^{-\Delta\Delta Ct}$ method.

TABLE 1 siRNA sequences for FAK and mTERF1 targeting.

siRNA name	Sequence
mTERF-siRNA1	Sense: 5′-UCUUGAACCAAAGAGAAAGUU-3′ Anti-sense: 5′-CUUUCUCUUUGGUUCAAGAUG-3′
mTERF-siRNA2	Sense: 5′-UAGUAAGUAAGUUUUUCAGUA-3′ Anti-sense: 5′-CUGAAAAACUUACUUACUAUG-3′
mTERF-siRNA3	Sense: 5′-AAAAUCUGCGGGAUCAUUGUG-3′ Anti-sense: 5′-CAAUGAUCCCGCAGAUUUUGU-3′
FAK-siRNA1	Sense: 5′-UGAAAGACCUUUAAUACUCGC-3′ Anti-sense: 5′-GAGUAUUAAAGGUCUUUCAUU-3′
FAK-siRNA2	Sense: 5′-UGAAAGACCUUUAAUACUCGC-3′ Anti-sense: 5′-GAGUAUUAAAGGUCUUUCAUU-3′
FAK-siRNA3	Sense: 5′-UUUUUCUAAUACUUCAUAGUU-3′ Anti-sense: 5′-CUAUGAAGUAUUAGAAAAAGA-3′
si-NC	Sense: 5′-UAUAUGACAGUAUAACGGCA-3′ Anti-sense: 5′-UGCCGUUAUACUGUCAUAUA-3′

TABLE 2 Primer sequences for the study.

Gene name	Primer sequence
FAK	Forward: 5′-ATCCCACACATCTTGCTGACTT-3′ Reverse: 5′-GCATTCCTTTTCTGTCCTTGTC-3′
mTERF1	Forward 5′-TATCCACGAGCAATAACACG-3′ Reverse 5′-TTACGGGTCAATCCAACTGAG-3′
Cyclin D1	Forward 5′-CGGAGGAGAACAAACAGATCAT-3′ Reverse 5′-AGGCGGTAGTAGGACAGGAAGT-3′
β-Actin	Forward 5′-CTCCATCCTGGCCTCGCTGT-3′ Reverse 5′-GCTGTCACCTTCACCGTTCC-3′

Immunofluorescence

Sterile coverslips were placed in a six-well tissue culture plate at one coverslip/well before cell culture. The subcultured HPASMCs were seeded at 1×10^5 cells/well to allow the cells to grow on the coverslips After various experiment condition exposures, the cells were then rinsed with PBS several times before fixation with 4% paraformaldehyde solution at room temperature for 10 min. The cells were then rinsed with PBS and cell membranes were permeabilized with 0.5% Triton X-100 solution at room temperature for 10 min. The cells were again rinsed with PBS followed by the removal of the coverslips which were then attached to the slides with neutral gums. The slides were placed in a wet box and incubated with 5% bovine serum albumin for 60 min to block the nonspecific binding sites. A goat anti-FAK (1:200) and calponin-1 (1:100) primary antibodies were added dropwise and the slides were then incubated overnight at 4°C. The next day, the slides were rinsed with PBS before the addition of Cy3-labeled corresponding anti-goat secondary antibodies. Following incubation in a 37°C incubator for 2 h, the slides were rinsed again with PBS. DAPI (5 μg/mL) was used to stain the nucleus and the expression of FAK and cyclin D1 in HPASMCs were then observed and photographed under the fluorescence microscope (Leica, Germany).

Mitochondria integrity analysis by flow cytometry

HPASMCs cultured in various conditions for 24 h were trypsinized and made into signal cell suspension (1×10^6/mL) in PBS. Then, 1 mL of Rhodamine 123 working solution was added into cell suspension and incubated in a 37°C incubator for 20 min. Cells were washed with PBS two times before being suspended in 0.5 mL of PBS. Flow cytometry analysis was performed with Attune NXT (Invitrogen, USA), with excitation wavelength at 488 nm, and emission wavelength at 529 nm. The data were analyzed by FlowJo V10.

Statistical analysis

Results were analyzed with GraphPad Prism5 software (GraphPad Software, USA). For the comparison among more than two groups, a two-way analysis of variance was performed. For the comparison between two groups, the student–Newman–Keuls q test was used. Differences at $p<0.05$ were statistically significant. All experiments were repeated more than three times.

RESULTS

In vitro culture of primary HPASMCs

The primary culture of HPASMCs isolated from human subjects was established as described in the Method section. In vitro cultured cells were then stained with actin and calponin-1, a smooth muscle cell marker for confirmation. As shown in Figure 1a, the cultured cells exhibited long spindle shape morphology with cluster-like parallel arrangement. A characteristic "peak-valley" growth pattern was observed when cells had grown to post-confluence. Immunohistochemistry staining with actin showed that the cells contained rich actin filament (Figure 1b). Immunofluorescent staining of smooth muscle cell marker calponin-1 (Figure 1c) demonstrated that these cells were indeed smooth muscle cells.

Knockdown FAK and MTEFR1 could inhibit cell proliferation

Three sets of siRNAs each targeting FAK and mTEFR1 were tested for their effect on reducing the expression levels of the genes. As shown in Figure 2a, three different siRNAs targeting FAK all significantly reduced the expression of FAK both assessed with RT-PCR and Western blot analysis, compared to the random control siRNA (si-NC), with si-FAK-3's effect being the most prominent. Similarly, as shown in Figure 2b, the three sets of siRNAs targeting mTERF1 also reduced the expression of mTERF1, with

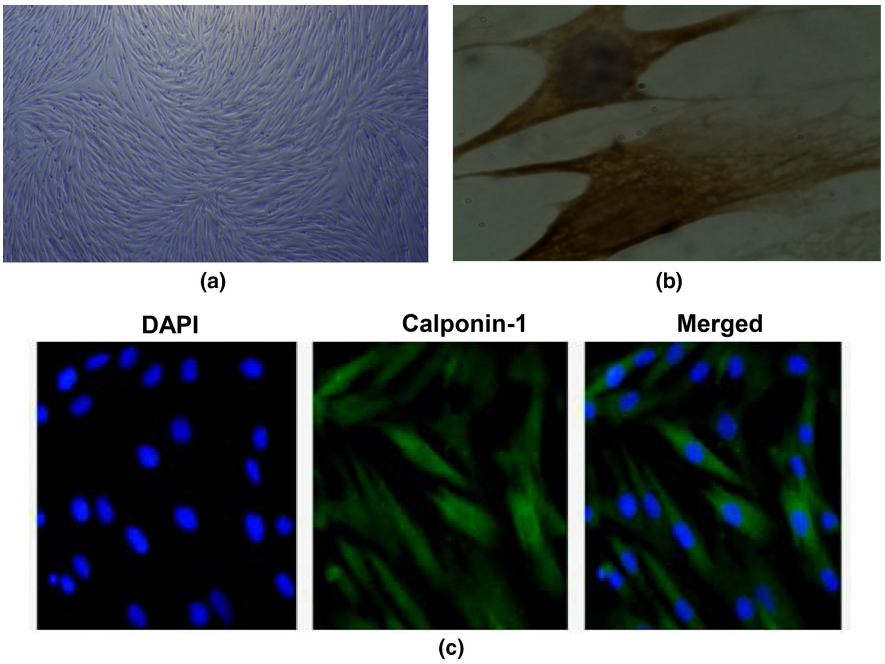

FIGURE 1 In vitro culture of primary HPASMCs. (a) Phase contrast microscopic picture of cultured HPASMCs (×100), when primary culture of HPASMCs reached confluence, a significant "peak and valley" was observed. (b) Immunohistochemistry image of HPASMCs stained with anti-Actin antibody (×200), Actin filament was observable. (c) Immunofluorescent image of HPASMC stained with anti-Calponin-1 antibody (×200). The expression of Calponin-1 protein was positive in HPASMCs. HPASMCs, human pulmonary artery smooth muscle cells.

si-mTERF1-1 being the most efficient. Thus, si-FAK-3 and si-mTERF1-1 were used in the subsequent experiments.

HPASMC proliferation was assessed in both normal and hypoxia conditions. As shown in Figure 2c, untreated HPASMCs grew significantly faster under hypoxia conditions than normal culture conditions (H-NC siRNA vs. N-NC siRNA; $p < 0.01$), suggesting that hypoxia promoted HPASMCs proliferation. However, when siRNA was used to knock down the expression of FAK, as shown in Figure 2c, the proliferation of HPASMCs was reduced after FAK knockdown in both normal and hypoxia conditions compared to the respective controls (N-siFAK vs. N-NC siRNA, H-siFAK vs. H-NC siRNA). Similarly, when siRNA was used to knock down the expression of mTREF1, reduced growth of cells in both normal and hypoxia conditions after mTREF1 knockdown were observed compared to the respective controls (N-simTREF1 vs. N-NC siRNA, H-simTREF1 vs. H-NC siRNA; Figure 2c).

The effect of FAK and mTREF1 knockdown on cyclin D1

It has been shown that FAK can modulate cell cycle progression through modulating cyclin D1.[5] We assess the effect of FAK knockdown on the expression of cyclin D1. As shown in Figure 3a top panel and Figure 3b left panel, FAK protein level in HPASMCs was significantly reduced in siRNA-treated cells under both normal and hypoxia conditions ($p < 0.01$). The mRNA levels of FAK expression were also significantly reduced in both conditions ($p < 0.01$; Figure 3c). Meanwhile, as shown in Figure 3a second panel and Figure 3b middle panel, after FAK knockdown, the protein level of cyclin D1 did not change much under normal culture conditions but reduced significantly under hypoxia conditions ($p < 0.01$). Similar changes were also observed for cyclin D1 mRNA levels (Figure 3c).

This phenotype was also observed with an immunofluorescent staining method. As shown in Figure 4a,b, FAK-specific siRNA treatment significantly reduced the FAK staining signal in HPASMCs in both normal and hypoxia conditions. The staining signal of cyclin D1 was reduced in HPASMCs only under hypoxia conditions, but not under normal culture conditions after FAK was knocked down by siRNA. The quantitation of the signal changes is shown in Figure 4c.

The knockdown of mTERF1 had a similar effect on cyclin D1 expression as FAK knockdown. As shown in Figure 3a third panel and Figure 3b right panel, the mTREF1 protein level in HPASMCs was significantly reduced in siRNA-treated cells under both normal and hypoxia conditions. The mRNA levels of mTREF1 expression were significantly reduced in both conditions as well (Figure 3c). At same time, shown in Figure 3a second panel and Figure 3b middle panel, mTERF1 siRNA treatment led to significant protein level reduction in cyclin D1 only under hypoxia conditions but not under normal conditions. The cyclin D1 mRNA levels also showed similar changes (Figure 3c).

Interestingly, we also observed a reduction in both mRNA and protein levels of mTERF1 after FAK knockdown under hypoxia conditions only (Figure 3). However, knocking down mTERF1 with siRNA had no impact on FAK expression under either condition. Our results

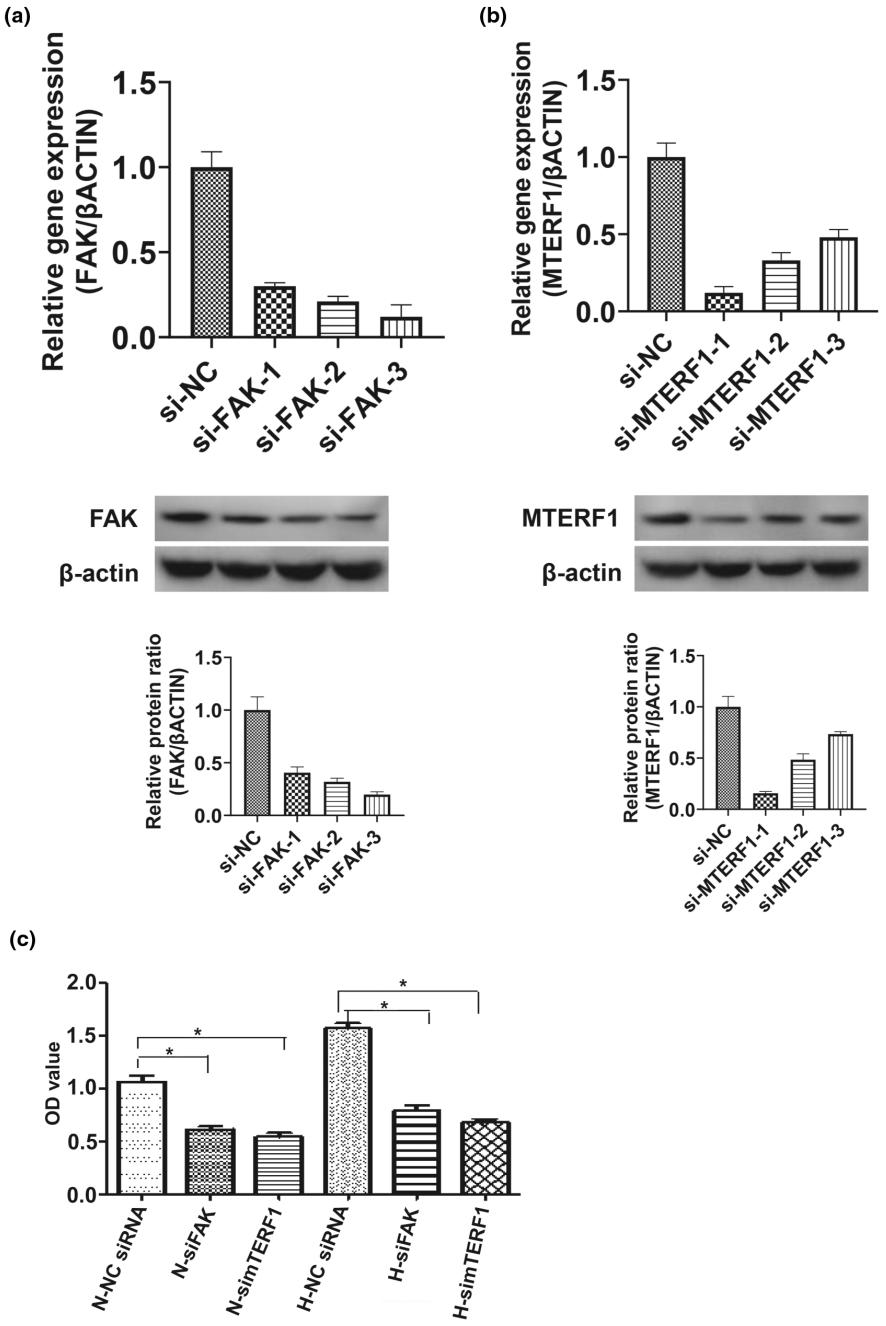

FIGURE 2 Knockdown FAK and mTEFR1 could inhibit cell proliferation. The effect of knocking down target genes (a) FAK and (b) mTEFR1 by three different siRNA constructs was assessed with RT-PCR and Western blot analysis. (c) The proliferation rate of cells indicated by absorbance at 450 nm upon knockdown of FAK and mTERF1 under normal and hypoxic conditions. *$p < 0.05$; H, hypoxia; N, normal; RT-PCR, real-time polymerase chain reaction.

suggested that mTERF1 may also be a downstream target gene of FAK, and the mTERF1 and cyclin D1 pathway may have an interplay in the response of FAK activation in HPASMCs under hypoxic conditions.

The effect of FAK and mTERF1 knockdown on mitochondrial function

Hypoxia conditions have a complicated impact on mitochondrial function. We studied whether the knockdown of FAK and mTERF1 had different impacts on mitochondrial function during normal and hypoxia conditions. Rhodamine 123 staining was used to assess the mitochondrial membrane potential of the HPASMCs cultured under different conditions. As shown in Figure 5, knockdown FAK or mTERF1 could cause the mitochondria to lose the membrane potential significantly under both normal and hypoxia conditions. This result indicated that both FAK and mTERF1 were important for mitochondria integrity.

DISCUSSION

In this study, we demonstrated that FAK inhibition led to reduced proliferation of primary HPASMCs under

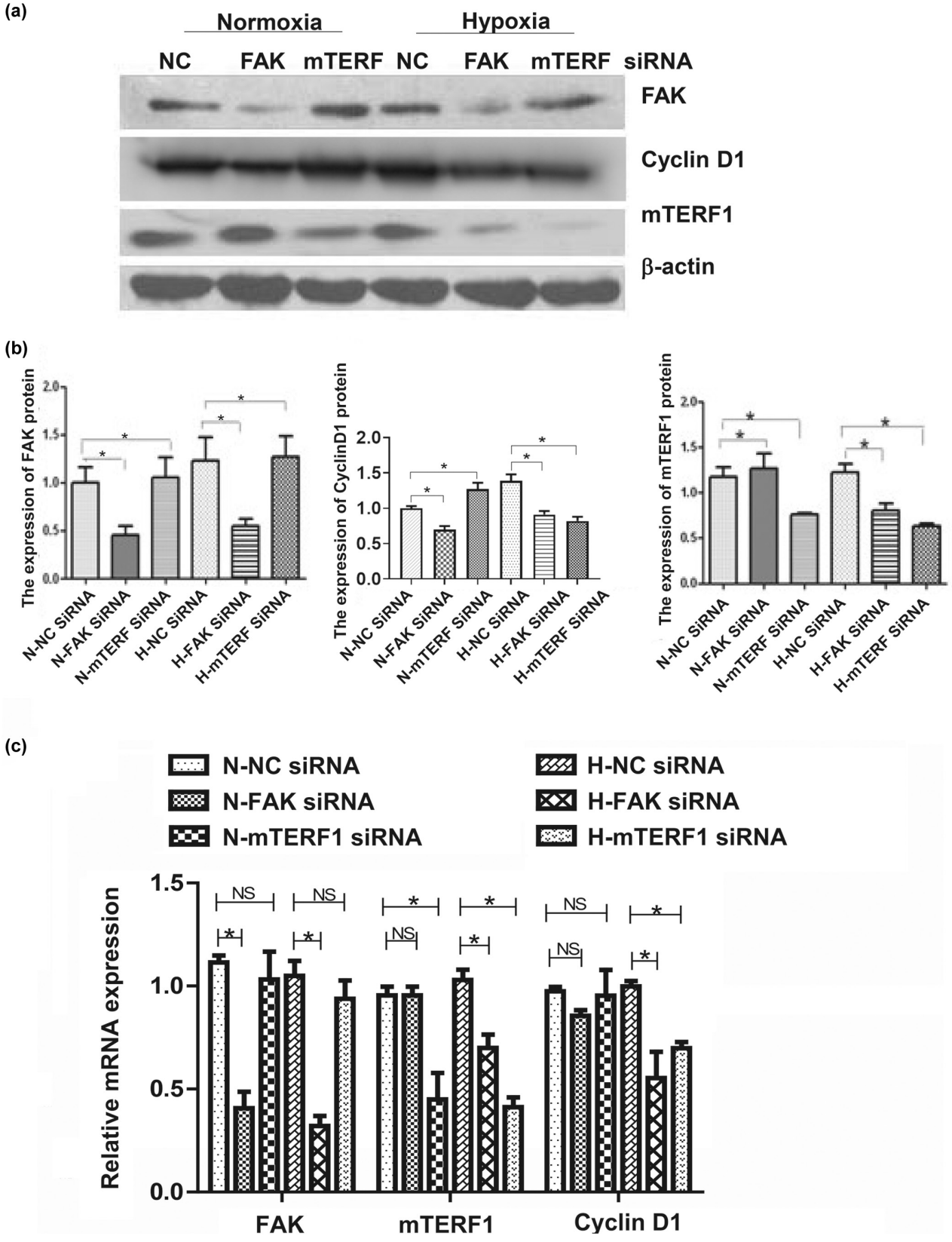

FIGURE 3 Effect of FAK and mTERF1 knockdown on the protein and mRNA expression levels of FAK, mTERF1, and cyclin D1 under hypoxic and normal conditions. (a) Representative Western blot images of FAK, mTERF1, and cyclin D1 expression in normal and hypoxia conditions treated with siRNA to FAK or mTERF1. β-Actin was used as the loading control. (b) Quantifying FAK, cyclin D1, and mTERF1 expression levels in the conditions shown in Western blot analysis. (c) The mRNA levels of FAK, mTERF1, and cyclin D1 treated with siRNA to FAK or mTERF1 in normal and hypoxic conditions $^*p < 0.05$; H, hypoxia; N, normal; NS, no significance.

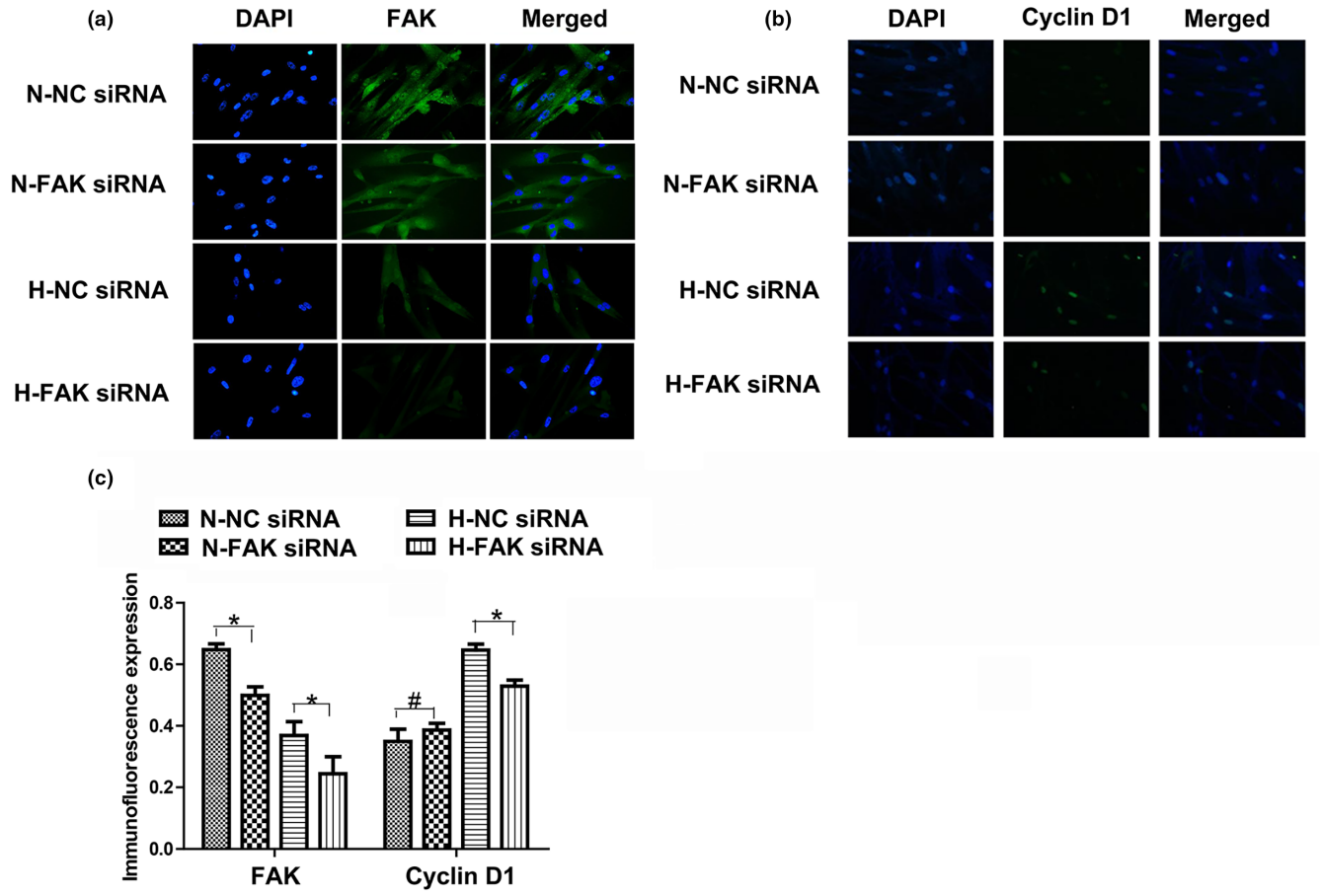

FIGURE 4 Immunofluorescence images for FAK and cyclin D1 staining on HPASMCs treated with siRNA to FAK in normal and hypoxia conditions. (a) The immunofluorescence staining image of FAK; (b) The immunofluorescence staining image of cyclin D1. (c) Quantitation of FAK and cyclin D1 expression levels, as shown in immunofluorescence staining. *$p < 0.05$; H, hypoxia; HPASMCs, human pulmonary artery smooth muscle cells; N, normal.

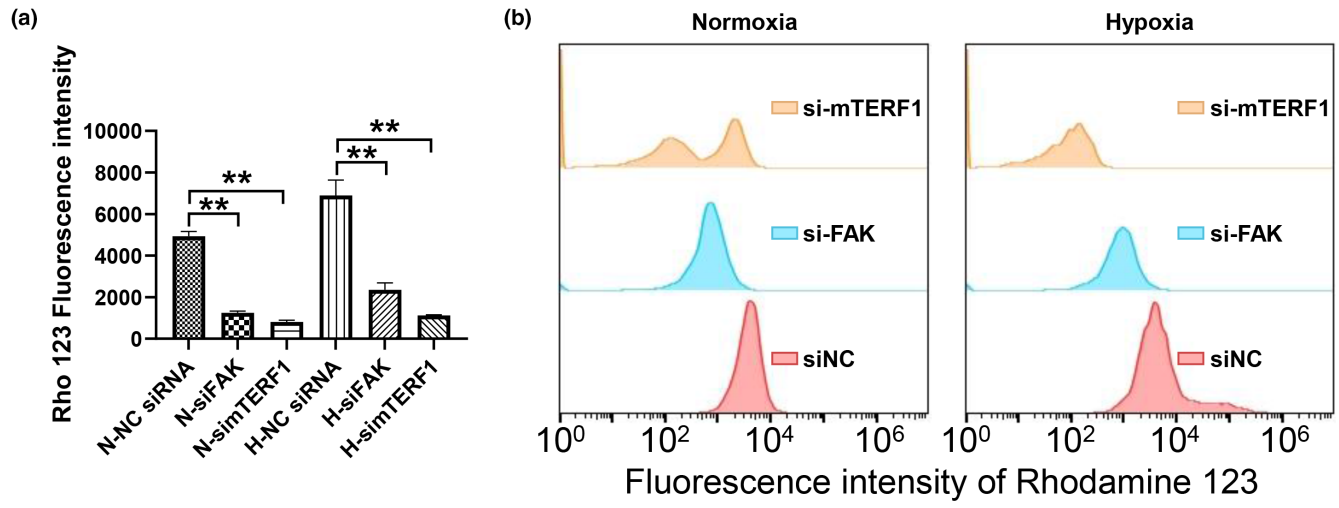

FIGURE 5 Flow cytometry analysis for HPASMCs stained with Rhodamine 123 to assess the mitochondria integrity. (a) Histogram graph for Rhodamine 123 staining on HPASMC under various treatments. (b) Quantitation of fluorescence intensity of Rhodamine 123 on HPASMC. **$p < 0.01$; HPASMCs, human pulmonary artery smooth muscle cells.

both normal and hypoxia conditions, while it only led to reduced expression of mTERF1 and cyclin D1 under hypoxia. Our results suggested that mTERF1 and cyclin D1 may be the downstream targets for FAK-mediated hypoxia response in HPASMCs, providing potential targets for PAH intervention.

Previous studies have shown that hypoxia can activate FAK to regulate the proliferation and migration of HPASMCs.[11,26] The mechanism of FAK in modulating HPASMCs during hypoxia response is not entirely clear. Studies have also shown that PAH is associated with mitochondrial abnormalities, including mitochondrial fragmentation.[12,17] Moreover, reduced cyclin D1 levels were shown to exert a protective effect on PAH.[6] However, no studies have investigated the link between FAK, mitochondria, and cyclin D1, and the effect of their interaction on cell proliferation. In this study, we showed that hypoxia promoted HPASMC proliferation, which is consistent with previous studies.[26] When FAK expression was reduced by siRNA, cell proliferation was hindered. Under hypoxia conditions, knocking down FAK in HPASMCs led to a reduction of the expressions of both mTERF1 and cyclin D1, which was not observed under normal culture conditions. A decrease in cyclin D1 expression was also observed in HPASMCs under hypoxia conditions when mTERF1 expression was knocked down by siRNA. The findings that knocking down both FAK and mTERF1 could significantly damage the mitochondria integrity under both normal and hypoxia conditions further underlie the importance of these two proteins to mitochondrial functions. These observations prompted us to propose a signaling pathway between FAK and mTERF1/cyclin D1 to influence cell proliferation of HPASMCs during hypoxia.

The mTERF1 is known to be a DNA-binding protein that functions as a transcriptional terminator for mtDNA by unwinding DNA, promoting the eversion of three nucleotides upon DNA binding.[27] Although the mTERF family is known to be vital for mitochondrial DNA replication, they are also found involved in other cellular processes. Overexpression of mTERF1 in HT29 cells is shown to promote cell proliferation, migration, invasion, and xenograft tumor formation. It is shown that mTERF1 regulates the AMPK/mTOR signaling pathway in cancer cells.[28]

It has been suggested that cyclin D1 inhibits mitochondrial metabolism by inhibiting phosphorylation of nuclear respiratory factor 1.[29] Cyclin D1 competes with hexokinase to suppress mitochondrial function by binding to mitochondrial voltage-dependent anion channels.[30] However, the exact interaction between mTERF1 and cyclin D1 under hypoxia still needs to be investigated.

AUTHOR CONTRIBUTIONS

C.L. designed the research. C.L., H.Y., Q.Lu., and Q.Li. performed the research and analyzed the data. C.L., H.Y., Q.Lu., and Q.Li. wrote the manuscript.

FUNDING INFORMATION

No funding was received for this work.

CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT AND CONSENT TO PARTICIPATE

The study was approved by Yueyang municipal Hospital of Hunan Normal University Ethics Committee (2015030206). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from individual participants.

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How to cite this article: Lin C, Yang H, Luo Q, Liu Q. FAK mediates hypoxia-induced pulmonary artery smooth muscle cell proliferation by modulating mitochondrial transcription termination factor 1/cyclin D1. *Clin Transl Sci*. 2024;17:e13767. doi:10.1111/cts.13767