OVEREXPRESED LNCRNA AC068039.4 CONTRIBUDES TO PROLIFERATION AND CELL CYCLE PROGRESSION OF PULMONARY ARTERY SMOOTH MUSCLE CELLS VIA SPOISING MIR-26A-5P/TRPC6 IN HYPOXIC PULMONARY ARTERIAL HYPERTENSION

Yuhan Qin, Boqian Zhu, Linqing Li, Dong Wang, Yong Qiao, Bo Liu, Erfi Luo, Jiantong Hou, Gaoliang Yan, and Chengchun Tang

ABSTRACT—Background: Hypoxic pulmonary hypertension (HPH) is a devastating and incurable disease characterized by pulmonary vascular remodeling, resulting in right heart failure and even death. Accumulated evidence has confirmed long non-coding RNAs (IncRNAs) are involved in hypoxia-induced pulmonary vascular remodeling in HPH. The exact mechanism of IncRNA in hypoxic pulmonary hypertension remains unclear. Methods: Microarray analysis was applied to investigate the profiles of IncRNA expression in pulmonary artery smooth muscle cells (PASMCs) cultured under hypoxia and normoxia condition. qRT-PCR was performed for the expression of IncRNAs, miRNA, and mRNAs, western blot analysis was employed for the detection of the expression of proteins. CCK-8 and transwell chamber assay were applied for the assessment of PASMC proliferation and migration, respectively. Besides, flow cytometry was performed for assessments of cell cycle progression. The binding between AC068039.4 and miR-26a-5p, miR-26a-5p, and TRPC6 3’UTR was detected by dual luciferase reporter assay. Results: A total of 1,211 IncRNAs (698 up-regulated and 513 down-regulated) were differently expressed in hypoxia-induced PASMCs. Consistent with microarray analysis, quantitative PCR verified that AC068039.4 was obviously up-regulated in hypoxia-induced PASMCs. Knocking down AC068039.4 alleviated proliferation and migration of PASMCs and regulated cell cycle progression through inhibiting cells entering the G0/G1 cell cycle phase. Further experiment indicated AC068039.4 promoted hypoxic PASMCs proliferation via sponging miR-26a-5p. Conclusion: In conclusion, downregulation of IncRNA AC068039.4 inhibited pulmonary vascular remodeling through AC068039.4/miR-26a-5p/TRPC6 axis, providing new therapeutic insights for the treatment of HPH.

KEYWORDS—Hypoxic pulmonary hypertension, IncRNA AC068039.4, miR-26a-5p, PASMCs, TRPC6

INTRODUCTION

Pulmonary hypertension (PH) is an incurable disease with complex pathogenesis and characterized by over-proliferation, apoptosis resistance of pulmonary artery smooth muscle cells (PASMCs), pulmonary vascular remodeling (PVR), and elevated pulmonary vascular resistance, resulting in increased pulmonary arterial pressure, right heart failure, and even death (1, 2). It is convincing that over-proliferation of PASMCs is the pathophysiological basis of PAR and plays a vital role in the occurrence and development of PH (3). Hypoxic pulmonary hypertension (HPH) is a common cause of PH in multiple hypoxia and lung diseases, such as chronic obstructive pulmonary disease, interstitial lung disease, sleep-disordered breathing, and chronic exposure to high altitude (4). Although clinical symptoms could be relieved under current treatments, no

specifically effective treatments for HPH have been developed and disease progression remains inevitable (5). Besides, no specific symptoms usually lead to delay in diagnosis and poor prognosis (6). Therefore, further exploration of the pathogenesis of HPH is of great significance for finding novel effective treatments.

Based on the development of next-generation sequencing technology, a large amount of IncRNAs, longer than 200 nucleotides and incapable of protein transcription, have been identified exert their role in diverse biological process (7–9). The “competitive endogenous RNA (ceRNA) hypothesis (microRNA sponge)” is a critical possible mechanism involving in gene regulation, indicating that IncRNA could sponge miRNA and participate in miRNA regulation, indirectly involves in the regulation of miRNA-targeted genes (10). ceRNA activity forms regulatory network across transcriptome on a large scale, immensely enlarging the functional genetic information and exert crucial role in pathogenesis of disease (10). To date, numerous IncRNAs have been identified to act as miRNA sponges to regulate the downstream gene expression, ultimately contributing to regulation of cellular events (11–15). The interaction between IncRNA and miRNA has been demonstrated in modulation of cell proliferation and apoptosis (16). Previous studies have identified the role of several emerging...
IncRNAs, such as MEG3 (17), LncRPT (18), Tug1 (19), MALAT1 (20), Hoxaas3 (21), CASC2 (22), is involved in the regulation of PASMCs proliferation, cell cycle progression and apoptosis of PASMCs, and modulation of PVR. However, the role of IncRNA in HPH remains largely unknown. The previous study identified a significantly expressed novel LncAC068039.4.

The previous study demonstrated that miR-26a was down-regulated in both plasma from PAH patients and lung tissues of monocrotaline (MCT)-induced rats (23). Store-operated calcium channels (SOCE)-induced store-operated calcium entry (SOCE) is the main cause of calcium imbalance in PASMCs under hyperoxia, transient receptor potential canonical 6 (TRPC6) is the main component of SOCE, up-regulated TRPC6 is related to PASMCs phenotype from contractile phenotype to the proliferative phenotype (26). The previous study demonstrated IncRNAs may participate in the regulation of TRPC6 expression (27).

Therefore, the current research aims to explore the role of IncRNA AC068039.4 in the regulation of pathogenesis of HPH. AC068039.4 was found up-regulated in hyperoxia-induced PASMCs. AC068039.4 interacted with and sponged miR-26a-5p, resulting in decreased degradation of TRPC6 and increased TRPC6 expression, ultimately contributing to over-proliferation of PASMCs. AC068039.4/miR-26a-5p/TRPC6 axis might provide a novel therapeutic target for HPH treatment.

**MATERIALS AND METHODS**

**Cells culture and ethics statement**

The human PASMCs used in the experiment were isolated from the distal pulmonary arterioles in three patients undergoing pulmonary lobectomy with similar clinical basic data and normal pulmonary artery pressure. This study was approved by the Medical Ethics Committee for Clinical Research of Zhongda Hospital (Nanjing, China). All subjects signed informed consent before the research. After removing the adventitia and intima of the pulmonary artery, the pulmonary artery smooth muscle was cut into small pieces of 1 × 1 mm², and then transferred to a culture flask. Isolated PASMCs were cultured in DMEM/F12 (HyClone, Calif) containing 10% fetal bovine serum (FBS) (Gibco, San Diego, NY), supplemented with 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO₂ (FBS) (Gibco, San Diego, NY), supplemented with 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO₂.

**Cell proliferation assay**

The cells were seeded in a 96-well plate at a density of 5 × 10³ cells per well, and 10 μL of CCK-8 reagent (KGA317, KeyGEN BioTECH, Nanjing, China) was added to each well, then the cells were incubated in a 37°C incubator for 4 h. A microplate reader (SYNERGY/H4, BioTek, USA) was used to detect the absorbance value of the cells at a wavelength of 450 nm (OD 450 nm) to analyze the proliferative activity.

**Cell migration assay**

Transwell chambers (Corning, USA) were used to perform cell migration assay to evaluate the migration potential of PASMCs according to the manufacturer’s introductions. The upper Transwell chamber, where PASMCs with different treatments were added, contains 0.5% FBS medium, while the lower chamber was filled with 10% FBS medium. After culturing for 18 h, cells were washed with PBS and stained with crystal violet at room temperature for 15 min and then dried. A microscope was used for observation (Olympus, Japan).

**Cell cycle progression**

Cells were collected after trypanosynthesis, centrifuged and resuspended in 1 mL cold PBS. Then, cells were washed twice with PBS and fixed with precooled 70% ethanol at 4°C overnight. The fixed cells were collected after centrifugation, and then resuspended in 500 μL of staining buffer, 100 μL of RNaseA was added, then add 25 μL propidium iodide, the suspension was subjected to a water bath at 37°C for half an hour. Finally, the cells were filtered through a 400 mesh sieve, flow cytometry was used to detect whether the cells are in the G0/G1, S or G2/M cell cycle.

**Measurements of [Ca²⁺],**

Calcium measurement was performed as previously described (28). In brief, fluo-4 AM fluorescent probes were synthesized, M200 PRO plate-reader was used to detect fluorescence intensity and semiquantitatively analyze changes in calcium ions in cells.

**Total RNA isolation and quantitative reverse transcription PCR**

Total RNA from PASMCs under different treatments was extracted with RNAsio Plus reagent (9109, TaKaRa, Shiga, Japan) according to the manufacturer’s protocol. Nanodrop 2000 (Thermo Scientific, Mass) was used to measure concentration of RNA. Equal amounts of total RNA were reversed transcribed into cDNA using Prime-Script RT kit (RR047A, Takara, Japan). RT-PCR was performed with the TB GreenTM Premix Ex Taq TM II (RR820A, Takara, Japan) and Primis 7500 SDS (Thermo Scientific, Mass). The CT values of the target genes were recorded, relative gene expression was calculated using the 2⁻DDCT method, and β-actin or U6 were used as internal references. Primers sequences are listed in Table 2.

**Western blot analysis**

Cell total protein extraction kit (KGP2100, KeyGEN BioTECH, Nanjing, China) was used to extract total cellular proteins, and the protein concentration was measured using the BCA Detection Assay Kit (KGP602, KeyGEN BioTECH, Nanjing, China) according to the manufacturer’s instructions. An equal amount (20 μg) of the extracted proteins were subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a PVDF membrane (Millipore, Billerica, Mass). Membranes were blocked in TBST containing 5% non-fat milk for 1 h and incubated with primary antibody overnight. Antibodies used in the experiment included anti-TRPC6 antibody (ab62461), anti-PCNA antibody (ab92552), anti-Cyclin A1 (ab53699), anti-Cyclin D1 (ab53699), anti-Cyclin E1 (ab133266), and anti-ACTIN antibody (ab8226) that were purchased from Sigma-Aldrich (St. Louis, MO). The bands were visualized with ECL Western Blotting Detection Reagents (KGP110, KeyGEN BioTECH, Nanjing, China).

**Table 1. Sequences of sh-AC068039.4, miR-26a-5p-mimic, and miR-26a-5p-inhibitor**

| Gene          | Sequence (5'-3')          |
|---------------|---------------------------|
| shAC068039.4-1 | AGAACATCAAGGAGAAATA       |
| shAC068039.4-2 | CCAACATGCAATTAGTTA        |
| shAC068039.4-3 | AATATGCTGATGTGAAAA        |
| miR-26a-5p mimic | UUCAGUUAUCGAGAAGGCUC     |
| miR-26a-5p inhibitor | AGGCTATCTCGTGAATCTTGAAGA  |
regulated lncRNAs) (fold change $>2.0$, $P$ value $<0.05$), indicating that these lncRNAs may be involved in the development of hypoxic pulmonary hypertension. In order to verify the reliability of the sequencing results, five significantly up-regulated lncRNAs and five down-regulated lncRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) (Fig. 1B), and the results indicated that they were consistent with the microarray analysis experiment. The expression of lncRNA AC068039.4 in hypoxia-induced PASMCs was significantly up-regulated compared with the normoxic group, and RNA expression of AC068039.4 was significantly increased with nearly three folds higher than that of the control group.

LncRNA AC068039.4 was upregulated in hypoxia-induced PASMCs and down-regulation of AC068039.4 alleviated hypoxia-induced proliferation and migration in PASMCs

Previous studies have shown that hypoxia can promote PASMCs proliferation and transwell. CCK-8 and transwell chamber analysis were used to investigate the effect of hypoxia on the proliferation and migration of PASMCs. As is shown in Figure 2A, CCK8 assay analysis showed that after hypoxia exposure 0, 24, 48, and 72 h, PASMCs proliferation capacity increased in a time-dependent manner. Figure 2B illustrated the transwell chamber analysis indicated that hypoxia significantly promoted the migration of PASMCs, which is also time-dependent. The results of microarrays analysis and PCR verification have shown that AC068039.4 is significantly up-regulated in hypoxia-induced PASMCs. Figure 2C further clarifies the effect of different hypoxia exposure times on the expression of AC068039.4 in PASMCs. Quantitative PCR indicated that the expression of AC068039.4 increased significantly under hypoxic conditions. After 48 h of hypoxia culture, the mRNA level of AC068039.4 was three times that of the normoxic control group. Lentivirus-mediated specific shRNA and lentivirus overexpressing AC068039.4 were synthesized and transfected into PASMCs to further clarify the role of AC068039.4 in hypoxia-induced proliferation and migration of PASMCs. Transfection with sh-AC068039.4 significantly decreased the expression of AC068039.4 mRNA and sh-AC068039.4–1 was used for further experiment for its best transfection efficiency. Transfection with lentivirus overexpressing AC068039.4 also effectively up-regulated the AC068039.4 mRNA expression (Fig. 2D). Down-regulation of AC068039.4 could effectively reduce the proliferation potential of PASMCs and effectively reversed the hypoxia-induced hyperproliferation of PASMCs while overexpression of AC068039.4 could further promote PASMCs proliferation (Fig. 2E). In addition, knockdown of AC068039.4 reversed the hypoxia-induced upregulation of PCNA protein expression in PASMCs; however, overexpression of AC068039.4 under hypoxia exposure further upregulated the hypoxia-induced PCNA protein level (Fig. 2F). Consistently with western blot analysis, qRT-PCR showed that the PCNA protein expression in PASMCs; however, overexpression of AC068039.4 under hypoxia exposure further upregulated the hypoxia-induced PCNA protein level (Fig. 2F). Consistently with western blot analysis, qRT-PCR showed that the PCNA protein expression in PASMCs; however, overexpression of AC068039.4 under hypoxia exposure further upregulated the hypoxia-induced PCNA protein level (Fig. 2F). Consistently with western blot analysis, qRT-PCR showed that the PCNA protein expression in PASMCs; however, overexpression of AC068039.4 under hypoxia exposure further upregulated the hypoxia-induced PCNA protein level (Fig. 2F).
hypoxia-induced PASMCs over-proliferation and elevated migration potential.

**LncRNA AC068039.4 was involved in hypoxia-induced PASMCs cell cycle progression**

Flow cytometry was used to clarify the role of AC068039.4 in the regulation of cell cycle progression; it is clear that hypoxia can promote the cell cycle transformation from G0/G1 phase to S + G2/M (29). Figure 3A and B indicated that down-regulation of AC068039.4 reduced the increased percentage of cells in the G2/M and S phases induced by hypoxia. In contrast, AC068039.4 overexpression increased the percentage of cells in the G2/M and S phase. Cyclin A/D/E are reported as key cell cycle proteins in S and G2/M phases, and widely regarded as cell proliferation markers (30). To determine the role of AC068039.4 on the regulation of cell cycle progression, western blotting and qPCR were performed to analyze the protein and mRNA expression levels of cyclin A1, cyclin D1, and cyclin E1. As illustrated in Figure 3C and D, both protein and mRNA levels of cyclin A1, cyclin D1, and cyclin E1 were reduced in PASMCs transfected with sh-AC068039.4 compared with negative control. Consistently, compared with negative controls, overexpression of AC068039.4 increased the expression levels of these proteins and mRNA. To sum up, these data indicated that AC068039.4 can promote cell proliferation through regulation of the cell cycle progression.
Fig. 2. **Down-regulation of AC068039.4 alleviated hypoxia-induced PASMCs proliferation and migration.** A, CCK8 assay analysis of PASMCs proliferation capacity after hypoxia exposure 0, 24, 48, and 72 h. B, Transwell chamber analysis of the migration potential of PASMCs cultured at 0, 24, 48, and 72 h of hypoxia. C, Quantitative PCR of AC068039.4 in PASMCs after 24, 48, and 72 h exposure to hypoxia compared with the normoxic group. D, qPCR analysis of validity of shRNA lentivirus against AC068039.4 and lentivirus overexpressing AC068039.4. E, CCK8 assay analysis of PASMCs proliferation capacity transfected with sh-AC068039.4 and OE-AC068039.4 respectively. (F) PCNA protein level and PCNA mRNA level (G) in hypoxia-induced PASMCs model transfected with sh-AC068039.4 and OE-AC068039.4. H, Transwell chamber analysis of migration potential of PASMCs transfected with sh- and OE-AC068039.4. Each experiment was repeated at least three times. * indicates statistical difference ($P < 0.05$) and ** represents $P < 0.01$. 

Copyright © 2020 by the Shock Society. Unauthorized reproduction of this article is prohibited.
LncRNA AC068039.4 promoted hypoxic PASMCs proliferation by interaction with miR-26a-5p

miRNA screens of plasma from PAH patients and MCT-induced PH rats demonstrated that miR-26a was significantly downregulated compared to healthy controls. In addition, circulating miR-26a was identified as a novel biomarker candidate for PAH and closely correlated with 6-min walk distance (23). qPCR analysis was conducted to detect the expression of miR-26 in PASMCs and demonstrated that miR-26a-5p significantly decreased as the hypoxia exposure time increased (Fig. 4A). Therefore, we investigated the effect of AC068039.4 on miR-26a-5p in hypoxia. qPCR analysis

**Fig. 3.** AC068039.4 was involved in hypoxia-induced PASMCs cell cycle progression. A, Cell cycle distribution was assessed by flow cytometry and analyzed quantitatively (B). C, Protein expression levels of cyclin A1, cyclin D1, and cyclin E1 were analyzed by western blotting following transfection with sh-AC068039.4 and OE-AC068039.4. D, mRNA levels of cyclin A1, cyclin D1, and cyclin E1 were analyzed by qPCR following transfection with sh-AC068039.4 and OE-AC068039.4. * and ** represent P < 0.05, P < 0.01 respectively.
showed reduced miR-26a-5p expression caused by hypoxia can be partially reversed after PASMCs was transfected with sh-AC068039.4 and miR-26a-5p was further downregulated in PASMCs transfected with OE-AC068039.4 compared with negative control, suggesting that miR-26a-5p could be regulated by AC068039.4 (Fig. 4B). Dual luciferase reporter gene assay was performed after cotransfection of mimic NC and miR-26a-5p mimic with WT-AC068039.4 and MUT-AC068039.4. The gene sequence of WT and MUT-AC068039.4 was listed in Figure 4C. Comparison to the mimic NC group, decreased luciferase activity was observed in cells cotransfected miR-26a-5p mimic and WT-AC068039.4, whereas no evident differences were detected when cotransfected miR-26a-5p mimic and MUT-AC068039.4, suggesting miR-26a-5p could bind to AC068039.4 (Fig. 4D). CCK8 analysis showed that the effect of inhibition of hypoxia-induced PASMCs proliferation was counteracted after miR-26a-5p transfection. Taken together, AC068049.4 could exert its role in hypoxia-induced PASMCs proliferation through regulation of miR-26a-5p.

LncRNA AC068039.4 promoted hypoxic PASMCs proliferation through AC068039.4/miR-26a-5p/TRPC6 axis

Previous studies have demonstrated TRPC6 plays a vital role in hypoxic pulmonary vascular remodeling and PASMCs proliferation and migration (28). Figure 5A and B illustrated that both mRNA and protein level of TRPC6 were significantly upregulated in PASMCs exposed to hypoxia condition. As shown in Figure 5C, the expression of TRPC6 protein decreased in PASMCs transfected with sh-AC068039.4, in contrast,
up-regulated TRPC6 protein was found in PASMCs transfected with OE-068039.4. The expression of TRPC6 mRNA is consistent with TRPC6 protein expression after transfection with sh-AC068039.4 and OE-AC068039.4 compared with negative control (Fig. 5D). Based on that AC068039.4 was involved in hypoxic PASMCs proliferation through interaction with miR-26a-5p, TRPC6 could be regulated by AC068039.4, we wonder whether miR-26a-5p exerted its role in regulation of hypoxia-
induced PASMCs proliferation through targeting TRPC6. Western blotting indicated the expression of TRPC6 protein decreased in PASMCs transfected with miR-26a-5p mimic compared with mimic NC, miR-26a-5p inhibitor could further up-regulate the expression of TRPC6 protein (Fig. 5E), which is consistent with qPCR (Fig. 5F). Dual luciferase report analysis was performed to confirm the interaction between miR-26a-5p and TRPC6. Bioinformatics prediction software showed that miR-425-5p had a potential binding site to the 3’-UTR of TRPC6 mRNA (Fig. 5G). Comparison to the mimic NC group, decreased luciferase activity was observed in cells co-transfected miR-26a-5p mimic and WT-TRPC6, cells co-transfected miR-26a-5p mimic and MUT-TRPC6 render unresponsive, suggesting TRPC6 is a target gene of miR-26a-5p (Fig. 5H).

Our group has demonstrated that overexpression of TRPC6 increases the basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_{\text{b}}\)). Semiquantitative analysis of basal [Ca\(^{2+}\)]\(_{\text{b}}\), by F/F0 was performed, as shown in Figure 5I, the basal [Ca\(^{2+}\)]\(_{\text{b}}\), decreased after transfection of sh-AC068039.4, in contrast, overexpression of AC068039.4 increased [Ca\(^{2+}\)]. These data suggested that AC0689.4 may exert its biological role through the AC068039.4/miR-26a-5p/TRPC6 axis.

**DISCUSSION**

In this present research, microarray analysis was performed to search for differentially expressed lncRNA. Real-time PCR identified the significantly upregulated AC068039.4 in hypoxia-induced PASMC, which might serve as a novel biomarker candidate in HPH. Downregulation of AC068039.4 inhibited proliferation and cell cycle progression of PASMCs. Combining prediction software with dual luciferase report analysis, AC068039.4 was detected to exert its role via sponging miR-26a-5p. Furthermore, miR-26 could bind to TRPC6 3’UTR, resulting in decreased degradation of TRPC6 and increased TRPC6 expression. Taken together, a novel AC068039.4/miR-26a-5p/TRPC6 axis may involve in the development of hypoxic pulmonary hypertension.

The excessive proliferation of PASMCs plays an essential role in PVR (31). LncRNAs are endogenously RNAs lacking protein-encoding function with more than 200 nucleotide length (8). Multiple IncRNAs have been confirmed participating in the development of PH through the regulation of the proliferation of PASMCs and pulmonary vascular remodeling (32–35). Emerging IncRNAs have been discovered involved in the regulation of cardiovascular disease (36, 37). Gu et al. (38) first performed IncRNA expression profiles of lung tissues of 5 CTEPH patients and 5 healthy control tissues by microarray, and 185 differentially expressed IncRNAs were found. Liu et al. (35) conducted a microarray analysis and found 36 up-regulated IncRNAs and 111 down-regulated IncRNAs in the pulmonary arteries of HPH rats. TCONS_00034812 was identified remarkably reduced in PA of HPH rats, while Schlosser et al. (39) demonstrated no significant changes of IncRNAs in plasma of PAH and healthy subjects. The potential reasons are as followed: First, only a small fraction of known IncRNAs were measured in Kenny Schlosser’s research, other potential IncRNA biomarker candidates may be overlooked. Besides, the product standardization of commercial RT-qPCR kits used in the study remains to be checked. Lastly, scaling up the plasma starting volume might improve the detection rate of IncRNAs. Different from the measurement of plasma IncRNAs expression, we measure the expression of cellular IncRNA in PASMC, which are abundant inside PASMCs. Different from previous studies, in the current research, microarray analysis was conducted to find out the differently expressed IncRNAs in hypoxia and normoxia-induced PASMCs. Significantly differential IncRNAs were selected for PCR verification. AC068039.4 was found three times higher in hypoxia-induced PASMCs. Furthermore, down regulation of AC068039.4 suppressed the proliferation and migration of PASMCs induced by hypoxia, AC068039.4 also involved in cell cycle progression, knocking down AC068039.4 decreased the percentage of cells in the S and G2/M phases through inhibition of PASMCs entering into G1 phase.

ceRNA (miRNA sponge) is one of the main mechanisms of IncRNA–miRNA interactions. LncRNAs function as miRNAs sponges or bait to isolate miRNAs through complementary base pairing, leading to the loss or reduction of miRNA function, further involve in post-transcriptional regulation (40). CHRF was identified as endogenous sponge through downregulation of miR-489 to participate in the regulation of cardiac hypertrophy (41). Furthermore, MALAT1 sponges miR-124-3p and promoted proliferation of PASMCs. MEG3 and H19 acted as miRNA sponges of miR-328 and miR-675 respectively in PH (17, 42). In the current research, miR-26a expression was significantly down-regulated in hypoxia-induced PASMCs. Luciferase reports assay confirmed the binding between AC068039.4 and miR-26a. We demonstrated that miR-26a is the target gene of AC068039.4, overexpression AC068039.4 promoted PASMCs proliferation through downregulation of miR-26a expression in hypoxia-induced PASMCs.

The increase of free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{b}}\)) concentration is a vital factor to stimulate pulmonary vascular contraction and proliferation of PASMCs (43). Store-operated Ca\(^{2+}\) canonical (SOC) results in intracellular calcium imbalance in PASMCs under hypoxia, causing pulmonary artery contraction and remodeling (44). SOC is mainly composed of transient receptor potential canonical channel (TRPC) proteins (45), of which TRPC6 is a major protein (46). Previous research indicated that chronic hypoxia upregulated the expression of TRPC6 in pulmonary vein smooth muscle cells (PVSMCs) and [Ca\(^{2+}\)]\(_{\text{b}}\), and thereby promoted the proliferation and migration of PVSMC (24). Yu et al. (47) demonstrated up-regulated TRPC6 promoted PASMCs proliferation. Bosentan demonstrated downregulation of TRPC6 inhibits the proliferation of PASMCs (48). Gene sequencing revealed single-nucleotide polymorphism (SNP) in TRPC6 promoter region differs in idiopathic PAH patients (49). Our research showed consistent increased TRPC6 expression in hypoxia-induced PASMCs, but the upstream regulatory mechanism of TRPC6 remains largely unknown.

microRNAs (miRNAs) are a class of highly conserved non-coding RNAs with 21 to 23 nucleotides, miRNAs exert their role through inhibition of downstream targeted mRNA or
promotion of the degradation of targeted mRNAs via RNA interference (50). miRNAs are essential in a variety of pathophysiological processes (51). miR-26a is regarded as a tumor suppressor (52). The current research demonstrated knocking down AC068039.4 significantly down-regulated the expression of TRPC6 in PASMCs under hypoxia condition. Besides, PASMCs transfected with miR-26a-5p mimic could reverse the up-regulated TRPC6 induced by hypoxia, miR-26a-5p inhibitor exerts converse role and further increases TRPC6 expression. It has been reported that overexpression of TRPC6 increases the basal $[Ca^{2+}]_i$. Semiquantitative analysis of basal $[Ca^{2+}]_i$ showed silencing AC068039.4 downregulated $[Ca^{2+}]_i$, while upregulation TRPC6 overexpression increased $[Ca^{2+}]_i$. Bioinformatics prediction software showed that miR-425-5p had a potential binding site to the 3'-UTR of TRPC6 mRNA. Luciferase reports assay confirmed that miR-26a could bind to TRPC6 3'UTR, suggesting that miR-26a may be involved in the upstream regulation of TRPC6. Consistently, Schlosser et al. (23) found circulating miR-26 decreased in plasma from PAH patients and monocrotaline (MCT)-induced PH rats. Interestingly, studies have shown that overexpression of miR-26a can inhibit TRPC6 expression and related downstream apoptotic pathway activation (53). Besides, miR-26a was downregulated in experimental atherosclerosis via targeting TRPC3 (54). These data indicated that AC068039.4/miR-26a-5p/TRPC6 axis might participate in the development of hypoxic pulmonary hypertension.

**CONCLUSION**

In conclusion, the current research demonstrated that AC068039.4 is up-regulated in PASMCs induced by hypoxia, AC068039.4 promoted PASMCs proliferation, migration and cell cycle progression through competitively combining with miR-26a-5p through ceRNA pattern. Decreased free miR-26a-5p bound to the downstream target gene TRPC6, resulting in decreased degraded TRPC6 and increased TRPC6 expression and contributing to increased $[Ca^{2+}]_i$ level, ultimately promoting the proliferation and migration of PASMCs, eventually contributing to pulmonary vascular remodeling. Figure 6 illustrated that AC068039.4/miR-26a-5p/TRPC6 axis plays a significant role in the regulation of hypoxia-induced PASMCs proliferation; it might provide a novel potential therapeutic insight for the treatment of HPH.

**ACKNOWLEDGMENTS**

The authors thank all subjects who participated in this study.

**REFERENCES**

1. Galie N, Humbert M, Vachiery JL, Gibbs S, Lang I, Torbicki A, Simonneau G, Peacock A, Vonk Noordegraaf A, Beghetti M, et al.: 2015 ESC/ERS guidelines for the diagnosis and treatment of pulmonary hypertension: The joint task force for the diagnosis and treatment of pulmonary hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT). Eur Heart J 37(1):67–119, 2016.
23. Schlosser K, White RJ, Stewart DJ: miR-26a linked to pulmonary hypertension.

22. Leisegang MS, Fork C, Josipovic I, Richter FM, Preussner J, Hu J, Miller MJ, et al.: Long noncoding RNAs and long noncoding RNAs.

21. Zhang H, Liu Y, Yan L, Wang S, Zhang M, Ma C, Zheng X, Chen H, Zhu D: Long noncoding RNAs in hypertensive rats: Long noncoding RNA XR007793 regulates pulmonary arterial hypertension.

20. Yoon JH, Abdelmohsen K, Gorespe M: Functional interactions among micro-RNAs and long noncoding RNAs.

19. Wang S, Cao W, Gao S, Nie X, Zheng X, Xing Y, Chen Y, Bao H, Zhu D: TUG1 regulates pulmonary arterial smooth muscle cell proliferation in pulmonary arterial hypertension.

18. Chen J, Guo J, Cui X, Dai Y, Tang Z, Qu J, Raj JH, Hu Q, Gou D: The long noncoding RNA LARPT is regulated by PDGF-BB and modulates the proliferation of pulmonary arterial smooth muscle cells. Am J Respir Cell Mol Biol 58:2(1):381–386, 2018.

17. Xing Y, Zheng X, Fu Y, Qi J, Li M, Wang S, Li S, Zhu D: Long noncoding RNA GAS5: a novel regulator of hypertension-induced vascular remodeling. Hypertension 68(3):736–748, 2016.

16. Yoon JH, Abdelmohsen K, Gorespe M: Functional interactions among micro-RNAs and long noncoding RNAs.

15. Tay Y, Kats L, Salmena L, Pandolfi PP: A ceRNA hypothesis: the classification.

14. Thenappan T, Ormiston ML, Ryan JJ, Archer SL: Pulmonary arterial hypertension: pathogenesis and clinical management. Circulation 107(1):20–31, 2003.

13. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, et al.: Coding-independent regulation of the competitive endogenous RNA and induces lymphoma in vivo. Cell 161(2):319–332, 2015.

12. Joo MS, Shin S-B, Kim EJ, Kim H, Kim SG: Ngf/long noncoding RNAs control muscle differentiation by functioning as a competing endogenous RNA. Cell 147(2):358–369, 2011.

11. Karreth FA, Reschke M, Ruaoco A, Ng C, Chapuy B, Léopold V, Sjoberg M, Keane TM, Verma A, La M, et al.: The BRAF pseudogene functions as a competitive endogenous RNA and induces lymphoma in vivo. Cell 161(2):319–332, 2015.

10. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP: A ceRNA hypothesis: the classification.

9(3):613–626, 2015.

8. Schlosser K, Hanson J, Villeneuve PJ, Dimitroulakos J, McIntyre L, Pilote L, et al.: Long noncoding RNA GAS5: a novel regulator of hypertension-induced vascular remodeling. Hypertension 68(3):736–748, 2016.

7. St Laurent G, Wallestedt C, Kapranov P: The landscape of long noncoding RNA classification. Trends Genet 31(5):239–251, 2015.

6. Galie N, Barbera JA, Frost AE, Ghofrani HA, Hoeper MM, McLaughlin VV, et al.: Initial use of ambrisentan plus tadalafil in pulmonary arterial hypertension. N Engl J Med 373(9):834–844, 2015.

5. Galie N, Barbera JA, Frost AE, Ghofrani HA, Hoeper MM, McLaughlin VV, et al.: Initial use of ambrisentan plus tadalafil in pulmonary arterial hypertension. N Engl J Med 373(9):834–844, 2015.

4. Thenappan T, Ormiston ML, Ryan JJ, Archer SL: Pulmonary arterial hypertension: pathogenesis and clinical management. Circulation 107(1):20–31, 2003.

3. Chang YT, Tseng CN, Tannenberg P, Eriksson L, Yuen K, de Jesus Perez VA, Lundberg J, Engquist M, Botstein JS, et al.: Perlecan heparan sulfate deficiency impairs pulmonary vascular development and attenuates hypoxic pulmonary hypertension. Cardiovasc Res 107(1):20–31, 2015.

2. Newman JW, Fanburg BL, Archer SL, Badesch DB, Barst RJ, Garcia JD, Kao PN, Knowles JA, Loyd JE, McGoone MD, et al.: Pulmonary arterial hypertension: future directions: report of a national heart, lung and blood institute/office of rare diseases workshop. Circulation 109(24):2947–2952, 2004.

1. St Laurent G, Wallestedt C, Kapranov P: The landscape of long noncoding RNA classification. Trends Genet 31(5):239–251, 2015.
47. Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigno DD, Thistlethwaite PA, Rubin LJ, Yuan JX: Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A* 101(38):13861–13866, 2004.

48. Kunichika N, Landsberg JW, Yu Y, Kunichika H, Thistlethwaite PA, Rubin LJ, Yuan JX: Bosentan inhibits transient receptor potential channel expression in pulmonary vascular myocytes. *Am J Respir Crit Care Med* 170(10):1101–1107, 2004.

49. Yu Y, Keller SH, Remillard CV, Safrina O, Nicholson A, Zhang SL, Jiang W, Vangala N, Landsberg JW, Wang JY, et al.: A functional single-nucleotide polymorphism in the TRPC6 gene promoter associated with idiopathic pulmonary arterial hypertension. *Circulation* 119(17):2313–2322, 2009.

50. Bartel DP: MicroRNAs: target recognition and regulatory functions. *Cell* 136(2):215–233, 2009.

51. Eulalio A, Huntzinger E, Izaurralde E: Getting to the root of miRNA-mediated gene silencing. *Cell* 132(1):9–14, 2008.

52. Zhang X, Xiao D, Wang Z, Zou Y, Huang L, Lin W, Deng Q, Pan H, Zhou J, Liang C, et al.: MicroRNA-26a/b regulate DNA replication licensing, tumorigenesis, and prognosis by targeting CDC6 in lung cancer. *Med Cancer Res* 12(11):1535–1546, 2014.

53. Zhang Y, Qin W, Zhang L, Wu X, Du N, Hu Y, Li X, Shen N, Xiao D, Zhang H, et al.: MicroRNA-26a prevents endothelial cell apoptosis by directly targeting TRPC6 in the setting of atherosclerosis. *Sci Rep* 5:9401, 2015.

54. Feng M, Xu D, Wang L: miR-26a inhibits atherosclerosis progression by targeting TRPC3. *Cell Biosci* 8:4, 2018.