Overexpressed lncRNA AC068039.4 contributes to proliferation and cell cycle progression of pulmonary artery smooth muscle cells via sponging miR-26a-5p/TRPC6 in hypoxic pulmonary arterial hypertension

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Research

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Abstract

Background: Hypoxic pulmonary hypertension (HPH) is a devastating and incurable disease characterized by pulmonary vascular remodeling, resulting to right heart failure and even death. Accumulated evidence has confirmed long coding RNAs (lncRNAs) are involved in hypoxia induced pulmonary vascular remodeling in HPH. The exact mechanism of lncRNA in hypoxic pulmonary hypertension remains unclear.

Methods: Microarray analysis was applied to investigate the profiles of lncRNA expression in pulmonary artery smooth muscle cells (PASMCs) cultured under hypoxia and normoxia condition. qRT-PCR was performed for the expression of lncRNAs, miRNA and mRNAs, western blot analysis was employed for detection the expression of proteins. CCK-8 and transwell chamber assay were applied for 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 were detected by dual luciferase reporter assay.

Results: A total of 1211 lncRNAs (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-26-5p. In addition, transient receptor potential canonical 6 (TRPC6) was confirmed to be a target gene of miR-26a-5p.

Conclusion: In conclusion, downregulation of lncRNA AC068039.4 inhibited pulmonary vascular remodeling through AC068039.4/miR-26a-5p/TRPC6 axis, providing new therapeutic insights for the treatment of HPH.

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 lncRNAs, 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" indicates that lncRNA could sequester targeted miRNA and participate in miRNA regulation, indirectly involves in the regulation of miRNA-targeted genes[10]. The interaction between lncRNA and miRNA has been demonstrated in modulation of cell proliferation and apoptosis[11]. Previous studies have identified the role of several emerging lncRNAs, such as MEG3[12], LncRPT[13], Tug1[14], MALAT1[15], Hoxaas3[16], CASC2[17], are involved in the regulation of PSMCs proliferation, cell cycle progression and apoptosis of PSMCs and modulation of PVR. However, the role of lncRNA in HPH remains largely unknown. The current microarray analysis identified a significantly expressed novel lncAC068039.4.

Previous study demonstrated that miR-26a was down-regulated in both plasma from PAH patients and lung tissues of monocrotaline (MCT)-induced rats[18]. Store-operated calcium channels (SOCE) induced store-operated calcium entry (SOCE) is the main cause of calcium imbalance in PSMCs under hypoxia, transient receptor potential canonical 6 (TRPC6) is the main component of SOCC, up-regulated TRPC6 is related to PSMCs proliferation and PVR[19, 20]. TRPC6 also involves in PSMCs phenotype from contractile phenotype to the proliferative phenotype[21]. Previous study demonstrated lncRNAs may participate in regulation of TRPC6 expression [22].

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

Methods And Materials

Cells culture and ethics statement

The Human PSMCs used in the experiment were isolated from the distal pulmonary arterioles in patients undergoing pulmonary lobectomy. 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 $1 \times 1 \text{ mm}^2$, and then transferred to a culture flask. Isolated PSMCs were cultured in DMEM/F12 (Hyclone, CA) containing 10% fetal bovine serum (FBS) (Gibco, San Diego, NY, USA), supplemented with 100U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. For hypoxic condition, PSMCs were incubated in the condition

containing 1% O₂,94% N₂ and 5% CO₂. 3–7 passages of PSMCs were used in the experiment. Cells were starved in serum-free DMEM/F12 medium for 24 h before all experiments.

RNA interference, construction of the lentiviral vector and cell transfection

Lentivirus encoding full length of AC068039.4 sequence, specific short hairpin RNA targeting AC068039.4 (sh-AC068039.4) tagged with Green fluorescent protein (GFP) and the negative control lentiviral vectors tagged with GFP were constructed by Genechem Co. (Shanghai, China). Recombinant lentiviral vectors noncoding miR-26a-5p mimic, mimic negative control (NC), miR-26a-5p inhibitor and inhibitor NC were also designed and synthesized by Genechem Co. to regulate the expression of miR-26a-5p. The sequences for each were listed in Table 1. The PSMCs were cultured in 6-well plates 24 h before transfection with a cell density of 70–80% and PSMCs were transfected following the manufacturer’s introductions. Transfection reagents were removed after 24 h, and the cells were further cultured in DMEM containing 5% FBS. 3–4 days after transfection, fluorescence expression was observed using fluorescence microscopy and transduction efficiency was assessed by GFP expression > 80%. PSMCs were collected used for subsequent experiments and shRNA with the best silencing effect tested by qPCR was used to perform the following experiment.

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

Gene	Sequence (5’-3’)
shAC068039.4-1	AGAACTACAAGGAGAAATA
shAC068039.4-2	CCACAATGCAATTATGTTA
shAC068039.4-3	AATAATGCCATGTGTGAAA
miR-26a-5p mimic	UUCAAGUAAUCCAGGAUAGGCU
miR-26a-5p inhibitor	AGCCTATCCTGGATTACTTGAA

Cell proliferation assay

The cells were seeded in 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 hours. 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) was used to perform cell migration assay to evaluate the migration potential of PSMCs 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 trypsinization, 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 500µL of staining buffer, 100 µl of RNaseA was added, then add 25µL 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.

Total RNA isolation and quantitative reverse transcription PCR

Total RNA from PASMCs under different treatments was extracted with RNAiso Plus reagent (9109, TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Nanodrop 2000 (Thermo Scientific, MA, USA) 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 Green™ Premix Ex Taq™ II (RR820A, Takara, Japan) and Prism 7500 SDS (Thermo Scientific, MA, USA). The CT values of the target genes were recorded, relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and β -actin or U6 were used as internal references. Primers sequences are listed in Tables 2.

Table 2
Sequences of the primers for quantitative PCR

Gene	Sequence (5'-3')
lncRNA AC068039.4	Forward: GTGCCGAGATTGCAGCCTCTG
	Reverse: AGACGCTCCTCACTTCCTAGACG
miR-26a-5p	Forward: CGCGTTCAAGTAATCCAGGA
	Reverse: AGTGCAGGGTCCGAGGTATT
	RT Primer: GTCGTATCCAGTGCAGGGTCCGAGGT
TRPC6	Forward: CATGACGGCTTTAGAACTTAGC
	Reverse: CTTCAAGTGTCTTCTGCACAGATC
PCNA	Forward: GAAGGTGTTGGAGGCACTCAAGG
	Reverse: GCAGCGGTAGGTGTCGAAGC
Cyclin A1	Forward: GGCACAGACCCAAAGCACACTAC
	Reverse: AACCTCCACCAGCCAGTCCAC
Cyclin D1	Forward: GCCCTCGGTGTCCTACTTCAAATG
	Reverse: TCCTCCTCGCACTTCTGTTCCTC
Cyclin E1	Forward: GTGTCCTGGATGTTGACTGCCTTG
	Reverse: CGCACCACTGATACCCTGAAACC
β -actin	Forward: GGCACCCAGCACAAATGAAG
	Reverse: CCGATCCACACGGAGTACTTG
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT

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 (KGP902, KeyGEN BioTECH, China) according to the manufacturer's instructions. An equal amount (20 μ g) of the extracted proteins were subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a PDVF membrane (Millipore, Billerica, MA, USA). 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) were purchased from Abcam (Cambridge, UK),

the next day the PVDF membrane was incubated with a secondary antibody (Beijing TDY Biotech Co., Ltd., Beijing, China) for 1 hour. An ECL detection kit (GE Healthcare, UK) was used for blots visualization using and bolts were exposed with X-ray film.

Dual luciferase reporter assay

The AC068039.4 full length and wild-type (WT) 3'-UTR of TRPC6 were synthesized and cloned to pmirGLO Dual-Luciferase Vector (Promega, Madison, WI, USA). The predicted binding site was mutant as MUT-AC068039.4 and Mut-TRPC6. WT or MUT plasmid and miR-26a-5p mimics were co-transfected into Human embryo kidney 293 (HEK293) cells for 24 h. Cells were harvested and the relative luciferase activity was determined by using a Dual-Luciferase Reporter Assay System (Promega).

Microarray analysis

Total RNA was extracted from PSMCs induced by hypoxia and controls, lncRNA microarray analysis was performed by Genechem Co. (Shanghai, China). The lncRNA microarray analysis identified 698 up-regulated lncRNAs and 513 down-regulated lncRNAs in hypoxia induced PSMCs compared with PSMCs cultured under normoxic condition (fold change > 2.0 and P value < 0.05).

Statistical Analysis

SPSS 22.0 software (SPSS, Chicago, IL, USA) was used for analysis. Data were expressed as mean $\pm$ standard deviation. Independent sample student's t test was used for the comparison between two groups, and one-way ANOVA followed by Dunnett's test was performed to evaluate differences among groups. $P < 0.05$ (*) was considered statistically significant, $P < 0.01$ (**) was regarded as highly significant difference. All experiments were performed at least three times.

Results

Differentially expressed profiles of lncRNAs in normoxia and hypoxia induced PSMCs

As shown in the schematic diagram in Fig. 1., microarrays analysis was used to capture the expression profile of lncRNAs in PSMCs cultured under normoxia and hypoxia. Hierarchical cluster analysis showed that the expression profiles of lncRNA in PSMCs induced by normoxia and hypoxia were different (Fig. 1A). Compared with the control group, a total of 1211 lncRNAs in PSMCs induced by hypoxia were significantly differently expressed (698 up-regulated and 513 down-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 PSMCs was significantly up-

regulated compared with the normoxic group, and RNA expression of AC068039.4 was significantly increased with nearly 3 folds higher than that of the control group.

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

Previous studies have shown that hypoxia can promote PSMCs proliferation and migration. CCK-8 and transwell chamber analysis were used to investigate the effect of hypoxia on the proliferation and migration of PSMCs. As is shown in Fig. 2A, CCK8 assay analysis showed that after hypoxia exposure 0, 24, 48, and 72 hours, PSMCs proliferation capacity increased in a time-dependent manner. Figure 2B illustrated the transwell chamber analysis indicated that hypoxia significantly promoted the migration of PSMCs, 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 PSMCs. Figure 2C further clarifies the effect of different hypoxia exposure time on the expression of AC068039.4 in PSMCs. Quantitative PCR indicated that the expression of AC068039.4 increased significantly under hypoxic conditions. After 48 hours 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 PSMCs to further clarify the role of AC068039.4 in hypoxia-induced proliferation and migration of PSMCs. 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 PSMCs and effectively reversed the hypoxia-induced hyperproliferation of PSMCs while overexpression of AC068039.4 could further promote PSMCs proliferation (Fig. 2E). In addition, Knockdown of AC068039.4 reversed the hypoxia-induced upregulation of PCNA protein expression in PSMCs, however, overexpression of AC068039.4 under hypoxia exposure further upregulated the hypoxia-induced PCNA protein level (Figs. 2F). Consistently with western blot analysis, qRT-PCR showed PCNA mRNA level significantly reduced after down-regulation of AC068039.4 and vice versa (Fig. 2G). In addition, the migration potential of PSMCs could be reduced after downregulation of AC068039.4, whereas upregulated AC068039.4 promoted PSMCs migration (Fig. 2H). These finding indicated that downregulation of AC068039.4 could alleviate hypoxia induced PSMCs over-proliferation and elevated migration potential.

LncRNA AC068039.4 was involved in hypoxia-induced PSMCs 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[23].

Figure 3A and 3B 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[24]. 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 Fig. 3C and 3D, both protein and mRNA levels of cyclin A1, cyclin D1 and cyclin E1 were reduced in PSMCs 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.

LncRNA AC068039.4 promoted hypoxic PSMCs 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-minute walk distance[18]. qPCR analysis was conducted to detect the expression of miR-21 in PSMCs 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 showed reduced miR-26a-5p expression caused by hypoxia can be partially reversed after PSMCs was transfected with sh-AC068039.4 and miR-26a-5p was further downregulated in PSMCs 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 co-transfection 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 were listed in Fig. 4C. Comparison to the mimic NC group, decreased luciferase activity was observed in cells co-transfected miR-26a-5p mimic and WT-AC068039.4, whereas no evident differences were detected when co-transfected 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 PSMCs proliferation was counteracted after miR-26-5p transfection. Taken together, AC068049.4 could exert its role on hypoxia-induced PSMCs proliferation through regulation of miR-26a-5p.

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

Previous studies have demonstrated TRPC6 plays a vital role in hypoxic pulmonary vascular remodeling and PSMCs proliferation and migration[25]. Figure 5A and 5B illustrated that both mRNA and protein level of TRPC6 significantly up-regulated in PSMCs exposed to hypoxia condition. As shown in Fig. 5C, the expression of TRPC6 protein decreased in PSMCs 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-regulated 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). 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 involves in the development of hypoxic pulmonary hypertension.

The neointimal and medial hypertrophy of the small and medium pulmonary arteries plays an essential role in PVR, mainly due to the excessive proliferation of PASMCs[26]. LncRNAs are endogenous RNAs lacking protein-encoding function with more than 200 nucleotide length, and were considered to be transcription junk before[8]. Multiple lncRNAs has been confirmed participating in the development of PH through the regulation of the proliferation of PASMCs and pulmonary vascular remodeling[27–30]. Benefit from the rapid development of next-generation sequencing technology, emerging lncRNAs have been discovered involved in the regulation of cardiovascular disease[31, 32]. Gu et al. firstly performed lncRNA expression profiles of lung tissues of 5 CTEPH patients and 5 healthy control tissues by microarray, and 185 differentially expressed lncRNAs were found[33]. Liu et al.[30] conducted a microarray analysis and found that 36 up-regulated lncRNAs and 111 down-regulated lncRNAs in the pulmonary arteries (PA) of HPH rats (fold change > 2.0 and P value < 0.05). TCONS_00034812 was identified remarkably reduced in PA of HPH rats (fold change = 8.97, P = 0.01). While Kenny Schlosser demonstrated no significant changes of lncRNAs in plasma of PAH and healthy subjects[34]. Different from previous studies, in the current research, microarray analysis was conducted to find out the

differently expressed lncRNAs in hypoxia and normoxia induced PSMCs. Significantly differential lncRNAs were selected for PCR verification, AC068039.4 was found 3 times higher in hypoxia induced PSMCs. Furthermore, down regulation of AC068039.4 suppressed the proliferation and migration of PSMCs 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 PSMCs entering into G1 phase.

Competing endogenous RNA (ceRNA) is one of the main mechanisms of lncRNA–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 involves in post-transcriptional regulation[35]. CHRF was identified as endogenous sponge through downregulation of miR-489 to participate in the regulation of cardiac hypertrophy[36]. Furthermore, MALAT1 served as ceRNA through sponging miR-124-3p. 1 and promoted proliferation and migration of PSMCs. MEG3 and H19 acted as miRNA sponges of miR-328 and miR-675 respectively in PH[37, 12]. In the current research, miR-26a expression was significantly down-regulated in hypoxia induced PSMCs. 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 PSMCs proliferation through downregulation of miR-26a expression in hypoxia induced PSMCs.

The increase of free intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) concentration is a vital factor to stimulate pulmonary vascular contraction and proliferation of PSMCs[38]. Store-operated Ca^{2+} canonical (SOCC) mediated store-operated calcium entry (SOCE) results to the imbalance of intracellular calcium in PSMCs under hypoxia, causing pulmonary artery contraction and remodeling[39]. SOCC is mainly composed of transient receptor potential canonical channel (TRPC) proteins[40], of which TRPC6 is a major protein constituting SOCC[41]. Previous research indicated that chronic hypoxia upregulate the expression of TRPC6 in pulmonary vein smooth muscle cells (PVSMCs) and $[\text{Ca}^{2+}]_i$, and thereby promoted the proliferation and migration of PVSMC[19]. Ying Yu et al. demonstrated up-regulated TRPC6 expression promoted PSMCs proliferation isolated from IPAH patients[42]. Bosentan exerts a medicinal effect on PSMCs involves the downregulation of TRPC6 and inhibits the proliferation of PSMCs[43]. Gene sequencing revealed higher frequency of -254 (C→G) of single nucleotide polymorphism (SNP) in TRPC6 promoter region in idiopathic PAH patients[44]. TRPC6 expression was significantly increased in hypoxia induced PSMCs, 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[45]. miRNAs are essential in a variety of pathophysiological processes[46]. miR-26a is regarded as a tumor suppressor, it could inhibit lung cancer cell proliferation, migration and invasion[47]. The current research demonstrated knocking down AC068039.4 significantly down-regulated the expression of TRPC6 in PSMCs under hypoxia condition. Besides, PSMCs transfected with miR-26a-5p mimic could reverse the up-regulated TRPC6 induced by hypoxia, 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 K and his colleagues found circulating miR-26 decreased in plasma from PAH patients and monocrotaline (MCT)-induced PH rats, indicating that miR-26a may be a reliable potential biomarker of PH[18]. Interestingly, studies have shown that overexpression of miR-26a can inhibit TRPC6 expression and related downstream apoptotic pathway activation, thereby participating in reversing ox-LDL-induced apoptosis[48]. Besides, miR-26a was downregulated in experimental atherosclerosis model both in vivo and in vitro via targeting TRPC3[49]. 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 PSMCs induced by hypoxia, AC068039.4 promoted PSMCs proliferation, migration and cell cycle progression through competitively combining with miR-26a-5p through ceRNA pattern. Decreased free miR-26a-5p binded to the downstream target gene TRPC6, resulting in decreased degraded TRPC6 and increased TRPC6 expression and contributing to increased SOCE and $[Ca^{2+}]_i$ level, ultimately promoting the proliferation and migration of PSMCs, 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 PSMCs proliferation, it might provide a novel potential therapeutic insight for the treatment of HPH.

Abbreviations

CCK-8: Cell counting kit-8; CeRNA: Competing endogenous RNA; FBS: Fetal bovine serum; HPH: Hypoxic pulmonary hypertension; LncRNA: Long non-coding RNA; MCT: Monocrotaline; miRNAs: microRNAs; PAs: Pulmonary arteries; PSMCs: Pulmonary artery smooth muscle cells; PVR: Pulmonary vascular remodeling; PVSMCs: Pulmonary vein smooth muscle cells; qRT-PCR: Quantitative real time polymerase chain reaction; SNP: Single nucleotide polymorphism; SOCC: Store-operated Ca^{2+} canonical; SOCE: Store-operated calcium entry; TRPC6: Transient receptor potential canonical 6; UTR: Untranslated region;

Declarations

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Availability of data and materials

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

Authors' contributions

YQ, BZ, GY, DW, YQ contributed to the conception and design of the work. YQ, BZ and LL performed the experiments, analyzed the data, and made the figures; YQ, BL, EL, JH analyzed the results; YQ drafted the paper. CT revised the work critically. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was authorized by the Zhongda Hospital, and obtained written informed consents from all the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Differentially expressed profiles of lncRNAs in PSMCs cultured under normoxia and hypoxia. (A) The hierarchical clustering of differentially expressed lncRNAs. 1211 differentially expressed lncRNAs (698 up-regulated and 513 down-regulated) from hierarchical clustering were identified between normoxia and hypoxia induced PSMCs. The expression level of lncRNA is expressed in red if it is above the average value of the gene in all samples. Conversely, green indicates that the expression level of lncRNA is below

the average value. B: Realtime-PCR validates significantly differently expressed lncRNAs, and lncRNA AC068039.4 significantly up-regulated in hypoxia-induced PSMCs.

Figure 2

Down-regulation of AC068039.4 alleviated hypoxia-induced PSMCs proliferation and migration. (A) CCK8 assay analysis of PSMCs proliferation capacity after hypoxia exposure 0, 24, 48, and 72 hours. (B) Transwell chamber analysis of the migration potential of PSMCs cultured at 0, 24, 48, and 72 hours

of hypoxia. (C) Quantitative PCR of AC068039.4 in PASMCs after 24, 48 and 72h 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 $P<0.01$.

Figure 3

AC068039.4 was involved in hypoxia-induced PSMCs 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 level 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.

Figure 4

AC068039.4 sponges to miR-26a-5p and promoted hypoxic PSMCs proliferation. (A) qRT-PCR analysis of miR-26-5p expression levels in hypoxia induced PSMCs at different hypoxia exposure time. (B) Expression of miR-26a-5p in PSMCs transfected with sh-AC068039.4, OE-AC068039.4 and negative control. (C) Gene sequence of wild type AC068039.4 and mutated AC068039.4 constructed into vector in luciferase reporter assay analysis. (D) Luciferase reporter assay analysis of the binding between miR-26a-5p and predicted binding sites in AC068039.4. (E) CCK8 assay analysis of the cell proliferation viability in hypoxia induced PSMCs transfected with sh-AC068039.4, miR-21 inhibitor and NC respectively. * and ** represent $P < 0.05$, $P < 0.01$ respectively.

Figure 5

AC068039.4 promoted hypoxic PSMCs proliferation through AC068039.4/miR-26a-5p/TRPC6 axis. (A)TRPC6 mRNA level in PSMCs exposure to hypoxia. (B)The expression of TRPC6 protein in hypoxia induced PSMC. The expression of TRPC6 protein level (C) and TRPC6 mRNA (D) in PSMCs transfected with sh-AC068039.4 OE-AC068039.4 and negative control. TRPC6 protein expression (E) and TRPC6 mRNA expression (F) in PSMCs transfected with miR-26a-5p mimic, mimic NC, miR-26a-5p inhibitor and

inhibitor NC. (G) The binding site between miR-26a-5p and TRPC6 mRNA 3'UTR predicted by bioinformatics software. (H) Luciferase reporter assay analysis of the binding between miR-26a-5p and predicted binding sites in TRPC6. * and ** represent $P<0.05$, $P<0.01$ respectively.

Figure 6

Working model: the activation of the AC068039.4/miR-26a-5p/TRPC6 axis plays a vital role in hypoxia induced PSMCs proliferation. AC068039.4 is up-regulated in PSMCs exposed to hypoxia, AC068039.4 promotes PSMCs proliferation, migration and cell cycle. AC068039.4 competitively binds miR-654-3p through ceRNA model, leading to reduced binding between free miR-654-3p and target TRPC6 mRNA. Increased expression of TRPC6 mediates the increase of SOCE, which promotes PSMCs proliferation and migration, eventually causing Pulmonary vascular remodeling.