MicroRNA-199a-5p Regulates the Proliferation of Pulmonary Microvascular Endothelial Cells in Hepatopulmonary Syndrome

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Key Words
MicroRNA-199a-5p • Caveolin-1 • Rac1 • Pulmonary microvascular endothelial cells • Proliferation • Hepatopulmonary syndrome

Abstract
Background/Aims: Pulmonary microvascular endothelial cell (PMVEC) proliferation and angiogenesis contribute to the development of hepatopulmonary syndrome (HPS). MicroRNA-199a-5p (miR-199a-5p) has emerged as a potent regulator of angiogenesis, and its expression levels significantly decrease in the serum of patients with hepatopathy. However, it has not been reported about whether miR-199a-5p might control PMVEC proliferation. Here, we described the miR-199a-5p governing PMVEC proliferation in HPS. Methods: PMVECs were treated with rat serum from common bile duct ligation (CBDL) or sham. MiR-199a-5p mimic or inhibitor was used to change the miR-199a-5p expression. Knockdown of caveolin-1 (Cav-1) was performed using siRNA. NSC-23766 was used to inhibit Rac1 activity. Gene and protein expressions were quantified by qRT-PCR and western blot. Cell proliferation was analyzed by 3H-TdR incorporation and CCK-8 assays. Stress fibers were detected by immunofluorescence. Results: CBDL rat serum induced the down-regulation of miR-199a-5p. Delivery of miR-199a-5p suppressed the CBDL rat serum-induced PMVEC proliferation whereas knockdown of miR-199a-5p promoted PMVEC proliferation. This was accompanied by a decrease and an increase in Cav-1 expression, respectively. Cav-1 siRNA abolished the enhancement of PMVEC proliferation induced by the miR-199a-5p inhibition. Although stress fibers were disrupted in Cav-1 deficient cells, NSC-23766 increased stress fibers and contributed to cell proliferation. Conclusions: CBDL rat serum induced down-regulation of miR-199a-5p in PMVECs, which led to an increase of Cav-1 gene expression. Increased Cav-1 expression, by inhibiting Rac1 activity, led to the formation of stress fibers, which contribute to PMVEC proliferation and thus the pathogenesis of HPS.

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Introduction

Hepatopulmonary syndrome (HPS), a liver-induced lung vascular disorder, occurs when microvascular alterations impair arterial oxygenation [1]. The circulating factors such as cytokines, chemokines, growth factors, and adhesion molecules, emanating from the diseased liver, affect the pulmonary vascular tone [2-4]. The microvascular alterations, featuring angiogenesis and intrapulmonary vascular dilation, are attributable to the pathogenesis of HPS [5]. Defining the mechanisms underlying the microvascular alterations of HPS could facilitate the development of effective medical therapies. Proliferation of pulmonary microvascular endothelial cells (PMVECs) due to imbalanced circulating factors has been considered as a critical factor contributing to microvascular alterations [6, 7]. Although many signaling pathways have been demonstrated to control PMVEC proliferation, micro-ribonucleic acids (miRs) associated with these pathways were recently identified in control of PMVEC proliferation, extensively improving our understanding of HPS pathogenesis.

MiRs are small (-22 nucleotides) non-coding RNAs that regulate gene expression by binding to a specific target mRNA at the 3' untranslated region (UTR), thereby repressing translation and/or degrading mRNA. MiRs target a cluster of genes, allowing them to orchestrate a broad variety of biological processes. Increasing evidence has revealed that miRs play an important role in the angiogenesis process. Certain miRs, such as miR-130, miR-135b and miR-221, have been shown to exert pro-angiogenic effects; while miR-199a-5p and miR-222 have been characterized to have antiangiogenic activities [8-11]. Among these miRs, miR-199a-5p was found to be down-regulated in hepatocellular carcinoma (HCC) tissues and hepatoma cell lines, and interestingly, decreased expression of circulating miR-199a-5p was also found in the serum of HCC patients [12, 13]. Furthermore, it has been reported that miR-199a-5p, as an effector of Akt and transforming growth factor-beta (TGF-β) signaling, participates in cell proliferation, motility and angiogenesis by targeting the 3' UTR of caveolin-1 (Cav-1) [14, 15]. Combining these observations, we hypothesized that miR-199a-5p is likely to be a critical mediator of HPS.

In the present study, we investigated the role of miR-199a-5p in CBDL rat serum-induced PMVEC proliferation. We identified Cav-1 as a direct and functional target of miR-199a-5p, mediating the role of miR-199a-5p in PMVEC proliferation. Thus, our study provided a potential therapeutic target for the reversal of PMVEC proliferation in the pathogenesis of HPS.

Materials and Methods

Animals

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All of the procedures performed on the rats were approved by the Third Military Medical University Committee on Animal Care. Male Sprague-Dawley rats (200-250 g, Laboratory Animal Center of the Third Military Medical University, Chongqing, China) were used for the experiments. Experimental HPS was induced by common bile duct ligation (CBDL), as previously described [16]. The control animals underwent sham operations. Serum was obtained at the end of 5 weeks. CBDL rat serum and sham rat serum were used for subsequent experiments.

Cell culture

PMVECs were isolated from the healthy rat lungs, as previously described [17]. Cells were cultured in endothelial cell medium (ECM) (Sciencell, California, San Diego, USA) containing 5% fetal bovine serum (FBS) (Invitrogen, California, USA), 100 U/ml penicillin and 100 μg/ml streptomycin and 1% endothelial cell growth supplement, and incubated in an atmosphere of 5% CO₂/95% air at 37 °C. When the cells grew to 80% confluence, concentration of FBS was changed from original 5% to 0.1%. After 24 h of synchronous growth, the PMVECs were suspended in ECM containing 5% CBDL or sham rat serum for 12 h and 24 h.
Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the TRIzon reagent (Invitrogen, California, USA) according to the manufacturers’ instructions. Reverse transcription of miRNAs, mRNAs was performed by miScript Reverse Transcription kit (Qiagen, Nasdaq, New York, USA). The cDNA served as the template, miR-199a-5p expression was detected using a miRNA-specific miScript Primer Assay in combination with the miScript SYBR Green PCR Kit, while Cav-1 mRNA level was analyzed by QuantiTect Primer Assay in combination with the miScript SYBR Green PCR kit. Amplification of the specific products was performed using the iCycleriQ Real-Time PCR Detection System. Expression levels of miR and mRNA were calculated using the 2^−ΔΔCt method, with U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous controls, respectively.

Western blot

Total cellular proteins were harvested in nondenaturing lysis buffer (CWBO, Beijing, China). Briefly, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to PVDF membranes. After blocking for 1 h, membrane was incubated with anti-Cav-1 (1:1000), anti-GTP Rac1, anti-total Rac1 (1:500, Abcam, Toronto, Canada), or anti-GAPDH antibody (1:1000, Sigma-Aldrich, St. Louis, Mo, USA) at 4°C overnight. Samples were then incubated with HRP-conjugated rabbit anti-goat IgG at a 1:5000 dilution for 2 h and were analyzed with the ECL system (Advansta, California, USA). The optical density of the immunoreactivity was measured and analyzed with an Alpha Imager (Bio-Rad, Hercules, California, USA).

Transfection

MiR-199a-5p mimic, miR-199a-5p inhibitor and Cav-1 siRNA were synthesized by Gene Pharma (Shanghai, China). At 24 h before transfection, cells were seeded in a 24-well plate at 1×10^5 cells/well. MiR-199a-5p mimic (50 nM) or miR-199a-5p inhibitor (100 nM), Cav-1 siRNA (100 nM) were transfected into the PMVECs using Lipofectamine 2000 (Invitrogen, California, USA). The efficacy of transfection was assessed using qRT-PCR and western blot.

Thymidine (³H-TdR) incorporation assay

The cells were supplemented with 1 μCi ³H-TdR during the last 6 h of the treatment period. The incorporation was stopped with cold PBS solution, and 0.25% trypsin was added to digest cells and to separate them from cell wall. Then, cells were washed with physiological saline, stabilized with 10% trichloroacetic acid, decolorized with absolute ethanol, dried for 30 min at 80°C, transferred to scintillation fluid, and counted with a liquid scintillation counter (counts/min, cpm).

Cell Counting Kit-8 (CCK-8) assay

At the end of serum treatment, cells in 96-well plates (100 μl of medium) were incubated with 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) for 2 h at 37°C. The absorbance was read at 450 nm using the varioskan flash multimode reader (Thermo, Massachusetts, USA).

Immunofluorescence

Cells were fixed with 4% formaldehyde for 15 min, peneamblized in 0.3% Triton X-100 for 15 min, and blocked in 10% goat serum for 30 min at 37°C. Cells were then incubated with anti-F-actin antibody (Novus, Littleton, Colorado, USA) at 1:200 dilutions at 4°C overnight and subsequently incubated with DyLight 594 affinipure goat anti-rabbit secondary antibody (Earthox, San Francisco, California, USA) at 1:500 dilutions for 1 h at 37°C. Nuclei were stained using 4'-6-diamidino-2-phenylindole (DAPI, Beyotime Inc., Shanghai, China). Images were obtained using a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Rac1 activation assay

The GTPase activity of Rac1 was determined using Rac1 activation assay kit (Cell Biolabs, California, San Diego, USA), according to the manufacturer’s instruction. Briefly, cells were lysed with lysis buffer. The lysates was treated with either GTPγS (positive control) or GDP (negative control). The treated lysates were then incubated with PAK PBD Agarose beads at 4°C for 1 h. The level of GTP-Rac1 was analyzed by western blot using an anti-Rac1 specific monoclonal antibody.
Results

CBDL rat serum decreased miR-199a-5p expression and enhanced PMVEC proliferation

To determine whether PMVECs displayed the same miR-199a-5p expression profile in HPS as that in HCC, we compared the expression levels of miR-199a-5p in PMVECs treated with either CBDL rat serum or sham rat serum. Relative expression levels of miR-199a-5p were reduced by 40% at 12 h and 78% at 24 h in CBDL rat serum-treated PMVECs, compared with sham rat serum-treated cells (Fig. 1A). We have recently demonstrated that CBDL rat serum strongly induces PMVEC proliferation [17]. Thus, we asked whether the reduced levels of miR-199a-5p could affect PMVEC proliferation. PMVECs were transiently transfected with...
miR-199a-5p mimic or inhibitor to alter the endogenous levels of miR-199a-5p. In sham rat serum-treated cells, both DNA synthesis level and viable cell number were significantly increased by the delivery of miR-199a-5p inhibitor. And in CBDL rat serum-treated cells, miR-199a-5p inhibitor also induced significant increases in DNA synthesis level and viable cell number. In contrast, the increases of both DNA synthesis level and viable cell number induced by CBDL rat serum were greatly inhibited by the delivery of miR-199a-5p mimic.
MiR-199a-5p negatively regulated Cav-1 expression in CBDL rat serum-treated PMVECs

Our following study was to elucidate the molecular mechanism about how miR-199a-5p negatively controls PMVEC proliferation. Cav-1 has recently been reported as a direct target of miR-199a-5p in lung fibroblast cells during inflammation [14]. Thus, we asked whether CBDL rat serum could result in an alteration in Cav-1 expression via miR-199a-5p. Exposure of PMVECs to CBDL rat serum significantly increased the expression levels of Cav-1 mRNA at 12 h and 24 h (1.7-fold and 2.3-fold over sham control, respectively) (Fig. 2A). Similarly, 1.9-fold and 2.4-fold increases in the expression levels of Cav-1 protein were
found in PMVECs after exposure to CBDL rat serum for 12 h and 24 h, respectively, compared with sham rat serum-exposed PMVECs (Fig. 2B). Next, we assessed whether the elevated expression of Cav-1 in CBDL rat serum-treated PMVECs is due to the reduced miR-199a-5p. Both expression levels of Cav-1 mRNA and protein were increased by miR-199a-5p inhibitor in the PMVECs treated with sham rat serum. More importantly, the up-regulation of Cav-1 expression induced by CBDL rat serum was significantly diminished by miR-199a-5p mimic (Fig. 2C and 2D). These results confirmed Cav-1 as a direct downstream target of miR-199a-5p in PMVECs, indicating that Cav-1 might be critically important in control of PMVEC proliferation induced by CBDL rat serum.
Cav-1 depletion attenuated the miR-199a-5p-dependent PMVEC proliferation

To test whether Cav-1 acts as a downstream mediator of miR-199a-5p signaling in the CBDL rat serum-induced PMVEC proliferation, PMVECs were subjected to Cav-1 siRNA. Transfection with Cav-1 siRNA reduced the expression levels of Cav-1 mRNA and protein more than 70%, compared to siControl transfection (Fig. 3A and 3B). Cav-1 depletion by Cav-1 siRNA strongly blocked the miR-199a-5p inhibitor-mediated increases of both DNA synthesis level and viable cell number in PMVECs exposed to either sham rat serum or CBDL rat serum (Fig. 3C and 3D). Moreover, Cav-1 siRNA further enhance the miR-199a-5p mimic-induced inhibition of cell proliferation, as the reduction of both DNA synthesis level and viable cell number mediated by miR-199a-5p mimic were lower in PMVECs transfected with Cav-1 siRNA than with control siRNA (Fig. 3C and 3D). All these demonstrated that Cav-1
functionally controls the CBDL rat serum-induced PMVEC proliferation mediated by miR-199a-5p.

**Cav-1 depletion led to the loss of stress fibers and the activation of Rac1**

Vascular remodeling implies cytoskeletal reorganization of endothelial cells. We have recently established that cytoskeletal changes contribute to the CBDL rat serum-induced PMVEC proliferation. Therefore, immunofluorescent images were captured to link Cav-1 to stress fiber formation, a major cytoskeletal change we described previously [17]. Few stress fibers were observed in the cytoplasm of PMVECs transfected with miR-199a-5p mimic. Dramatic central actin stress fibers were detected in PMVECs transfected with miR-199a-5p inhibitor. Cav-1 siRNA-transfected cells showed the remarkable reduction of these central stress fibers, particularly in the condition of miR-199a-5p inhibitor delivery (Fig. 4A). Small GTPases of the Rho family are important controller of intracellular stress fiber formation. Constitutive activation of Rac1 resulted in almost void of intracellular stress fibers. In contrast, inhibition of Rac1 strengthened actin structures in microvascular endothelial cells [18]. Here, we assessed the activity of Rac1. Following the delivery of miR-199a-5p inhibitor, Cav-1 siRNA induced nearly 1.6-fold increases in GTP-bound Rac1 levels compared with siRNA control in sham rat serum-treated cells, and 1.8-fold increases were in CBDL rat serum-treated cells compared with siRNA control. After transfection with miR-199a-5p mimic, 1.7-fold increase in Rac1 activity was observed in Cav-1 siRNA-expressing cells treated with CBDL rat serum compared with siRNA control cells (Fig. 4B). These indicate the potential involvement of Cav-1 in miR-199a-5p-regulated PMVEC proliferation via the control of Rac1 activity and stress fiber formation.

**Rac1 inhibitor abrogated the inhibitory effects of Cav-1 depletion on stress fiber formation and PMVEC proliferation**

Furthermore, we introduced the inhibition of Rac1 activity to check the effects of Rac1 on both stress fiber formation and PMVECs proliferation. Rac1 activity was significantly diminished after the administration of Rac1 chemical inhibitor NSC-23766 (Fig. 5A). Central actin stress fibers remained continuously low in Cav-1 siRNA-expressing cells either treated with sham or CBDL rat serum. NSC-23766 treatment strongly increased stress fiber formation in Cav-1 siRNA-expressing cells, especially in response to CBDL rat serum (Fig. 5B). Consistent with these changes, NSC-23766 recovered the CBDL rat serum induced PMVEC proliferation which was completely inhibited by Cav-1 siRNA, as shown by the prominent increases in both DNA synthesis level and viable cell number (Fig. 5C and 5D).

**Discussion**

In earlier studies, we and other groups demonstrated that PMVEC proliferation and angiogenic regulators in the lung vasculature triggered angiogenesis and contributed to intrapulmonary vasodilation in experimental HPS [4, 17]. In this study, we focused on the novel concept that microRNA alteration resulting from the HPS microenvironment could contribute to the proliferation of PMVECs. We found that CBDL rat serum induced the down-regulation of miR-199a-5p, and this reduction of miR-199a-5p was related to the increase in PMVEC proliferation. These results were in agreement with previous observations that miR-199a-5p mimics negatively regulated angiogenic response in multiple myeloma [11], and miR-199a-5p down-regulation triggered PMVEC proliferation during hypoxia in the lungs [19, 20]. Our findings provided another direct evidence that the decreased miR-199a-5p expression contributed to the CBDL rat serum-induced PMVEC proliferation, further extending miR-199a-5p as a relevant contributor in the pathogenesis of HPS.

Cav-1 was experimentally validated to be one target of miR-199a-5p, which acts as a downstream of ERK and Akt signaling activation [15]. Our previous study confirmed that ERK and Akt activation occurs in the lungs of CBDL rats. Thus, we hypothesized that miR-
199a-5p might function as a potential control of cell proliferation and that such a function might depend on its regulation of Cav-1 [21-23]. Our present study found that CBDL rat serum increased Cav-1 expression, and this increase of Cav-1 was due to the decreased miR-199a-5p levels so that miR-199a-5p-mediated inhibition of Cav-1 expression was abrogated. Furthermore, we demonstrated that Cav-1 functions to promote PMVEC proliferation, particularly in response to CBDL rat serum.

How does this miR-199a-5p-mediated control of Cav-1 regulate PMVEC proliferation? In the pathophysiological process of the CBDL rat serum-induced PMVEC proliferation, we previously established that cytoskeletal changes might play an important role [17]. Here, we showed that miR-199a-5p strongly represses the formation of central actin stress fibers in PMVECs while Cav-1 highly promotes stress fiber formation. This finding suggested that the potential role of Cav-1 in the CBDL rat serum-induced PMVEC proliferation is to increase stress fiber formation.

Dynamic regulation of cytoskeleton is essential for the integration of cell signals that initiate cell proliferative properties. Cav-1 is a component of a negative feedback loop that regulates cytoskeletal dynamics by regulating the polyubiquitylation and subsequent degradation of Rac1 GTPase. Cav-1 associates with the hypervariable C-terminal domain of Rac1 through its scaffolding domain [24]. Cav-1 depletion led to the activation of Rac1 and consequently induced a Rac1 phenotype, increased cell spreading and loss of polarity [25-27]. In this present study, Cav-1 depletion increased Rac1 activity despite miR-199a-5p knockdown. Otherwise, Rac1 inhibitor abolished the Cav-1 depletion-induced inhibition of stress fiber formation and promoted the proliferation of siCav-1-deficient cells. This finding emphasized that inactivation of Rac1 was involved in the induction of PMVEC proliferation by increasing stress fiber formation.

In summary, our findings provided a direct support that CBDL rat serum-induced PMVEC proliferation was, at least in part, mediated by miR-199a-5p. Cav-1 acted as the major downstream factor of miR-199a-5p in both the inhibition of Rac1 activity and the facilitation of stress fiber formation. This study depicted an important role of the Cav-1-Rac1 signaling-modulated stress fiber formation in the promotion of PMVEC proliferation, suggesting the potential therapeutic significance of miR-199a-5p for HPS.

**Abbreviations**

HPS (hepatopulmonary syndrome); PMVECs (pulmonary microvascular endothelial cells); MiRs (micro-ribonucleic acids); MiR (199a-5p-microRNA-199a-5p); Cav-1 (caveolin-1); CBDL (common bile duct ligation); UTR (untranslated region); HCC (hepatocellular carcinoma); TGF-β (transforming growth factor-beta); qRT-PCR (quantitative real time polymerase chain reaction); GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

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**Disclosure Statement**

All authors declare no conflicts of interest.

**References**

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