Flavopiridol Mitigates the Progression of Monocrotaline-Induced Pulmonary Hypertension in Rats by Targeting Cyclin-dependent Kinase 9

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Research Article

Keywords: Pulmonary hypertension, Cyclin-dependent kinase 9 (CDK9), Flavopiridol, Proliferation, Apoptosis

Posted Date: April 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-415596/v1

Version of Record: A version of this preprint was published at Cardiovascular Drugs and Therapy on January 28th, 2022. See the published version at https://doi.org/10.1007/s10557-021-07285-w.

Abstract

Purpose: To investigate the role of cyclin-dependent kinase 9 (CDK9) and the therapeutic potential of CDK9 inhibitor (flavopiridol) in monocrotaline (MCT)-induced pulmonary hypertension.

Methods: In vivo experiments, pulmonary hypertension rats were established by a single intraperitoneal injection of MCT (60 mg/kg) for 2 weeks and treated with flavopiridol (5 mg/kg, i.p., twice a week) or vehicle for 2 weeks. In vitro experiments, human pulmonary artery smooth muscle cells (HPASMCs) were treated with flavopiridol (0.025-1μM) or vehicle under hypoxic condition. Hemodynamic recording, right ventricle and lung histology, isolation of pulmonary arterial tissues were performed. The expressions of CDK9, RNA polymerase II, c-Myc, Mcl-1 and survivin were determined by qRT-PCR and western blotting, proliferation and apoptosis of PASMCs were also assayed.

Results: CDK9 was upregulated in both rat pulmonary arterial tissues and HPASMCs. Upregulation of CDK9 increased the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNA pol II) on serine-2, promoting the expression of prosurvival and antiapoptotic proteins (c-Myc, Mcl-1 and survivin). Furthermore, treatment with flavopiridol (5 mg/kg) significantly alleviated pulmonary artery remodeling and partially reversed the progression of monocrotaline-induced pulmonary hypertension. Consistently, flavopiridol (0.5 μM) treatment decreased the proliferation and induced the apoptosis of cultured HPASMCs under hypoxic conditions. As a result of CDK9 inhibition and subsequent inhibition of RNA pol II CTD phosphorylation at serine 2, flavopiridol decreased c-Myc, Mcl-1 and survivin expressions in isolated pulmonary small arteries, leading to cell growth inhibition and apoptosis.

Conclusion: Flavopiridol mitigates the progression of monocrotaline-induced pulmonary hypertension in rats by targeting cyclin-dependent kinase 9.

Introduction

Pulmonary hypertension (PH) is a devastating cardiopulmonary disorder with poor prognosis and limited curative options. Despite receiving treatment, approximately 10%-15% of patients with PH die within 1 year of medical follow-up [1, 2]. The remodeling of small pulmonary arteries is an important pathological characteristic of PH [3, 4]. Recently, a novel cancer-like concept for PH has emerged [5-7], as some similarities between cancer and PH have been confirmed. For instance, similar to the hallmarks of cancer cells, pulmonary artery smooth muscle cells (PASMCs) are characterized by overproliferation and resistance to apoptosis [8,9]. However, the underlying mechanism of pulmonary vascular remodeling are not fully understood.

Cyclin-dependent kinases (CDKs) can be divided into the partially overlapping classes of cell cycle regulators (e.g., CDK1, 2, 4, 6, and 7) and transcriptional regulators (e.g., CDK7, 8, 9, and 10-13) [10]. CDK9 is a key catalytic subunit of positive transcription elongation factor b (P-TEFb) that promotes transcriptional elongation by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNA pol II) at serine-2 [11]. Activation of CDK9 can enhance the expressions of several signal-responsive

proteins that regulate proliferation and apoptosis, such as proto-oncogenes protein (c-Myc) and antiapoptotic proteins (Mcl-1 and survivin) [12, 13]. The dysregulation of CDK9 activity or expression has been shown to be associated with several diseases, including cancer, cardiac hypertrophy and acquired immunodeficiency syndrome (AIDS), etc [11, 14, 15]. Compared to normal tissue, CDK9 overexpression and/or hyperactivity has been observed in several types of cancer (e.g., melanoma) [14, 16-18]. The upregulation of proto-oncogenes proteins and antiapoptotic proteins (e.g., c-Myc, Mcl-1 and survivin) in cancer cells has been confirmed to contribute to their overproliferation and resistance to apoptosis [19, 20]. Intriguingly, enhanced c-Myc, Mcl-1 and survivin expressions have also been observed in PH patients and PH model PASMCs [21, 22]. However, whether the enhanced proliferation and resistance to apoptosis of PASMCs in PH is due to the CDK9-mediated overexpression of these targeted genes remains unknown.

Additionally, the inhibition of CDK9 by selective blocker or shRNA leads to a block in transcriptional elongation and the suppression of short-living antiapoptotic proteins (e.g., Mcl-1), resulting in an increased rate of apoptosis and antitumor effects [13, 18, 20]. Flavopiridol is one of the most studied CDK9 inhibitors and has been tested in antitumor and cardiac hypertrophy clinical trials [23-25]. Flavopiridol displays potent activity against CDK9, and its mechanism of action is believed to involve the inhibition of CDK9-mediated transcriptional elongation[24]. Flavopiridol is known to bind tightly to the ATP binding site of CDK9 in a noncompetitive manner to inhibit the CDK9-mediated phosphorylation and activation of RNA pol II, thereby affecting RNA pol II-dependent transcription. Mcl-1, Bcl-2 and Bcl-xL have been shown to be inhibited by flavopiridol treatment, and it is possible that these events are mediated through CDK9 inhibition [26, 27].

Several proteins, including hexamethylene-bisacetamide-induced protein 1/2 (HEXIM 1/2), La-related protein 7 (LARP7) and methylphosphate capping enzyme (MePCE) have been shown to negatively regulate the kinase activities of CDK9 or P-TEFb and have also been reported to be involved in CDK9-related diseases [28, 29]. For example, LARP7 downregulation has been confirmed in several cancers, which subsequently potentiates CDK9-mediated transcriptional elongation and promoted cancer growth and metastasis [30]. However, whether these regulatory proteins are involved in the pathological process of PH remain unknown.

In the present study, we investigated the roles and underlying mechanism of CDK9 in the cancer-like phenotype (overproliferation and resistance to apoptosis) of PASMCs in pulmonary hypertension rats, and sought to elucidate whether the CDK9 inhibitor (flavopiridol) could influence the pathogenic progress of pulmonary hypertension through targeting CDK9.

Materials And Methods

Experimental animals and pulmonary artery hypertension models

Adult young male Sprague-Dawley rats (200-250 g, 2-3 months old) were purchased from the Experimental Animal Center of Weitonglihua Co., Ltd. (Beijing, China). Monocrotaline (MCT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 1.0 N HCl at a concentration of 40

mg/ml, adjusted to pH 7.4 with 1.0N NaOH and diluted with distilled water. The rats were intraperitoneally injected with MCT (60 mg/kg) or vehicles (Normal saline), and once PH was established (2 weeks after MCT injection), the rats with pulmonary hypertension were randomly divided into two groups and treated with either flavopiridol (5 mg/kg, i.p., twice a week) or vehicle (0.01% DMSO) for 2 weeks. All experiments involving animals were performed with the approval of the Animals Care and Use Committee of Huazhong University of Science and Technology (Wuhan, Hubei, China). Up to three rats were housed per cage, and the animals were provided free access to water and food with constantly maintained room temperature and humidity under a 12-12 h light-dark cycle.

Assessment of right ventricle function and hypertrophy

The right ventricle systolic pressure (RVSP) of rats was recorded as previously reported[31]. Briefly, at the end of experiment, the rats from each group were anesthetized (pentobarbital, 50 mg/kg, i.p.) and intubated, then the diaphragm was surgically exposed through the abdomen, and a 25-gauge needle connected to a pressure transducer (AD Instrument) was inserted into the right ventricle (RV) through the diaphragm. The RVSP was continuously recorded for 10-15 mins using a PowerLab data acquisition system (AD Instruments). Following hemodynamic recording, the heart and lung were removed en bloc. Then, the right ventricle (RV) and left ventricle plus interventricular septum (LV+S) were separated and weighted to calculate the RV hypertrophy index (RV/ [LV+S]).

Histology and morphometric analysis

The collected lungs were perfused with normal saline and fixed in 4% paraformaldehyde overnight. Then, representative cross-sections of the lungs that included the peripheral and the central pulmonary arteries were sampled and embedded in paraffin blocks. Serial sections (5 μm-thick) were then prepared and stained with hematoxylin and eosin to assess vascular pathology. The percent wall thickness (WT %) of arteries (15-150 μm) was calculated using the following formula as previously described [3]: WT%=2×WT/external diameter (ED) ×100. The percent wall area of arteries was also measured.

Preparation of pulmonary arterial tissue

The PH and control rats were anesthetized with pentobarbital sodium (200 mg/kg, i.p.), and the hearts and lungs were removed after a midline thoracotomy. Intrapulmonary arteries from the lung lobes were aseptically excised and placed in PBS solution, after which the adherent fat and connective tissues were carefully removed under a dissecting microscope. The adventitia was scraped using forceps and the endothelium was carefully removed. Then, the pulmonary arterial tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C for use in the subsequent experiments.

Cell culture and in vitro experiments

Human pulmonary artery smooth muscle cell line (HPASMCs, passage 4-6, Lonza, USA) were cultured in DMEM at 37°C under a humidified 5% CO_2. For in vitro hypoxic experiments, HPASMCs were cultured in

an incubator equilibrated with 3% O_2 (in $N_{2)}$, while control cells were cultured in an incubator equilibrated with room air (21% O_2). Flavopiridol (0.025-1μM in vehicle) or an equal volume of vehicle (0.01% DMSO) was added to DMEM during the final 24 h of exposure to normoxic or hypoxic conditions.

Protein preparation and Western blotting

HPASMCs were lysed in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysate was centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatants were then used as protein samples. Equal amounts of protein (30 μg) from each cell type was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Munich, Germany). After blocking with 5% skim milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated at 4°C overnight with the following antibodies: anti-CDK9 (CST, 1:500), anti-RNA pol II (Abcam, 1:1000), anti-phospho-RNA pol II (Ser-2) (Abcam, 1:1000)， anti-Mcl-1 (Abcam, 1:1000), anti-c-Myc (Abcam, 1:1000), anti-survivin (CST, 1:500) and anti-β-actin (Abcam, 1:3000). The protein levels were normalized to β-actin. The gel bands were visualized with Amersham ECL prime Western blotting detection reagent (GE Healthcare, Little Chalfont, UK), and then the band density was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Real time PCR

Total RNA from rat pulmonary arteries and HPASMCs were isolated using Trizol and reverse transcribed with gene-specific primers. Small pulmonary arteries (<1000 μm) from PH or control rats were used for RNA extraction. Real-time PCR was performed as previously described [3]. Specific primers are listed in Table. 1.

Cell counting kit-8 (CCK8)

HPASMCs were seeded in a 96-well plate at a final density of 3000 cells/well [9] and incubated in growth media supplemented with flavopiridol (0.025-1μM) for 24 h. Subsequently, the medium was replaced with fresh medium containing 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitropheny)-5-(2, 4-disulfophenyl)-2H-tetrazole monosodium salt (CCK8, Dojindo, Japan) solution per 100 μl of medium. Then, after 2h of incubation at 37℃, the plates were read at 450 nm using a microplate reader (Thermo, USA). Ten wells were used for each treatment.

5-Ethynyl-2'-deoxyuridine (EDU)

5-Ethynyl-2'-deoxyuridine (EDU, RIBOBIO, China) is a thymidine analog that incorporates into replicating DNA in place of thymine during cell proliferation. The specific activity of the cellular DNA in response to the Apollo fluorescent probe dye is an indicator of cell proliferation. The cells were seeded in a 96-well plate at a density of 3×10^3 cells/well[9], with each group being assayed in triplicate. After 12 h of synchronization, the cells in each group were treated for 24 h according to the experiment design, and the

medium was replaced with medium containing 25 μM EDU as well as the stimulating factor and the inhibitor. Subsequently, after incubating for 18 h, the cells were stained, photographed and counted as the mean of five randomly selected fields under ×400 magnification.

Statistical analysis

All experiments were performed in triplicate independent experiments. The results are presented as the mean ± S.E.M., except where noted. Student's *t*-test and one-way ANOVA were used to assess the significance of differences between two or among multiple groups, respectively. Statistical significance was defined at $p<0.05$ for all tests.

Results

CDK9 Is Overexpressed in PH

First, we measured CDK9 expression in rats with monocrotaline (MCT)-induced pulmonary hypertension by RT-PCR and Western blotting. As shown in Fig.1A, CDK9 mRNA expression was significantly increased in pulmonary arteries isolated from MCT-exposed rats (~2.4 fold) compared to that of the control group. Consistent\with the upregulation of CDK9 mRNA expression, CDK9 protein expression in the MCT group was also significantly upregulated compared to the control group (from 0.95 ± 0.07 to 1.47 ± 0.06, Fig. 1B). Similarly, in vitro experiment, CDK9 mRNA expression was also markedly increased upon exposure to hypoxia in HPASMCs for 24 h (~2.1-fold, Fig. 1C), comparing with the normoxic HPASMCs. Interestingly, CDK9 protein expression in the hypoxia group rapidly decreased upon the hypoxia treatment (1-3 h) but was followed by a gradual increase over time and was ~1.5 fold greater than observed in the control group at 24 h (Fig. 1D).

Flavopiridol Mitigates the Progression of MCT-induced PH in Rats

To further investigate the functional role of CDK9 in pulmonary hypertension, we assessed whether CDK9 inhibition by flavopiridol treatment could mitigate the progression of MCT-induced PH in rats. As expected, in Fig. 2A-B, the baseline RVSP in the MCT group (35.1 ± 2.3 mmHg) was significantly higher than that in the control group (17.7 ± 0.7 mmHg), and CDK9 inhibition by flavopiridiol (5 mg/kg) treatment significantly decreased the RVSP (24.4 ± 1.3 mmHg, $P<0.05$) of MCT-treated rats when compared to the untreated MCT rats (35.1 ± 2.3 mmHg). Additionally, as shown in Fig. 2C-D, MCT promoted a significant increase in the RV hypertrophy index (0.57 ± 0.01, $P<0.05$) compared to that observed in the control group (0.27 ± 0.01), while flavopiridol mitigated PH, as the RV hypertrophy index was significantly lower in the MCT-treated rats (0.45 ± 0.01, $P<0.05$) than in the untreated MCT rats (0.57 ± 0.01).

Flavopiridol Attenuates the Vascular Remodeling of MCT-induced PH in Rats

Since the remodeling of small pulmonary arteries is an important pathological characteristic of PH [2, 8, 9], we next assessed whether a reduction in pulmonary vascular remodeling of small pulmonary arteries

could account for improved hemodynamics in flavopiridol-treated rats. As shown in Fig. 3A-B, pulmonary hypertension in MCT-treated rats was associated with an increase in the media wall thickness of pulmonary arteries, which was significantly attenuated after flavopiridol treatment. Furthermore, as shown in Fig. 3C, immunofluorescence analysis of lung tissue indicated that compared to the control group, the expression of PCNA (marker of cell proliferation) in the pulmonary arteries was significantly increased in the MCT-treated rats and decreased when these rats were administrated CDK9 inhibitor (flavopiridol).

Flavopiridol Inhibites the Overproliferation and Promotes the Apoptosis of HPASMCs under Hypoxia in vitro

The uncontrolled proliferation and resistance to apoptosis of PASMCs are the predominant factors of pulmonary vascular remodeling [2, 9]. Hypoxia is an important stimulus for HPASMCs proliferation and PH. Therefore, the effects of flavopiridol on the proliferation and apoptosis of hypoxia-treated and control HPASMCs were investigated. As shown in Fig. 4A, the cell viability of HPASMCs in the hypoxia group (3% O_2, 131.5 ± 2.9%) was significantly higher than that of the control group (21% O_2, 100.2 ± 1.6 %). Furthermore, flavopiridol strongly inhibited HPASMCs proliferation in a concentration-dependent manner in the hypoxia group, especially when flavopiridol used at 0.5 μM, which caused an ~50% decrease in cell viability, whereas flavopiridol minimally influenced HPASMCs cell growth in the control group. Thus, flavopiridol treatment could reverse the hypoxia-induced overproliferaion of HPASMCs.

Additionally, in agreement with the CCK-8 assay results, as shown in Fig. 4B, the number of apoptotic HPASMCs was remarkably decreased under hypoxic conditions (7.0 ± 0.4%) compared to that observed in the normoxic control (9.4 ± 0.5%), while flavopiridol (0.5 μM) significantly promoted the apoptosis of hypoxia-treated HPASMCs (from 7.0 ± 0.4% to 33.3 ± 1.7%, ~5 fold) but only minimally increased apoptosis of untreated HPASMCs (from 9.4 ± 0.5% to 15.2 ± 1.2%, ~1.6 fold). Furthermore, hypoxia significantly increased the percentage of EdU positive cells (40.8 ± 2.0%) compared to that observed in the normoxic control group (28.9 ± 2.0%), and flavopiridol dramatically reduced the hypoxia-induced HPASMCs over-proliferation (from 40.8 ± 2.0% to 19.0 ± 1.1%, Fig. 4C).

Flavopiridol Inhibites CDK9-mediated Transcriptional Elongation and Suppresses the Expressions of downstream Prosurvival and Antiapoptotic Proteins of PASMCs in PH

As flavopiridol was previously reported to be a potent CDK9 inhibitor [23], we further examined whether flavopiridol could inhibit CDK9 expression or kinase activity. As shown in Fig. 5, compared to that observed in the control group, CDK9 expression was significantly upregulated in MCT group, and flavopiridol had no effect on CDK9 expression. Subsequently, we examined the effect of flavopiridol on CDK9 kinase activity by measuring the cellular levels of RNA polymerase II phosphoforms at Ser-2 which is the specific site for RNA polymerase II phosphorylation by CDK9. The results presented in Fig. 5A-B indicate that significant higher phosphorylation of RNA polymerase II at Ser-2 occurred in MCT group (0.89 ± 0.04, $P<0.05$) than in the control group (0.42 ± 0.05). Interestingly, flavopiridol significantly

reduced the phosphorylation of RNA polymerase II at Sr-2 in isolated pulmonary arterial tissues (from 0.89 ± 0.04 to 0.56 ± 0.07) but it had no effect on the total protein levels of RNA polymerase II in isolated pulmonary arterial tissue.

CDK9-mediated transcription of several prosurvival and antiapoptotic proteins (e.g., c-Myc Mcl-1 and survivin) plays an important role in proliferation and apoptosis resistance of cancer cells [19, 20], and the aforementioned prosurvival and antiapoptotic proteins also involved in the pathology of PH [21, 22]. Thus, the effects of flavopiridol on the expressions of c-Myc, Mcl-1 and survivin were examined. As shown in Fig. 5C-E, the mRNA and protein expressions of c-Myc, Mcl-1 and survivin in isolated pulmonary arterial tissues of MCT-treated rats were significantly upregulated compared to that of the control group.

CDK9-related Negative Regulators are Downregulated in PH

Emerging evidence as suggested that some CDK9-related negative regulators (e.g., HEXIM 1/2, LARP7 and MePCE) also involved in CDK9-related disease, e.g., cancers [28, 30]. Next, we examined whether these negative regulators also involved in pulmonary hypertension. As shown in Fig. 6, compared to the control group, decreased LARP7, HEXIM 1, and MePCE mRNA expression (~0.4, 0.7, 0.5 fold respectively) were detected in pulmonary arterial tissues isolated from MCT-exposed rats, while no significant difference was observed in HEXIM 2 mRNA expression between the control and MCT-treated groups.

Discussion

In the present study, we firstly revealed that CDK9 was upregulated in isolated pulmonary arterial tissues from MCT-induced PH rats in vivo and in hypoxic cultured HPASMCs in vitro. This upregulation of CDK9 was associated with increased CDK9-mediated phosphorylation of the C-terminal domain (CTD) of RNA pol II at serine-2. Secondly, several downstream prosurvival and antiapoptotic proteins (c-Myc, Mcl-1 and survivin) were also upregulated at both the mRNA and protein levels. These molecular changes in pulmonary arterial tissues coincident with pathogenic enhancement of pulmonary vasculature remodeling and elevated pulmonary arterial pressure. Furthermore, flavopiridol significantly alleviated pulmonary artery remodeling and reversed the progression of pulmonary hypertension through the inhibition of CDK9-meditated transcription elongation. Taken together, these findings elucidated the potential role and underlying mechanism of CDK9 in the pathogenesis of pulmonary vasculature remodeling under PH and flavopiridol can partially reverse pathogenic vasculature remodeling in PH rats by targeting CDK9.

Growing evidence indicates that pulmonary hypertension shares several similar pathogenic characteristics with the cancers [6, 7], with one of the most important similarities being that both cancer cells and PH-associated vascular cells, especially PASMCs, exhibiting a proproliferative and antiapoptotic phenotype [3, 6]. In our present study, the level of a biomarker of cell proliferation (PCNA) in pulmonary arteries from MCT-induced PH rats were increased, and a significant hypertrophic media layer harboring proliferative PASMCs was observed in pulmonary small arteries. This overproliferation phenotype of vascular cells, especially PASMCs, will lead to an increasing thickness of the pulmonary artery media

wall, consequently inducing PH and right ventricle failure. Moreover, HPASMCs overproliferation and resistance to apoptosis were also induced under hypoxic (3%) conditions, similar to that observed in cancer cells exposed to a hypoxic environment [32]. Interestingly, this abnormal overproliferative and antiapoptotic phenotype of PAMSCs can be reversed by treatment with the antitumor agent flavopiridol in both in vivo PH model and in vitro HPASMCs, suggesting that reversing these cancer-like phenotypes may be a promising therapeutic strategy to combat PH.

CDK9 has been extensively investigated as a target for cancer therapy, and CDK9 upregulation has been shown to play a crucial role in the proproliferative and antiapoptotic phenotype of cancer cells [18, 20]. CDK9 overexpression has been reported in osteosarcoma, melanoma, gastric cancer, etc [16, 17, 33]. CDK9 inhibition blocks transcriptional elongation, thereby suppressing the expression of antiapoptotic proteins, such as MCL-1, BIRC5[13]. CDK9 inhibitors such as flavopiridol, SNS-032, or roscovitine have proven efficient through the downregulation of anti-apoptotic genes XIAP and MCL-1[16, 26, 34]. Although several signal-responsive genes that regulate proliferation and apoptosis, such as proto-oncogenes proteins (c-Myc), antiapoptotic proteins (Mcl-1 and survivin), have been attributed to CDK9 related abnormal transcriptional elongation, and elevated CDK9 expression was also reported in cancer cells [12, 18, 20], its potential roles in PH pathogenesis remain largely unknown.

Flavopiridol is a broadly specific CDK inhibitor with a distinct preference for CDK9. The K_i values of flavopiridol for CDK9/CycT (3 nM) was previously shown to be approximately 10-fold lower than those for other CDKs (40-70 nM) [35]. CDK9 inhibition by flavopiridol can remarkably attenuate tumor growth in vitro and in vivo [26, 27]. As previously observed in cancer cells, CDK9 upregulation was also detected in both pulmonary arterial tissues from MCT-induced PH rats and hypoxia-treated HPASMCs. Additionally, CDK9 inhibition by flavopiridol attenuated the overproliferation and promoted the apoptosis of PASMCs, thereby alleviating pulmonary vasculature remodeling and reversing the progression of pulmonary hypertension in rats, which is like its antitumor effects. Taken together, these results show that CDK9 upregulation also contributes to the proproliferative and anti-apoptotic phenotype of PH PASMCs, which is like that observed in cancer cells.

As a catalytic subunit of P-TEFb, CDK9 activation can promote transcriptional elongation by phosphorylating RNA polymerase II CTD. In the present study, consistent with its potent inhibition of CDK9 kinase activity observed in cancer cells [26], flavopiridol also significantly reduced the phosphorylation on RNA polymerase II CTD at Ser-2 in isolated PH pulmonary arterial tissues, indicating a potential role of flavopiridol in reversing pulmonary vessel remolding. Furthermore, the overexpression of several short-lived prosurvival and antiapoptotic proteins, such as c-Myc, Mcl-1 and survivin has been reported to contribute to overproliferation and resistance to apoptosis in cancer cells [19, 20], and these proteins were preferentially depleted by the inhibition of CDK9-mediated transcriptional elongation [13]. In agreement with the results of other studies, both mRNA and protein levels of c-Myc, Mcl-1 and survivin were also significantly increased in our isolated PH pulmonary arterial tissues, which could be remarkably decreased by flavopiridol treatment. Therefore, our results suggested that the antiproliferative and

proapoptotic effects of flavopiridol on pulmonary arteries and PASMCs are mediated by the inhibition of CDK9-mediated transcriptional elongation and these expressions of these downstream proteins.

In addition to the changes of CDK9 expression or activity，CDK9/P-TEFb related regulators may also be involved in CDK9-related disease (e.g. cancers) [28, 30, 36]. As previously reported, LARP7, HEXIM1 or HEXIM2 and MePCE, bind to 7SK small nuclear RNA (snRNA) to form the 7SK small nuclear ribonucleoprotein (7SK snRNP) complex, which plays a role in inhibiting CDK9/P-TEFb activity [29, 30]. LARP7 was previously reported to suppress CDK9/P-TEFb activity, and LARP7 knockdown or inhibition can cause an increase in CDK9 activity [30]. HEXIM1/2 can inhibit CDK9 kinase activity in a 7SK snRNA - dependent manner [29]. MePCE was reported to stabilize 7SK snRNA to facilitate the inhibition of CDK9/P-TEFb activity [37]. In agreement with these findings, in our present study, these components of the 7SK snRNP complex [LARP7, HEXIM1 (not HEXIM2) and MePCE] were significantly decreased in isolated PH pulmonary arteries, suggesting that these CDK9-related negative regulators may also be involved in the pathology of pulmonary hypertension. Additionally, previous studies have reported that overexpression of these CDK9-related negative regulators (e.g., LARP7) decreases CDK9/P-TEFb kinase activity to reverse the progression of CDK9-related diseases [30, 36], thus, the underlying mechanisms of these CDK9-related negative regulators in pulmonary hypertension requires further investigation.

However, like other anticancer agents, flavopiridol (0.5μM) still cytotoxic toward the control group HPASMCs, demonstrating that some therapeutic strategies are needed to reduce the usage of flavopiridol to treat pulmonary hypertension. In the present study, the three assayed CDK9-related negative regulators (LARP7, HEXIM1 and MePCE) were downregulated in isolated PH pulmonary arterial vessels. Furthermore, whether the overexpression of these proteins combined with CDK9 inhibition (flavopiridol) can exert synergistic inhibitory effects toward CDK9-mediated transcriptional elongation to yield a more pronounced suppression of the development of pulmonary hypertension and decrease side effects will be investigated in our further studies.

There were several limitations of the present study. First, due to the technical simplicity and reproducibility advantages, an MCT-induced pulmonary hypertension model was used in our present study, but the differences between MCT models and human pulmonary hypertension should be taken into consideration. MCT not only induces PH but also affects both the right and left ventricles as well as other organs (liver and kidney injuries), which may affect the progression of pulmonary hypertension [38]. In subsequent studies, analyses of pulmonary artery samples from pulmonary hypertension patients may be more convincing to evaluate the associated role of CDK9. Second, flavopiridol is a pan-CDK9 inhibitor and may alter transcription via other mechanisms in addition to CDK9 inhibition or could inhibit other CDKs, although with lower efficacy [35]. Thus，it would be more convincing if a higher specific CDK9 inhibitor (e.g., BAY-1143572) was used in subsequent studies [39]. Third, P-TEFb comprise CDK9 and cycin T (T1, T2a or T2b), and cyclin T1 is the primary CDK9 partner cyclin (approximately 80%) [37]. In addition, cyclin T1 has also been reported to be overexpressed in cancer tissues compared to normal tissues, enhancing transcriptional elongation and promoting tumor malignances [40]. However, whether

cyclin T1 is upregulated and synergies with CDK9 upregulation to facilitate the development of pulmonary hypertension requires further investigation.

In summary, CDK9 upregulation and the subsequent enhanced activation of transcriptional elongation, which leads to increased downstream prosurvival and antiapoptotic proteins expression, may be associated with the mechanisms involved in pulmonary artery remodeling and facilitate the progression of pulmonary hypertension. Flavopiridol significantly attenuated the pulmonary artery remodeling, reversed the progression of pulmonary hypertension by inhibiting CDK9-meditated transcription elongation. These findings may provide novel insights in the pathological mechanism of pulmonary hypertension, and CDK9 inhibition, like flavopiridol treatment, should be considered a potential novel therapeutic strategy for pulmonary hypertension.

Declarations

Authors' Contributions WKM, QJ conception and design of research study; QJ , ZQH and NNS performed experiments; QJ, ZQH analysed the data and interpreted results of experiments; WKM, QJ wrote the manuscript; All authors read and approved the final manuscripts.

Funding This work was supported by grant 81470251 (to Weike Mao) from the National Natural Science Foundation of China (Beijing, China).

Availability of Data and Material No, some restrictions will apply.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

Ethics Approval All experiments involving animals were performed with the approval of the Animals Care and Use Committee of Huazhong University of Science and Technology (Wuhan, Hubei, China).

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Tables

Table 1 Primer sequences of genes

Genes	Primer sequences(5’-3’)
Human-CDK9	Sense:ATGGCAAAGCAGTACGACTCG
	Antisense:GCAAGGCTGTAATGGGGAAC
Human-GAPDH	Sense:5’-GAAGGTGAAGGTCGGAGT-3’
	Antisense:5’-GAAGATGGTGATGGGATTTC-3’
Rat-CDK9	Sense:5’-CCTCCGGCACTCGTTGGCTG-3’
	Antisense:5’-GATTTCGGCTCTGGTTGGT-3’
Rat-c-Myc	Sense:5’-CAACGTCTTGGAACGTCAGA-3’
	Antisense:5’-TCATCTGCTTGAACGGACAG-3’
Rat-Mcl-1	Sense:5’-TCATCTCCCGCTACCTGC-3’
	Antisense:5’-ACTCCACAAACCCATCCC-3’
Rat-survivin	Sense:5’-ACCACCGGATCTACACCTTCAAGA-3’
	Antisense:5’-ATTCTCGGTAGGGCAGTGGATGAA-3’
Rat-Larp7	Sense:5’-ACGGTATATGTGGAGTTGC-3’
	Antisense:5’-AACAACATTGCCACATTTCC-3’
Rat-HEXIM1	Sense:5’-CAGAATTGAGCTGCTTGGA-3’
	Antisense:5’-CCAGTGGATAAGTCCTCCC-3’
Rat-HEXIM2	Sense:5’-AGACGAAGACTTCTGGTGG-3’
	Antisense:5’-AATCTCCCTTCCTGGTTGC-3’
Rat-MePCE	Sense:5’-TCGTCACGGGTAACTATGTC-3’
	Antisense:5’-CGGAACATTCTCTTCAGCC-3’
Rat-GAPDH	Sense:5’-GCCCATCACCATCTTCCAGGAG-3’
	Antisense:5’-GAAGGGGCGGAGATGATGAC-3’

Figures

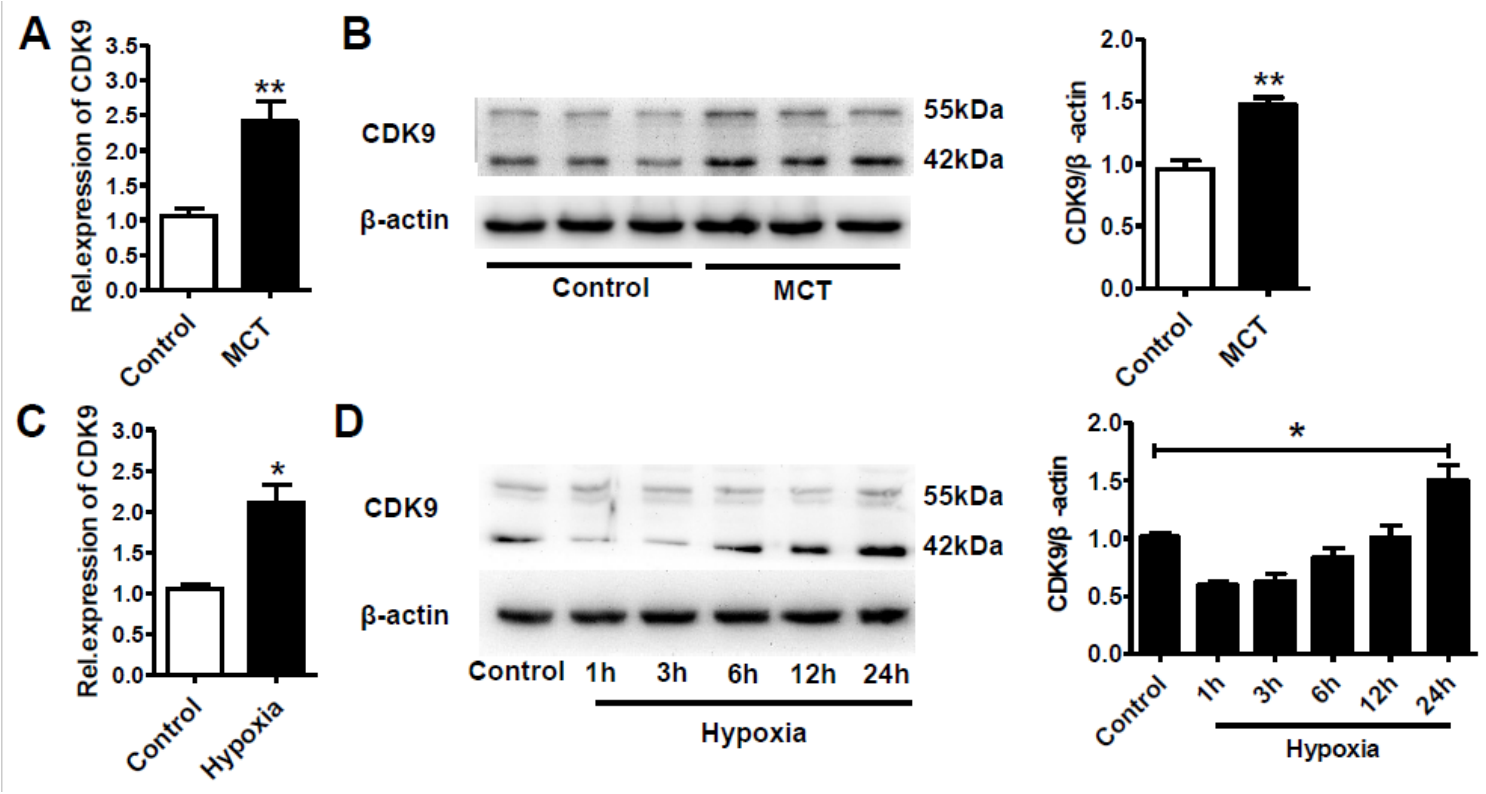

Figure 1

CDK9 Is Overexpressed in PH

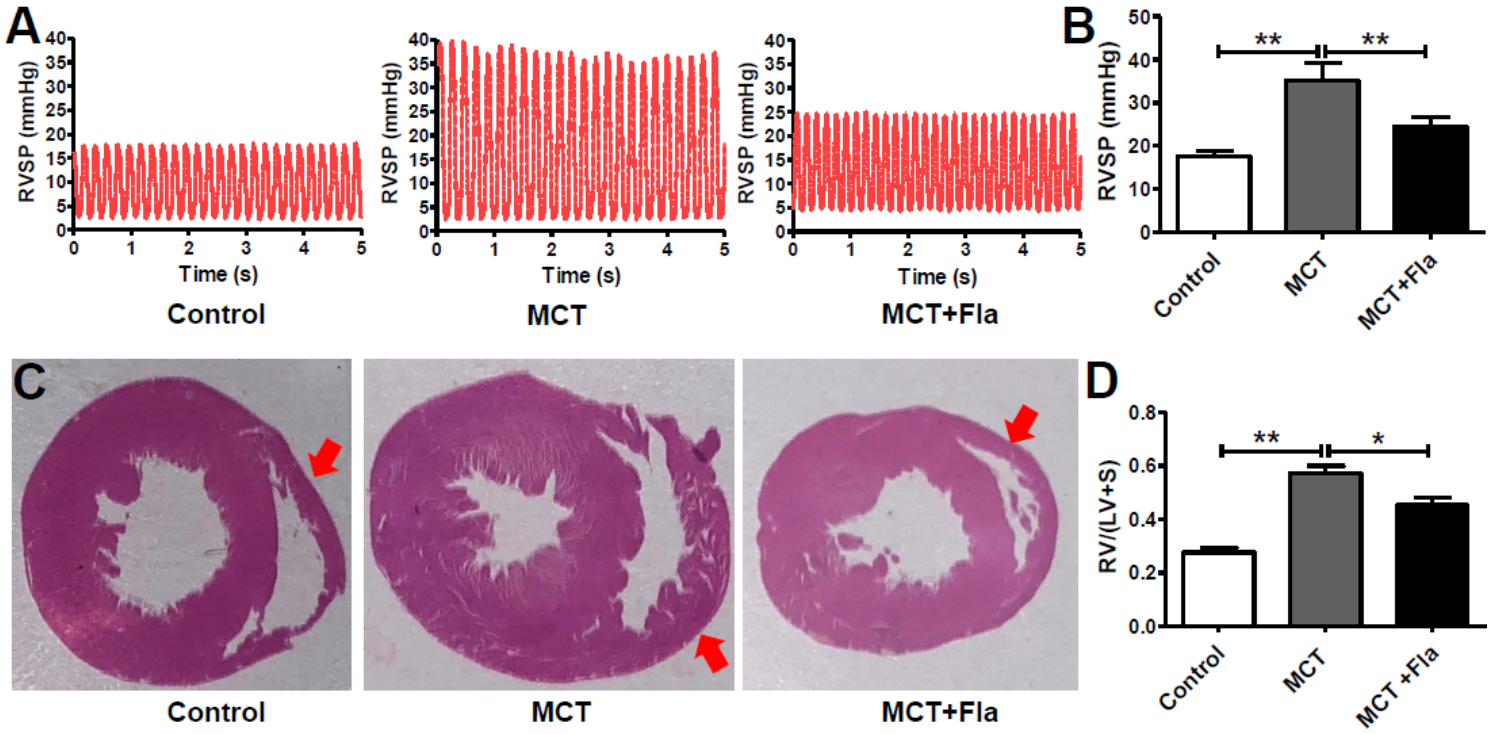

Figure 2

Flavopiridol Mitigates the Progression of MCT-induced PH in Rats

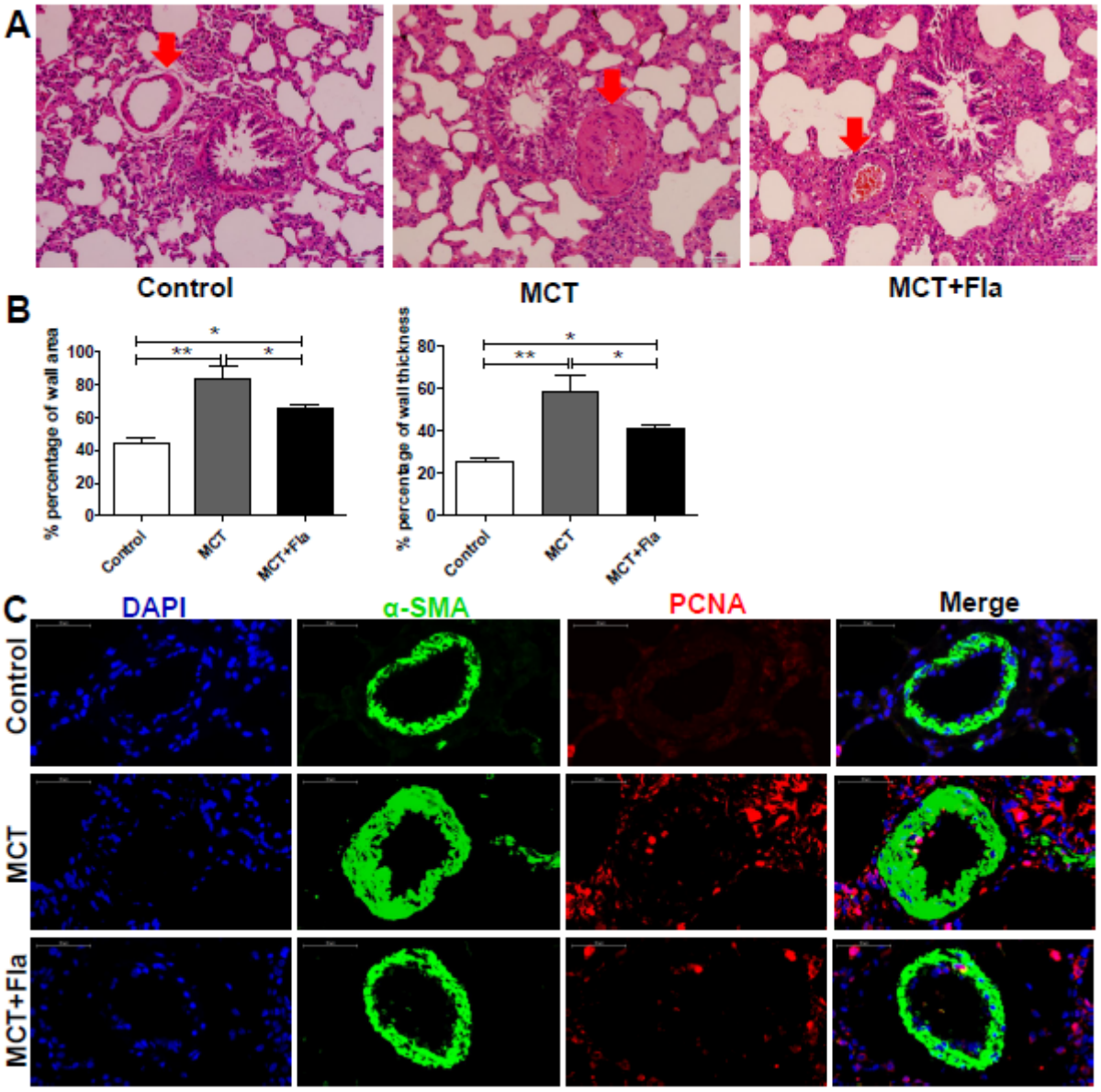

Figure 3

Flavopiridol Attenuates the Vascular Remodeling of MCT-induced PH in Rats

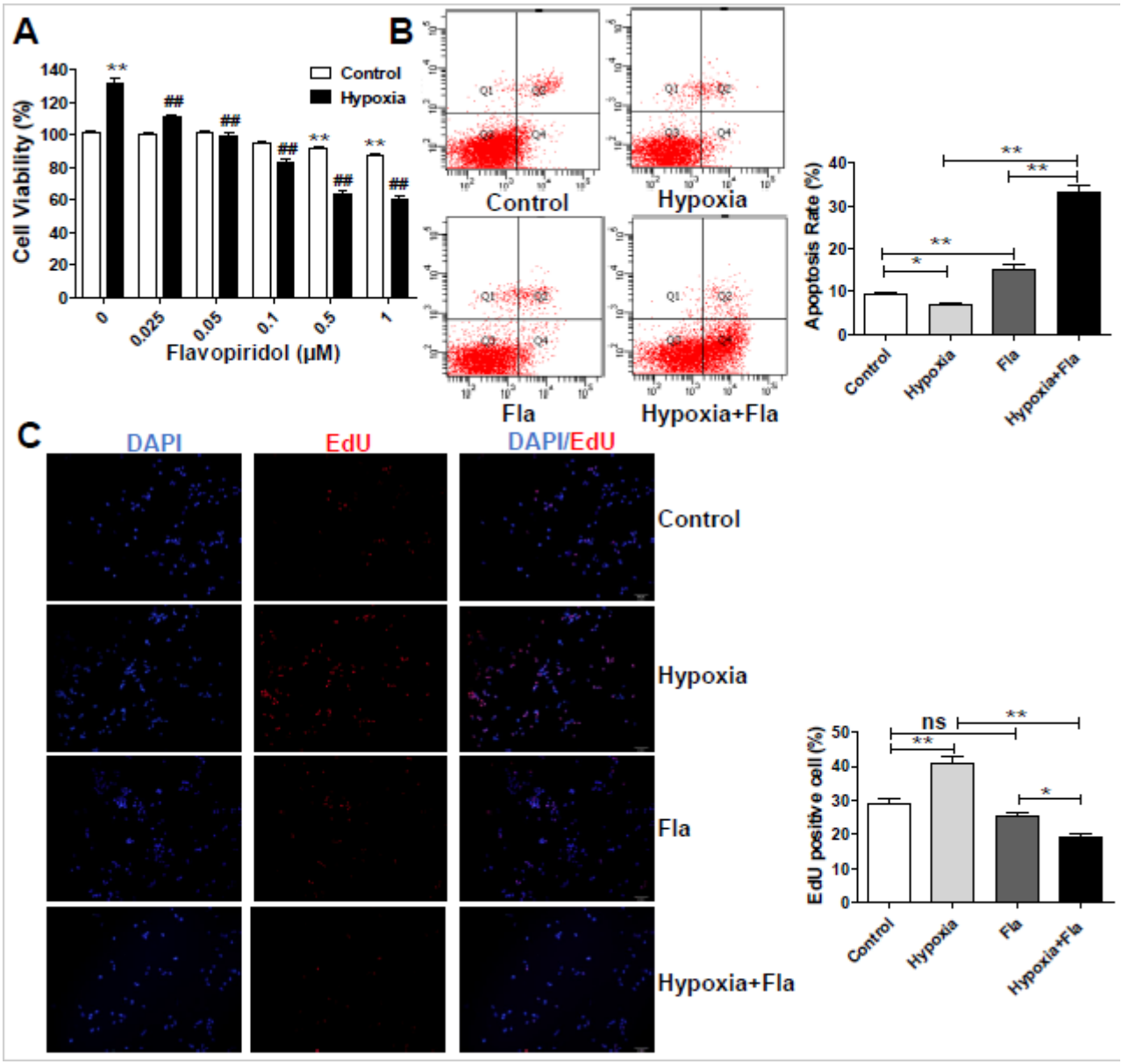

Figure 4

Flavopiridol Inhibites the Overproliferation and Promotes the Apoptosis of HPASMCs under Hypoxia in vitro

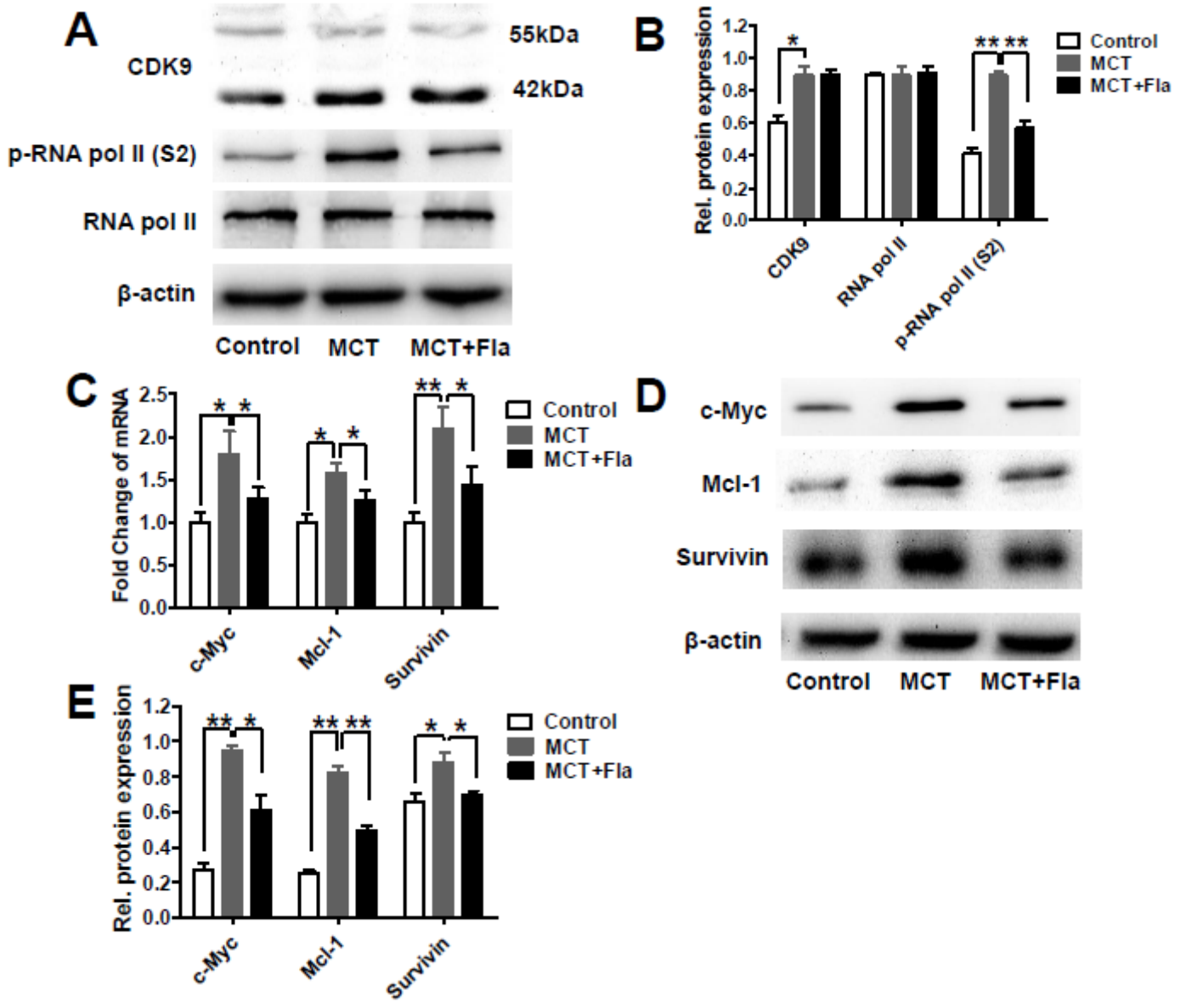

Figure 5

Flavopiridol Inhibites CDK9-mediated Transcriptional Elongation and Suppresses the Expressions of downstream Prosurvival and Antiapoptotic Proteins of PASMCs in PH

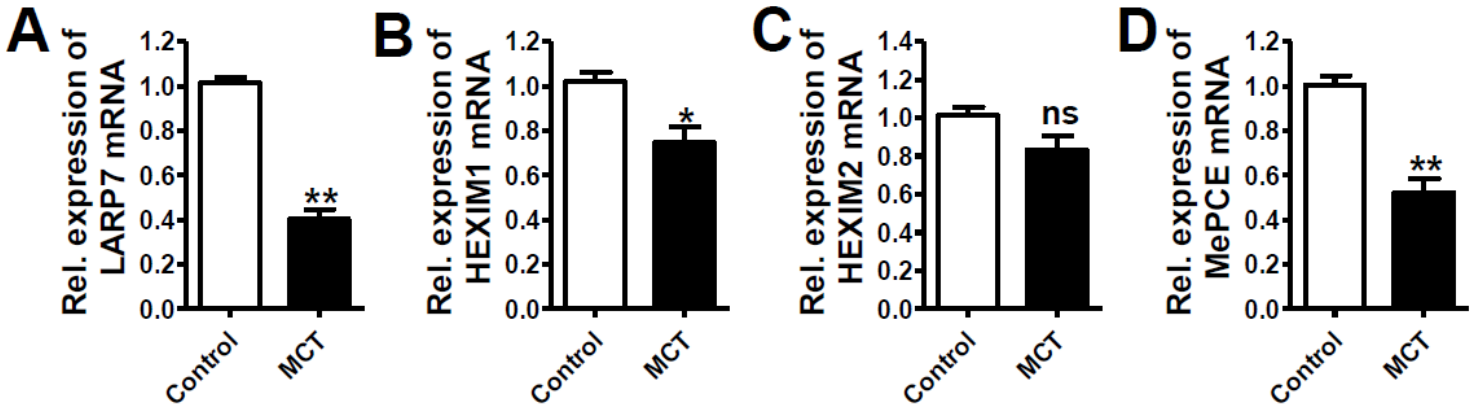

Figure 6

CDK9-related Negative Regulators are Downregulated in PH