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miR-221-3p is upregulated in acute pulmonary embolism complicated with pulmonary hypertension and promotes pulmonary arterial smooth muscle cells growth

--Manuscript Draft--

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Dear Editor,

On behalf of all coauthors, I am submitting our manuscript entitled "***miR-221-3p is upregulated in acute pulmonary embolism complicated with pulmonary hypertension and promotes pulmonary arterial smooth muscle cells proliferation and migration***" for your consideration for publication in "***Open Medicine"***. The highlights of our manuscript are as follows:

1. miR-221-3p is upregulated in the serum of APE-PH patients.
2. miR-221-3p promotes proliferation of PASMCs.
3. miR-221-3p promotes migration of PASMCs.
4. miR-221-3p targets and inhibits PTEN expression.
5. miR-221-3p promotes proliferation and migration of PASMCs by targeting PTEN.

Thank you for your consideration. I am looking forward to receiving your assessment of our manuscript.

Sincerely yours,

Li Zhou

zhoulizzzl@163.com

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miR-221-3p is upregulated in acute pulmonary embolism complicated with pulmonary hypertension and promotes pulmonary arterial smooth muscle cells growth

Lei Tang[1], Shuai Niu[1], Jinwei Xu[2], Wei Lu[3], Li Zhou[3]*

[1]Vascular surgery department, Hebei General Hospital, Shijiazhuang City, 050000, China

[2]Respiratory medicine department, Hebei General Hospital, Shijiazhuang City, 050000, China

[3]Respiratory medicine department, The Third Hospital of Hebei Medical University, Shijiazhuang City, 050000, China

Correspondence: Li Zhou, Respiratory medicine department, The Third Hospital of Hebei Medical University, No.139 Ziqiang Road, Qiaoxi District, Shijiazhuang City, 050000, Hebei Province, China.

Email: zhoulizzzl@163.com

Tel: 0311-87819669

Running title: miR-221-3p promotes PASMCs proliferation

Abstract

Pulmonary arterial smooth muscle cells (PASMCs) functions are associated with the pathogenesis of pulmonary hypertension (PH) which is a life-threatening complication of acute pulmonary embolism (APE). This study sought to explore the expression pattern of microRNA (miR)-221-3p in APE-PH patients and its role in PASMCs proliferation and migration. The clinical data and venous blood of APE-PH patients were collected. The expression levels of miR-221-3p and phosphatase and tensin homolog (PTEN) in serum were determined, followed by receiver operator characteristic curve analysis of miR-221-3p diagnostic efficacy. PASMCs were transfected with miR-221-3p mimics and PTEN-overexpressed vector, followed by assessment of cell viability, proliferation, and migration through cell counting kit-8, 5-ethynyl-2'-deoxyuridine, Transwell, and wound healing assays. The binding between miR-221-3p and PTEN 3'UTR region was testified by the dual-luciferase assay. miR-221 was upregulated in the serum of APE-PH patients and presented with good diagnostic efficacy with 1.155 cutoff value, 66.25% sensitivity, and 67.50% specificity. miR-221 was negatively correlated with PTEN in APE-PH patients. miR-221 overexpression facilitated PASMCs proliferation and migration *in vitro*. miR-221-3p bound to PTEN 3'UTR region to decrease PTEN protein levels. PTEN overexpression abolished the promotive role of miR-221-3p in PASMCs. Overall, miR-221-3p targeted PTEN3 to facilitate PASMC proliferation and migration.

Key words: Acute pulmonary embolism; Pulmonary hypertension; Pulmonary artery smooth muscle cells; Proliferation; Migration

1 Introduction

Pulmonary embolism (PE) is a common contributor to cardiovascular fatalities, clinically characteristic of breathlessness, pleuritic chest pain, and syncope [1]. Prompt diagnosis and management are of paramount importance since over 70% of deaths from acute PE (APE) happen within the first hour [2]. However, due to its non-specific clinical symptoms, its diagnosis lacks a standardized approach, largely reliant on radiological imaging [3]. Even worse, pulmonary hypertension (PH) is considered to be the most life-threatening complication that prevails in 2–4% PE patients [4]. It can cause sustained obstruction and elevated pulmonary vascular resistance and even right ventricular dysfunction, increasing the mortality risk of PE [5]. Currently, there is no identification of the causative mechanism underlying APE-PH. For this reason, it is of urgent demand to identify molecules that play a role in the diagnosis and treatment of APE-PH.

Despite a variety of pathologic factors related to PH, pulmonary arterial smooth muscle cells (PASMCs) hyperplasia is a cardinal inducer of pulmonary vascular remodeling which is the common feature of PH development [6]. Like other vascular smooth muscle cells (VSMCs), PASMCs are quiescent in most times, however once activated by environmental cues, such as hypoxia, they can change their phenotypes, contributing to development, tissue injury, and vessel remodeling. There is compelling evidence that increased proliferative and migrative phenotypes of PASMCs play a critical role in the process of PH [7]. MicroRNAs (miRNAs) are well-studied small non-coding RNAs with about 22 nucleotides in length and an ability to promote messenger RNA (mRNA) degradation through interaction with the 3'untranslated region (UTR) [8]. They participate in various cellular processes, such as apoptosis, proliferation, migration, and differentiation, and further affect the progression of human diseases [9,10]. A number of miRNAs have been identified to be involved in PASMCs phenotypic switching, indicating their role in the prevention of PH [11]. In this context, we exploited a miRNA-based approach to expound on the pathogenesis of APE-PH and provide novel targets.

miR-221 has been found to be elevated in the plasma of PH patients, suggesting its clinical significance in PH diagnosis [12]. Moreover, miR-221-3p overexpression is likely to

accelerate the proliferation of PASMCs, highlighting its participation in PH process [13]. However, its role in APE-PH remains unknown. Phosphatase and tensin homolog (PTEN) is deemed as a dual phosphatase with both protein and lipid phosphatase properties and exerts tumor-suppressive and metabolic functions [14]. PTEN overexpression inhibits lung injury and inflammation in the context of APE [15]. Besides, PTEN dysregulation contributes to the pathogenesis of PH secondary to chronic obstructive pulmonary disease [16]. Most importantly, our bioinformatic data nominated PTEN as a downstream target gene of miR-221-3p, indicating their close relationship in APE-PH. Given the currently available evidence, we hypothesized that miR-221-3p plays a role in APE-PH by targeting PTEN, hoping to provide a rationale for the diagnosis and management of APE-PH.

2 Methods

2.1 Clinical samples

A total of 80 cases of APE patients complicated with PH (with an average age of 67.34 ± 3.66, 44 males, 36 females) who received treatment in Hebei General Hospital from June 2019 to November 2021 were included in this study and 80 healthy individuals at the same time period were included as the controls. Our study was ratified by the Ethics Committee of Hebei General Hospital.

Inclusion criteria were as follows: (1) APE is diagnosed according to the relevant criteria specified by the European Society of Cardiology [17] and PH is diagnosed according to a previous review [18]. (2) with a color-coded Doppler sonography (1.7-3.4 MHz, Vivid7, General Electric Company, Boston, MA, USA) to evaluate pulmonary arterial systolic pressure (PASP) on admission, under APE in quiet state, PASP > 30 mm Hg or mean pulmonary arterial pressure > 25 mm Hg; under APE in active state, mean pulmonary arterial pressure > 30 mm Hg; (3) embolism in the lower extremity vein; (4) no chronic obstructive pulmonary disease, pulmonary tuberculosis and other pulmonary diseases; (5) no mental disorder and speech and hearing impairments; (6) with complete clinical data.

Exclusion criteria were as follows: (1) with a history of surgery for lung diseases; (2) with heart, liver and kidney complications; (3) with immune or malignant tumor complications; (4) with severe infection complications.

Patients and healthy individuals were all collected for 5 mL of fasting venous blood. The blood samples were centrifuged to separate the serum and sequenced by quantitative real-time polymerase chain reaction (qRT-PCR) to determine the serum target genes.

2.2 Cell culture

Human PASMCs were procured from Sciencell (San Diego, CA, USA) and cultured in a smooth muscle cell medium with a humidified 5% air and 37℃. Cells were detached with 0.05% trypsin/ethylene diamine tetraacetic acid (Invitrogen, Carlsbad, CA, USA) and then passaged. The cells of the third to fifth generations were used for the subsequent assays.

2.3 Cells transfection

miR-221-3p mimic (miR-mimic) and its corresponding control (mimic-NC) were obtained from GenePharma (Shanghai, China) and transfected into PASMCs using Lipofectamine 3000 (Invitrogen). To overexpress PTEN, the complementary DNA (cDNA) sequence of PTEN was amplified and cloned into the pcDNA3.1 plasmid to construct pcDNA3.1-PTEN (oe-PTEN), and the plasmid was transfected into PASMCs using Lipofectamine 3000 in a similar way, with the pcDNA3.1 empty vector serving as the negative control (oe-NC). Then, 48 h after transfection, cells were collected and analyzed by qRT-PCR to determine transfection efficiency.

2.4 Cell counting kit-8 (CCK-8) assay

The viability of PASMCs after different treatments was evaluated with the help of the CCK-8 assay kits. PASMCs with different treatments were loaded into the 96-well plates at a density of 4×10^4 cells/well. Each well was added with 10 μL of CCK-8 reagent for 2 h incubation at 37℃, followed by determining the absorbance at a wavelength of 450 nm.

2.5 5-ethynyl-2'-deoxyuridine (EdU) assay

PASMCs were placed in 96-well plates (1×10^5 cells/well) and incorporated with 50 μM EdU medium (100 μL/well, Thermo Fisher Scientific, Waltham, MA, USA) for 2 h (5% CO_2, 37℃). According to the manufacturer's protocol, cells were treated with 4% paraformaldehyde for 30 min and stained with 100 μL Apollo staining solution (RiboBio,

Guangdong, China) at ambient temperature for 30 min. Next, 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific) was used for 30 min nuclear staining. Afterwards, stained cells were visualized using a fluorescence microscope (Olympus, Tokyo, Japan) and results were shown as percentages.

2.6 Tanswell assay

First, 100 μL of cell suspension containing 5×10^4 PASMCs was transferred to the apical chamber with serum-free medium. The Transwell chamber was preserved in a 24-well plate containing 500 μL complete medium (Dulbecco's modified Eagle medium + 10% fetal bovine serum). After 24 h incubation, non-migrative cells were removed by cotton swabs. Migrative cells were treated with 10 min fixation with 4% methanal solution and 15 min staining with 0.4% crystal violet. Migrative PASMCs were observed and counted under an optical microscope.

2.7 Wound healing assay

PASMCs were cultured in the 6-well plates until formation of a monolayer. A wound gap was created with a sterile pipette tip. All monolayer cells were scratched by the same sterile pipette tip to create scratches, with scratches towards the same direction. Following that, cells were rinsed with phosphate buffered saline twice to remove the detached cells. After 24 h incubation, the distance of cell migration was measured to determine the migration rate.

2.8 qRT-PCR

The total RNA was extracted from PASMCs or the serum by applying the TRIzol reagent. Then, 1 μg of total RNA was converted into the cDNA with the help of a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific). qRT-PCR was conducted by applying a SensiFAST SYBR No-ROX kit (Bioline, Taunton, MA, USA) on the CFX RTPCR system (Bio-Rad, Hercules, MA, USA). The relative account of gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [19], with U6 small nuclear RNA [20] as the control gene for miR-221-3p and GAPDH for mRNAs. PCR primers are exhibited in Table 1.

2.9 Western blot assay

The total protein was separated from PASMCs through the treatment of radioimmunoprecipitation assay reagent (Abcam, Cambridge, MA, USA), with a bicinchoninic acid kit (Beyotime, Shanghai, China) to quantify protein concentration. Then,

being separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 20 μg of protein was transferred onto polyvinylidene fluoride membranes. After being blocked by 5% skim milk, the overnight incubation was performed with primary antibodies against PTEN (1:1000, ab170941, Abcam) and GAPDH (1: 10000, ab181602, Abcam), followed by 1 h incubation at ambient temperature with secondary antibody against immunoglobulin G (1: 5000, ab6721, Abcam). Western blots were visualized with an enhanced chemiluminescence assay kit (Pierce, Rockford, IL, USA) and quantified with the help of ImageJ software (NIH, Bethesda, MD, USA).

2.10 Dual-luciferase reporter assay

The binding site of miR-221-3p and PTEN was predicted on the StarBase database (http://starbase.sysu.edu.cn/) [21]. PTEN 3'UTR containing the binding site of miR-221-3p was amplified from human genome DNA and cloned into the pmirGLO vector (Promega, Madison, WI, USA) to form the wild-type vector (PTEN-WT). Next, the above 3'UTR was treated with site-directed mutation to form the mutant-type vector (PTEN-MUT). PTEN-WT or PTEN-MUT was co-transfected into PASMCs with miR-221-3p mimic or mimic NC. A dual-luciferase assay kit was used to determine luciferase activity 48 h after transfection.

2.11 Statistical analysis

All data were processed with SPSS21.0 statistical software (IBM SPSS Statistics, Chicago, IL, USA) and GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) for statistical analysis and graphing. Data were examined to be normally distributed with homogeneity of variance. Data between two panels were compared using the *t* test and data among multiple panels were compared using one-way or two-way analysis of variance (ANOVA), and the Tukey's multiple comparison test was used for the post-hoc test. $P < 0.05$ denotes statistical significance and $P < 0.01$ denotes highly statistical significance.

3 Results

3.1 miR-221-3p is upregulated in APE-PH patients and has good diagnostic efficacy

We determined the expression pattern of miR-221-3p in the serum of APE-PH patients and observed higher expression levels of miR-221-3p in the patient group ($P < 0.01$, Fig 1A). The

receiver operator characteristic (ROC) curve revealed the area under the curve (AUC) of 0.6929, the cutoff value of 1.155, the sensitivity of 66.25%, and the specificity of 67.50% (Fig 1B). These data indicated that miR-221-3p was upregulated in APE-PH patients and helped the diagnosis of APE-PH.

3.2 miR-221-3p overexpression promotes the proliferation of PASMCs

To provide insights into the role of miR-221-3p in APE-PH, we cultured PASMCs *in vitro* and transfected miR-221-3p mimic into cells, resulting in miR-221-3p upregulation ($P < 0.01$, Fig 2A). The CCK-8 assay showed that the viability of PASMCs was increased after miR-221-3p overexpression ($P < 0.01$, Fig 2B). The EdU assay revealed that the proliferation potential of PASMCs was enhanced after miR-221-3p overexpression ($P < 0.01$, Fig 2C). These findings elicited that miR-221-3p overexpression promoted the proliferation of PASMCs.

3.3 miR-221-3p overexpression promotes the migration of PASMCs

Meanwhile, we performed Transwell and wound healing assays to assess the change in cell migration potential. The results revealed that the number of migrative cells and migration distance were increased secondary to miR-221-3p overexpression ($P < 0.01$, Fig 3A-B), suggesting that miR-221-3p overexpression promoted the migration of PASMCs as well.

3.4 miR-221-3p targets and inhibits PTEN expression

The downstream target genes of miR-221-3p were obtained from the StarBase database, among which PTEN has been unveiled to be downregulated in APE [15]. The dual-luciferase reporter assay validated the binding relationship between miR-221-3p and PTEN. The mutated sequence cannot bind to miR-221-3p, leading to no reduction in luciferase activity, but wild-type sequence can bind to miR-221-3p and further markedly reduced the luciferase activity ($P < 0.05$, Fig 4 A). In addition, the expression levels of PTEN were found to be diminished in the serum of APE-PH patients and further reduced in PASMCs in response to miR-221-3p overexpression ($P < 0.05$, Fig 4 B-C). Moreover, miR-221-3p expression was negatively correlated with PTEN in the serum of patients ($P < 0.05$, Fig 4 D). Collectively, these findings suggested that miR-221-3p can target and inhibit PTEN expression.

3.5 PTEN overexpression reverses the promotive role of miR-221-3p in the proliferation-migration cascade of PASMCs

At last, we validated the above mechanism in the proliferation and migration of PASMCs. PASMCs cells were transfected with PTEN-overexpressed vector ($P < 0.05$, Fig 5A) and combined with miR-221-3p mimic. Our experiments showed that after PTEN overexpression, cell potentials for proliferation ($P < 0.05$, Fig 5B-C) and migration ($P < 0.05$, Fig 5D-E) were reduced. The above data elicited that miR-221-3p enhanced the proliferation-migration cascade of PASMCs by targeting PTEN.

4 Discussion

Pulmonary hypertension (PH) is a rare complication of APE but represents an important risk for morbidity and mortality [4]. Central to the pathogenesis of this disease is pulmonary vascular remodeling which is a result of abnormal proliferation and migration of PASMCs [6]. MiRNAs signatures play a crucial role in the onset and development of PH by regulating PASMCs functions [11]. It remains necessary to identify new miRNAs that are essential for PASMCs functions, thus providing novel insights into APE-PH diagnosis and treatment. The unique aspect of our study is to determine the serum level of miR-221-3p in APE-PH patients and analyze its diagnostic efficacy and further evaluate the role of the miR-221-3p/PTEN axis in the proliferation-migration cascade of PASMCs at cellular level.

Knockdown of miR-221 is likely to inhibit the proliferation of VSMCs from rat aortas, nominating it as a potential target for proliferative vascular diseases [22]. Moreover, miR-221 has been shown a 4-fold upregulation in the plasmid of APE patients relative to health individuals [12]. As a member of the miR-221 family, miR-221-3p overexpression can dramatically elevate the proliferation and migration of aortic VSMCs, thereby mediating vascular remodeling [23]. miR-221-3p acts as a pro-proliferative miRNA in PASMCs in the case of PH [13]. It has been also shown to be increased in the right ventricle secondary to PH, highlighting its significance for PH-associated cardiac function [24]. Furthermore, since PH shows several analogous features with carcinogenesis [25], the carcinogenic activity of miR-221-3p in several subtypes of lung cancer [26,27] indicates its close relationship with PH pathogenesis. In our cohorts, we uncovered the upregulation of miR-221-3p in the serum of APE-PH patients. The ROC analysis to evaluate the diagnostic efficacy of miR-221-3p in

APE-PH showed the AUC of 0.6929, the cutoff value of 1.155, the sensitivity of 66.25%, and the specificity of 67.50%, suggesting that serum miR-221-3p > 1.155 is beneficial for APE-PH diagnosis.

Subsequently, we cultured PASMCs *in vitro* and overexpressed miR-221-3p in cells. The CCC-8 and EdU assays revealed that miR-221-3p overexpression promotes the proliferation of PASMCs. The Transwell and wound healing assays showed the role of miR-221-3p overexpression in enhancing the migration of PASMCs. It has been uncovered that hypoxia upregulates the expression levels of miR-221-3p in PASMCs [13]. Therefore, we conjectured the upstream mechanism of miR-221-3p in APE-PH is associated with hypoxia. To understand the downstream mechanism of miR-221-3p in PASMCs proliferation and migration, we used the StarBase database and the dual-luciferase assay to confirm that there was a binding relationship between miR-221-3p and PTEN. Curcumin is known to elevate PTEN expression to play a protective role in APE rats [15]. Hypoxia contributes to the pathogenesis of PH through the miR-214/PTEN axis [16]. The silencing of PTEN is associated with PH-induced cardiopulmonary vascular remodeling [28]. Accordingly, we noticed the downregulation of PTEN in the serum of APE-PH patients and the decrease in PTEN expression levels in PASMCs caused by miR-221-3p overexpression. Besides, miR-221-3p expression was negatively correlated with PTEN expression in the serum of APE-PH patients. Collectively, our findings suggested that miR-221-3p bound to PTEN3' UTR to repress PTEN expression.

On the other hand, PTEN is known to interact with multiple genes, such as transforming growth factor-beta1, methyltransferase-like 3, and YTH domain family 2, aiming to regulate the proliferation and migration of PASMCs [29,30]. Thereafter, we overexpressed PTEN in PASMCs, followed by rescue experiments with miR-221-3p mimic. Our results revealed that PTEN overexpression moderated the promotion of PASMCs proliferation and migration caused by miR-221-3p overexpression. However, excessive PASMCs proliferation and migration just represent a part of PH pathological features. We did not explore the role of the miR-221-3p/PTEN axis in the regulation of other APE-PH pathological features. In addition, we only explored the role of the miR-221-3p/PTEN axis at cellular level, and whether other downstream targets of miR-221-3p participate in PASMCs proliferation and migration

remains unknown. Future studies are needed to investigate the role of the downstream targets of miR-221-3p in PASMCs proliferation and migration and clinically analyze the association between miR-221-3p and symptoms of APE or PH.

4 Conclusions

In a nutshell, our study for the first time unraveled that miR-221-3p decreases the protein levels of PTEN by binding to PTEN3'UTR, thus accelerating the proliferation-migration cascade of PASMCs. Besides, miR-221-3p boasts good diagnostic efficiency in APE-PH. Our findings may provide novel theoretical knowledge for the diagnosis and management of APE-PH.

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Author contributions: Lei Tang, and Shuai Niu conceived the study. Jinwei Xu, Wei Lu, and Li Zhou analyzed and interpreted patient data. Jinwei Xu wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures and Figure Legends

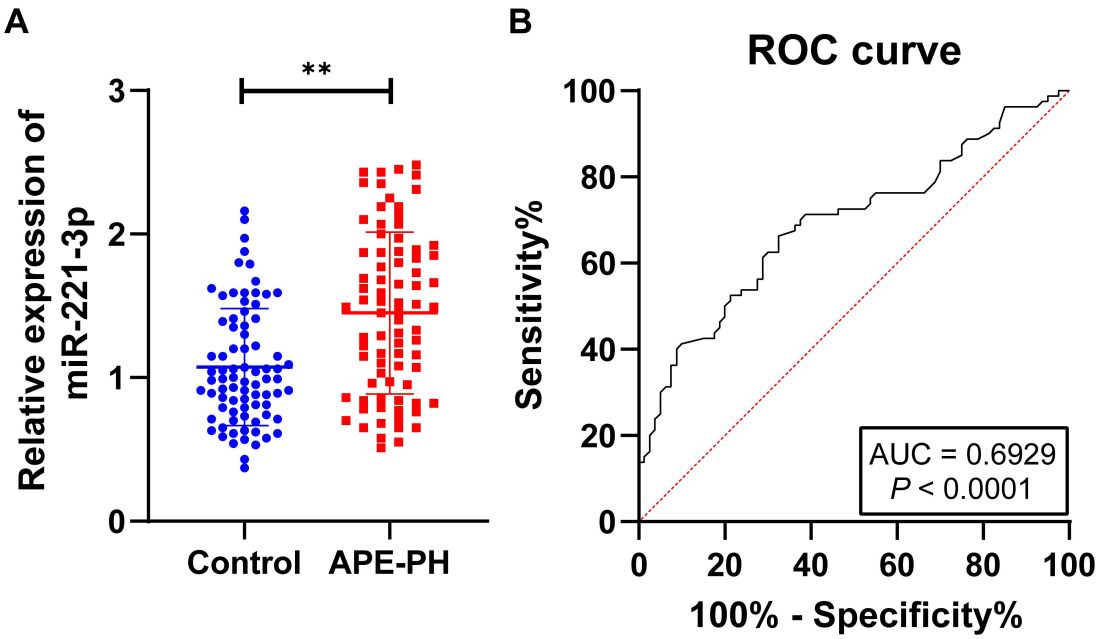


Fig 1 miR-221-3p is upregulated in APE-PH patients and helps the diagnosis of APE-PH. First, 80 APE-PH patients were selected, with 80 healthy individuals as the controls. A: miR-221-3p expression levels in the serum were determined by qRT-PCR, N=80; B: Diagnostic effect of serum miR-221-3p for APE-PH was analyzed by the ROC curve, AUC: area under the curve. Data in panel A were analyzed by the *t* test. ** $P < 0.01$.

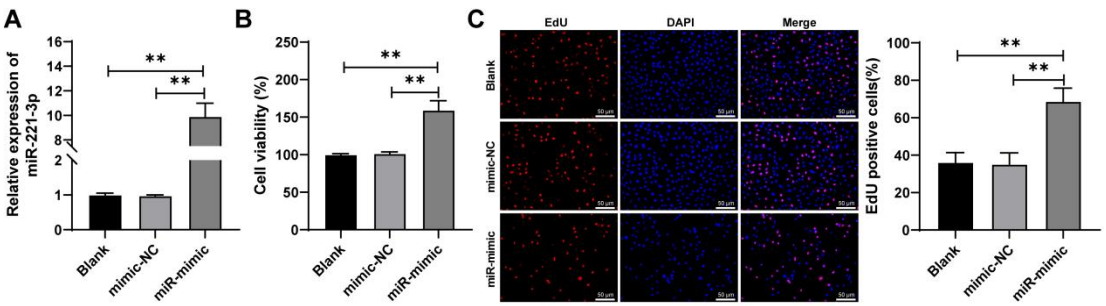


Fig 2 miR-221-3p overexpression promotes the proliferation of PASMCs. PASMCs were transfected with miR-221-3p mimic (miR-mimic), with mimic-NC as the negative control. A: miR-221-3p expression levels in PASMCs were determined by qRT-PCR; B: Cell viability was evaluated by the CCK-8 assay; C: Cell proliferation potential was evaluated by EdU assay. Cell experiments were replicated 3 times independently. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. ** $P < 0.01$

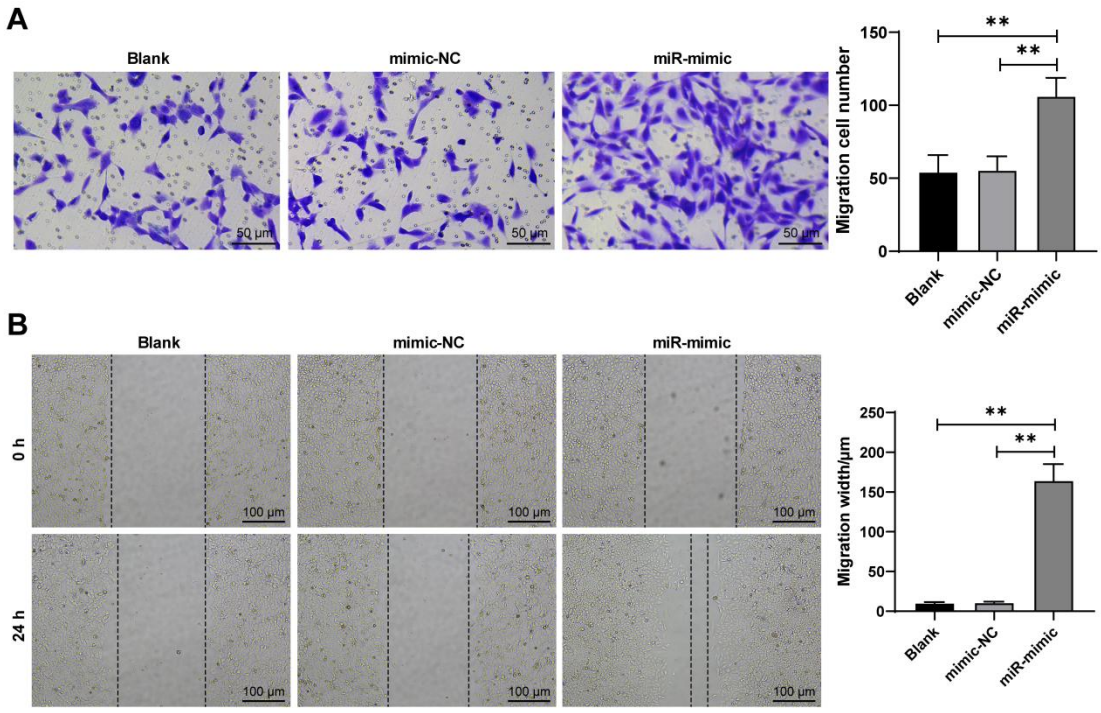
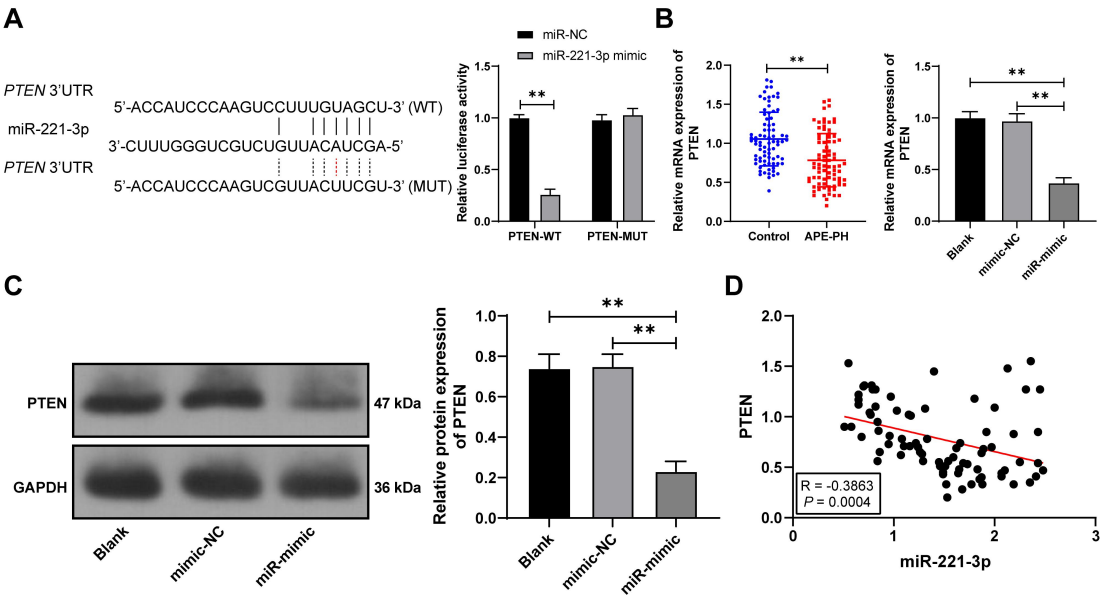
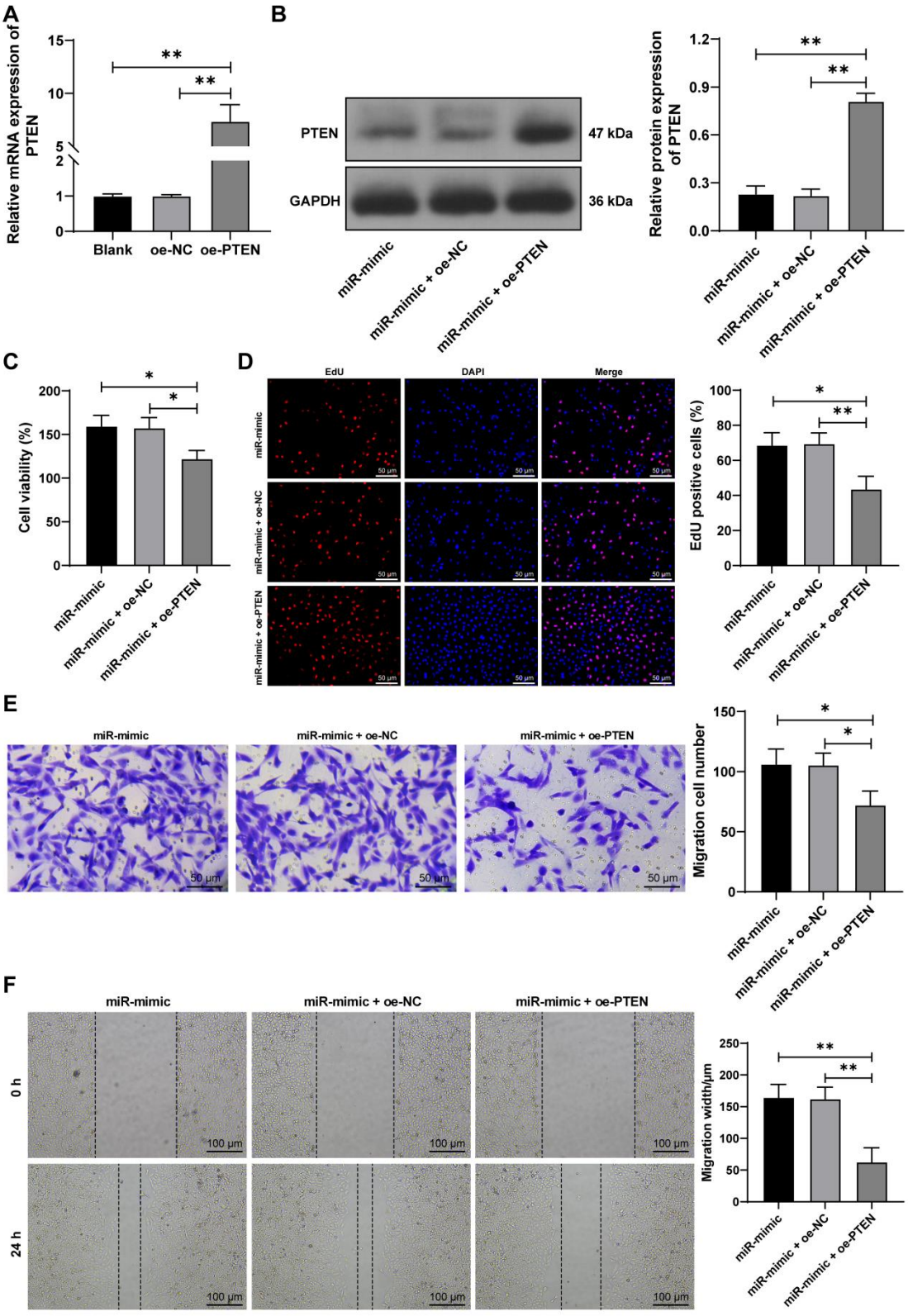


Fig 3 miR-221-3p overexpression promotes the migration of PASMCs. PASMCs were transfected with miR-221-3p mimic (miR-mimic), with mimic-NC as the negative control. A: The number of migrative cells was measured by Transwell assay; B: Migration distance was determined by wound healing assay. Cell experiments were replicated 3 times independently. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. ** $P < 0.01$.



389 **Fig 4** miR-221-3p targets and inhibits PTEN expression. A: The binding relationship between
390 miR-221-3p and PTEN 3'UTR was analyzed by the dual-luciferase reporter assay; B: PTEN
391 mRNA levels in the serum (N=80) and PASMCs were determined by qRT-PCR; C: PTEN
392 expression levels in PASMCs with different treatments were determined by Western blot
393 assay; D: Pearson analysis uncovered a negative correlation between miR-221-3p and PTEN
394 expression in the serum. Cell experiments were replicated 3 times independently. Data in
395 panel A were analyzed by two-way ANOVA and data in panel B (histogram) and C were
396 analyzed by one-way ANOVA, followed by Tukey's multiple comparison. test. Data in panel
397 B (scatter diagram) were analyzed by the *t* test. ** $P < 0.01$.



398 **Fig 5** PTEN overexpression reverses the promotive role of miR-221-3p in the proliferation
399 and migration of PASMCs. PTEN-overexpressed vector (oe-PTEN) was transfected into
400 PASMCs, with empty vector (oe-NC) as the negative control, followed by rescue experiments
401 with miR-221-3p mimic. A-B: PTEN expression levels in PASMCs were determined by

qRT-PCR and Western blot assay; C: Cell viability was assessed by the CCK-8 assay; D: Cell proliferation was assessed by EdU assay; E: The number of migrative cells was determined by Transwell assay; F: The migration distance was measured by wound healing assay. Cell experiments were replicated 3 times independently. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison. test. ** $P < 0.01$.

Tables and Table Captions

Table 1 qPCR primers

Gene	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
PTEN	CAGCCATCATCAAAGAGATCG	GGATATTGTGCAACTCTGCAATTA
GAPDH	CTCAACTACATGGTTTAC	CCAGGGGTCTTACTCCTT
U6	TCGCTTCGGCAGCACATATACT	GCTTCACGAATTTGCGTGTCATC
miR-221-3p	GCCGAGAGCTACATTGTCTG	CTCAACTGGTGTCGTGGA