SCUBE1 inhibits pulmonary artery smooth muscle cell proliferation and migration in acute pulmonary embolism by modulating BMP7 (#91224)

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SCUBE1 inhibits pulmonary artery smooth muscle cell proliferation and migration in acute pulmonary embolism by modulating BMP7

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Objectives: Activated platelets after acute pulmonary embolism (APE) can release bioactive factors to promote pulmonary artery smooth muscle cell (PASMC) proliferation and migration. SCUBE1 was reported to be able to participate in platelet-platelet interactions, possibly involved in the activation of platelets in early onset thrombi. This study was intended to investigate the expression changes of SCUBE1 in PASMCs after APE and the underlying mechanism. **Methods:** The plasma of APE patient and healthy control was collected. The hyperproliferative model of PASMCs was established by using PDGF as a stimulator. Cell counting kit-8 (CCK-8), Transwell, wound healing, Western blot and co-IP assays were used to investigate the regulation of SCUBE1-mediated BMP7 on PDGF-induced PASMC proliferation and migration. **Results:** Increased of SCUBE1 was found in APE patient plasma and PDGF-induced PASMCs. Interference of SCUBE1relieved PDGF-induced proliferation, migration and decreased PCAN expression. Mechanism studies demonstrated that SCUBE1 bound with BMP7 and positively BMP7 expression. Enhanced of BMP7 abolished the impact of SCUBE1 silencing on proliferation and migration of PASMCs after PDGF treatment. **Conclusion:** In the PDGF-induced proliferation of PASMCs, the expression of SCUBE1 was upregulated and the expression of BMP7 was downregulated, SCUBE1 silencing may inhibit the PDGF-induced proliferation and migration of PASMCs by restraining BMP7.

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Abstract

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Keywords: Acute pulmonary embolism, pulmonary artery smooth muscle cell, SCUBE1, proliferation, migration

1 Introduction

Acute pulmonary embolism (APE) is the collective term for a group of disorders or clinical syndromes in which various emboli occlude the pulmonary arterial system as the cause of their morbidity [1, 2]. APE is a common and severe life-threatening condition associated with myocardial infarction, stroke and known as the three major cardiovascular diseases. The rapid progression of the onset of APE, the variety of symptoms, the high case fatality rate of severe illness, and the common phenomenon of diagnosis and treatment error, increasingly become the life-threatening medical difficulty of people in today's era [3, 4]. Although diagnostic techniques are constantly improving, approximately 30% of patients still die without being diagnosed with acute pulmonary embolism [5]. At present, clinical according to the degree of embolization is commonly used drug treatment modalities are divided into thrombolytic therapy and anticoagulant therapy, but thrombolytic side effects are also more obvious, and patients need to face the risk of bleeding. Therefore, clear diagnosis and seeking for therapeutic drugs and targets become effective means to reduce the case fatality of acute pulmonary embolism.

With the development of biological research, researchers have found that platelet-derived growth factor (PDGF), a major bioactive factor released by activated platelets after APE, has a strong vasoconstrictor effect and is also able to promote the proliferation of pulmonary artery smooth muscle cells (PASMCs) and promote the transformation of fibroblasts into smooth muscle cells, which leads to the remodeling of the pulmonary vasculature [6, 7]. PASMCs are located in the tunica media of the pulmonary artery wall and, under pathological stimuli, can migrate from the tunica media to the intima, the interior of which initiates the abnormal proliferation of PASMCs through a series of cell signal transduction systems [8]. Therefore, it is important to elucidate the molecular mechanisms and signaling pathways that lead to abnormal proliferation and migration in PASMCs.

Signal peptide cub epidermal growth factor domain containing protein 1 (SCUBE1) is an activated platelet surface expressed and secreted glycoprotein in early embryonic development stage, which can promote the interaction between platelets and support platelet matrix adhesion [9]. A study indicated that SCUBE1 was upregulated in renal tumor tissues, which could be a promising biomarker in renal cell cancer [10]. In aneurysmal subarachnoid hemorrhage (aSAH), SCUBE1 level are correlated with increasing severity and poor outcomes of aSAH patients [11]. Some foreign scholars have reported that SCUBE1 expression is high in acute pulmonary embolism patients and extremely low in healthy people, suggesting that SCUBE1 is promising as a biomarker for the early diagnosis of APE [12]. However, there are few studies on SCUBE1 in APE, and whether it is involved in PASMC proliferation and migration remains to be clarified.

In the current study, we aim to decipher the role and regulator network of SCUBE1 in the proliferation, migration of PASMCs and provide a theoretical basis for the research of SCUBE1 on treatment of APE.

2 Methods and materials

2.1 Study subjects

Study subject acute pulmonary embolism (APE) patients who visited the The Second Affiliated Hospital of Xiamen Medical College were selected as the case group, and those who had no previous history of allergy and were in good health were selected as the control group. There were no significant differences in age and gender comparisons between the two groups. All patients with APE were diagnosed by computed tomography pulmonary arteriography and without any treatment before the diagnosis was confirmed. And exclusion of patients with concomitant right ventricular dysfunction of other causes, and other causes of SCUBE1 gain, such as acute coronary syndrome, acute myocardial infarction, acute ischemic cerebrovascular disease, peripheral artery disease, or other ischemic diseases. Venous blood (3 mL) was drawn from the APE and healthy control groups, respectively, and the blood was centrifuged, and the upper plasma was stored in a −80°C freezer for further use. All samples obtained in this study were approved by the

ethics committee of the Xiamen Medical College and abided by the ethical guidelines of the Declaration of Helsinki, and have received written informed consent.

2.2 Cell culture and treatment

Human PASMCs purchased from ATCC (VA, USA) were seeded into 6-well plates and cultured in complete medium (DMEM/F12 medium + 10% FBS) at 37°C and 5% CO_2. For proliferation induction, PASMCs were added with different concentrations of PDGF (10, 20, or 40 ng/mL). To grow the cells to 70% - 80%, the cells were divided into control group, PDGF group (PDGF (20 ng/mL) was added into PASMCs for 12 h of stimulation), PDGF + sh-NC group (24 h after PASMCs were transfected with sh-NC, PASMCs were stimulated with 20 ng/mL PDGF for 12 h) and PDGF + SCUBE1 group (24 h after PASMCs were transfected with sh-SCUBE1, PASMCs were stimulated with 20 ng/mL PDGF for 12 h). According to the Lipofectamine 2000 reagent instructions, sh-SCUBE1 and sh-NC were transfected into PASMCs and then PASMCs was cultured for 48h.

2.3 RT-qPCR

MolPure® Cell/Tissue Total RNA Kit was used to Total RNA extraction (Yi Sheng Biotechnology, shanghai, China). A prime script RT-PCR Kit (Takara, Dalian, China) was preformed to reverse transcribed into cDNA. The cDNA synthesized in the previous step was used as the amplification template for real-time PCR, and the relevant reaction system was subsequently configured according to the SYBR Premix Ex TaqTM Ⅱ kit and amplified using a fluorescence quantitative PCR machine. At the end of the reaction, the CT value was read and recorded, using a $2^{-\Delta\Delta CT}$ method was used to analyze the experimental results.

2.4 Western blot assay

After transfection treatment, PASMC were added to RIPA lysate containing enzyme inhibitors and left on ice for 15min. After the supernatant was collected by centrifugation, the concentration of each protein sample was calculated after establishing a curve based on the absorbance of the standard. Protein lysates were diluted according to protein concentration to ensure that the same amount of sample (20ug) was loaded into each well, and then analyzed on a 10% SDS-PAGE gel. The separating glue was removed for wet

membrane transfer. At this end, 5% skim milk powder blocking solution was incubated for 2h, followed by the addition of the corresponding primary antibody (SCUBE1, PANA, GAPDH, BMP7) for overnight incubation at 4 °C. The next day, the primary antibody was recovered and the membrane was incubated with horseradish peroxidase-linked secondary antibody for 1h. Finally, the developer solution was added to the ChemiDoc Touch imaging system for exposure and photography.

2.5 CCK-8

PASMC suspension was added to each well of a 96 well plate and allowed to seed 4×10^3 cells. After 24h, discard the old medium and PASMCs were added to fresh medium with or without PDGF, and experiments were terminated after different stimulation time points (24 h, 48 h, 72 h). Each well was subsequently treated with 10 μL of CCK8 solution for 2 h and the absorbance of each well was measured at a dual wavelength of 450 nm by a microplate reader.

2.6 EDU assay

PASMCs were seeded into 24 well plates and cultured overnight, followed by the addition of a final concentration of 10 μM of EDU working solution and incubation at 37℃ for 2h. Upon completion of EDU labelling of cells, PASMCs were fixed by adding 4% paraformaldehyde for 15 min. After PASMCs were washed 3 times with 3% BSA, permeabilization solution of 0.3% Triton X-100 in PBS was added to each well and incubated for 15 min. Then, 100μl of Click reaction solution was added to each well and incubated in a wet box at room temperature for 30 min under light. Finally, a 1:10 dilution of DAPI staining solution was added to each well, and PASMCs were observed and photographed under a fluorescence microscope.

2.7 Wound healing assay

PASMCs were seeded into 6 cm dishes at the appropriate density after digestion and centrifugation, and medium was changed once in 2-3 days to observe cell growth to 80-90% confluence. A scratch was formed by scratching the bottom of the dish with a 200 μL sterile pipette tip. Detached cells were rinsed with PBS. Medium with or without PDGF was added into culture dish. Images were taken with an inverted microscope within 24 h

after scratching. The relative distance of cell migration into the scratched area was measured, and the percentage of healing was calculated.

2.8 Transwell assay

PASMC suspensions were made by digesting the cells to a final concentration of 5×10^5/mL. Pipetting 200 μL of the above cell suspension was added to the upper chamber of the well and 650 μL of the medium containing 10% FBS was added to the lower chamber of the chamber. After that, the chambers were put into a 5%CO_2 incubator at 37°C and incubated for 24 h. Subsequently, the remaining cells on the upper chamber surface were gently wiped away with a disposable cotton swab, 1 mL of 4% paraformaldehyde was added, fixed for 20 min, and the chambers were stained with 0.5% crystal violet dye solution for 20 min. Five fields were selected for pictures under an inverted microscope, and the stained cells were counted.

2.9 Statistical analysis

The raw data obtained from experiments on this subject were represented as mean ± standard (SD). The statistical method for data comparison between the two groups was unpaired t-test by using SPSS 20.0. When P value < 0.05, we considered that the difference was statistically significant.

3. Results

3.1. SCUBE1 was highly expressed in patients with pulmonary embolism and in PDGF-induced PASMCs

To verify the expression status of SCUBE1 in the plasma of patients with acute pulmonary embolism (APE), we collected total RNA from SCUBE1, and applied real time-PCR to detect the expression levels of SCUBE1 mRNA in the corresponding plasma. The results showed that the mRNA levels of SCUBE1 were all highly expressed in the plasma of patients with APE compared with healthy controls (Figure 1A). Furthermore, pulmonary artery smooth muscle cells (PASMCs) play an important role in the process of APE. We stimulated cells with different concentrations of PDGF (10, 20 or 40 ng/mL) to study the expression of SCUBE1 in PASMCs. Western blotting indicated that PDGF intervention

could promote SCUBE1expression in PASMCs, and the strongest effect was seen on SCUBE1 expression at 20 ng/mL PDGF (Figure 1A).

3.2 Interference of SCUBE1 restrained PDGF-induced proliferation of PASMCs

To investigate whether SCUBE1 is involved in ape by regulating PASMC proliferation, we applied shRNA to interfere with the expression of SCUBE1 for proliferation experiments. The results of Western blotting indicated that sh-SCUBE1 transfection showed better interference ability in PASMCs (Figure 2A). CCK-8 assays uncovered that interference of SCUBE1 prominently reduced PASMC proliferation (Figure 2B). Besides, we further applied EDU assay to assess cell proliferation. We can clearly observe that cell proliferation was higher in the PDGF group than in the control group and lower in the PDGF + sh-SCUBE1 group than in the PDGF + sh-NC group (Figure 2C). As an important protein regulating cell proliferation, the expression of PCNA was evaluated by Western blotting. The results demonstrated that PDGF could promote the expression of PCNA, while the level of PCNA in the PDGF + sh-SCUBE1 group after inhibition of SCUBE1 was lower than that in the PDGF + sh-NC group (Figure 2D).

3.3 Interference of SCUBE1 restrained PASMC migration

After PASMCs were transfected with SCUBE1 shRNA, Transwell assay and Wound-healing assay were applied to detect the migration ability of PASMCs in each group. Though Transwell assay, we found that SCUBE1 silencing leads to the decrease of PASMC migration ability, and triggered a decline in the number of cells passing through the chamber (Figure 3A). Meanwhile, wound-healing assay results further demonstrated that SCUBE1 interference prominently suppressed the migration ability of PASMCs (Figure 3B).

3.4 BMP7 was targeted by SCUBE1

We further want to study the biological mechanism of SCUBE1 in APE progression. A previous study reported that BMP7 was a potential downstream target of SCUBE1.Western blotting assays demonstrated that compared to the control, PDGF treatment caused an obvious increased of BMP7 in PASMCs (Figure 3A). Through co-IP assay analysis, we found that BMP7 existed in complexes precipitated with antibody against SCUBE1 and

SCUBE1 existed in complexes precipitated with antibody against BMP7 in comparison with control IgG (Figure 3B and C). Furthermore, the results of RT-qPCR uncovered that elevated of SCUBE1 enhanced BMP7 expression level and interference of SCUBE1 reduced BMP7 expression level in PASMCs (Figure 3D).

3.5 SCUBE1 contributed to PASMC proliferation and migration by regulating BMP7 expression

To study whether BMP7 participate in SCUBE1-mediated biological behavior in PASMCs, sh-SCUBE1 was transfected alone or together with BMP7 into PASMCs. As expected, western blotting indicated that inhibition of SCUBE1 conspicuously inhibited BMP7 expression, which was restored by transfection with BMP7 (Figure 5A). Though CCK-8 and EDU assays, we observed that introduction of BMP7 reversed the impact of SCUBE1 silencing on cell proliferation in PASMCs (Figure 4B and C). Surprisingly, the decreased PCAN expression was observed in PASMCs after SCUBE1 silencing, whereas BMP7 elevated could inverted these change (Figure 4D). In addition, the migration ability of SCUBE1-depleted PASMCs was enhanced, which was abrogate by BMP7 upregulation (Figure 5E and F)

4 Discussion

APE is a common complex cardiovascular disease caused by multiple pathogenic factors, with pulmonary vascular remodeling as the main feature. In recent years, some drugs have been confirmed to improve clinical outcomes, but the overall prognosis of this disease remains poor, and the underlying pathogenesis is not fully understood. Vascular remodeling occurs locally in the pulmonary arteries of the embolized segment after the occurrence of APE, and the hyperproliferation and apoptosis inhibition of PASMCs is one of the important mechanisms of pulmonary vascular remodeling. For how to inhibit PASMC proliferation and promote their apoptosis is the research hotspot of ape therapy at present.

An increasing number of scholars have focused their eyes on the aberrant proliferation of PASMCs. It has been shown that miR-106b-5p expression was downregulated in PDGF-

induced PASMCs and APE mouse models and that targeting NOR1 inhibited cell proliferation and migration [13]. DUSP1 level was enhanced by miR-34a-3p silencing to further promote APE development, manifested by accelerating mPAP elevation and pulmonary artery wall thickening in vivo, and promoting PASMC proliferation and migration in vitro [14]. A recent study uncovered that let-7b-5p was a key regulator of proliferation and migration in PASMCs, which could suppress IGF-1 expression to impede PASMC proliferation and migration [15]. Janus kinase 3 inhibition by JANEX-1 helped to ameliorate the proliferation of PASMCs induced by PDGF through the STAT3/VEGF/FAK signaling pathway in APE [16]. In the present study, our data indicated that elevation of SCUBE1 in patient plasma may be used for the early diagnosis of APE. Cellular functional studies revealed that SCUBE1 interference remarkably abrogated the promoting effect of PDGF on proliferation and migration of PASMCs.

Recently, a large body of evidence has implicated SCUBE1 in cardiovascular disease, such as acute ischemia stroke, APE, deep vein thrombosis and acute coronary syndrome [12, 17, 18]. Importantly, SCUBE1 acts as a key adhesion protein through its EGF like domain to form cross homophilic bridges on activated platelets during thrombus formation, thereby contributing to the progression of acute thromboembolic disease [9, 12, 19]. In addition, SCUBE1 was highly expressed in peritubular capillaries after renal I/R injury and enhanced proliferation of epithelial cells through BMP7 signaling [20, 21]. Earlier studies suggested that bone morphogenetic proteins (BMPs) are involved in vascular progenitor cells during angiogenesis, acting by regulating the growth, differentiation, and turnover of vascular cell populations [22, 23]. There was evidence strongly suggested that dysregulation of bone forming protein signaling was associated with abnormal proliferation and migration of PASMCs. For instance, BMP2 was found to be selectively upregulated in pulmonary arteries exposed to hypoxia, and it could inhibit PASMC proliferation and promote apoptosis [24]. BMP4 contributes to chronic hypoxic pulmonary hypertension by promoting the proliferation, migration and vascular remodeling of PASMCs [25, 26]. Importantly, in PASMCs after monocrotaline pyrrole (MCTP) stimulation, upregulation of BMP7 was found in PASMCs, which enhanced proliferation

and PCAN expression by inhibiting BMPR2 and elevating ActIIα levels [27]. Herr, we demonstrate that BMP7 was a target of SCUBE1, silencing of SCUBE1 contributed to enhance BMP7 expression. SCUBE1 knockdown suppressed the impact of PDGF on proliferation and migration of PASMCs through silencing of BMP7.

5 Conclusions

PDGF treatment resulted in increased expression of SCUBE1, which in turn promoted BMP7 expression. SCUBE1 might contribute to the PDGF-induced proliferation of PASMCs by positively regulating BMP7, which in turn promoted the occurrence and development of APE. The above results provide novel targets and molecular markers for ape therapy, thereby favoring the prevention and treatment of malignant pulmonary vascular disease-APE.

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Figure 1

SCUBE1 was overexpressed in ~~in~~ the plasma of patients with APE and PASMCs after PDGF stimulation.

A: RT-qPCR was used to analyze the relative expression of SCUBE1 in SCUBE1 (n = 50). B: Western blotting was used to analyze relative expression of SCUBE1 in PASMCs after different concentration of PDGF (10, 20 or 40 ng/mL). *P < 0.05.

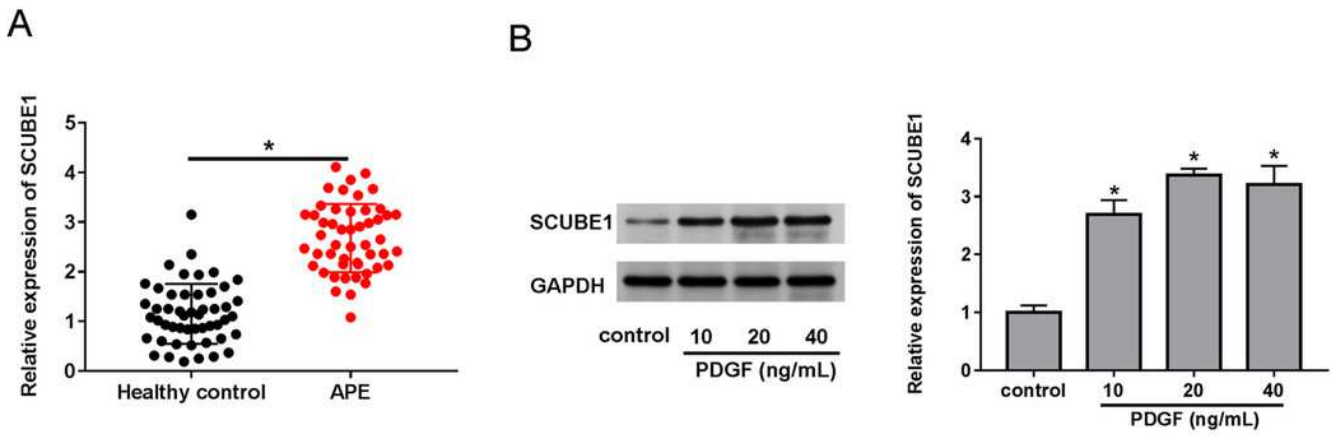


Figure 2

PDGF-induced proliferation of PASMCs was decreased by SCUBE1 silencing.

sh- SCUBE1 or sh-NC was transfected into PASMCs. A: Western blotting detection of SCUBE1 level in PASMCs after treatment. B: CCK-8 assay detection of PASMC proliferation after treatment. C: EDU assay detection of PASMC proliferation after treatment. D: Western blotting detection of PCNA level in PASMCs after treatment. *P < 0.05.

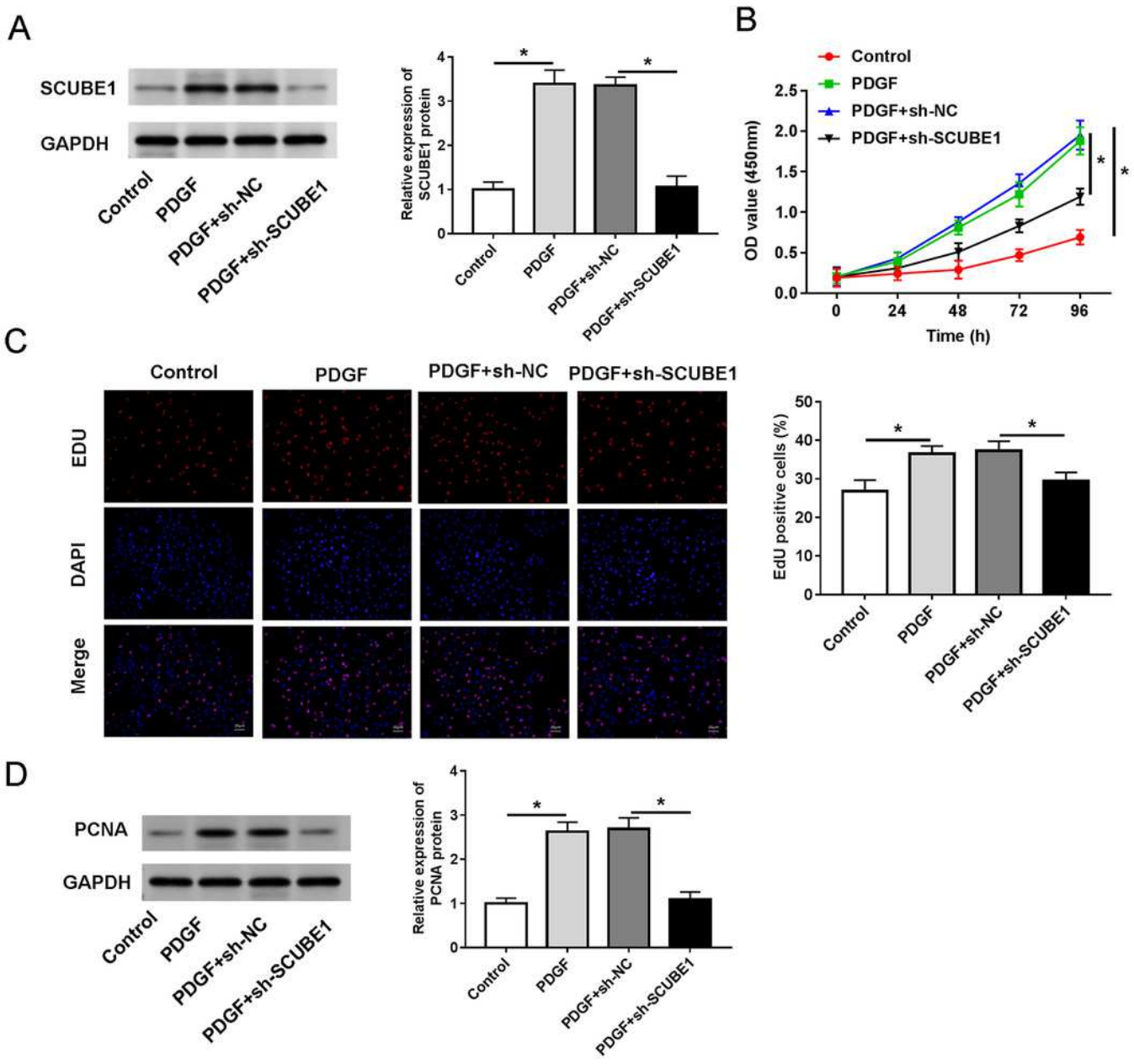

A
SCUBE1
GAPDH
Control
PDGF
PDGF+sh-NC
PDGF+sh-SCUBE1
Relative expression of SCUBE1 protein
B
Control
PDGF
PDGF+sh-NC
PDGF+sh-SCUBE1
OD value (450nm)
Time (h)
C
Control
PDGF
PDGF+sh-NC
PDGF+sh-SCUBE1
EDU
DAPI
Merge
EdU positive cells (%)
D
PCNA
GAPDH
Relative expression of PCNA protein

Figure 3

PDGF-induced migration of PASMCs was decreased by SCUBE1 silencing.

sh- SCUBE1 or sh-NC was transfected into PASMCs. A: Transwell migration assay detection of PASMCs migration. Scale bar: 100 μm. B: wound-healing assay detection of PASMCs migration. Scale bar: 100 μm. *P < 0.05.

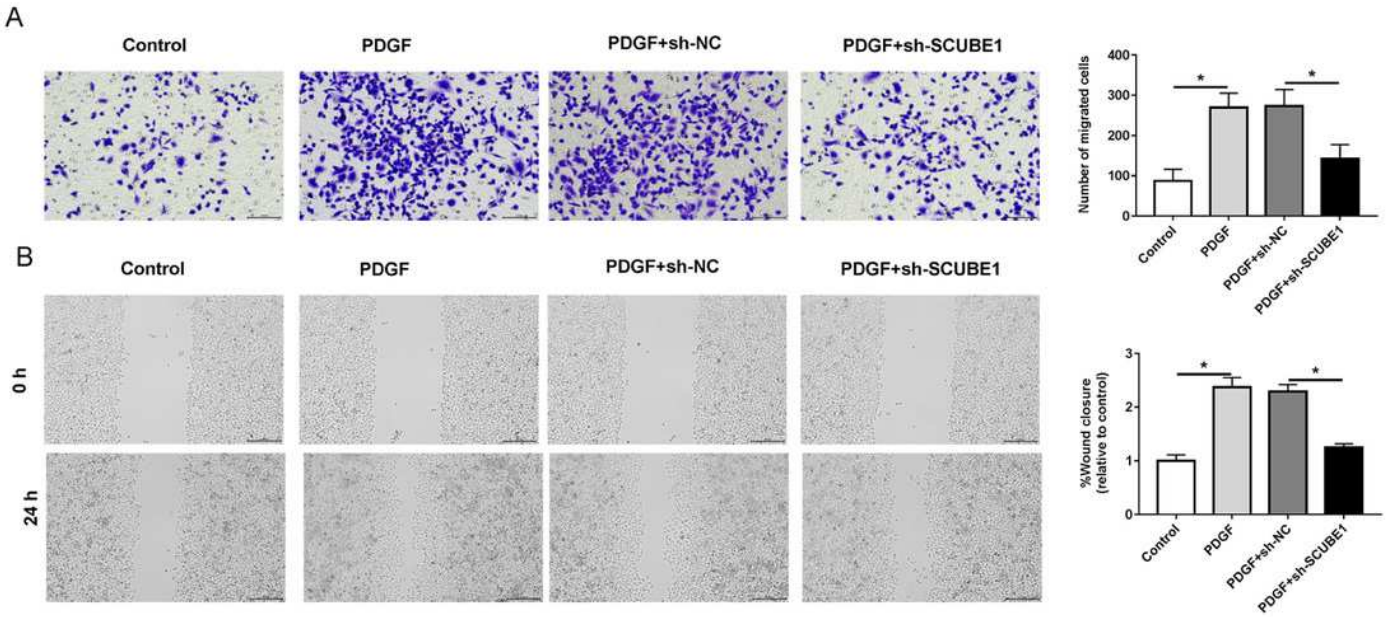


Figure 4

SCUBE1 bound with BMP7.

A: Western blotting detection of BMP7 level in PASMCs after PDGF treatment. B and C: CoIP validation of the relationship of SCUBE1 and BMP7. D: Western blotting detection of BMP7 level in PASMCs after SCUBE1 overexpression or knockdown. *P < 0.05.

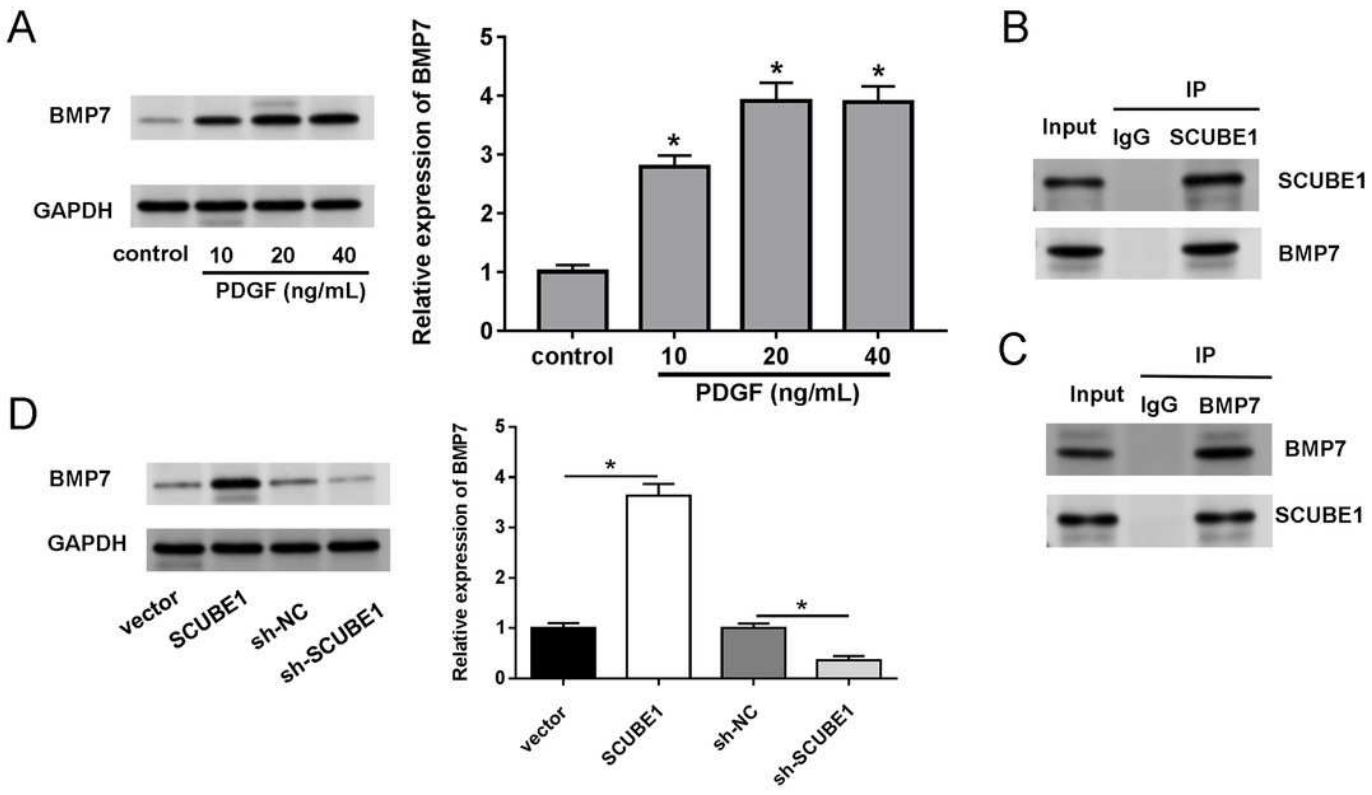
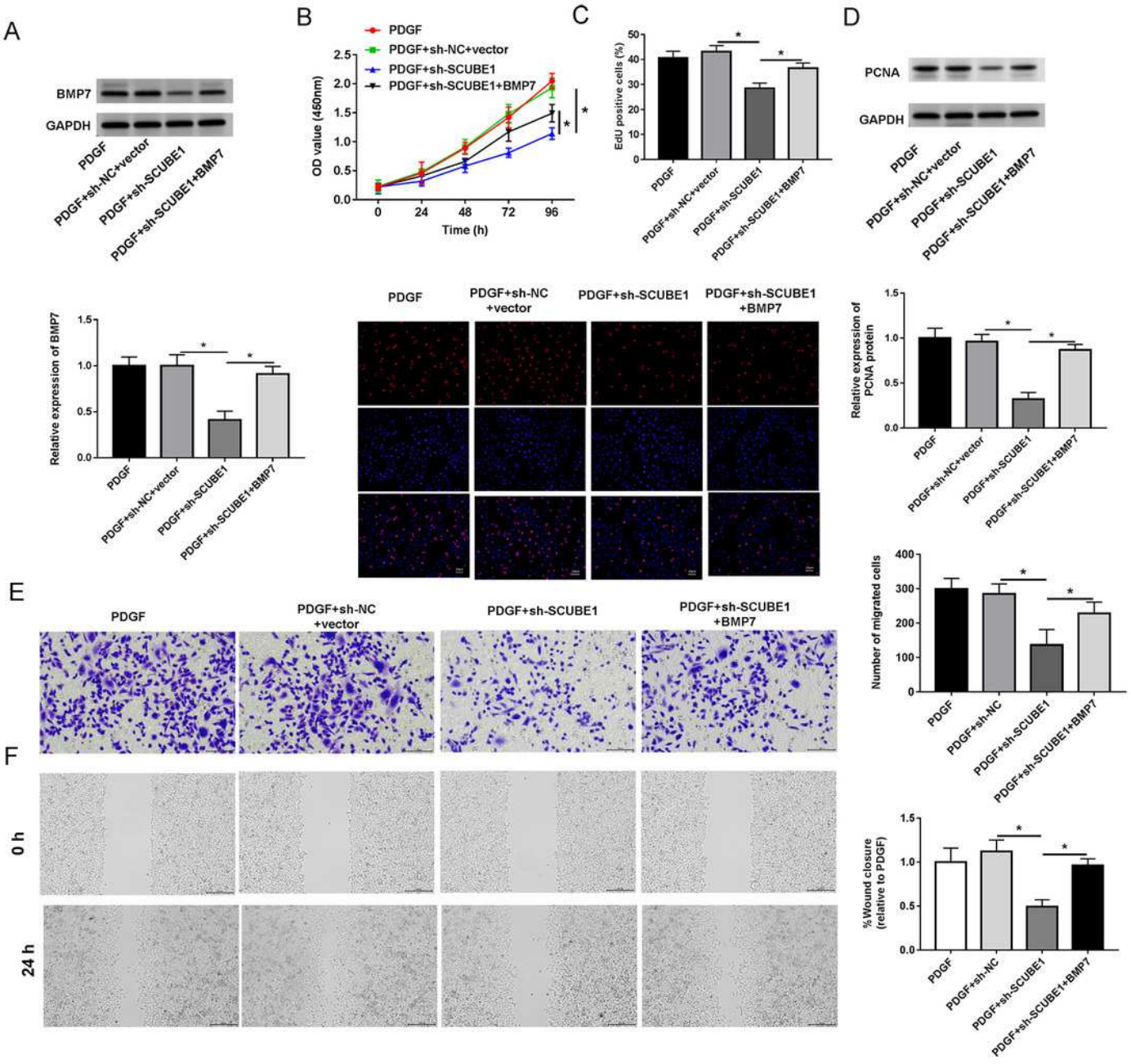


Figure 5

SCUBE1 interference accelerated proliferation and migration in PDGF-induced PASMCs by reducing BMP7.

A: Western blotting detection of BMP7 level in PASMCs. B: CCK-8 and EDU detection of PASMCs proliferation. D: Western blotting detection of PCAN level in PASMCs. E and F: Transwell and Wound-healing assay detection of PASMCs migration *P < 0.05.


A
BMP7
GAPDH
PDGF
PDGF+sh-NC+vector
PDGF+sh-SCUBE1
PDGF+sh-SCUBE1+BMP7
B
PDGF
PDGF+sh-NC+vector
PDGF+sh-SCUBE1
PDGF+sh-SCUBE1+BMP7
OD value (450nm)
Time (h)
C
EdU positive cells (%)
D
PCNA
GAPDH
Relative expression of BMP7
Relative expression of PCNA protein
PDGF+sh-NC +vector
PDGF+sh-SCUBE1 +BMP7
E
Number of migrated cells
F
0 h
24 h
% Wound closure (relative to PDGF)