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COP9 signalosome subunit 6 mediates PDGF -induced pulmonary arterial smooth muscle cells proliferation

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ABSTRACT

Up-regulation of mammalian COP9 signalosome subunit 6 (CSN6) and consequent reduction of SCF ubiquitin ligase substrate receptor β-transduction repeat-containing protein (β-TrCP) have been shown to be associated with cancer cells proliferation. However, it is unclear whether CSN6 and β-TrCP are also involved in PDGF-induced pulmonary arterial smooth muscle cells (PASMCs) proliferation. This study aims to address this issue and further explore its potential mechanisms. Our results indicated that PDGF phosphorylated Akt, stimulated PASMCs proliferation; while inhibition of PDGF receptor (PDGFR) by imatinib prevented these effects. PDGF further up-regulated CSN6 protein expression, this was accompanied with β-TrCP reduction and increase of Cdc25A. Inhibition of PDGFR/PI3K/Akt signaling pathway reversed PDGF-induced such changes and cell proliferation. Prior transfection of CSN6 siRNA blocked PDGF-induced β-TrCP down-regulation, Cdc25A up-regulation and cell proliferation. Furthermore, pre-treatment of cells with MG-132 also abolished PDGF-induced β-TrCP reduction, Cdc25A elevation and cell proliferation. In addition, pre-depletion of Cdc25A by siRNA transfection suppressed PDGF-induced PASMCs proliferation. Taken together, our study indicates that up-regulation of CSN6 by PDGFR/PI3K/Akt signaling pathway decreases β-TrCP by increasing its ubiquitinated degradation, and thereby increases the expression of Cdc25A, which promotes PDGF-induced PASMCs proliferation.

1. Introduction

Pulmonary arterial hypertension (PAH) is a devastating vascular disease characterized by progressive elevation of pulmonary arterial pressure. It is well accepted that the common pathological mechanisms of PAH include persistent pulmonary vasoconstriction, pulmonary vascular remodeling and thrombosis in situ [1]. Pulmonary vascular remodeling characterized by intima thickening and media hyperplasia plays an important role in the pathological progress of PAH [2]. The major cellular mechanism underlying pulmonary vascular remodeling is excessive pulmonary arterial smooth muscle cells (PASMCs) proliferation [3]. Therefore, it is important to explore the molecular mechanisms responsible for PASMCs proliferation and search strategies to ameliorate pulmonary vascular remodeling and thus to prevent and treat PAH.

Mammalian COP9 signalosome (CSN), a multi-protein complex, consists of eight subunits (CSN1–CSN8) [4] and is involved in many cellular processes, including protein degradation, cell cycle progression, signal transduction and tumorigenesis [5–8]. CSN complex regulates Cullin-Ring ubiquitin ligase (CRL) activity, thereby coordinating CRL-mediated ubiquitination activity [9–11]. Skp1-Cullin-F-box (SCF) ubiquitin ligase is CRLs, regulated by CSN that has been found to stabilize F-box proteins [12,13]. F-box proteins are SCF ubiquitin ligase substrate receptors, which recognize the SCF ubiquitin ligase substrates and facilitate the degradation of these substrates [11]. Cullin is deneddylated by the CSN [14]. Cullin deneddylation has been found to prevent the autocatalytic degradation of SCF ubiquitin ligase substrate adapter, thus maintaining the stability of substrate receptor and promoting SCF ubiquitin ligase activity in vivo [7,12,13,15].

CSN6, a subunit of CSN, contains a MPN domain, which is homologous to that of CSN5 [15,16]. CSN5 plays an important role in cullin deneddylation via linking its Jab1/MPN metalloenzyme (JAMM) motif to the metalloprotease and removing Nedd8 from cullin [14,17,18]. However, the biochemical function of the CSN6 MPN domain has not been defined yet. CSN6 has been speculated to play a role in structural integrity of the CSN complex and cullin deneddylation catalyzed by

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CSN5 [19]. Study has found that CSN6 is over-expressed in several cancers and correlates with poor prognosis [20–22]. Recently, CSN6 has been shown to positively/negatively regulate CRL activity, such as RING-containing E3 ligase COP1, SCF ubiquitin ligase SCF^{Fbw7} and SCF^{Skp2}, leading to coordinating CRL-mediated degradations and stabilities of CRL substrates [23–25]. Study in colorectal cancer has found that up-regulation of CSN6 by EGF decreases SCF ubiquitin ligase substrate receptor β-transduction repeat-containing protein (β-TrCP) by promoting its autocatalytic degradation, thereby inhibiting SCF ubiquitin ligase-mediated degradation of β-catenin and promoting cell proliferation [22]. However, it is unclear whether up-regulation of CSN6 inhibits the expression of SCF ubiquitin ligase substrate receptor β-TrCP and is associated with PDGF-induced PASMCs proliferation.

Cdc25A, a member of dual phosphatase family, plays an important role in assisting both G1/S and G2/M progression by dephosphorylating and activating CDK2 and CDK1 [26,27]. Over-expression of Cdc25A has been frequently described in multiple cancer samples, which is highly associated with the malignancy and poor prognosis in cancer patients [28,29]. Studies has shown that $SCF^{β\text{-}TrCP}$ ubiquitin ligase plays a critical role in degradation of Cdc25A during proliferation, in which the F-box protein β-TrCP binds to the DSG motif of Cdc25A in a manner dependent on phosphorylation of Ser82 within the motif, and thereby targets Cdc25A for degradation [30,31]. Recent study has indicated that suppression of β-TrCP by small interfering RNA, which recognizes SCF ubiquitin ligase substrate Cdc25A and facilitates its ubiquitinated degradation, is found to be associated with up-regulation of Cdc25A in lung cancer [32]. However, it remains uncertain whether suppression of β-TrCP by up-regulation of CSN6 facilitates Cdc25A ubiquitinated degradation and subsequently promotes cell proliferation.

Recent studies have reported that activation of PI3K/Akt signaling pathway increases the expression of CSN6 in several cancer cells [21,33,34]. However, it is unknown whether activation of PI3K/Akt signaling pathway up-regulates CSN6 in PDGF-stimulated PASMCs. Furthermore, CSN6 has been found to decrease β-TrCP by promoting its autocatalytic degradation, thereby stabilizing SCF ubiquitin ligase substrate in colorectal cancer cells [22]. However, it is unclear whether up-regulation of CSN6 decreases β-TrCP by increasing its ubiquitinated degradation, thereby increasing the lifetime of Cdc25A in PDGF-stimulated PASMCs. Moreover, study has shown that up-regulation of Cdc25A mediateses ET or Ang II-induced rat aortic VSMCs proliferation [35]. However, the effect of Cdc25A on PDGF-stimulated PASMCs proliferation remains unclear. To address these issues, primary cultured PASMCs were stimulated with PDGF, the effect of CSN6 on PASMCs proliferation was examined, and its molecular mechanisms were further explored.

2. Materials and methods

2.1. Cell isolation and culture

The isolation of primary PASMCs from main pulmonary arteries of male Sprague-Dawley rats (100–150 g) was performed using the previously reported method. All animal care and procedures were performed in accordance with Xi'an Jiaotong University Animal Care Policy following the Guide for the Care and Use of Laboratory Animals [36]. All experimental protocols used in this study were reviewed and approved by the Laboratory Animal Care and Use Committee of Xi'an Jiaotong University. Briefly, rats were anesthetized with intraperitoneal injections of 10% chloral hydrate (3 ml/kg of body weight), and then soaked in 75% alcohol for 3 min. The hearts and lungs were excised from the chest cavity and rinsed with phosphate-buffered saline (PBS) (4 °C). Segments of the main pulmonary arteries were rapidly removed free from connective and fat tissues and cleaned in PBS (4 °C) under aseptic conditions. The smooth muscle of the pulmonary arteries was dissociated away from the adventitia and intima by scraping off with a fine forceps, then cut into small pieces (about 0.5–1 mm^2) and placed into a culture flask.

PASMCs were cultured in complete medium including Dulbecco's Modified Eagle Medium (DMEM)/High glucose (Gibco, Grand Isle, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, HangZhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. After 2 weeks, cells were passaged by trypsinization using 0.25% trypsin (Invitrogen, Carlsbad, CA, USA). Immunofluorescence staining with a specific antibody against α-smooth muscle actin (α-SMA, Sigma-Aldrich, St. Louis, MO, USA) was performed to identify the purity of PASMCs. All PASMCs at passage 4–8 were used. Before each experiment, cells were starved overnight by seeding with medium containing 1% FBS to minimize serum-induced effect. PDGF (Peprotech, Rocky Hill, NJ, USA) was dissolved in distillated water at 1000 μg/ml as the stock solution and performed to stimulate cell proliferation. Imtinib (Aladdin, Shanghai, China), a small molecular used to inhibit PDGF receptor (PDGFR), was dissolved in distillated water at 100 μM as stock solution. LY294002 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) at 100 mM as stock solution and applied to inhibit the activity of phosphatidylinositol 3 kinase (PI3K). MG-132 (Selleckchem, Houston, TX, USA), used as a proteasome inhibitor, was dissolved in DMSO with the stock concentration of 10 mM.

2.2. BrdU incorporation assay

BrdU incorporation assay was evaluated using BrdU ELISA Kit (Maibio, Shanghai, China) as recommended by the manufacturer's instructions to determine the proliferation of PASMCs. Approximately 5×10^3 cells were seeded in 96-well plates and starved (1% FBS in DMEM) overnight after adhering for 24 h. Thereafter, BrdU labeling solution was added to each well at the end of different treatments and then incubated for 2 h at 37 °C. Subsequently, the medium was carefully aspirated, FixDenat solution was added for 30 min at room temperature to denature the cells, and then anti-BrdU monoclonal antibody conjugated to peroxidase was added and incubated for 90 min at room temperature. Finally, the antibody conjugate was removed and then substrate solution was added to each well for 10 min. The absorbance was collected at 370 nm with a microplate reader (Bio-Rad, Richmond, CA, USA). The blank corresponded to 100 μl of culture medium with or without BrdU.

2.3. Small interfering RNA transfection

Sequence-specific or non-targeting control small interfering RNA (siRNA) (GenePharm, Shanghai, China) were transfected into PASMCs using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions to silence the expressions of CSN3, CSN5, CSN6 and Cdc25A proteins. Briefly, primary PASMCs were seeded into 6-well plates at a density of 5×10^5 cells/well. Subsequently, siRNA and Lipofectamine were diluted in serum-free DMEM separately and incubated at room temperature for 5 min. Diluted siRNA was then mixed with diluted Lipofectamine and incubated at room temperature for 20 min. Finally, primarily cultured cells at 30–50% confluence were transfected with the complex of siRNA and Lipofectamine for 6 h in serum-free DMEM and cultured in complete DMEM for 48 h in 5% CO2 at 37 °C. The knockdown efficiency of siRNA transfection was determined by western blotting.

2.4. Western blotting

The primarily cultured PASMCs were harvested, washed three times with PBS (4 °C) and lysed with ice-cold RIPA buffer supplemented with 1% henylmethanesulfonyl fluoride (PMSF), protease inhibitors and phosphatase inhibitors for 30 min on ice. Following centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatants were collected as total protein and protein concentrations were evaluated using BCA protein