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Knockdown of AMPK α 2 Promotes Pulmonary Arterial Smooth Muscle Cells Proliferation via mTOR/Skp2/p27^{Kip1} Signaling Pathway

Rui Ke ¹, Lu Liu ¹, Yanting Zhu ¹, Shaojun Li ¹, Xinming Xie ¹, Fangwei Li ¹, Yang Song ¹, Lan Yang ¹, Li Gao ² and Manxiang Li ^{1,*}

¹ Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, China; ke19870912@163.com (R.K.); liulu290@126.com (L.L.); zhuyanting0816@163.com (Y.Z.); wdm19870914@163.com (S.L.); xiexinming1986@163.com (X.X.); lifangwei80@163.com (F.L.); wyszhl@163.com (Y.S.); yanglanmed@163.com (L.Y.)

² Division of Allergy and Clinical Immunology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA; lgao2@jhmi.edu

* Correspondence: manxiangli@hotmail.com; Tel./Fax: +86-29-8532-3850

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Abstract: It has been shown that activation of adenosine monophosphate-activated protein kinase (AMPK) suppresses proliferation of a variety of tumor cells as well as nonmalignant cells. In this study, we used post-transcriptional gene silencing with small interfering RNA (siRNA) to specifically examine the effect of AMPK on pulmonary arterial smooth muscle cells (PASMCs) proliferation and to further elucidate its underlying molecular mechanisms. Our results showed that knockdown of AMPK α 2 promoted primary cultured PASMCs proliferation; this was accompanied with the elevation of phosphorylation of mammalian target of rapamycin (mTOR) and S-phase kinase-associated protein 2 (Skp2) protein level and reduction of p27^{Kip1}. Importantly, prior silencing of mTOR with siRNA abolished AMPK α 2 knockdown-induced Skp2 upregulation, p27^{Kip1} reduction as well as PASMCs proliferation. Furthermore, pre-depletion of Skp2 by siRNA also eliminated p27^{Kip1} downregulation and PASMCs proliferation caused by AMPK α 2 knockdown. Taken together, our study indicates that AMPK α 2 isoform plays an important role in regulation of PASMCs proliferation by modulating mTOR/Skp2/p27^{Kip1} axis, and suggests that activation of AMPK α 2 might have potential value in the prevention and treatment of pulmonary arterial hypertension.

Keywords: AMPK; pulmonary arterial smooth muscle cells; proliferation; mTOR; Skp2

1. Introduction

Pulmonary arterial hypertension (PAH) is a common clinical syndrome characterized by sustained elevation of pulmonary vascular resistance and increased pulmonary arterial pressure, leading to right heart failure and ultimately death [1,2]. Pulmonary vascular remodeling characterized by thickening of all layers of vascular wall is a hallmark of all types of PAH [3]. Pulmonary arterial smooth muscle cells (PASMCs) proliferation *in situ* and migration to intima is critical to the pathogenesis of pulmonary vascular remodeling [4]. The deregulation of multiple signaling pathways in PASMCs is convinced to be associated with excessive proliferation of PASMCs [5,6]. Therefore, strategies either to enhance the function of anti-proliferative cascades or to inhibit pro-proliferative signaling pathways are important in the amelioration of pulmonary vascular remodeling and therefore the management of PAH.

Adenosine monophosphate-activated protein kinase (AMPK) is a key metabolic and redox sensor that has emerged as a critical regulator of cell proliferation and apoptosis [7]. AMPK exists as a heterotrimeric complex with a catalytic subunit α and two regulatory subunits β and γ [8].

Pathological changes associated with increased AMP to ATP ratio, such as nutrient deprivation, hypoxia, ischemia, heat shock and oxidative stress, cause AMPK activation [8,9]. AMPK is also found to be activated by several chemical compounds [10,11] and signaling pathways [12] independent of energy imbalance. Activation of AMPK regulates diverse cellular processes including cell migration, proliferation, apoptosis and survival [13]. Studies have shown that AMPK activation suppresses proliferation of a variety of tumor cells as well as nonmalignant cells, which indicates that activation of AMPK is a promising strategy for the treatment of cancer as well as other human diseases [14–16].

Recent studies have employed AMPK activators such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and metformin to demonstrate that activation of AMPK suppresses PSMCs proliferation [17,18]. Studies in animal model have also found that activation of AMPK by metformin inhibits the development of PAH by suppressing pulmonary vascular remodeling [19]. However, downstream targets regulated by AMPK to confer its roles in PAH are not fully clarified yet; in addition, pharmacological modulation of AMPK is not entirely specific for AMPK and has off-target effects [20,21]. RNA interference (RNAi) is a simple and effective method for silencing gene expression and has great advantages over traditional approaches for examining the role of a specific molecule in the development of a particular disease [22]. In the present study, we used post-transcriptional gene silencing with small interfering RNA (siRNA) to selectively inhibit AMPK in primary cultured PSMCs, and specifically examined the effect of AMPK on PSMCs proliferation, and the molecular mechanisms underlying this effect were further explored.

2. Results

2.1. Knockdown of Adenosine Monophosphate-Activated Protein Kinase $\alpha 2$ (AMPK $\alpha 2$) Promotes Pulmonary Arterial Smooth Muscle Cells (PSMCs) Proliferation

To specifically examine the effect of AMPK on PSMCs proliferation, siRNA-mediated knockdown of either AMPK $\alpha 1$ or AMPK $\alpha 2$ was performed. As depicted in Figure 1A,B, transfection of the corresponding specific siRNA reduced the levels of AMPK $\alpha 1$ and AMPK $\alpha 2$ protein by 78% and 79%, respectively ($p < 0.01$ versus control). Non-targeting siRNA transfection did not reduce AMPK $\alpha 1$ and AMPK $\alpha 2$ protein expression. Figure 1C indicates that PSMCs lacking AMPK $\alpha 2$ protein exhibited an elevated cells proliferation assessed by BrdU incorporation assay, which was 1.46-fold increase over control ($p < 0.05$), while transfection of PSMCs with AMPK $\alpha 1$ siRNA or non-targeting siRNA did not affect cells proliferation. These results suggest that knockdown of AMPK $\alpha 2$ induces PSMCs proliferation.

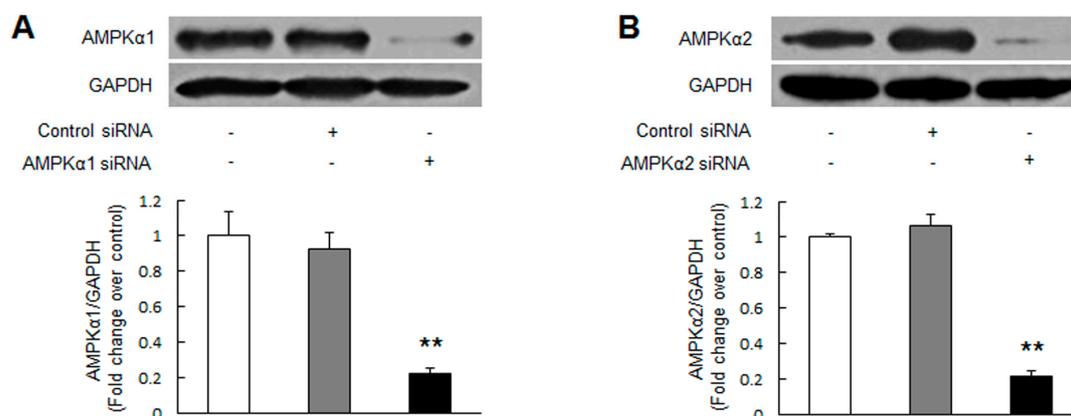


Figure 1. Cont.

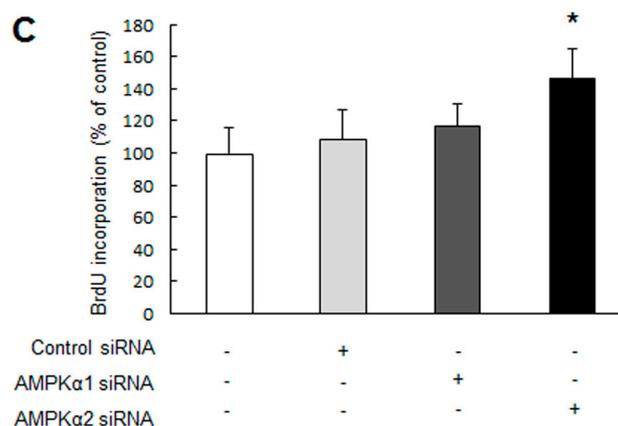


Figure 1. Knockdown of adenosine monophosphate-activated protein kinase $\alpha 2$ (AMPK $\alpha 2$) promotes pulmonary arterial smooth muscle cells (PASMCs) proliferation. Primary cultured PASMCs were transfected with AMPK $\alpha 1$ or AMPK $\alpha 2$ sequence-specific small interfering RNA (siRNA) and non-targeting siRNA. Protein levels of AMPK $\alpha 1$ (A) and AMPK $\alpha 2$ (B) were determined by immunoblotting ($n = 3$ in each group); (C) PASMCs were transfected with indicated siRNA, cells proliferation was examined by BrdU incorporation assay ($n = 4$ in each group). +, with indicated siRNA transfection; -, without siRNA transfection; * $p < 0.05$ versus control; ** $p < 0.01$ versus control.

2.2. Knockdown of AMPK $\alpha 2$ Increases the Phosphorylation of Mammalian Target of Rapamycin (mTOR), Upregulates S-Phase Kinase-Associated Protein 2 (Skp2) and Downregulates p27^{Kip1}

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that positively regulates cell growth, proliferation, and survival [23]. Several groups have reported that activation of AMPK targets on mTOR to suppress its function and further inhibits a variety of cells proliferation [24–26]. To investigate whether knockdown of AMPK causes activation of mTOR in PASMCs, AMPK $\alpha 2$ was silenced by siRNA transfection and mTOR activity was measured using immunoblotting. Figure 3A indicates that the phosphorylation of mTOR, which is an indicator of mTOR intrinsic catalytic activity, was elevated in AMPK $\alpha 2$ knockdown PASMCs with a 1.99-fold increase compared with control ($p < 0.01$), suggesting that knockdown of AMPK $\alpha 2$ causes unrestrained mTOR activation in PASMCs.

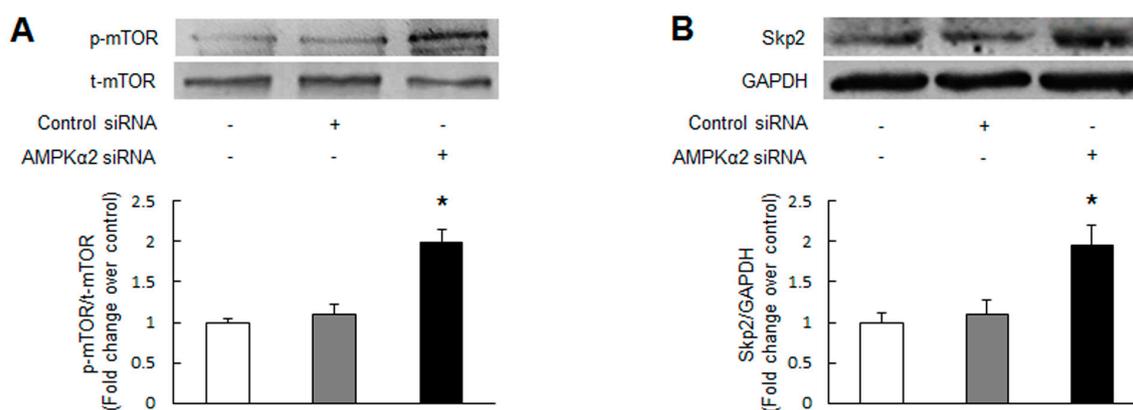


Figure 2. Cont.

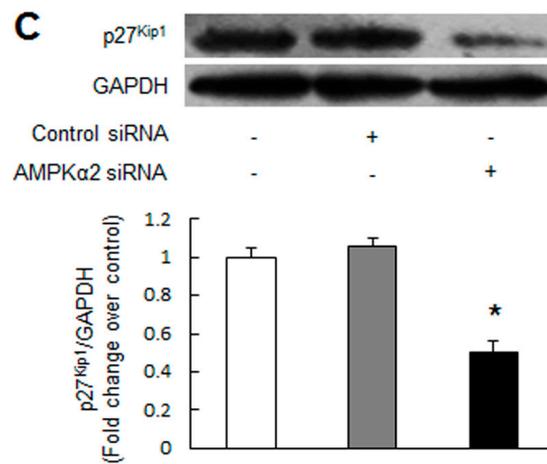


Figure 3. Knockdown of AMPKα2 increases the phosphorylation of mammalian target of rapamycin (mTOR), upregulates S-phase kinase-associated protein 2 (Skp2) and downregulates p27^{Kip1}. Primary cultured PSMCs were transfected with AMPKα2 specific siRNA and non-targeting siRNA. Phosphorylation of mTOR (A) and protein levels of Skp2 (B) and p27^{Kip1} (C) were determined by immunoblotting ($n = 3$ in each group). +, with indicated siRNA transfection; –, without siRNA transfection; * $p < 0.01$ versus control.

p27^{Kip1} is one of the cyclin-dependent kinases (CDKs) inhibitors that plays a critical role in regulating cell cycle progression in mammalian cells [27]. It has been shown that inhibition of S-phase kinase-associated protein 2 (Skp2)-mediated p27^{Kip1} degradation underlies AMPK suppression of several types of cells proliferation [17,28,29]. Here, we examined whether the changes of Skp2 and p27^{Kip1} are also associate with the effect of AMPKα2 knockdown on PSMCs proliferation. As shown in Figure 3B, cells lacking AMPKα2 by siRNA transfection demonstrated a 1.95-fold increase in Skp2 protein expression ($p < 0.01$ versus control). Non-targeting siRNA transfection did not affect Skp2 protein level. Figure 3C indicates that protein level of p27^{Kip1} was reduced in AMPKα2 knockdown PSMCs, which was 0.50-fold decrease compared with control ($p < 0.01$), while non-targeting siRNA transfection did not change p27^{Kip1} expression. These findings suggest that knockdown of AMPKα2 upregulates Skp2 and downregulates p27^{Kip1} in PSMCs.

2.3. mTOR Mediates AMPKα2 Knockdown-Induced Skp2 Upregulation, p27^{Kip1} Downregulation and PSMCs Proliferation

Studies have shown that mTOR plays an important role in regulating Skp2 and p27^{Kip1} in malignant cells [30–32]. To determine whether mTOR mediates the effect of AMPKα2 knockdown on Skp2 and p27^{Kip1} modulation in PSMCs, silencing of mTOR was applied. Figure 4A indicates that mTOR specific siRNA transfection reduced mTOR protein level to 28 percent of control ($p < 0.01$), while non-targeting siRNA did not affect mTOR protein expression. Figure 4B,C show that AMPKα2 knockdown notably upregulated Skp2 expression ($p < 0.01$ versus control siRNA transfection) and reduced p27^{Kip1} protein level ($p < 0.05$ versus control siRNA transfection), while pre-silencing of mTOR decreased Skp2 expression from 1.87-fold to 0.89-fold over control siRNA and increased p27^{Kip1} level from 0.50-fold to 1.19-fold over control siRNA, respectively ($p < 0.01$ versus control siRNA + AMPKα2 siRNA transfection). We also observed that silencing of mTOR alone reduced Skp2 and increased p27^{Kip1} protein level ($p < 0.05$ versus control siRNA transfection), suggesting that there is a basal function of mTOR in PSMCs. These results suggest that activation of mTOR is one of major protein responsible for AMPKα2 knockdown-induced Skp2 upregulation and p27^{Kip1} downregulation.

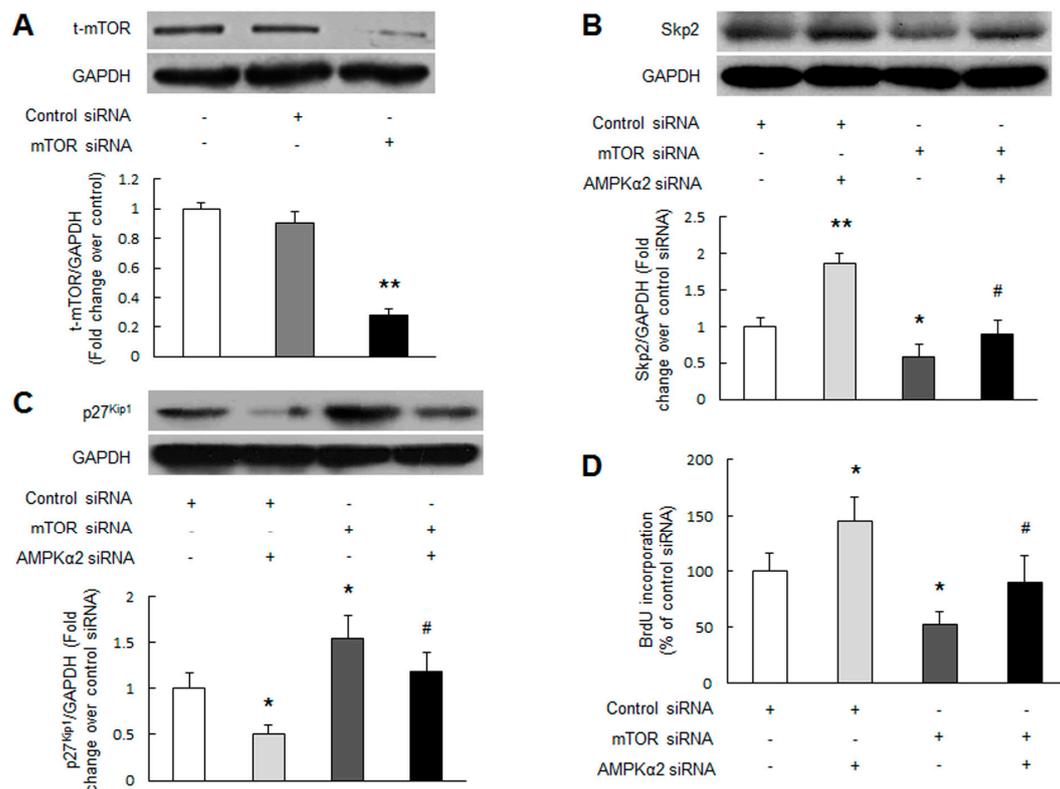


Figure 4. mTOR mediates AMPK α 2 knockdown-induced Skp2 upregulation, p27^{Kip1} downregulation and PASCs proliferation. (A) Primary cultured PASCs were transfected with mTOR sequence-specific siRNA and non-targeting siRNA. mTOR protein level was measured using immunoblotting ($n = 3$ in each group); (B–D) PASCs were transfected with non-targeting siRNA or mTOR specific siRNA, and followed by transfection with or without AMPK α 2 siRNA. Protein level of Skp2 (B) and p27^{Kip1} (C) were determined using immunoblotting ($n = 3$ in each group). Cell proliferation was examined by BrdU incorporation assay (D) ($n = 4$ in each group). +, with indicated siRNA transfection; –, without siRNA transfection; * $p < 0.05$ versus control siRNA transfection; ** $p < 0.01$ versus control siRNA transfection; # $p < 0.01$ versus control siRNA + AMPK α 2 siRNA transfection.

To further confirm whether activation of mTOR mediates AMPK α 2 knockdown-induced PASCs proliferation, BrdU incorporation rate was determined in PASCs transfected with non-targeting siRNA or mTOR siRNA followed with or without AMPK α 2 knockdown. Figure 4D shows that AMPK α 2 knockdown triggered a 1.45-fold increase in cells proliferation ($p < 0.05$ versus control siRNA transfection), while pre-silencing of mTOR reduced PASCs proliferation to 0.90-fold over control siRNA ($p < 0.01$ versus control siRNA + AMPK α 2 siRNA transfection). Silencing of mTOR alone reduced basal PASCs proliferation ($p < 0.05$ versus control siRNA transfection). Together, these observations indicate that mTOR mediates AMPK α 2 knockdown-induced PASC proliferation by regulating Skp2 and p27^{Kip1}.

2.4. Skp2 Mediates AMPK α 2 Knockdown-Induced p27^{Kip1} Reduction and PASCs Proliferation

Skp2 is the main rate-limiting regulator of p27^{Kip1} ubiquitylation and degradation in various cell types [28,29]. To examine whether Skp2 mediates AMPK α 2 knockdown-induced p27^{Kip1} reduction, depletion of Skp2 was applied. Figure 5A indicates that Skp2 specific siRNA transfection reduced Skp2 protein level to 25 percent of control ($p < 0.01$), while non-targeting siRNA did not affect Skp2 protein level. Figure 5B shows that AMPK α 2 knockdown significantly reduced p27^{Kip1} protein level ($p < 0.05$ versus control siRNA transfection), while prior depletion of Skp2 abrogated the reduction of

p27^{Kip1}, which increased from 0.50-fold to 1.30-fold over control siRNA ($p < 0.01$ versus control siRNA + AMPK α 2 siRNA transfection). Loss of basal Skp2 alone also increased p27^{Kip1} protein level ($p < 0.05$ versus control siRNA transfection). These results suggest that Skp2 specifically mediates AMPK α 2 knockdown-induced p27^{Kip1} reduction.

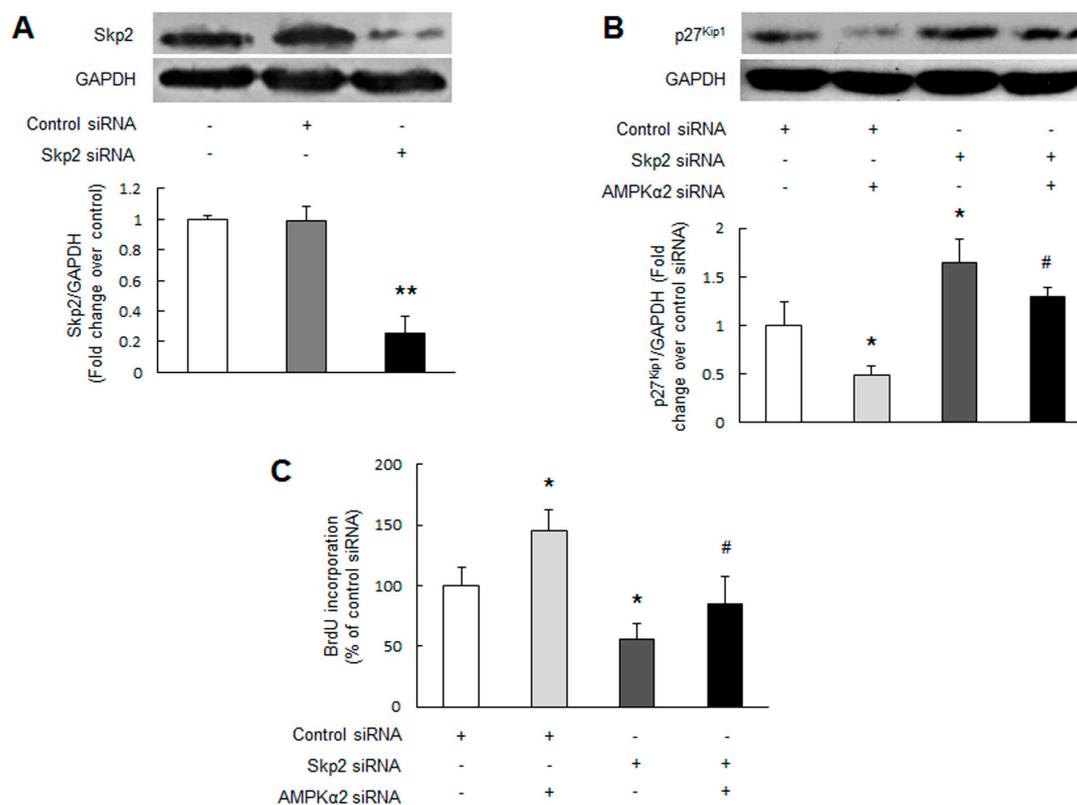


Figure 5. Skp2 mediates AMPK α 2 knockdown-induced p27^{Kip1} reduction and PSMCs proliferation. (A) Primary cultured PSMCs were transfected with Skp2 sequence-specific siRNA and non-targeting siRNA, the silencing efficiency was assessed by immunoblotting ($n = 3$ in each group); (B,C) PSMCs were transfected with non-targeting siRNA or Skp2 specific siRNA, and followed with or without AMPK α 2 siRNA transfection. Protein level of p27^{Kip1} was examined using immunoblotting (B) ($n = 3$ in each group). Cell proliferation was measured by BrdU incorporation assay (C) ($n = 4$ in each group). +, with indicated siRNA transfection; -, without siRNA transfection; * $p < 0.05$ versus control siRNA transfection; ** $p < 0.01$ versus control siRNA transfection; # $p < 0.01$ versus control siRNA + AMPK α 2 siRNA transfection.

To further verify the involvement of Skp2 in AMPK α 2 knockdown-induced PSMCs proliferation, PSMCs were transfected with non-targeting siRNA or Skp2 siRNA followed with or without AMPK α 2 knockdown. Figure 5C demonstrated that AMPK α 2 knockdown markedly stimulated PSMCs proliferation ($p < 0.05$ versus control siRNA transfection), while prior depletion of Skp2 inhibited PSMCs proliferation induced by AMPK α 2 knockdown, which declined from 1.45-fold to 0.85-fold over control siRNA ($p < 0.01$ versus control siRNA + AMPK α 2 siRNA transfection). Lacking Skp2 protein alone also reduced basal PSMCs proliferation ($p < 0.05$ versus control siRNA transfection). Collectively, these results suggest that Skp2-induced p27^{Kip1} reduction mediates AMPK α 2 knockdown-stimulated PSMCs proliferation.

3. Discussion

In the present study, we have provided direct evidence that knockdown of AMPK α 2 by siRNA stimulates primary cultured PSMCs proliferation; this effect is coupled to the particular activation

of mTOR and subsequent upregulation of Skp2 and reduction of p27^{Kip1}. The study suggests that activation of AMPK α 2 might have potential value in the management of PAH by modulation of pulmonary vascular remodeling.

AMPK is a crucial energy sensor that regulates cellular and whole-body energy balance [8]. Although best known for its effects on metabolism, AMPK has been recently found to have many other functions such as regulation of cell proliferation, apoptosis and migration [13]. Emerging studies have shown that activation of AMPK benefits a variety of cancer as well as other diseases by suppressing proliferation of tumor cells as well as nonmalignant cells [14–16]. In this study, we used post-transcriptional gene silencing with siRNA to specifically examine the effect of AMPK on PSMCs proliferation. Our results demonstrated that knockdown of AMPK α 2 isoform promotes PSMCs proliferation, indicating the anti-proliferative action of AMPK α 2 in PSMCs. AMPK has numerous targets in pathways involved in growth regulation [33]. mTOR, a key positive regulator of cell growth, proliferation, and survival, has been proved to be one of the most characterized downstream effectors of AMPK [24–26]. mTOR controls proteins synthesis required for cell proliferation and is implicated in a variety of human disorders, including cancer, cardiovascular disease, autoimmunity, metabolic disorders and inherited disease [23,34,35]. Recent studies have also found that mTOR activation promotes PSMCs proliferation and is associated with pulmonary vascular remodeling in PAH [36,37]. mTOR is negatively regulated by tuberous sclerosis complex 1 (TSC1)/TSC2 [38]. In the majority of cells under stress conditions, activation of AMPK phosphorylates TSC2 leading to TSC1/TSC2-dependent suppression of mTOR to inhibit cell proliferation [38,39]. The current study indicated that knockdown of AMPK α 2 caused unrestrained mTOR activation and increased PSMCs proliferation. Loss of mTOR with siRNA abolished AMPK α 2 knockdown-induced PSMCs proliferation. This indicates that mTOR might be a direct target of AMPK α 2 in regulation of PSMCs proliferation.

Mammalian cell proliferation is delicately regulated by cell-cycle regulatory proteins including CDKs and CDK inhibitors [40]. Skp2, a F-box component of Skp1/Cullin/F-box protein (SCF)-type ubiquitin ligase, is responsible for the ubiquitination and proteasomal degradation of cell-cycle regulators such as the CDK inhibitor p27^{Kip1} [41]. Upregulation of Skp2 has been observed in various malignant tumors [42,43]. Moreover, tumors overexpressing Skp2 are strongly associated with low p27^{Kip1} levels and poor disease-free and overall survival [44]. Inhibition of Skp2 prevents p27^{Kip1} degradation and suppresses proliferation of many cell types [45,46]. p27^{Kip1} as a CDK inhibitor binds to and prevents the activation of cyclin E-CDK2 and cyclin D-CDK4 complexes, therefore blocks the G1-S transition of cell cycle and suppresses cell proliferation [47,48]. Our study showed that PSMCs lacking AMPK α 2 exhibited a significant elevation of Skp2 protein level; this was accompanied with reduction of p27^{Kip1} and proliferation of PSMCs. Silencing of mTOR attenuated the changes of Skp2 and p27^{Kip1} caused by AMPK α 2 siRNA transfection. Our results further indicated that inhibition of Skp2 by siRNA abolished AMPK α 2 knockdown-induced p27^{Kip1} reduction and therefore suppressed PSMCs proliferation. Taken together, these results suggest that Skp2/p27^{Kip1} pathway lays to the downstream of mTOR in mediating the effect of AMPK α 2 on PSMCs proliferation. Encouragingly, our preliminary study in rat PAH model found that similar mechanisms were also existed *in vivo* (date not shown here).

PAH is a common clinical syndrome with high mortality and requires a longer-term management [1]. The present study demonstrates that knockdown of AMPK α 2 stimulated PSMCs proliferation, suggesting that enhancing AMPK α 2 activity might be a novel therapeutic strategy in the prevention and treatment of PAH. Metformin is an *in vitro* synthetic AMPK agonist which has been commonly used in clinic to treat type 2 diabetes with wide clinical experience and safety record [49]. We have recently found that activation of AMPK α 2 by metformin suppresses PSMCs proliferation [50], together with our current findings, suggesting that metformin might be used in clinical treatment of PAH. Yet, this still needs to be tested in patients with PAH.

4. Materials and Methods

4.1. Cell Culture

Rat PSMCs were obtained from main pulmonary arteries from 4- to 5-week-old Sprague-Dawley rats using the method described by Wu *et al.* [17]. All animal procedures were approved and carried out in accordance with the Laboratory Animal Care Committee of Xi'an Jiaotong University. Briefly, rats were euthanized by CO₂ overdose. Main pulmonary arteries were rapidly dissociated and cleaned under sterile conditions. Then, the adventitia and endothelium of pulmonary arteries were carefully scraped off and the remaining smooth muscle was minced (about 1 mm²) and placed into a culture flask. PSMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM)/High glucose (Gibco, Grand Isle, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, HangZhou, China) and 1% antibiotics (complete DMEM) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were fed every 2–3 days and subcultured by trypsinization using 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) till reaching 80% confluence. All PSMCs used were from passages 4 and 6. The purity and identity of PSMC were determined by immunostaining with an antibody against α -smooth muscle actin (α -SMA, Sigma-Aldrich, St. Louis, MO, USA) as described previously [17].

4.2. siRNA Transfection

Transfection of PSMCs with siRNA (GenePharm, Shanghai, China) was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacture's protocols. PSMCs were seeded into 6-well plates and cultured till reaching 30%–40% confluence. Cells were then transfected with 100 μ M siRNA plus 5 μ L of Lipofectamine for 6 h in serum-free DMEM and sequentially cultured in complete DMEM for 48 h in a 37 °C, 5% CO₂ humidified incubator. The silencing efficiency was determined by immunoblotting.

4.3. BrdU Incorporation Assay

Cells proliferation was determine by BrdU incorporation assay according to the instructions of BrdU ELISA Kit (Maibio, Shanghai, China). Briefly, aliquots of 5×10^3 cells were plated on 96-well plates and serum deprived (1% FBS in DMEM) overnight. After different treatments, cells were labeled by addition of BrdU for 2 h at 37 °C. After removing the labeling medium, cells were fixed and denatured by FixDenat solution and then incubated with anti-BrdU monoclonal antibody conjugated to peroxidase for 90 min at room temperature. Finally, substrate solution was added in each well and the absorbance at 370 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA). Absorbance was normalized to initial reading to verify equal cell numbers at the start of assay.

4.4. Immunoblotting

The cultured PSMCs were washed twice with ice-cold phosphate buffered saline (PBS) and lysed on ice in RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride and proteinase inhibitors. The lysates were then centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatant was collected as sample protein. Equal amount of protein from each sample was loaded and resolved on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) using semidry transfer. The membranes were then blocked and probed with polyclonal or monoclonal primary antibodies against phosphor-mTOR, total-mTOR, Skp2, p27^{Kip1} (Cell Signaling Technology, Beverly, MA, USA; 1:1000 dilution), AMPK α 1 and AMPK α 2 (Proteintech Group, Chicago, IL, USA; 1:500 dilution) as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich; 1:2000 dilution). After incubating with primary antibodies, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich; 1:5000 dilution). Immunoreactive bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) and quantified using Quality One software (Bio-Rad). Sample loadings were normalized by immunoblotting with GAPDH or related total protein.

4.5. Statistics

Statistical analysis was carried out using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean \pm standard deviation. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistical significant was determined at the level of $p < 0.05$.

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Author Contributions: Rui Ke and Manxiang Li designed experiments. Rui Ke, Lu Liu, Yanting Zhu, Shaojun Li, Xinming Xie and Fangwei Li performed experiments. Rui Ke, Lu Liu and Yanting Zhu analyzed data. Yang Song, Lan Yang and Li Gao contributed reagents or other essential materials and helped to revise the manuscript. Rui Ke and Manxiang Li wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMPK	adenosine monophosphate-activated protein kinase
CDKs	cyclin-dependent kinases
mTOR	mammalian target of rapamycin
PAH	pulmonary arterial hypertension
PASMCs	pulmonary arterial smooth muscle cells
RNAi	RNA interference
Skp2	S-phase kinase-associated protein 2
siRNA	small interfering RNA
TSC	tuberous sclerosis complex

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