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Wild-type p53-induced phosphatase 1 promotes vascular smooth muscle cell proliferation and neointima hyperplasia after vascular injury via p-adenosine 5′-monophosphate-activated protein kinase/mammalian target of rapamycin complex 1 pathway

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Objectives: Vascular smooth muscle cell (VSMC) proliferation is a crucial cause of vascular neointima hyperplasia and restenosis, thus limiting the long-term efficacy of percutaneous vascular intervention. We explored the role of wild-type p53-induced phosphatase 1 (Wip1), a potent regulator of tumorigenesis and atherosclerosis, in VSMC proliferation and neointima hyperplasia.

Methods and results: Animal model of vascular restenosis was established in wild type C57BL/6J and VSMC-specific Tuberous Sclerosis 1 (TSC1)-knockdown mice by wire injury. We observed increased protein levels of Wip1, phospho (p)-S6 Ribosomal Protein (S6)$^{Ser235/236}$, p-4EBP1$^{Thr37/46}$ but decreased p-adenosine 5′-monophosphate-activated protein kinase (AMPK)α^{Thr172} both in carotid artery at day 28 after injury and in VSMCs after 48 h of platelet derived growth factor-BB (PDGF-BB) treatment. By using hematoxylin-eosin staining, Ki-67 immunohistochemical staining, cell counting kit-8 assay and Ki-67 immunofluorescence staining, we found Wip1 antagonist GSK2830371 (GSK) or mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin both obviously reversed the neointima formation and VSMC proliferation induced by wire injury and PDGF-BB, respectively. GSK also reversed the increase in mRNA level of *Collagen I* after wire injury. However, GSK had no obvious effects on VSMC migration induced by PDGF-BB. Simultaneously, TSC1 knockdown as well as AMPK inhibition by Compound C abolished the vascular protective and anti-proliferative effects of Wip1 inhibition. Additionally, suppression of AMPK also reversed the declined mTORC1 activity by GSK.

Conclusion: Wip1 promotes VSMC proliferation and neointima hyperplasia after wire injury via affecting AMPK/mTORC1 pathway.

Keywords: AMPK, mTORC1, vascular restenosis, VSMC proliferation, Wip1

Abbreviations: 4EBP1, 4E-binding protein 1; AKT, protein kinase B; AMPK, adenosine 5′-monophosphate-activated protein kinase; CCK-8, cell counting kit-8; GSK, GSK2830371; HE, hematoxylin and eosin; mTORC1, mammalian target of rapamycin complex 1; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PPM1D, protein phosphatase magnesium-dependent 1 delta; Rapa, rapamycin; TSC1, tuberous sclerosis 1; VSMC, vascular smooth muscle cell; Wip1, wild-type p53-induced phosphatase 1

INTRODUCTION

Vascular restenosis badly harms the curative effect of revascularization, despite the fact that novel treatments especially percutaneous interventions of coronary, carotid and other peripheral arteries have made great progress over the past 20 years [1,2]. Therefore, revealing the underlying mechanisms and searching for novel therapeutic targets of restenosis are to be urgently solved. The initiating step of vascular restenosis is de-endothelization and compression of atherosclerotic plaque into vascular wall via mechanical injury [3]. Whereafter, activated platelet and inflammatory cells gather at damaged area and secrete several cytokines, such as platelet-derived growth factor

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(PDGF), interleukin-6 and interleukin-1β, and so forth [3]. These processes initiate subsequent proliferation and migration of vascular smooth muscle cells (VSMCs) and lead to neointima hyperplasia, vascular remodeling and restenosis [3]. As the major components of vascular wall, VSMCs play a crucial role in maintaining normal function of blood vessels (e.g. self-repairment) but also in neointima formation owing to the high proliferative activity [4]. Accordingly, preventing excessive VSMC proliferation is emerging as a potent method for restenosis treatment.

Numerous studies have shown that, in mammalian bodies, VSMCs maintain a balance between proliferation and apoptosis. Under certain pathological conditions, VSMCs switch to a synthetic growth phenotype, which is characterized by excessive proliferation and migration [5,6]. The contractile-to-synthetic growth transition is a multifactorial process that involves many signaling pathways including cytokines, protein kinases, transcription factors and regulatory RNA molecules, and so forth. Though treatments based on aforementioned pathways, such as phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) pathways, a classical pathway regulating VSMC proliferation [7,8], have been introduced, the morbidity of restenosis still reached 10% [2]. Therefore, unraveling the novel underlying mechanism regulating VSMC proliferation is of great significance for preventing vascular restenosis.

The wild-type p53-induced phosphatase 1 (Wip1), a member of the PP2C family of Ser/Thr protein phosphatases, is also addressed as protein phosphatase magnesium-dependent 1 delta (PPM1D) [9]. Overexpressed in various tumors, Wip1 is originally emerging as an oncogene regulating tumorigenesis via its regulation of apoptosis [10,11]. At the molecular level, regulation of ATM in the mammalian target of rapamycin (mTOR) and P53 is discovered to play a key role in Wip1-mediated tumorigenesis [12]. Many studies have found that Wip1 also participates in other biological processes, such as neurogenesis [13], organismal aging [14] and haematopoietic stem cell homeostasis [15]. Wip1 is recently discovered to exert a pro-atherosclerotic role in the pathological process of atherosclerosis, during which autophagy, cholesterol efflux and macrophage migration are involved [16,17]. In the above study, adenosine 5′-monophosphate-activated protein kinase (AMPK) is also confirmed to be involved in ATM-dependent regulation of mTOR [16]. Vascular restenosis has similar pathological processes to atherosclerosis, such as VSMC proliferation and migration [18], reminding us that Wip1 may also be a potent functional regulator in vascular restenosis. However, whether Wip1 is associated with VSMC proliferation, neointima hyperplasia and restenosis after vascular injury is largely unknown.

In the current study, we describe a regulatory role for Wip1 in vascular restenosis, in which AMPK/mTORC1 pathway is involved. By using a Wip1 antagonist GSK2830371 (GSK), we found that Wip1 inhibition suppresses VSMC proliferation and neointima hyperplasia, accompanied by enhanced AMPK phosphorylation and decreased mTORC1 activity. Further study shows GSK fails to prevent VSMC proliferation and neointima hyperplasia in mice with tuberous sclerosis 1 (TSC1) knockdown, and thus leading to enhanced restenosis. Our research shows that Wip1-dependent modulation of VSMC proliferation and neointima hyperplasia by means of AMPK/mTORC1 may be identified as a potential therapeutic intervention for vascular restenosis.

METHODS

Experimental animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee and the Ethic Committee of The General Hospital of Western Theater Command (Chengdu, Sichuan, China). Wild-type C57BL/6J mice were purchased from Dashuo Animal Science and Technology (Chengdu, Sichuan, China). We generated VSMC-specific TSC1-knockdown mice by crossing mice carrying floxed Tsc1 alleles ($Tsc1^{fl/fl}$) with sm22cre mice. By using mixed strain $Tsc1^{fl/fl}$sm22cre$^{-/-}$ and C57BL/6 $Tsc1^{WT/WT}$sm22cre$^{+/+}$ (Jackson Laboratories, Cambridge, Massachusetts, USA), we finally got TSC1-loss mice with $Tsc1^{fl/fl}$sm22cre$^{+/-}$ genetype ($Tsc1^{KD}$). Littermate mice ($Tsc1^{WT/WT}$sm22cre$^{+/+}$, $Tsc1^{fl/WT}$sm22cre$^{+/-}$, $Tsc1^{fl/fl}$sm22cre$^{-/-}$) were used as controls ($Tsc1^{WT}$). Eight-week to 10-week-old male mice were housed in the following conditions: 22–25 °C,12-hour light/dark cycle, periodic air changes and free access to food and water. The common carotid artery of mouse was injured with wire just near to the carotid bifurcation as described in the literature [19]. GSK (25 mg/kg) [20] was administered via intraperitoneal injection for consecutive 21 days immediately after injury. Rapamycin (Rapa; 5 mg/kg) [21] was given via drinking water starting immediately after injury for consecutive 21 days. Compound C (300 mg/kg) was administered via subcutaneous Alzet osmotic minipumps (Durect Corporation, Cupertino, California, USA) for 28 days immediately after injury [22]. Equivalent physiological saline was used as controls for both GSK, Rapa and Compound C. Mice were deeply anesthetized with pentobarbital (100 mg/kg) after experiments and perfused with physiological saline.

Culture and treatment of vascular smooth muscle cell

VSMC culture was prepared as described in our previous research [23]. Briefly, VSMCs were isolated from aortas of 8- to 10-week-old mice after digestion with 0.25% trypsin (Beyotime Institute of Biotechnology, Shanghai, China). Then, the VSMCs were cultured at 37 °C in complete medium [Dulbecco's modifed Eagle's medium (DMEM; HyClone, Carlsbad, California, USA) supplemented with fetal calf serum (10%; Invitrogen, Carlsbad, California, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml)] in humidifed 5% CO_2 atmosphere. VSMCs were collected via centrifugation and then cultured in complete medium at 37 °C with 5% CO_2 for further use. Twenty-four hours of serum starvation was done in VSMCs under exponential growth conditions before all experiments. All VSMC experiments were from passages 3 to 10. Recombined human platelet derived growth factor-BB (PDGF-BB) (30 ng/ml; R&D Systems, Minneapolis, Minnesota, USA) [24], GSK (50 μmol/l; ChemieTek, Indianapolis, Indiana, USA) [20], Rapa (100 nmol/l; Sigma, St Louis, Missouri, USA) [25] and CC (15 μmol/l; MCE, Shanghai, China) [26] were utilized to treat the cultured VSMCs.

Immunohistochemical staining

Common carotid arteries were harvested, perfused with physiological saline, fixed with 4% paraformaldehyde and embedded in paraffin. The artery sections (4–5 μm) were obtained at 80-μm intervals and stained with hematoxylin and eosin. Five artery sections per sample were collected for staining. Finally, the areas of intima and media of the carotid artery were analyzed by utilizing Image-Pro Plus software. For immunohistochemical staining, artery sections were incubated at 4 °C with relative primary antibodies against Ki-67 (Cell Signaling Technology, Danvers, Massachusetts, USA) overnight after being blocked. The sections were subsequently followed by a secondary antibody incubation and counterstained with mayer hematoxylin.

Immunofluorescent staining

For immunofluorescent staining, cell culture media was removed from the six-well plate, and the cell plates were washed by PBS for three times. VSMCs were fixed and then rinsed by PBS, methanol, 0.5% triton X-100T and 1% blocking solution. Primary antibodies against SM α-actin and Ki-67 (Cell Signaling Technology) were diluted (1 : 1000) and incubated with VSMCs at 4 °C overnight in dark. VSMCs were washed with PBS and then incubated in dark with Alexa Fluor 594F(ab′)-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (1: 2500; Molecular Probes Inc., Eugene, Oregon, USA), respectively, for 1 h. Subsequently, VSMCs were incubated in the room temperature with DAPI (5 mg/ml; VECTOR Labs, Burlingame, California, USA) for 5 s and determined by using immunofluorescent microscopy (Leica MPS 60, Germany).

Immunoblotting

Extraction of carotid arteries and VSMCs were lysed with RIPA buffer (Beyotime Institute of Biotechnology) and then subjected to immunoblot analysis as described in our previous study [27]. Antibodies against Wip1, phospho (p)-S6 Ribosomal Protein (S6)$^{Ser235/236}$, total S6, p-4E-binding protein 1(4EBP1)$^{Thr37/46}$, total 4EBP1, TSC1, p-AMPKα^{Thr172}, AMPKα, p-AKT^{Thr308}, total AKT and β-actin were purchased from Cell Signaling Technology.

Real time qRT-PCR

Total RNA was extracted using Trizol agent (Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol by using common carotid arteries or VSMCs. The cDNA from 1 μg total RNA was synthesized with Bestar qPCR RT Kit (DBI Bioscience, Ludwigshafen, Germany). Real time qRT-PCR was performed in ABI Prism 7700 Sequence Detector (Applied Biosystems, Carlsbad, California, USA) by using 2× SyberGreen mixture (DBI Bioscience). *Actb* was utilized as a housekeeping gene and the classical ΔΔCt method was used to normalize gene expression. Primers used for each gene were listed as following:

1. *Wip1* (F, 5′-AGC GCA TGT AGG TGA CTC TG-3′; R, 5′-ACT CGG TTC ACT CCA GAC TT-3′)
2. *Collagen I* (F, 5′-GAG TAC TGG ATC GAC CCT AAC CA-3′; R, 5′-GAC GGC TGA GTA GGG AAC ACA-3′) [28]
3. *Collagen III* (F, 5′-TCC CCT GGA ATC TGT GAA TC-3′; R, 5′-TGA GTC GAA TTG GGG AGA AT-3′) [28]
4. *Actb* (F, 5'-TCC TTC TTG GGT ATG GAA-3'; R, 5'-AGG AGG AGC AAT GAT CTT GAT CTT-3') [29]

Cell counting kit-8 assay

VSMC proliferation was analyzed by using a cell counting kit (Solarbio, Beijing, China) according to the manufacturer's instruction. In brief, VSMCs were incubated in 96-well plates (0.5×10^4 cells per well) with physiological saline, GSK, PDGF-BB or PDGF-BB with GSK and then cultured for 48 h. Ten microliters of CCK-8 agent was then added to each well and incubated with VSMCs for another 2 h at 37 °C. By using a microplate reader, VSMC proliferation was finally determined via calculating the relative absorbance at 450 nm.

Wound-healing assay

VSMCs were seeded in six-well plates (1×10^5 cells per well) and serum-deprived for 24 h. Subsequently, the VSMCs were incubated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 24 h. The rates of wound closure were evaluated by using direct microscopic visualization followed with a reference point in the wound field at the bottom, thus permitting photographing of the same spot each time. The remaining cell-free areas were analyzed at 24 h after injury [23].

Transwell assay

For the migration assay, VSMCs were seeded in a upper transwell chamber (Millipore, Darmstadt, Germany) with 8-μm pores in each membrane and incubated with or without GSK (50 μmol/l) for 8 h in a 24-well plate (1×10^5 cells per well). The lower wells of the chamber were filled with serum-free DMEM with or without PDGF-BB (30 ng/ml). Non-migrated cells were then wiped off from the inside of chamber membrane. VSMCs on the lower surface were fixed with 4% paraformaldehyde, washed with PBS for three times and then stained with 1% crystal violet before placed on glass slides. Finally, VSMCs in five randomly selected fields per well were counted under a microscope.

Statistical analysis

Data are presented as mean ± S.D. Unpaired Student' *t*-test was applied to compare two independent groups if they are normally distributed. Two-way or multivariate analysis of variance (ANOVA) with an appropriate post hoc test was preformed to compare means that involve two or more factors, respectively. $P < 0.05$ was considered to be statistically significant. All tests were two-sided.

RESULTS

Inhibition of wild-type p53-induced phosphatase 1 ameliorates wire injury-induced neointima hyperplasia and restenosis

We harvested the bilateral common carotid arteries at 28 days after wire injury. To assess the role of Wip1 on

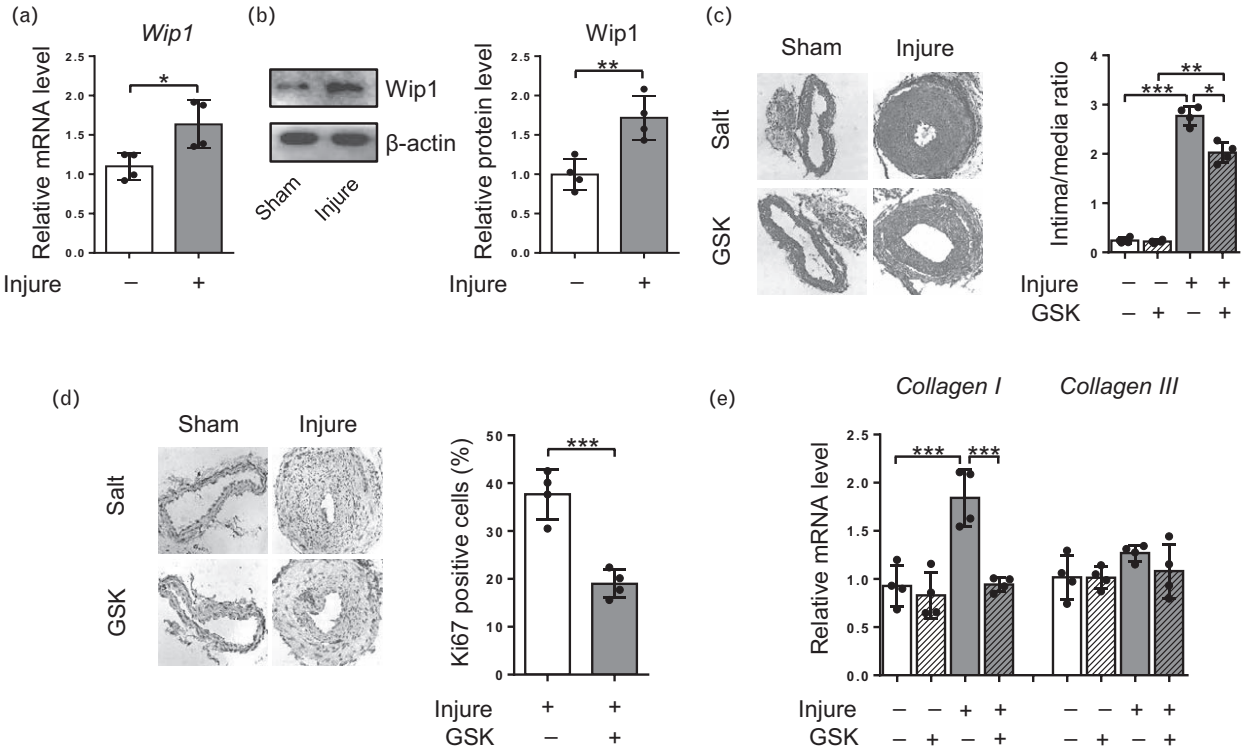

FIGURE 1 Wip1 inhibition ameliorates neointima hyperplasia and vascular restenosis after wire injury. (a) The relative mRNA level of *Wip1* in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury was determined by qRT-PCR ($n = 4$). (b) The protein expression of Wip1 and β-actin in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury were analyzed by immunoblotting ($n = 4$). (c) Representative HE staining of carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with or without GSK (25 mg/kg) treatment (left) and corresponding quantification for ratio of intima/media (right) ($n = 4$). Magnification 200×. (d) Immunohistochemistry staining of Ki-67 (brown) in sections of carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with or without GSK treatment (left) and corresponding quantification for Ki-67 positive cells within neointima (right) ($n = 4$). Magnification 200×. (e) The relative mRNA levels of *Collagen I* and *Collagen III* in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with or without GSK treatment were determined by qRT-PCR ($n = 4$). Data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the two marked groups, respectively. GSK, GSK2830371; HE, hematoxylin and eosin; Wip1, wild-type p53-induced phosphatase 1.

restenosis, we first determined whether vascular Wip1 expression is altered after wire injury. As a result, we found increased mRNA (Fig. 1a) and protein (Fig. 1b) level of Wip1 in the injured carotid artery, reminding the potential role of Wip1 to promote neointima hyperplasia during the restenosis development. Next, we detected the effects of Wip1 on morphological changes induced by wire injury. As the data showed, obvious lumen narrowing and elevated intima/media ratio were observed in arteries harvested at 28 days after injury, which were significantly ameliorated by Wip1 inhibition via utilizing a specific Wip1 antagonist (GSK) (Fig. 1c). Analysis of Ki-67-stained arteries also showed that the Ki67-positive area was significantly less in injured carotid arteries after GSK treatment (Fig. 1d). Enhanced synthesis and secretion of extracellular matrix, such as type I collagen and type III collagen constitute an important part of vascular restenosis [4], therefore, we further investigated whether Wip1 inhibition affected collagen deposition. As we supposed, the mRNA level of *Collagen* I in injured carotid arteries was significantly increased compared with those that received sham operation, which was reversed by GSK (Fig. 1e). However, there was no obvious difference in vascular mRNA level of *Collagen III* between carotid arteries received wire injury or sham operation. Taken together, these data indicate that Wip1 induction by mechanical damage promotes neointima formation and collagen synthesis, thus resulting in vascular restenosis.

Wild-type p53-induced phosphatase 1 inhibition suppresses vascular smooth muscle cell proliferation induced by platelet derived growth factor-BB

To further explore whether VSMC proliferation or migration is involved in Wip1 ablation-mediated protection against vascular restenosis, we tested the alteration of Wip1 in VSMC treated with PDGF-BB, a crucial factor that is released after vascular injury and modulates VSMC proliferation and migration [3]. Consistent with in-vivo experiment, the mRNA (Fig. 2a) and protein (Fig. 2b) level of Wip1 in VSMC increased evidently after treatment of PDGF-BB. CCK-8 assay showed that the increase of VSMC viability after completing PDGF-BB treatment was abolished by GSK (Fig. 2c), suggesting the role of Wip1 ablation in blocking VSMC proliferation. The result of Ki-67 immunofluorescence staining further supported this finding. We observed significantly increased Ki-67 positive VSMCs after 48 h of PDGF-BB treatment, which was then reversed by Wip1 inhibition (Fig. 2d). We next explored the biological function of Wip1 in modulating VSMC migration caused by PDGF-BB. As the wound healing assay showed, 24 h of

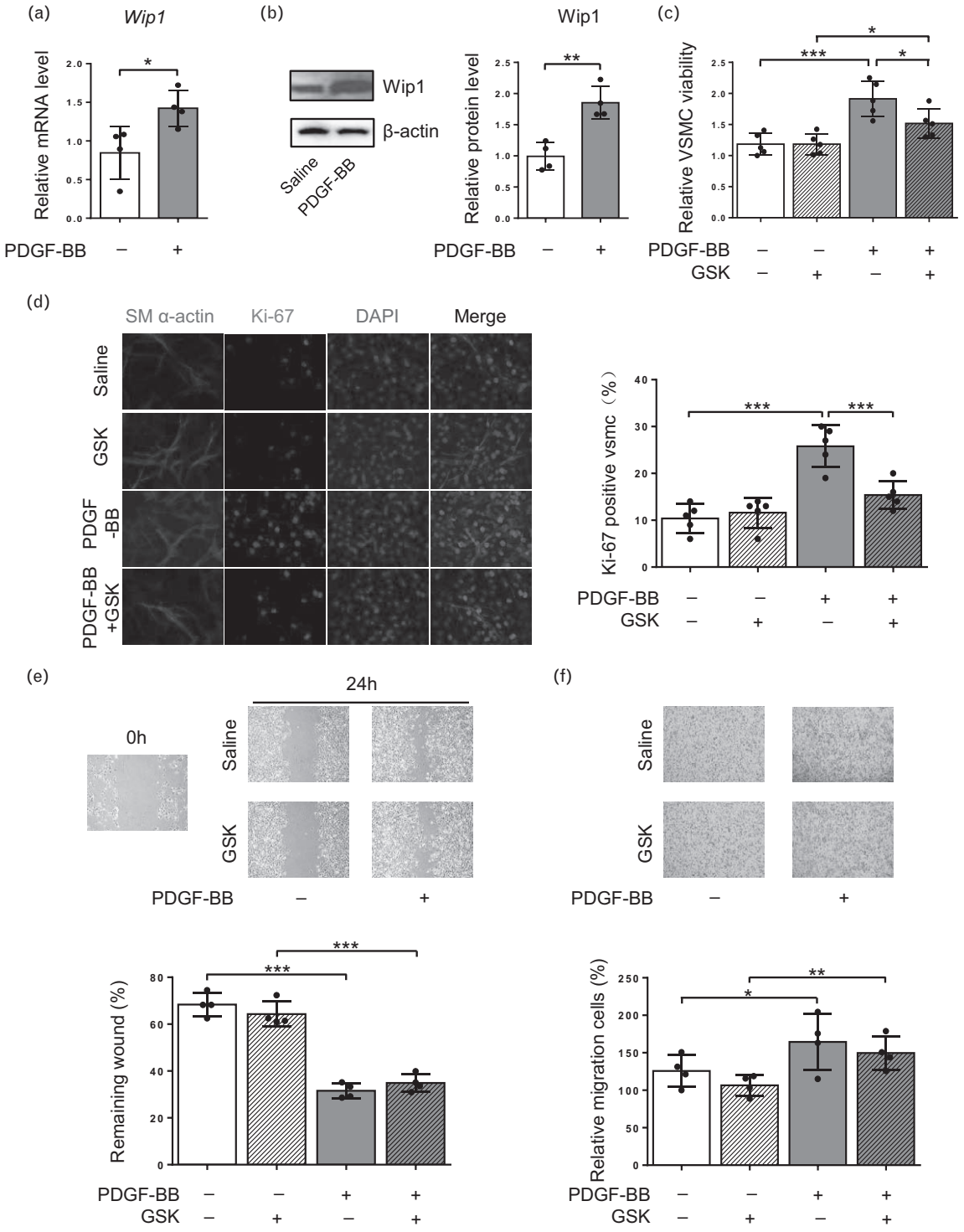

FIGURE 2 Wild-type p53-induced phosphatase 1 inhibition suppresses vascular smooth muscle cell proliferation but not migration induced by PDGF-BB. (a) The relative mRNA level of *Wip1* was detected by qRT-PCR in VSMCs after 48 h of physiological saline or PDGF-BB (30 ng/ml) treatment ($n = 4$). (b) The protein expression of Wip1 and β-actin were determined by immunoblotting in VSMCs after 48 h of physiological saline or PDGF-BB treatment ($n = 4$). (c) VSMC proliferation was analyzed via CCK-8 assay. VSMCs were incubated with physiological saline, GSK (50 μmol/l), PDGF-BB or PDGF-BB with GSK for 48 h. Then, the absorbance at 450 nm was obtained ($n = 5$). (d) VSMCs treated as above described were stained with SM α-actin (red), Ki-67 (green) and DAPI (blue). Representative images (left) and corresponding quantification of Ki-67 positive VSMCs (right) were shown ($n = 5$). Magnification 400×. (e) Migration of VSMCs treated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 24 h was measured via wound healing assay. Representative images (upper panel) and corresponding quantification of healing rates (lower panel) were shown ($n = 4$). Magnification 100×. (f) VSMCs were treated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 8 h and migration was assessed by transwell assay. Representative images (upper panel) and corresponding quantification of migration cells (lower panel) were shown ($n = 4$). Magnification 100×. Data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the two marked groups, respectively. GSK, GSK2830371; PDGF-BB, platelet derived growth factor-BB; VSMCs, vascular smooth muscle cells; Wip1, wild-type p53-induced phosphatase 1.

PDGF-BB exposure led to accelerated wound healing whether or not GSK was applied (Fig. 2e). Simultaneously, GSK also failed to reduce the number of migrated VSMCs caused by PDGF-BB (Fig. 2f). These results suggest that Wip1 inhibition does not affect VSMC migration caused by PDGF-BB. Therefore, Wip1 inhibition prevents vascular restenosis and neointima hyperplasia via preventing VSMC proliferation instead of migration.

Wild-type p53-induced phosphatase 1 ablation suppresses vascular smooth muscle cell proliferation and neointima hyperplasia via reducing mammalian target of rapamycin complex 1 activity

mTORC1 is a crucial complex for energy metabolism and plays diverse roles in cardiovascular function and diseases including vascular neointima hyperplasia [30–32]. mTORC1 is also confirmed to play an essential role in phenotypic conversion of VSMC induced by PDGF-BB [33]. As previous studies showed that Wip1 aggravated vascular diseases, such as atherosclerosis and hypertension partly via Atm-dependent modulation of mTOR pathway [16], we next investigated the potential role of mTORC1 pathway in the observed reduction of VSMC proliferation by Wip1 inhibition.

As the data showed, the phosphorylation level of S6 (Ser235/236) and 4EBP1 (Thr37/46), two main effectors of mTORC1 complex, were significantly increased in carotid arteries that received wire injury (Fig. 3a). Inhibition of Wip1 reversed the increased phosphorylation of S6 and 4EBP1 induced by wire injury (Fig. 3a). In turn, inhibition of mTORC1 activity by Rapa was also sufficient to exert the vascular protection against restenosis (Fig. 3b). However, there were no significant difference in intima/media ratio between wire injury + Rapa, wire injury + GSK and wire injury + GSK + Rapa group. The results of in-vitro experiments showed that the increased expression of p-S6$^{Ser235/236}$ and p-4EBP1$^{Thr37/46}$ after PDGF-BB treatment were also abolished by Wip1 inhibition (Fig. 3c). Similarly, Wip1 deficiency failed to further lowered PDGF-BB-induced VSMC proliferation on the basis of mTORC1 inhibition by Rapa (Fig. 3d). These data reminded us that mTORC1 inhibition might act downstream of Wip1 ablation to exert the vascular protection against restenosis induced by wire injury.

Mammalian target of rapamycin complex 1 activation blocks the inhibitory effects of wild-type p53-induced phosphatase 1 ablation on vascular smooth muscle cell proliferation and neointima hyperplasia

To further assess the accurate roles of mTORC1 pathway in Wip1-induced neointima hyperplasia, we utilized an animal model (*Tsc1*^{KD}) with specific TSC1 knockdown in VSMC to recapitulate excessive mTORC1 activity [34]. We firstly validated the vascular knockdown efficiency of TSC1 and found TSC1 knockdown led to significant increase in protein level of p-S6$^{Ser235/236}$ and p-4EBP1$^{Thr37/46}$ in common carotid arties (Fig. 4a) as well as VSMCs (Fig. 4c). In accordance with previous studies [31,33]. TSC1 knockdown further accelerated vascular restenosis induced by wire injury, which was not reversed by GSK (Fig. 4b). In-vitro trials also showed that excessive mTORC1 activation almost totally blocked the inhibitory effect of Wip1 ablation on PDGF-BB-induced VSMC proliferation (Fig. 4d). As previous study demonstrated that mTORC2 knockdown had no effect on phenotypic alteration of VSMCs induced by PDGF-BB [33], we did not further investigate whether mTORC2 was involved in Wip1-mediated vascular protection. In summary, our results suggest Wip1 deficiency inhibits VSMC proliferation, neointima hyperplasia and restenosis via controlling mTORC1 activity.

Wild-type p53-induced phosphatase 1-dependent control of p-adenosine 5′-monophosphate-activated protein kinase, but not protein kinase B, mediates promotion of vascular smooth muscle cell proliferation via affecting mammalian target of rapamycin complex 1 activity

As Wip1 inhibition induced declined mTORC1 activity and proliferative capacity in VSMC, we further explored the upstream molecular mechanism of mTORC1. mTORC1 signaling is known to be modulated by several growth factors and stress-associated kinases, such as AMPK and AKT [31]. The current study reveals that PDGF-BB treatment was associated with declined phosphorylation of AMPKα at site Thr172 in VSMC, which was reversed after incubation with GSK (Fig. 5a). Despite the fact that PDGF-BB also induced phosphorylation of AKT at site Thr308, the increase in AKT phosphorylation was not ameliorated by GSK (Fig. 5a). Next, we used an AMPKα antagonist, Compound C to incubate with VSMCs and found that the GSK-induced decrease in protein expression of p-S6$^{Ser235/236}$ and p-4EBP1$^{Thr37/46}$, the two major effectors of mTORC1 signaling after PDGF-BB treatment was abolished by CC (Fig. 5b). Simultaneously, we observed higher protein expression of p-S6$^{Ser235/236}$ and p-4EBP1$^{Thr37/46}$ after Compound C treatment in PDGF-BB-treated VSMC with or without GSK when comparing with control group (Fig. 5b). Additionally, immunofluorescence staining of Ki-67 showed that the inhibitory effect of GSK on PDGF-BB-induced VSMC proliferation was also abolished by Compound C (Fig. 5c). These data indicate an intermediary role of AMPK, but not AKT, toward suppression of mTORC1 signaling and VSMC proliferation by Wip1 inhibition.

p-Adenosine 5′-monophosphate-activated protein kinase dephosphorylation promotes vascular mammalian target of rapamycin complex 1 activation and impairs the protective capacity of wild-type p53-induced phosphatase 1 inhibition to ameliorate vascular restenosis

Our analysis revealed a crucial role of Wip1-dependent AMPK dephosphorylation in modulating VSMC proliferation via inducing mTORC1 activity. Therefore, we next explored whether our in-vitro results would be also translated into in-vivo effects of AMPK on Wip1-dependent

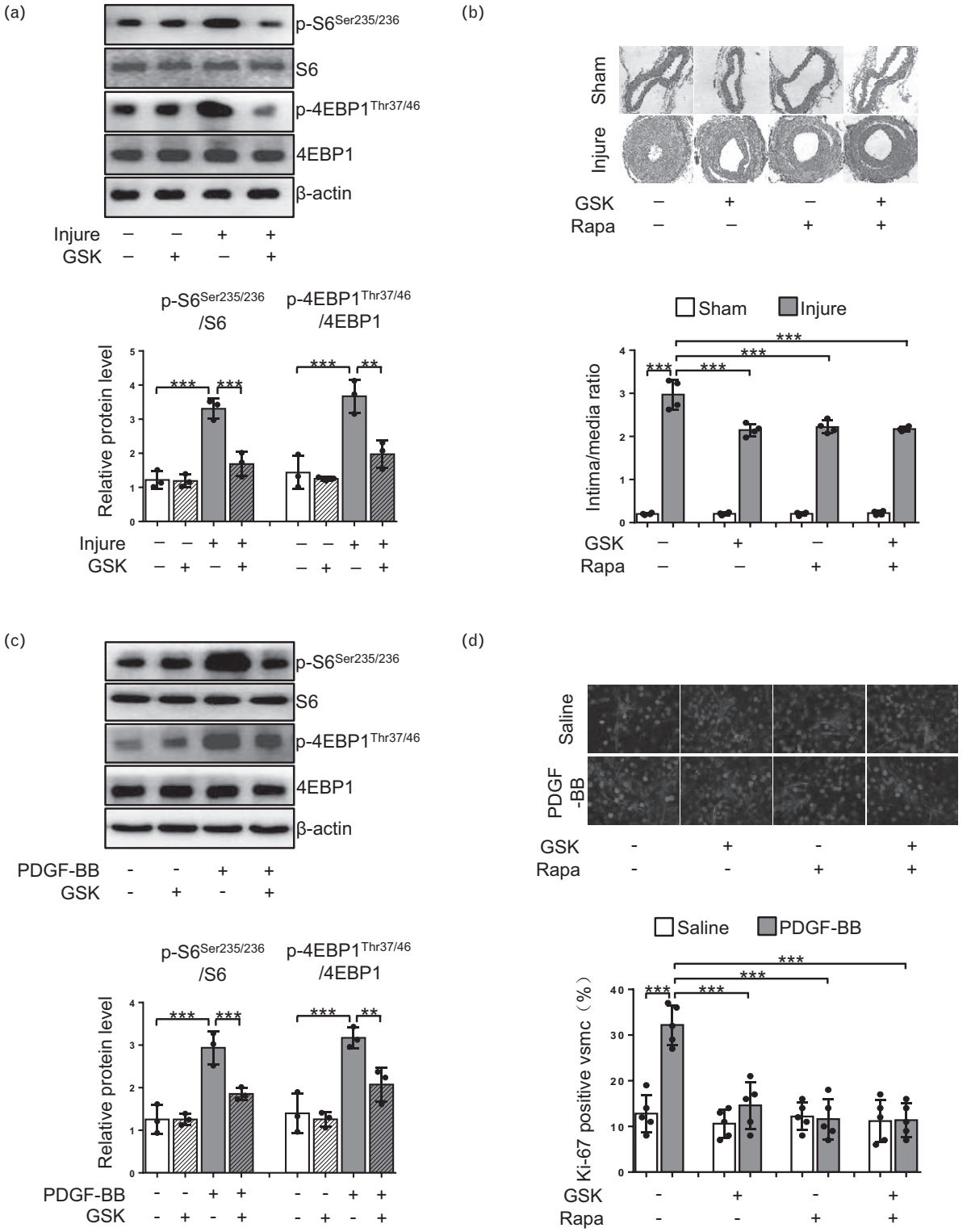

FIGURE 3 Mammalian target of rapamycin complex 1 activity is positively regulated by wild-type p53-induced phosphatase 1 in vascular smooth muscle cells treated by PDGF-BB and carotid arteries after wire injury. (a) The protein expression of p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with or without GSK treatment were analyzed by immunoblotting. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (b) Representative HE staining of carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with physiological saline, GSK, Rapa (5 mg/kg) or GSK plus Rapa treatment (upper panel) and corresponding quantification for ratio of intima/media (lower panel) were shown ($n = 4$). Magnification 200×. (c) The protein expression of p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin were evaluated by immunoblotting in VSMCs incubated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 48 h. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (d) VSMCs treated by physiological saline, GSK, Rapa or GSK plus Rapa for 48 h with or without PDGF-BB incubation were stained with SM α-actin (red), Ki-67 (green) and DAPI (blue). Representative images (upper panel) and corresponding quantification of Ki-67 positive VSMCs (lower panel) were shown ($n = 5$). Magnification 400×. Data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the two marked groups, respectively. GSK, GSK2830371; rapa, rapamycin; PDGF-BB, platelet derived growth factor-BB; VSMCs, vascular smooth muscle cells; Wip1, wild-type p53-induced phosphatase 1.

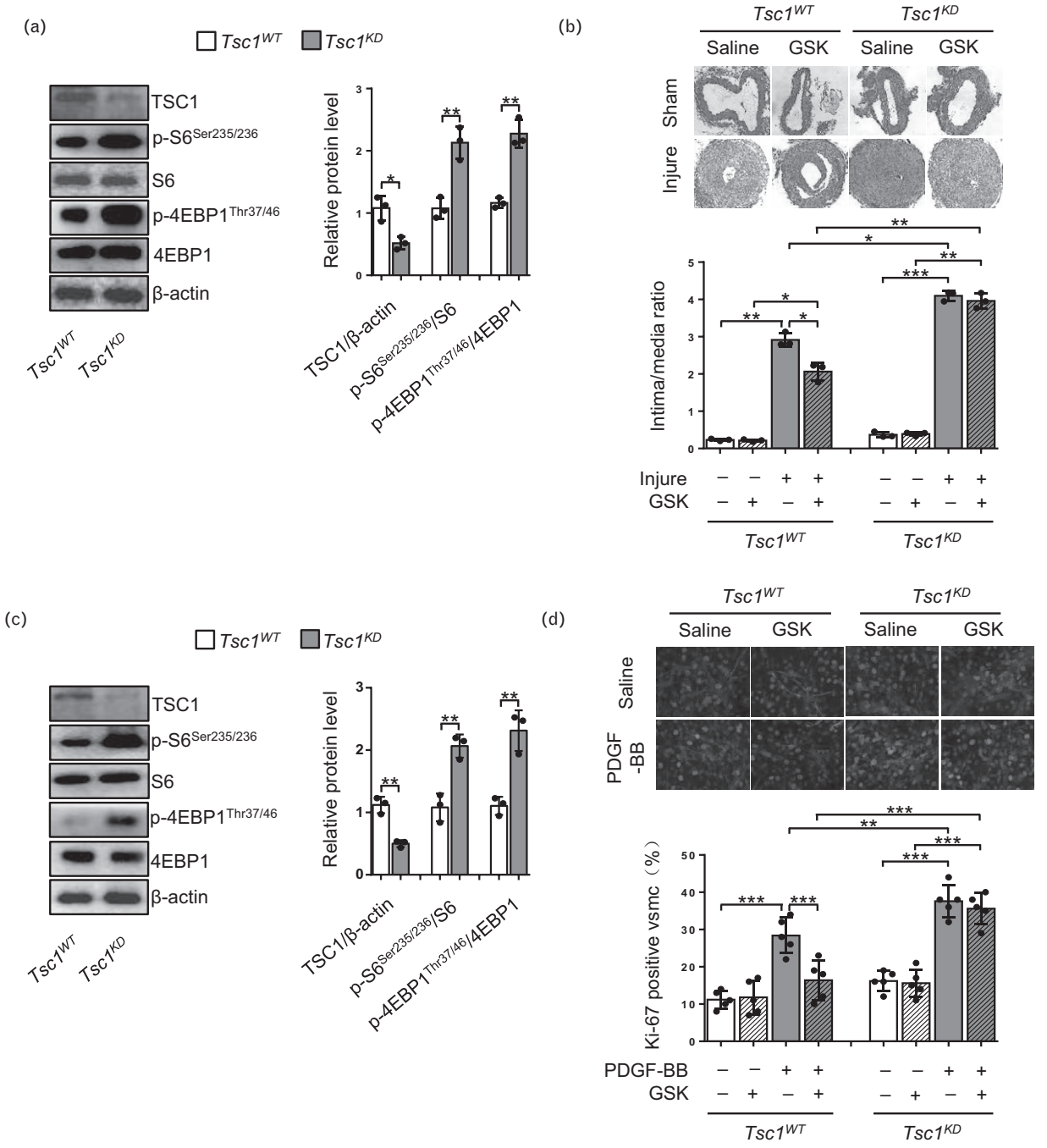

FIGURE 4 Tuberous sclerosis 1 knockdown abolished the repressive effects of wild-type p53-induced phosphatase 1 inhibition on vascular smooth muscle cell proliferation and neointima hyperplasia. (a) The protein expression of p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin in common carotid arteries from *Tsc1*^{WT} or *Tsc1*^{KD} mice. Representative bands (left) and corresponding quantification (right) were shown ($n=3$). (b) Representative HE staining of carotid arteries from *Tsc1*^{WT} or *Tsc1*^{KD} mice at day 28 after sham operation or wire injury with or without GSK treatment (upper panel) and corresponding quantification for ratio of intima/media (lower panel) were shown ($n=3$). Magnification 200×. (c) The protein expression of p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin in VSMCs isolated from *Tsc1*^{WT} or *Tsc1*^{KD} mice. Representative bands (left) and corresponding quantification (right) were shown ($n=3$). (d) VSMCs isolated from *Tsc1*^{WT} or *Tsc1*^{KD} mice were incubated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 48 h and then stained with SM α-actin (red), Ki-67 (green) and DAPI (blue). Representative images (upper panel) and corresponding quantification of Ki-67-positive VSMCs (lower panel) were shown ($n=5$). Magnification 400×. Data are shown as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ denote statistical comparison between the two marked groups, respectively. GSK, GSK2830371; PDGF-BB, platelet derived growth factor-BB; VSMCs, vascular smooth muscle cells; Wip1, wild-type p53-induced phosphatase 1.

promotion of mTORC1 activation, neointima hyperplasia and vascular restenosis. As the results showed, the decreased vascular AMPKα phosphorylation at site Thr172 induced by wire injury was recovered by GSK treatment (Fig. 6a). Importantly, AMPKα dephosphorylation prevented Wip1 inhibition-mediated decrease in vascular S6 and 4EBP1 phosphorylation after wire injury (Fig. 6b), thus providing direct evidence for the intermediary role of AMPK in Wip1-mediated mTORC1 signaling activation. In line with aforementioned molecular

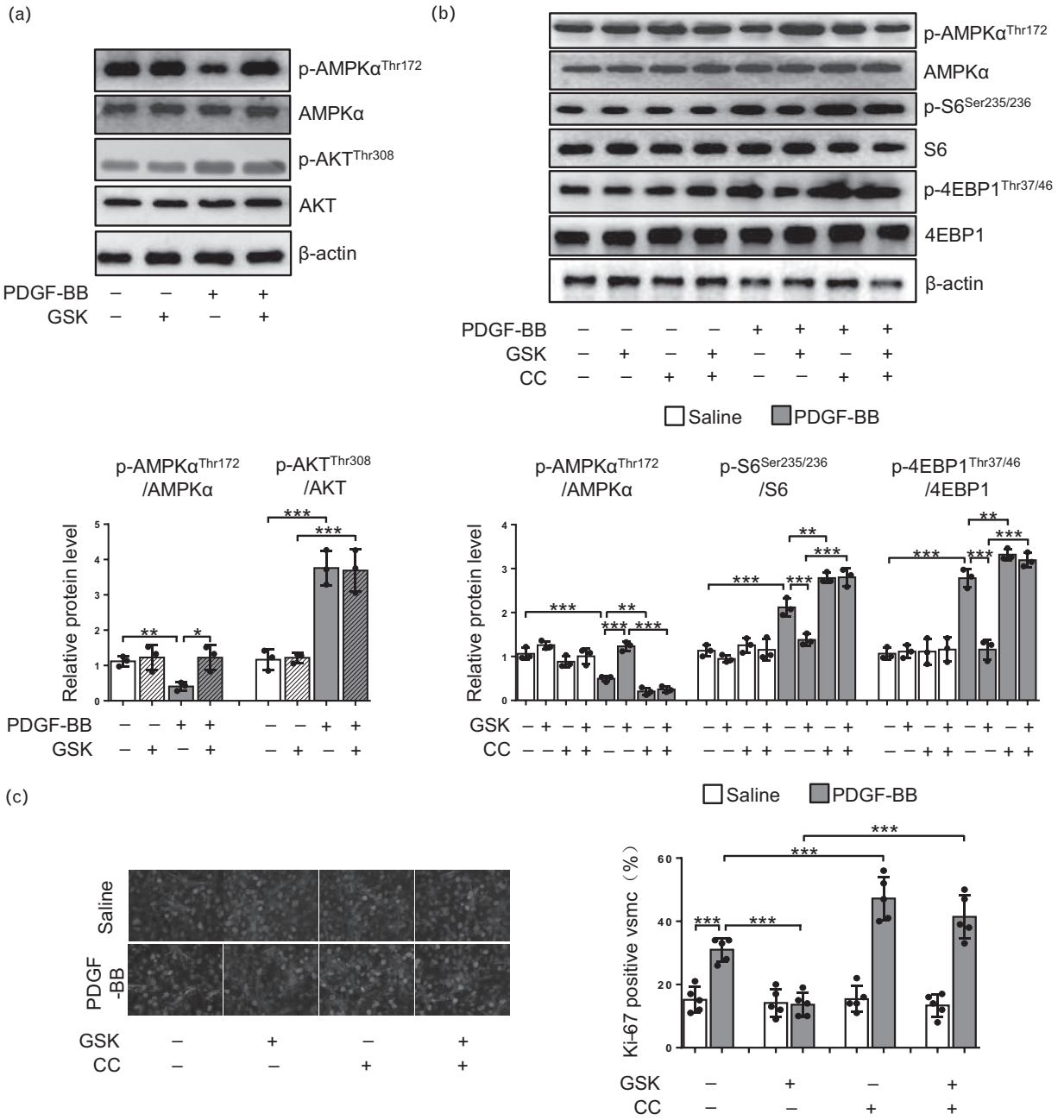

FIGURE 5 p-Adenosine 5′-monophosphate-activated protein kinase dephosphorylation blocked the inhibitory role of wild-type p53-induced phosphatase 1 ablation in vascular smooth muscle cell proliferation induced by PDGF-BB. (a) The protein expression of p-AMPKα^{Thr172}, AMPKα, p-AKT^{Thr308}, AKT and β-actin were evaluated by immunoblotting in VSMCs incubated with physiological saline, GSK, PDGF-BB or PDGF-BB plus GSK for 48 h. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (b) The protein expression of p-AMPKα^{Thr172}, AMPKα, p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin were evaluated by immunoblotting in VSMCs treated by physiological saline, GSK, CC (15 μM) or GSK plus CC for 48 h with or without PDGF-BB. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (c) VSMCs treated by physiological saline, GSK, CC or GSK plus CC for 48 h with or without PDGF-BB incubation were stained with SM α-actin (red), Ki-67 (green) and DAPI (blue). Representative images (left) and corresponding quantification of Ki-67-positive VSMCs (right) were shown ($n = 5$). Magnification 400×. Data are shown as mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the two marked groups, respectively. CC, Compound C GSK, GSK2830371; PDGF-BB, platelet derived growth factor-BB; VSMCs, vascular smooth muscle cells; Wip1, wild-type p53-induced phosphatase 1.

alterations, the declined intima/media ratio (Fig. 6c) and mRNA level of *Collagen I* (Fig. 6d) by GSK after wire injury were also reversed by Compound C. Accordingly, our results support the role of AMPK/mTORC1 pathway in preventing VSMC proliferation and neointima hyperplasia mediated by Wip1 inhibition.

DISCUSSION

Vascular percutaneous intervention is a widely applied therapeutic strategy for atherosclerosis-associated diseases, such as coronary heart disease. However, vascular restenosis is becoming a severe complication that limiting its

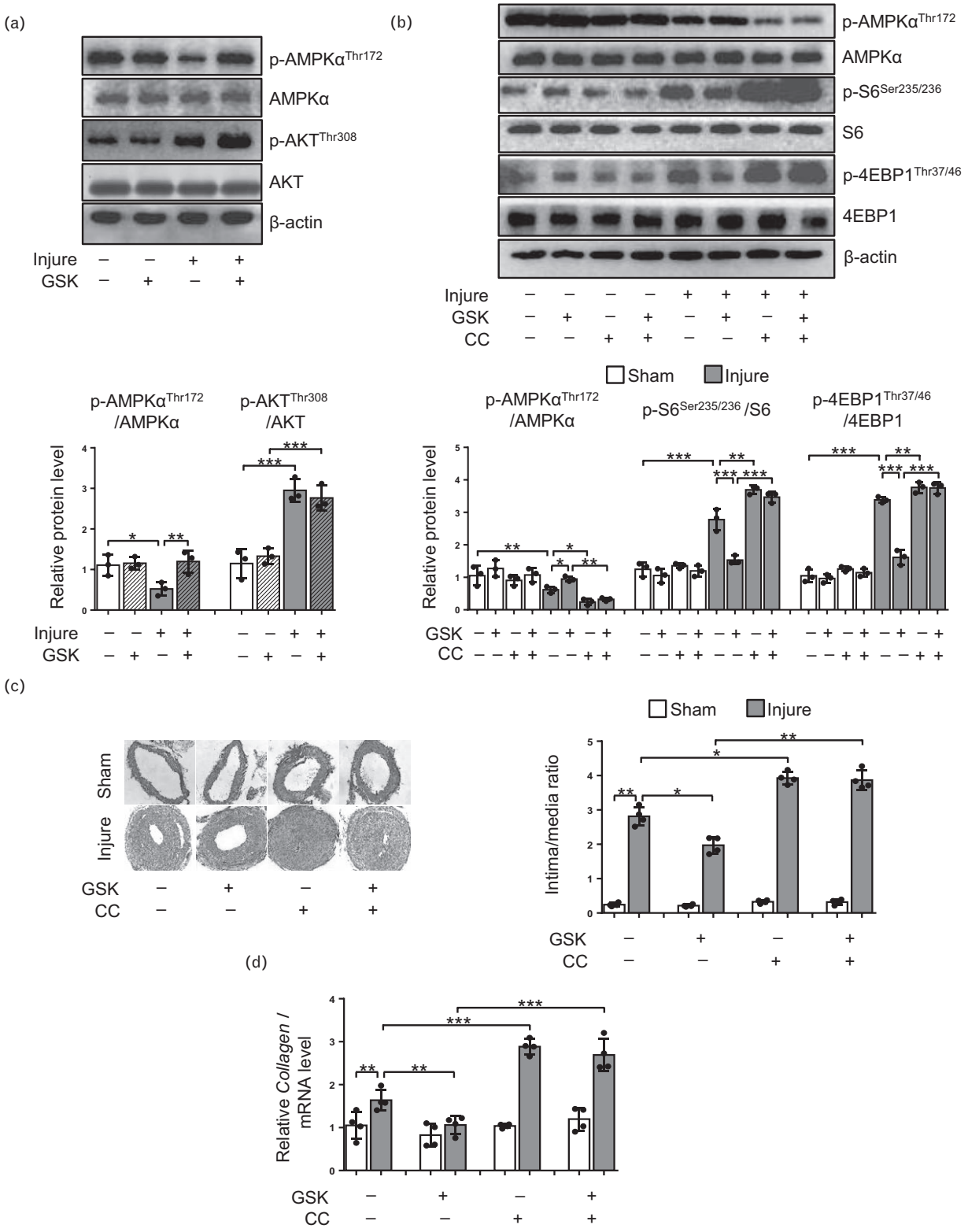

FIGURE 6 p-Adenosine 5′-monophosphate-activated protein kinase dephosphorylation impaired the protection of wild-type p53-induced phosphatase 1 inhibition against vascular restenosis by wire injury. (a) The protein expression of p-AMPKα^{Thr172}, AMPKα, p-AKT^{Thr308}, AKT and β-actin were evaluated by immunoblotting in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with or without GSK treatment. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (b) The protein expression of p-AMPKα^{Thr172}, AMPKα, p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1 and β-actin were evaluated by immunoblotting in carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with physiological saline, GSK, CC (300 mg/kg) or GSK plus CC treatment. Representative bands (upper panel) and corresponding quantification (lower panel) were shown ($n = 3$). (c) Representative HE staining of carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with physiological saline, GSK, CC or GSK plus CC treatment (left) and corresponding quantification for ratio of intima/media (right) were shown ($n = 4$). Magnification 200×. (d) The relative mRNA level of Collagen I in carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury with physiological saline, GSK, CC or GSK plus CC treatment was determined by qRT-PCR ($n = 4$). Data are shown as mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the two marked groups, respectively. CC, Compound C GSK, GSK2830371; VSMCs, vascular smooth muscle cells; Wip1, wild-type p53-induced phosphatase 1.

clinical efficacy. Though several drugs have been applied in stent therapy, the occurrence and development of restenosis and associated underlying mechanisms are still unsolved [35]. Our current study is focused on the roles of Wip1 inhibition in suppressing VSMC proliferation and neointima hyperplasia. We first observed increased expression of Wip1 in both carotid arteries that received wire injury and VSMCs that exposed to PDGF-BB. Inhibition of Wip1 significantly suppressed VSMC proliferation and ameliorated vascular restenosis. Next, we demonstrated that mTORC1 signaling, characterized by downstream molecules including S6 and 4EBP1, was positively regulated by Wip1. We also revealed that excessive mTORC1 activation counteracted the vascular protective effects of Wip1 inhibition against wire injury-induced restenosis, thus identifying mTORC1 as a crucial mediator of Wip1. Finally, the aforementioned effects of Wip1 ablation could be abolished by AMPK dephosphorylation. The findings that Wip1 ablation suppresses neointima hyperplasia of common carotid arteries after wire injury *in vivo* and VSMC proliferation *in vitro* indicate that Wip1 is a potential target for the treatment of vascular restenosis.

Identified as a positive regulator of tumorigenesis initially [36], Wip1 is recently proved to be also involved in vascular diseases, such as atherosclerosis [16,17,37]. As an essential part of systemic and local inflammation, macrophage exert bi-directional roles in initiation and progression of atherosclerosis and vascular restenosis [38]. Previous research found that Wip1 deficiency induced macrophage chemotaxis *in vitro* [17]. However, this study did not further explore either which subset of macrophage altered or the chemotaxis could be replicated *in vivo*. Another study confirmed the antiatherogenic role of Wip1 deficiency in suppression of macrophage conversion into foam cells *in vivo* via regulating autophagy and cholesterol efflux, despite the fact that Wip1-deficient mice with enhanced immune response [16]. As vascular restenosis and atherosclerosis share similar pathological processes, such as the above-mentioned macrophage function, these studies remind us that Wip1 inhibition might protect against vascular stenosis.

Our research is aimed to confirm the regulatory role of Wip1 in neointima hyperplasia and mechanical damage-associated restenosis. Vascular restenosis is a complex and multilayered process involving endothelial dysfunction, VSMC proliferation, migration and extracellular matrix deposition as well as inflammatory responses [4]. In correspondence with the roles of Wip1 in proliferation of different cell lines, such as tumor cells [36], stem cells [15] and islet cells [14], we found a positive regulatory role of Wip1 in VSMC proliferation, which constituted the basis of cellular dynamics in the occurrence and development of vascular restenosis. The effects of Wip1 on migration of different cells are multivariate according to previous studies [17,39,40]. Interestingly, our current research showed that Wip1 did not display significant inhibition or promotion on PDGF-BB-induced VSMC migration. This might be partly elucidated by the fact that the critical pathways modulating migration, such as PI3K/AKT [17] were not affected by Wip1 in VSMC. On the basis of these findings, we speculated that Wip1 inhibition exerted a valid role in preventing neointima hyperplasia in the development of vascular restenosis possibly through suppressing VSMC proliferation.

Previous studies demonstrate that the regulatory role of Wip1 in stem cell function, lipid metabolism and atherosclerosis appears to be dependent on an mTOR signaling [15,16]. mTOR is an atypical serine/threonine kinase that functions through interacting with relative proteins and constituting two distinct complex, mTORC1 and mTORC2 [30]. mTORC1 modulates protein synthesis, cell proliferation, autophagy, mitochondrial biogenesis and metabolism through the phosphorylation of p70S6K and 4EBP1, whereas mTORC2 promotes cell survival and polarity via the phosphorylation of its downstream target AKT [30]. It is confirmed that mTORC1 activation leads to higher activity of VSMC proliferation [33], whereas mTORC1 inhibition suppresses neointima hyperplasia and restenosis [41,42]. Consistent with aforementioned researches, our current study showed that mTORC1 activity was positively regulated by Wip1. More importantly, the restoration of mTORC1 activity by using a genetic strategy almost blocked the repression of restenosis by Wip1 ablation. To further confirm that Wip1 inhibition ameliorates restenosis via repressing VSMC proliferation in an mTORC1-dependent manner, we utilized PDGF-BB to treat cultured VSMCs isolated from $Tsc1^{KD}$ mice. Data showed that TSC1 knockdown-induced mTORC1 activation significantly accelerated the proliferative activity in VSMC even under the condition of Wip1 inhibition. Though mTORC2 pathway is also proved to modulate restenosis, this may be achieved via its role in mediating endothelial cell migration instead of VSMC proliferation [33,43]. These observations revealed a close connection between mTORC1 signaling, VSMC proliferation and the development of vascular restenosis at the downstream of Wip1.

Responding to growth factors (such as PDGF-BB) and stress, the activity of mTORC1 signaling then alters via PI3K/AKT and AMPK pathway [30]. AMPK phosphorylation induced by 5-aminoimidazole-4-carboxamide ribonucleoside [44] and adiponectin [42] is confirmed to suppress PDGF-BB-induced VSMC proliferation and transformation of VSMC phenotype in a mTORC1-dependent manner. In the current study, AMPK phosphorylation displayed opposite trend to mTORC1 signaling in VSMC after treated by PDGF-BB. More importantly, the inhibitory effects of Wip1 inhibition on VSMC proliferation and mTORC1 activation were abolished by Compound C-induced AMPK dephosphorylation. This reminds us that AMPK may act as an intermediate between Wip1 and mTORC1, which is also supported by a recent research that Wip1 deficiency leads to increased AMPK phosphorylation as well as declined phosphorylation of mTORC1 downstream target S6 and 4EBP1 [16]. Several lines of evidence demonstrate that AMPK activation also results in the repression of mTORC1 signaling, thus ameliorating neointima hyperplasia after vascular injury [45,46]. Accordingly, our current study further showed that AMPK dephosphorylation led to accelerated vascular restenosis and mTORC1 activation, which could not be ameliorated by Wip1 inhibition. PI3K/AKT pathway is also activated by PDGF-BB and thus promotes VSMC proliferation and neointima formation [47,48]. This is

in consistent with enhanced AKT phosphorylation at site Thr308 in both PDGF-BB-treated VSMCs and damaged arteries in our current study. However, the alteration of AKT phosphorylation was not affected by Wip1 inhibition. Therefore, Wip1 activation of mTORC1 acts in a AMPK-dependent and AKT-independent manner to promote VSMC proliferation and vascular restenosis.

Taken together, the current study revealed a repressive role of Wip1 inhibition in VSMC proliferation, neointima hyperplasia and vascular restenosis through an AMPK/mTORC1-dependent signaling pathway. Drug-eluting stents, such as taxol has been increasingly applied to treat stenosis, but it also causes subsequent in-stent thrombosis and neoatherosclerosis [49]. Wip1 inhibition suppresses both restenosis and atherosclerosis, therefore, restoration of AMPK/mTORC1 signaling pathway is thus worth testing in preventing vascular restenosis via local delivery of Wip1 inhibitor. These data help us to better understand the role of Wip1 during the occurrence and development of restenosis and also provide a novel candidate target for restenosis prevention and treatment in the future.

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Conflicts of interest

There are no conflicts of interest.

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