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Inhibition of PIKfyve Ameliorates the Proliferation and Migration of Vascular Smooth Muscle Cells and Vascular Intima Hyperplasia By Reducing mTORC1 Activity

Min Gu, MD, Zhen Wang, MD, PhD, Feifei Feng, MD, Yongjian Yang, MD, PhD, Xiongshan Sun, MD, PhD, and Dachun Yang, MD, PhD

Abstract: This study was designed to investigate the role and mechanism of PIKfyve in the proliferation and migration of vascular smooth muscle cells (VSMCs) and vascular intima hyperplasia. We first observed increased protein levels of PIKfyve, phospho (p)-S6 Ribosomal Protein (S6)$^{Ser235/236}$, p-4EBP1$^{Thr37/46}$ in VSMCs after 24 hours of platelet-derived growth factor (PDGF)-BB treatment. By using cell counting kit-8 assay, Ki-67 immunofluorescence staining and wound healing assay, we found that PIKfyve inhibition ameliorated the enhanced activity of VSMC proliferation and migration induced by PDGF-BB. Silencing PIKfyve also suppressed the phosphorylation of S6 and 4EBP1 (2 major effectors of mammalian target of rapamycin complex 1), glucose consumption, activity of hexokinase, and LDH in PDGF-BB-challenged VSMCs. After rescuing the phosphorylation of S6 and 4EBP1 by silencing *Tsc1*, the suppressive effects of PIKfyve inhibition on glucose utilization, proliferation, and migration in VSMCs were abolished. The animal model of vascular restenosis was established in C57BL/6J mice by wire injury. We found the expression of PIKfyve was increased in carotid artery at day 28 after injury. Reducing the activity of PIKfyve alleviated vascular neointima hyperplasia after injury. In conclusion, targeting PIKfyve might be a novel effective method to reduce the proliferation and migration of VSMCs and vascular restenosis by affecting mammalian target of rapamycin complex 1–mediated glucose utilization.

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From the Department of Cardiology, The General Hospital of Western Theater Command, Chengdu, Sichuan, China.

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M. Gu and Z. Wang contributed equally to this article.

Correspondence: Dachun Yang, MD, PhD or Xiongshan Sun, MD, PhD, Department of Cardiology, The General Hospital of Western Theater Command, Chengdu, Sichuan 610083, China (e-mail: yangdc71@126.com or shan19910927@sina.com).

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INTRODUCTION

Cardiovascular diseases (CVDs) are major causes of death and disability in the world.[1] Percutaneous vascular intervention is an important therapy for atherosclerosis of coronary, carotid, and other peripheral arteries. However, vascular restenosis is a major complication of vascular intervention, which causes severe adverse cardiovascular events and largely limits the curative effect of interventions.[2] Responding to mechanical damage of vascular endothelium, activated platelet, inflammatory, and immune cells gather at damaged site and secrete several cytokines or growth factors, such as platelet-derived growth factor (PDGF) and interleukin-6. These growth factors or inflammatory mediators further trigger excessive migration and proliferation of vascular smooth muscle cells (VSMCs), leading to neointima hyperplasia and restenosis.[3] However, the underlying molecular mechanisms of vascular neointima hyperplasia are still not completely understood. Therefore, revealing the potential mechanisms of vascular restenosis is of great significance to develop novel therapeutic targets.

Energy metabolism plays a crucial role in vascular physiological and pathological processes. Several types of cells from blood vessels will undergo a transition of lipid-to-glucose utilization under pathological conditions, including endothelial cells, VSMCs, and fibroblasts.[4–6] Recent studies have also found that the phenotype switching of VSMCs is also driven by this metabolic transition, which is characterized by overactivation of glycolysis and declined lipid metabolism.[7] For example, polypyrimidine tract-binding protein 1 acts upstream of pyruvate kinase muscle to promote glycolysis in both endothelial cells and vascular adventitial fibroblasts.[8,9] Our previous study suggest that the increased polypyrimidine tract-binding protein 1 level is also associated with enhanced proliferation of VSMC,[10] which further proves the crucial role of glucose metabolism in the phenotype switching of VSMCs. However, the precise mechanism that mediating the glucose metabolism during the phenotype transition of VSMC remains largely unknown.

Phosphatidylinositol 3-phosphate 5-kinase type III (PIKfyve) is an evolutionarily conserved lipid kinase, which synthesizes PtdIns5P and PtdIns (3, 5)P_2 to exert its

biological role.[11] As a phosphoinositide (3) phosphate (PI3P)–binding protein, PIKfyve plays a crucial role in normal endosome/lysosome formation transition and is thus important for autophagy regulation.[12,13] PIKfyve is also a regulatory factor of inflammation by affecting macrophage and neutrophil homeostasis.[14–16] In addition, PIKfyve also participates in glucose metabolism, such as glucose uptake and glucose intolerance.[17,18] Inhibition of PIKfyve can also suppress the proliferation and migration of cancer cells, lymphoma cells, fibroblasts, etc.[19–21] PIKfyve is expressed in mammalian muscle, blood vessel, heart, lung, intestine, and other tissues.[16] Recent studies have shown that PIKfyve also plays an important role in CVDs such as vascular calcification and cardiac hypertrophy.[22,23] However, the regulatory role of PIKfyve in VSMC proliferation and migration and vascular intima hyperplasia is unclear.

In this study, we describe a regulatory role for PIKfyve in vascular intima hyperplasia in a mammalian target of rapamycin complex 1 (mTORC1)–dependent manner. In vitro, inhibition of PIKfyve suppressed the glycolysis, proliferation, and migration of VSMC induced by PDGF-BB by reducing mTORC1 activity. Reversing mTORC1 activity abolished the suppressive effects of PIKfyve inhibition on VSMC proliferation, migration, and glucose metabolism. In addition, the PIKfyve inhibitor YM201636 suppressed neointima hyperplasia after vascular injury in vivo. Our study suggests that PIKfyve may become a novel potential therapeutic target for vascular restenosis.

METHODS

Animals

C57BL/6J mice were purchased from the Dashuo Animal Science and Technology (Chengdu, Sichuan, China). Mice of 8–10 weeks old were housed as following described: 22–25°C, 12-hour light/dark cycle, periodic air changes, and free access to food and water. 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). The injury of common carotid artery was performed as described in our previous research.[24] In brief, mice were first anesthetized with pentobarbital (40 mg/kg, intraperitoneal) and received a small midline incision in the neck area. Then, the left common carotid artery was isolated. The internal carotid artery and proximal common carotid artery were temporarily occluded with clamps. The external carotid artery was ligated. Then, a guidewire was inserted into the common carotid artery and passed 3 times with rotation backward and forward to denude the endothelium. Finally, the vascular clamps were removed, and the incision of neck was finally closed. Mice of sham group only received a small midline incision in the neck area, which was then closed without any treatment on artery. YM201636 (2 mg/kg) application was begun at the day of operation by oral administration for 14 consecutive days. Finally, mice were deeply anesthetized with pentobarbital (100 mg/kg, intraperitoneal), followed by decapitation, and then, carotid arteries were removed from the sacrificed mice for further experiments.

Isolation, Culture, and Treatment of VSMCs

VSMCs were obtained from thoracic aortas of 8- to 10-week-old mice after isolation and digestion with 0.25% trypsin (HyClone, Carlsbad, CA). The VSMCs were cultured in complete medium [Dulbecco's modified Eagle's medium (DMEM; HyClone) containing fetal bovine serum (10%; Invitrogen, Carlsbad, CA), penicillin (100 units/mL) and streptomycin (100 mg/mL)] in humidified 5% CO_2 atmosphere. VSMCs were obtained through centrifugation and then cultured with complete medium at 37°C with 5% CO_2 for further use. Recombined human platelet-derived growth factor-BB (PDGF-BB) (30 ng/mL; R&D Systems, Minneapolis, MN) was used for the treatment of cultured VSMCs.

Transfection of siRNA

We conducted siRNA transfection with Lipofectamine 2000 according to the manufacturer's instructions. In brief, VSMCs were transfected with target-specific *Pikfyve* or *Tsc1* siRNA (50 nmol/L; Ruibo, Guangdong, China), and scramble siRNA was used as a nonspecific control. *Pikfyve* siRNA (siPikfyve) sequence: 5′-GUUGUCAAUGGCUUUGUUU-3'[6]. *Tsc1* siRNA (siTsc1) sequence: 5′-GCUUUGACUCUCCCUUCUA-3'.

CCK-8 Assay

VSMC proliferation was analyzed by using a cell counting kit (CCK) (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 DMSO or PDGF-BB (30 ng/mL) for 24 hours. Ten microliters of CCK-8 agent was then added to each well and incubated with VSMCs at 37°C for another 2 hours. By using a microplate reader, VSMC proliferation was finally determined by calculating the relative absorbance at 450 nm.

Immunofluorescent Staining

Immunofluorescent (IF) staining was performed as previously described.[24] Cell plates were washed by PBS for 3 times, fixed with 4% paraformaldehyde for 30 minutes, and then blocked in 1% blocking solution. Plates were then incubated in dark with primary antibody against Ki-67 (cat 9129S, 1: 200; Cell Signaling Technology, Danvers, MA) at 4°C overnight. VSMC plates were then washed and subsequently stained with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (cat 4412S, 1: 2500; Cell Signaling Technology) in dark for 1 hour. Next, VSMC plates were stained with DAPI (cat MX4208, 5 mg/mL; VECTOR Labs, Burlingame, CA) for 5 minutes in room temperature. For PIKfyve staining, the artery sections were incubated with anti-PIKfyve antibody (cat sc-100408, 1: 50) and then followed by a secondary antibody (1:300; Invitrogen). Nuclei were stained with DAPI. Images were obtained by using an IF microscopy (Leica MPS 60; Wetzlar, HD, Germany). The fluorescence intensity was measured by using Image J software (Bethesda, MA).

Wound Healing Assay

VSMCs were cultured in 6-well plates (1×10^5 cells per well) and serum deprived for 12 hours. A scratch was conducted by using a 10-μL sterile pipette in the middle area of cells. VSMCs were then treated with DMSO or PDGF-BB (30 ng/mL) for 24 hours. Images were taken at regular intervals over a 24-hour period. The rates of wound closure were directly evaluated by using microscopic visualization followed with a reference point at the bottom of the wound field, thus permitting photographing of the same spot each time.

Quantitative RT-PCR

Total RNA of carotid arteries or VSMCs were extracted by using Trizol agent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The cDNA of 1 μg total RNA was synthesized with DBI qPCR kit (DBI Bioscience, Ludwigshafen, Germany). Real-time qRT-PCR was conducted in ABI Prism 7700 Sequence Detector (Applied Biosystems, Carlsbad, CA) by using 2 × SyberGreen mixture (DBI Bioscience). The relative expression of target gene mRNA was normalized to *Gapdh*. Primers used for each gene were listed as follows: *Pikfyve*: F: 5′-ATGGCCACAGATGACAAGAGTTCC-3'; R: 5′-CAGACTGTGTTCTTGAAGGG-3'; *α-SMA*: F: 5′-GTCCCAGACATCAGGGAGTAA-3'; R: 5′-TCGGATACTT CAGCGTCAGGA-3'; *Calponin*: F: 5′-TCTGCACATTTTAAC CGAGGTC-3'; R: 5′- GCCAGCTTGTTCTTTACTTCAGC-3'; *SM-MHC*: F: 5′- AAGCTGCGGCTAGAGGTCA-3'; R: 5′-CCCTCCCTTTGATGGCTGAG-3'[10]; *Gapdh*: F: 5′-AATGG ATTTGGACGCATTGGT-3'; and R: 5′- TTTGCACTGGTA CGTGTTGAT- 3'.

Western Blot Analysis

Western blot analysis was performed as previously described.[24] Extractions of carotid arteries or VSMCs were lysed with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). Antibodies against phospho (p)-S6 Ribosomal Protein $(S6)^{Ser235/236}$ (cat 4858S, 1: 1000), total S6 (cat 2217S, 1: 2000), p-4E–binding protein 1 $(4EBP1)^{Thr37/46}$ (cat 2855S, 1: 1000), total 4EBP1 (cat 9644S, 1: 2000), and GAPDH (cat 2118S, 1: 8000) were purchased from Cell Signaling Technology. Antibody against PIKfyve (cat sc-100408, 1: 500) was purchased from Santa Cruz (CA). The quantitative analysis was conducted by using Image J software (Bethesda, MA).

Glucose Consumption

Glucose consumption of VSMCs was assessed by calculating the difference between the glucose concentration in the collected medium before and after 24 hours of treatment. The glucose concentration was obtained by using a glucose kit (Solarbio) by calculating the relative absorbance at 505 nm.

HK Activity Assay

The hexokinase (HK) activity of VSMCs was measured by using a HK assay kit according to the manufacturer's protocol (Solarbio). VSMCs were broken by ultrasonic (power 200 W) in ice bath for 3 seconds. The process of homogenate was then repeated for 30 times with an interval of 10 seconds. After centrifuged at 8000*g* for 10 minutes at 4°C, the HK activity of cell supernatant was analyzed by calculating the relative absorbance at 490 nm.

LDH Activity Assay

Lactate dehydrogenase (LDH) activity was measured by using a LDH assay kit according to the manufacturer's protocol (Solarbio). Cells were lysed by an ultrasonic homogenizer and then centrifuged at 8000*g* at 4°C for 10 minutes. The LDH activity of cell supernatant was analyzed by calculating the relative absorbance at 490 nm. The OD values of the experimental group were normalized to those of the control group.

Immunohistochemical Staining

Histological changes of carotid arteries were analyzed by using standard hematoxylin and eosin (HE) and Ki-67 staining as previously described.[24] The artery sections (4 μm) were stained with hematoxylin and eosin and then analyzed by using Image-Pro Plus software (Bethesda, MD). For Ki-67 staining, artery sections were blocked and then stained with primary antibody against Ki-67 (cat bsm-33070M, 1: 250; Bioss, Beijing, China) at 4°C overnight. Sections were then incubated with a secondary antibody and subsequently counterstained with Mayer hematoxylin.

Statistical Analysis

Data are presented as mean ± SD. Unpaired Student's *t* test was applied to compare 2 independent groups if they are normally distributed. Two-way or multivariate analysis of variance with an appropriate *post hoc* test was preformed to compare means that involve 2 or more factors. SPSS 20.0 was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

RESULTS

Silencing PIKfyve Inhibits the Proliferation and Migration of VSMC Induced by PDGF-BB

PDGF-BB is a classical stimulator to induce the proliferation and migration of VSMC.[25] We first monitored the expression level of PIKfyve after PDGF-BB treatment. The mRNA and the protein level of PIKfyve was both increased in VSMCs exposed to PDGF-BB (Figs. 1A, B), suggesting that PIKfyve might play a role in the phenotypic regulation of VSMC. Then, we used siRNA of *Pikfyve* (siPikfyve) to silence PIKfyve expression and found siPikfyve significantly reduced protein expression of PIKfyve **Supplemental Digital Content** (see **Figure S1**, http://links.lww.com/JCVP/A779). CCK-8 assay showed that silencing PIKfyve restored the increased cell viability induced by PDGF-BB (Fig. 1C). This indicates that PIKfyve might either promote proliferation or reduce apoptosis in VSMCs. The result of Ki-67 immunofluorescence staining further supported this finding. The ratio of Ki-67–positive VSMCs was increased after 24 hours of

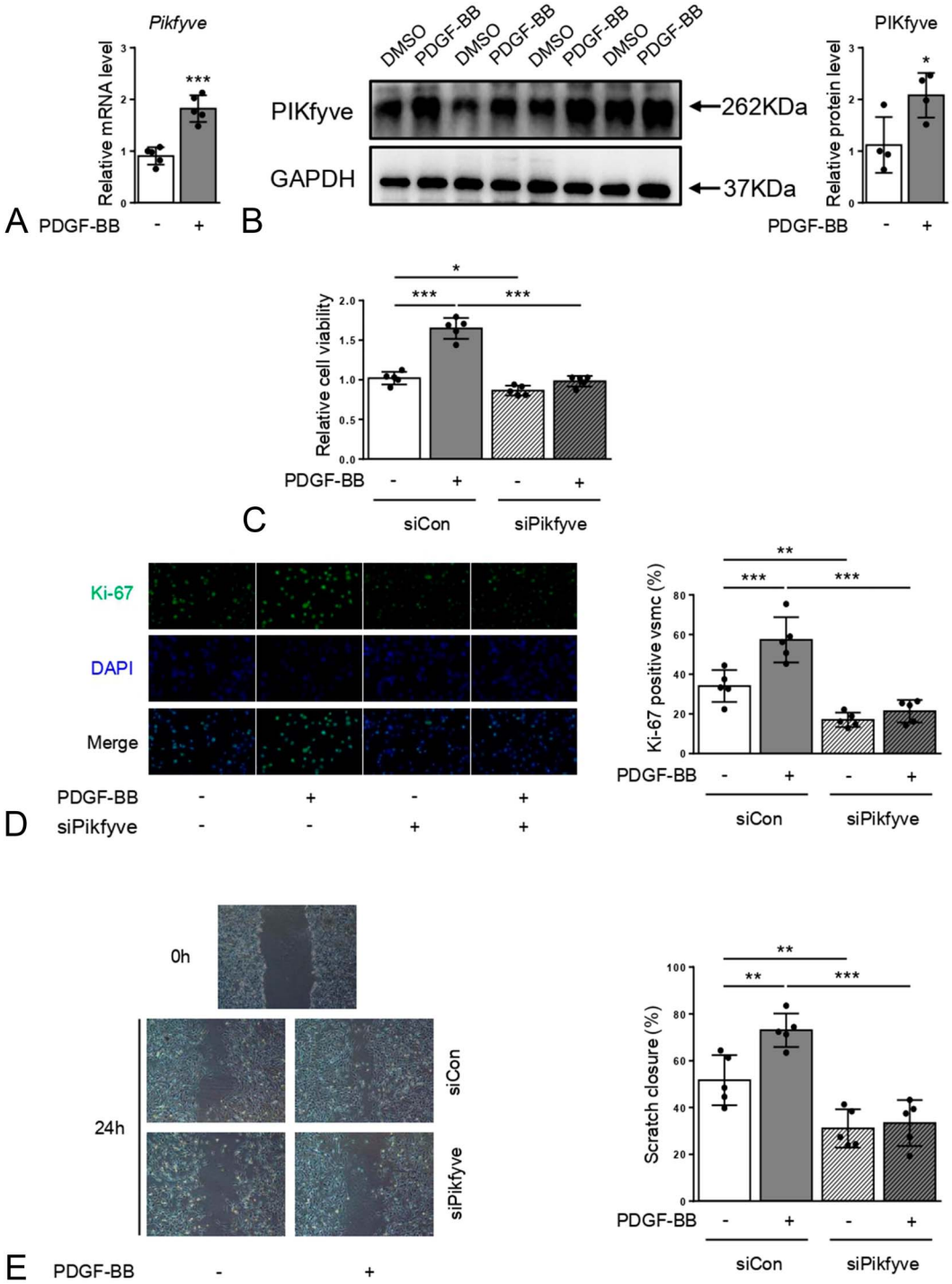

FIGURE 1. PIKfyve inhibition suppresses the proliferation and migration of VSMCs induced by PDGF-BB. A, The relative mRNA level of *Pikfyve* in VSMCs exposed to PDGF-BB for 24 hours was determined by qRT-PCR (n = 5). B, The protein expression level of PIKfyve in VSMCs after treatment of PDGF-BB for 24 hours was analyzed by immunoblotting (n = 4). C, VSMC proliferation was measured by using CCK-8 assay. VSMCs were transfected with siCon or siPikfyve and then incubated with or without PDGF-BB for 24 hours. The absorbance at 450 nm was obtained (n = 5). D, VSMCs treated as abovementioned were stained with 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, Cell migration was analyzed by using wound healing assay. Representative images (left) and corresponding quantification of healing rates (right) were shown (n = 5) (magnification 100×). Data are shown as mean ± SD. Post hoc: LSD test for C, D, and E. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ denote statistical comparison between the 2 marked groups, respectively.

PDGF-BB treatment. Inhibition of PIKfyve significantly suppressed the Ki-67–positive area with or without PDGF-BB treatment, indicating the proliferation of VSMC was attenuated after silencing PIKfyve. In addition, there was no significant difference in the ratio of Ki-67–positive VSMCs between DMSO + siPikfyve and PDGF-BB + siPikfyve groups (Fig. 1D). We also explored the role of PIKfyve in VSMC migration challenged by PDGF-BB. As the wound healing assay showed, 24 hours of PDGF-BB exposure accelerated the wound healing rate of VSMCs, which was restored by silencing PIKfyve (Fig. 1E). We also investigated the role of PIKfyve in PDGF-BB–induced VSMC phenotypic switch by determining the expression levels of VSMC differentiation marker genes such as *α-SMA*, *calponin*, and *SM-MHC*. Transfection of siPikfyve abolished PDGF-BB–induced decrease in the mRNA levels of *α-SMA*, *Calponin*, and *SM-MHC* **Supplemental Digital Content** (see **Figure S2**, http://links.lww.com/JCVP/A780). These results suggest that silencing PIKfyve can suppress the proliferation, migration, and phenotypic switch of VSMCs.

Inhibition of PIKfyve Suppresses PDGF-BB–Induced Glucose Utilization in VSMCs

VSMC proliferation consumes glucose for metabolic purposes.[26] Glycolytic flux is critical in the phenotypic shift of VSMC that occurs during neointima hyperplasia.[27,28] HK is the first rate-limiting enzyme of aerobic glycolysis that catalyzes the phosphorylation of glucose to glucose 6-phosphate while LDH catalyzes lactate dehydrogenation to pyruvate.[29] Therefore, we tested whether PIKfyve affected the glucose metabolism in VSMCs. As the results showed, we found that PDGF-BB caused enhanced activity of HK and LDH and increased glucose consumption in VSMCs (Figs. 2A–C). Inhibition of PIKfyve in VSMCs by siRNA significantly suppressed glucose consumption and the activity of HK and LDH with or without PDGF-BB incubation. Simultaneously, PDGF-BB failed to further affected glucose metabolism after silencing PIKfyve in VSMCs (Figs. 2A–C). These results indicate that PIKfyve can promote glucose utilization in VSMCs.

Silencing PIKfyve Inhibits Glycolysis By Reducing mTORC1 Activity in VSMCs

mTORC1 is a crucial protein complex for energy metabolism and plays an important role in proliferation and migration of cells.[30] To investigate whether mTORC1 plays a role in PIKfyve-mediated proliferation and migration of VSMCs, we first investigated the effect of PIKfyve on mTORC1 activity. S6 (site Ser235/236) and 4EBP1 (site Thr37/46) are 2 main effectors reflecting mTORC1 activity.[31] As our data showed, PDGF-BB exposure led to increased expression of p-S6$^{Ser235/236}$ and p-4EBP1$^{Thr37/46}$ in VSMCs. However, the enhanced phosphorylation of S6 and 4EBP1 induced by PDGF-BB was rescued by the inhibition of PIKfyve. It is noteworthy that silencing PIKfyve still inhibited the activity of mTORC1 in VSMCs even in the absence of PDGF-BB (Fig. 2D).

Glucose metabolism in vascular diseases, such as atherosclerosis and vascular aging, is closely related to the alteration of mTORC1 activity.[32,33] To determine the regulatory effect of mTORC1 on PIKfyve-mediated glucose metabolism in VSMCs, we used the siRNA of *Tsc1* (a crucial factor acts upstream of mTORC1)[24] to restore mTORC1 activity and then analyzed the indexes of glucose metabolism. All the results demonstrated that rescuing mTORC1 activity abolished the inhibitory effects of silencing PIKfyve on glucose consumption and activity of HK and LDH in PDGF-BB–challenged VSMCs (Figs. 3A–C). Therefore, mTORC1 is essential for the regulation of PIKfyve on glucose utilization in VSMCs.

PIKfyve Inhibition Suppresses the Proliferation and Migration of VSMCs in a mTORC1-Dependent Manner

Activation of mTORC1 is implicated in VSMC proliferation both in vivo and in vitro.[24] Because mTORC1 is a major signal that mediates PIKfyve-induced glycolysis in proliferated VSMCs, we next confirmed whether restoration of mTORC1 activity by siTsc1 also abolished the inhibitory effect of silencing PIKfyve on PDGF-BB–induced phenotypic switch of VSMCs. As shown in our results, knockdown of TSC1 by siRNA significantly enhanced phosphorylation of S6 and 4EBP1 in VSMC **Supplemental Digital Content** (see **Figure S3**, http://links.lww.com/JCVP/A781). mTORC1 activation by transfection of siTsc1 markedly increased PDGF-BB–induced proliferation and migration of VSMCs (Figs. 3D, E). Together, these results suggest that mTORC1 is a critical downstream factor of PIKfyve for modulating glucose metabolism, proliferation, and migration in VSMCs.

Inhibition of PIKfyve Activity Ameliorates Neointima Hyperplasia After Vascular Endothelial Injury

To explore whether PIKfyve affects the proliferation and migration of VSMCs in vivo, we assessed the effect of PIKfyve in an established model of neointima hyperplasia induced by vascular wire injury.[34] To analyze the role of PIKfyve in vascular intima hyperplasia, qPCR and Western blot were used to detect the content of PIKfyve in carotid artery. After 28 days of injury, we observed increased mRNA and protein level of PIKfyve in vessels (Figs. 4A, B). We found that PIKfyve was also increased within neointima after injury by using IF staining **Supplemental Digital Content** (see **Figure S4**, http://links.lww.com/JCVP/A782), indicating PIKfyve might also plays a role in vascular intima hyperplasia. An antagonist of PIKfyve, named YM201636, was then applied for 14 days as soon as the injury operation was completed. As our data showed, obvious lumen narrowing and the elevated intima/media ratio were observed in arteries harvested at 28 days after injury, which were significantly ameliorated by YM201636 (Fig. 4C). Analysis of Ki-67–stained arteries also showed that inhibition of PIKfyve activity reduced the Ki-67–positive area within neointima of the injured carotid artery (Fig. 4D). These data indicate that PIKfyve inactivation protects from the formation of neointima lesion by inhibiting the proliferation and migration of VSMCs.

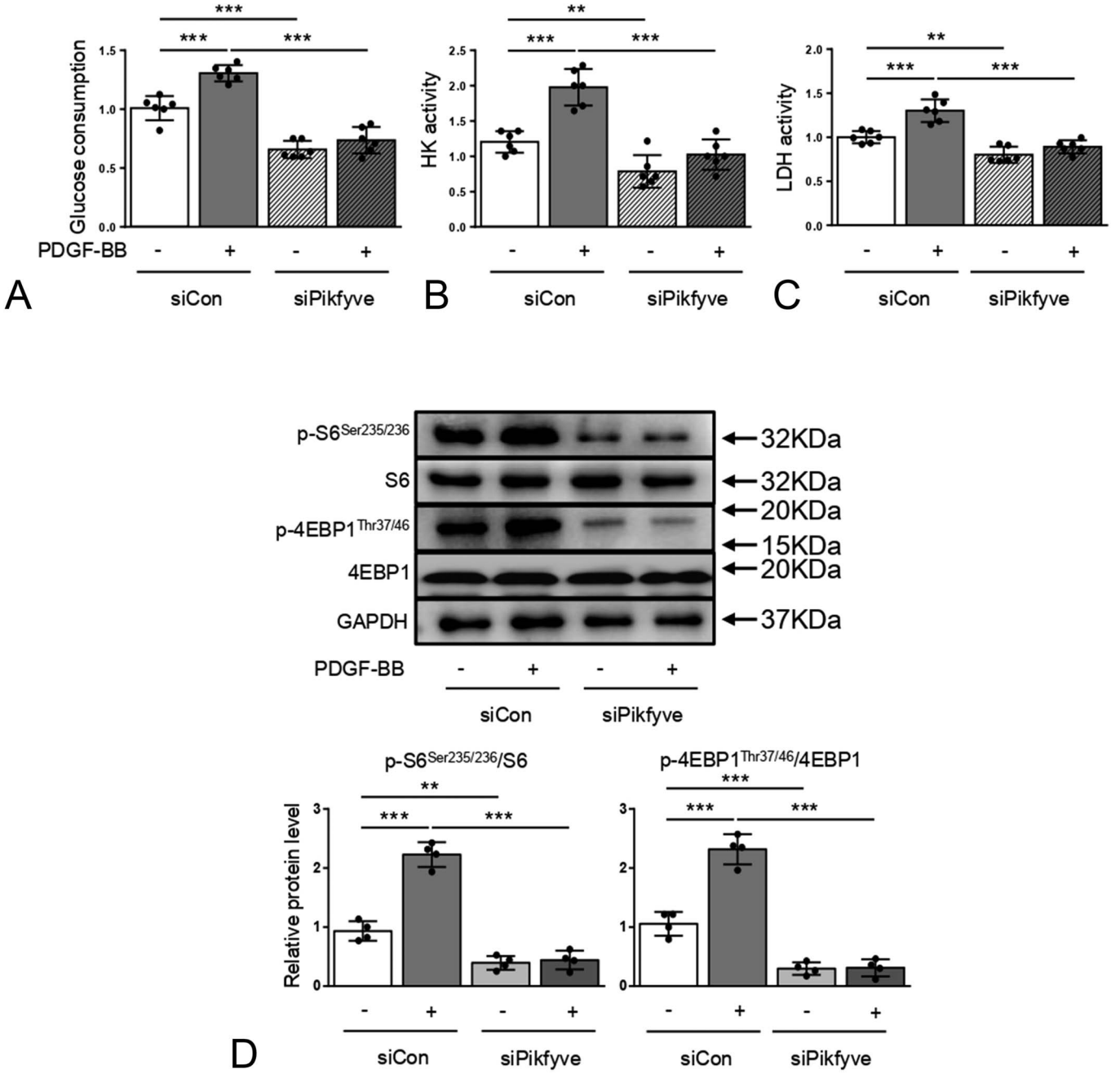

FIGURE 2. Silencing PIKfyve inhibits glucose utilization and mTORC1 activity. VSMCs were transfected with siCon or siPikfyve and then incubated with or without PDGF-BB for 24 hours. Glucose consumption (A), activity of HK (B), and LDH (C) in VSMCs treated as abovementioned were analyzed (n = 6). D, The protein expression of p-S6$^{Ser235/236}$, S6, p-4EBP1$^{Thr37/46}$, 4EBP1, and GAPDH were evaluated by immunoblotting in VSMCs. Representative bands (upper panel) and corresponding quantification (lower panel) were shown (n = 4). Data are shown as mean ± SD. Post hoc: LSD test for A, B, C, and D. $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the 2 marked groups, respectively.

DISCUSSION

Vascular percutaneous interventions are critical therapeutic strategies for atherosclerosis-associated diseases, including coronary heart disease and carotid artery stenosis. However, vascular restenosis largely limits its clinical efficacy and prognosis. The major pathological changes associated with vascular restenosis are neointima hyperplasia resulting from proliferation and migration of VSMCs.[35] Despite drug-eluting stents have got great progress, the occurrence of vascular restenosis and associated underlying mechanisms are still unsolved.[36] In this study, we confirmed that PIKfyve was a novel regulator in VSMC proliferation, migration, and

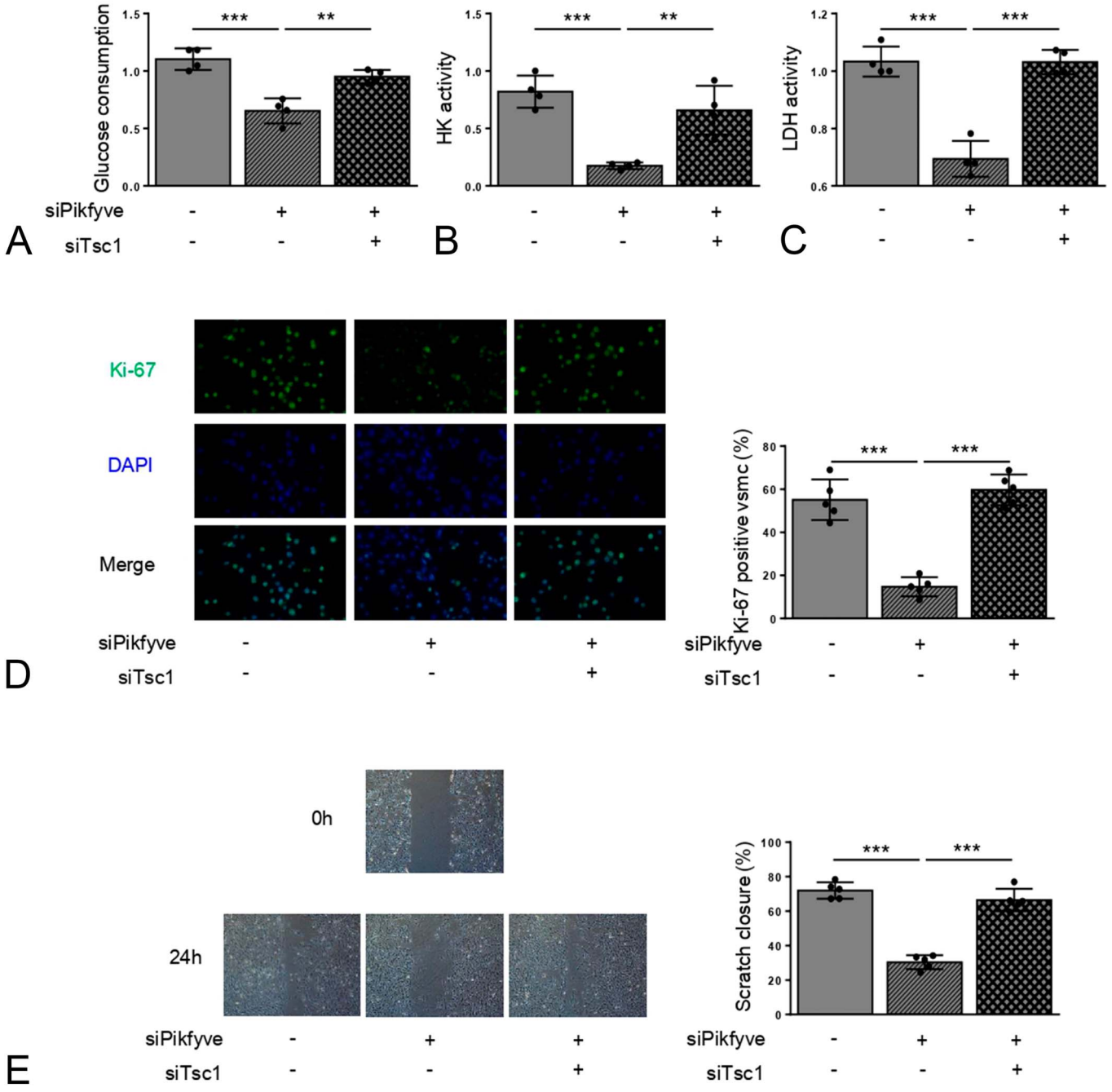

FIGURE 3. PIKfyve inhibition suppresses glucose utilization, proliferation, and migration in PDGF-BB–challenged VSMCs by reducing mTORC1 activity. VSMCs were transfected with siCon, siPikfyve, or siTsc1 and then incubated with or without PDGF-BB for 24 hours. Glucose consumption (A), activity of HK (B), and LDH (C) in VSMCs treated as abovementioned were analyzed (n = 4). D, VSMCs treated as above mentioned were stained with 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, Cell migration was analyzed by using wound healing assay. Representative images (left) and corresponding quantification of healing rates (right) were shown (n = 5) (magnification 100×). Data are shown as mean ± SD. Post hoc: LSD test for A, B, C, D, and E. $^{**}P < 0.01$ and $^{***}P < 0.001$ denote statistical comparison between the 2 marked groups, respectively.

neointima hyperplasia. PIKfyve was elevated in PDGF-BB–challenged VSMCs in vitro or injured arteries in vivo. Silencing PIKfyve inhibited the proliferation and migration of VSMCs through suppression of mTORC1 activity and subsequent glycolytic flux. Finally, we demonstrated that inhibition of PIKfyve protected against vascular intima hyperplasia.

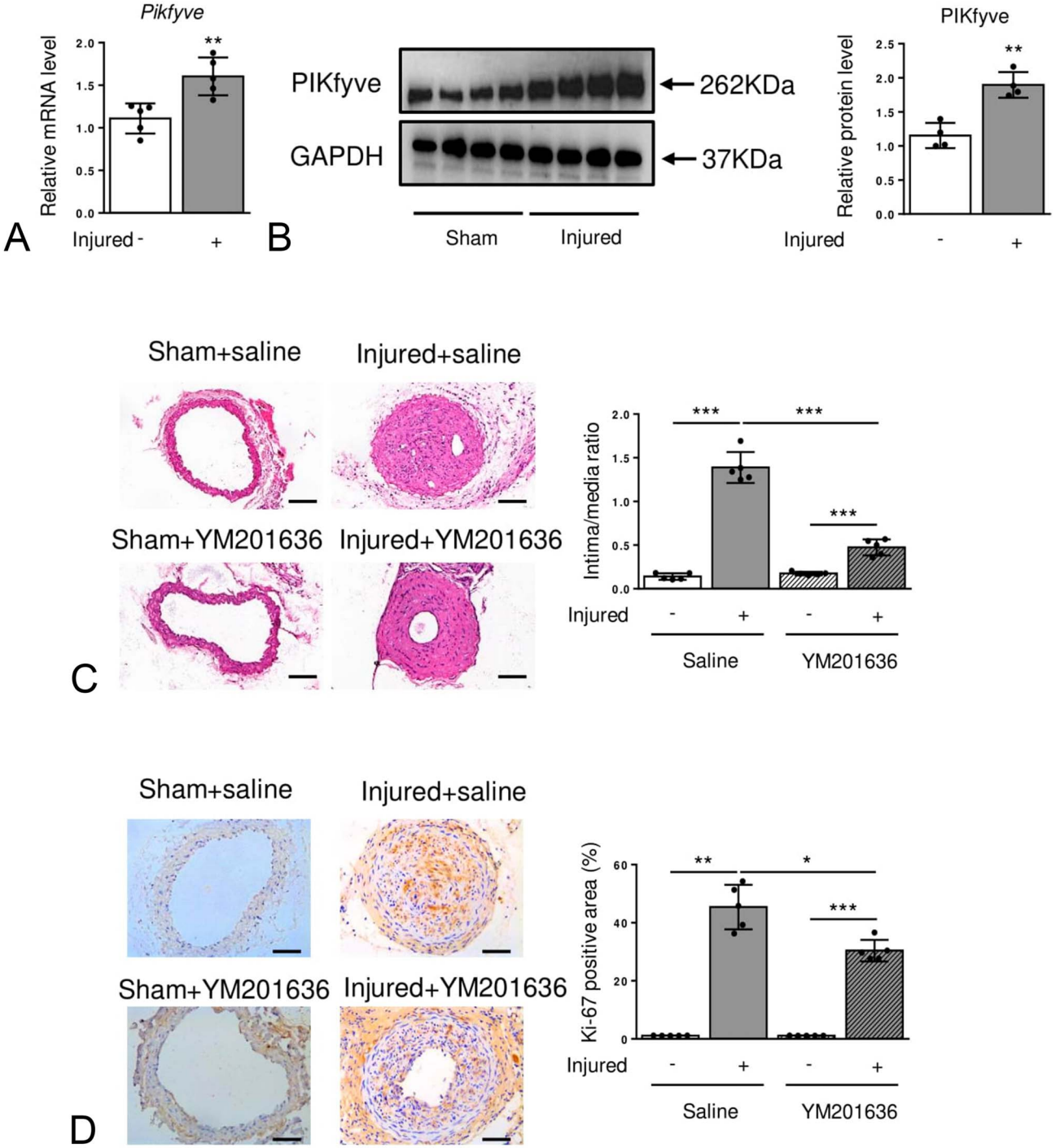

FIGURE 4. Inactivation of PIKfyve ameliorates neointima hyperplasia after vascular endothelial injury. A, The relative mRNA level of *Pikfyve* in common carotid arteries from C57BL/6J mice at day 28 after sham operation or wire injury was determined by qRT-PCR (n = 5). B, The relative protein level of PIKfyve 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 with or without YM201636 (2 mg/kg) treatment at day 28 after sham operation or wire injury (left) and corresponding quantification for the ratio of intima/media (right) were shown (n = 5). Scale bar, 50 μm (magnification 100×). D, Immunohistochemistry staining of Ki-67 (brown) in sections of carotid arteries from C57BL/6J mice with or without YM201636 treatment at day 28 after sham operation or wire injury (left) and corresponding quantification for Ki-67–positive cells within neointima (right) were shown (n = 5). Scale bar, 50 μm (magnification 100×). Data are shown as mean ± SD. Post hoc: LSD test for C and Dunnett T3 test for D. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ denote statistical comparison between the 2 marked groups, respectively.

As a conserved lipid kinase that synthesizes PI5P and PI (3,5) P2, PIKfyve is implicated in pleiotropic cellular processes, such as membrane trafficking, remodeling the actin cytoskeleton, and tyrosine kinase receptor signaling.[37] PIKfyve is also considered as a regulator for proliferation and migration in a series of cell types. For example, inhibition of PIKfyve by using YM201636 suppresses the growth of liver cancer by inhibiting liver cancer cell proliferation.[21] Another study has confirmed that PIKfyve activity is essential for the proliferation of epithelial cells.[19] In addition, PIKfyve is also implicated in cancer cell migration and invasion.[19] Whether PIKfyve plays a regulatory role in the proliferation and migration of VSMCs is not clear. In this study, we observed increased expression of PIKfyve in VSMCs after PDGF-BB treatment. Silencing PIKfyve significantly inhibited the proliferation and migration of VSMCs induced by PDGF-BB. This suggests that the upregulation of PIKfyve expression can promote the proliferation and migration of VSMCs. However, the underlying mechanism is still unclear.

It is becoming increasingly clear that VSMCs can change the metabolic pattern to meet the bioenergetic demand under pathological conditions. The phenotypic transition of VSMCs is confirmed to be driven by a metabolic switch summarized as enhanced glycolysis and declined fatty acid oxidation during several vascular diseases, such as hypertension, atherosclerosis, pulmonary hypertension, vascular calcification, and aneurysms.[7] In this study, we also verified this metabolic change by increased glucose consumption and enhanced activity of glycolytic enzymes in PDGF-BB–challenged VSMCs. Previous study has demonstrated that PIKfyve and its downstream signaling cascade are essential for glucose homeostasis in adipocytes and participate in glucose uptake in skeletal muscle.[17,18] Whether PIKfyve is involved in PDGF-BB–induced metabolic switch in VSMCs remains unknown. Interestingly, the enhanced glycolysis induced by PDGF-BB was totally terminated after inhibition of PIKfyve in this study. Therefore, PIKfyve ablation leads to declined glucose utilization, which might be an unrevealed mechanism for preventing the pathological phenotypic transition in VSMCs.

PIKfyve exerts its normal function partly by regulating the activity of mTORC1.[16,38] mTOR is an atypical serine/threonine kinase that exerts biological effects by forming 2 complex, mTORC1 and mTORC2.[31] mTORC1 plays a vital role in cell growth, proliferation, migration, autophagy, and mitochondrial biogenesis by its downstream effectors, S6 and 4EBP1.[39] It is well established that mTORC1 also promotes glucose consumption and oxidation in various tissues, including cardiovascular systems.[40–42] In this study, Western blotting analysis demonstrated that PIKfyve deficiency significantly decreased the activity of mTORC1. Thus, we hypothesized that PIKfyve directly affects mTORC1 activity to induce glucose utilization. To validate this hypothesis, we silenced the expression of *Tsc1*, a key upstream negative regulator of mTORC1, to restore the activity of mTORC1.[43] We also demonstrated that knockdown of TSC1 could rescue mTORC1 activity of VSMCs in our previous study.[24] Further study revealed that rescuing mTORC1 activity promoted glycolysis even in PIKfyve-deficient VSMCs, indicating that mTORC1 is essential for PIKfyve-mediated modulation of glycolysis. It is confirmed that TSC1 knockdown-induced mTORC1 activation causes excessive proliferation of VSMCs.[24,30] However, little is known about the role of mTORC1 in PIKfyve-mediated regulation of VSMC proliferation and migration. This study showed that TSC1 ablation–induced mTORC1 activation abolished the suppressive effect of silencing PIKfyve on PDGF-BB–induced phenotypic switch in VSMCs. All these data suggest that mTORC1 acts downstream of PIKfyve to promote glycolysis, proliferation, and migration of VSMCs.

Several lines of evidence suggest that PIKfyve is also implicated in CVDs. Inhibition of PIKfyve is confirmed to prevent heart against hypertrophy and fibrotic remodeling.[22,44] miR32-5p is positively correlated with VSMC calcification by targeting PIKfyve, thus leading to arterial stiffness.[23] In addition, PIKfyve deficiency impairs lysosomal homeostasis in macrophage and causes systemic inflammation,[16] which is also a core part during the development of vascular restenosis.[45] Because the vascular expression of PIKfyve was also increased after vascular injury in this study, we further speculated that PIKfyve might exert certain role in vascular restenosis. HE assay demonstrated that PIKfyve inactivation by YM201636 ameliorated neointima hyperplasia and vascular lumen stenosis after vascular injury. In accordance with HE staining, Ki-67 immunohistochemistry assay also showed that PIKfyve inhibition reduced the number of proliferating cells within neointima. All these data indicate that PIKfyve inhibition attenuates neointima hyperplasia and inhibits cell proliferation within neointima after injury.

CONCLUSIONS

In summary, we revealed that the increased protein level of PIKfyve enhanced glycolysis, proliferation, and migration of VSMCs and aggravated neointima hyperplasia after vascular injury. Inhibition of PIKfyve prevented against phenotypic transition of VSMCs by reducing mTORC1-mediated glucose utilization, which indicates that PIKfyve may be a novel target for the prevention and treatment of vascular restenosis.

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