Supplemental Material

Title： PI3Kγ regulates vascular smooth muscle cell phenotypic modulation and neointimal formation through CREB/YAP signaling

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Running title: PI3Kγ regulates VSMC phenotypic modulation.

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Supplemental Materials and Methods

Cell culture and treatment

Primary VSMCs were prepared from the thoracic aorta of mice using an explant method as previously described.[1] VSMCs were cultured in DMEM/F12 containing 10% FBS, 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2. All experiments were performed on cells at passage 3 to 6. Before reagents treatment, VSMCs were starved for 48 hours to achieve quiescence. For intervention experiments, PI3Kγ specific inhibitor AS605240 (1 μM) was reported to selectively inhibited PI3Kγ enzymatic activity,[2] thus, quiescent cells were pretreated with AS605240 (1 μM) for 60 minutes prior to 10% FBS stimulation.

For PI3Kγ, CREB and YAP knockdown, recombinant lentiviruses respectively expressing the short hairpin RNA (shRNA) targeting Pik3cg (PI3K p110γ), CREB and YAP were prepared and used to infect VSMCs as previously described.[3] A scrambled shRNA served as a negative control (NS-KD), according to the manufacturer`s protocol at a multiplicity of infection (MOI) of 100 in medium containing polybrene (5 mg/ml). Cell experiments were performed on quiescent cells 5 days later after infection.

For YAP and CREB ectopic expression, plasmid pcDNA3.1-YAP containing mouse YAP gene (pcYAP) and plasmid pcDNA3.1-CREB containing mouse CREB gene (pcCREB) or its control plasmid pcDNA3.1 was transfected into cultured VSMCs using transfection reagent Lipofectamine 3000 (Invitrogen) according to the manufacturer`s protocol. Total RNA and protein were extracted 48h after transfection.

Western blotting

Whole cell lysates were prepared with RIPA buffer containing protease inhibitors and quantified using the Bardford Protein Assay (Bio-Rad). Equal amounts of denatured cell lysates were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membrane. After blocking, proteins were detected with the corresponding primary antibodies and subsequently with appropriate HRP-conjugated secondary antibodies. Immunoreactivity was visualized with enhanced chemiluminescence detection reagents and recorded using ChemiDoc imaging system (Bio-Rad). Protein expression levels were normalized to β-actin.

Quantitative reverse transcription–polymerase chain reaction（qRT-PCR）

Quantitative RT-PCR was performed as previously described.[1] Total RNA was isolated from VSMCs using TRIzol Reagent and reverse-transcribed into cDNA using PrimeScript RT Master Mix. Subsequently, quantitative PCR was performed using the SYBR Premix Ex Taq kit in an iCycler Real-Time PCR Detection System (Bio-Rad). mRNA expression were analyzed by normalizing to GAPDH, and the value was calculated by the 2-ΔΔCt method. The primer sequences were used as follows: SM22α, 5'-CGGCCTTTAAACCCCTCACC-3' (forward) and 5'-GACTGCACTTCTCGGCTCAT-3' (reverse); Calponin, 5'-GCAGTGGACACACGCATTTT-3' (forward) and 5'-AACAACTGGCCCCA AGACTC -3' (reverse); YAP, 5'-CCCTCGTTTTGCCATGAACC-3' (forward) and 5'-TCCGTATTGCCT GCCGAAAT-3' (reverse); CREB, 5'-TGTACCACCGGTATCCATGC-3' (forward) and

5'-ACGCCATA A CAACTCCAGGG-3' (reverse); GAPDH 5'-CCCTTAAGAGGGATGCTGCC-3' (forward) and 5'-ACTGTGCCGTTGAATTTGCC-3' (reverse).

PI3Kγ Activity Assay

PI3Kγ activity was evaluated by measured the production of phosphatidyl inositol-3,4,5-triphosphate (PIP3) and the phosphorylation of Akt (ser473). PIP3 production was measured using a PIP3 Mass ELISA kit according to the manufacturer's protocol and p-Akt and total Akt was detected by Western blotting using corresponding antibodies. Briefly, cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 100mM NaCl, 10mM NaF, 10mM iodoacetamide, 1mM sodium orthovanadate, 1% NP-40 and 1 mM PMSF). After centrifugation, supernatant was subjected to immunoprecipitation with a monoclonal antibody to PI3K p110γ, followed by incubation with a PI3K reaction mixture containing $PI(4,5)P_2$ substrate and ATP for 3 hours at 37°C. After adding kinase stop solution, the absorbance was measured at 450nm in a microplate reader.

Chromatin Immunoprecipitation (CHIP)

CHIP was carried out using SimpleCHIP ® Plus Sonication Chromatin IP kit (Cell Signaling Technology) according to the manufacturer's protocol. Briefly, VSMCs were cross-linked with 1% formaldehyde and sonicated to acquire soluble chromatin with DNA fragments. Then chromatin was incubated with anti-CREB. Anti-IgG was used as control. The protein-DNA complex was precipitated by protein G magnetic beads. A series of procedures including the immunoprecipitates were eluted, protein-DNA cross-link was revised and DNA was purified. Then DNA fragments were used for Real time-PCR amplification and the primer for the promoter region (-1476 / -1469) of the YAP gene is:
5'-GCGTGTCTTAACTAGAGGAA-3'(forward) and 5'- CGAACCTGAGCCTTCTTAG-3' (reverse).

Cell proliferation assays

Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assays and BrdU incorporation assays as previously described.[4] For CCK-8 assay, suspended cells were seeded into 96-well plates at a density of 3×10^3 cells per well, allowed to attach and grow overnight. After serum-starvation for 48 hours, quiescent cells pretreated with or without AS605240 60 minutes were treated with 10% FBS. Afterwards, the cells were incubated with CCK-8 solution for 60 min, and the absorbance at 450nm was detected using a microplate reader.

For BrdU incorporation assay, VSMCs were treated as mentioned above, and then cells were incubated with BrdU-labeling solution for 4 hours. After fixation and denature, a BrdU antibody was added to detect the incorporated BrdU in the synthesized DNA of proliferating cells. Cells proliferation was evaluated by the absorbance at 450nm measured by a microplate reader.

Cell migration assays

VSMC migration was evaluated by wound-healing and Transwell migration assays as

previously described.[4] Briefly, about ~90% confluence, VSMCs were serum-starved for 48 hours prior to scratch wounding. The VSMCs pretreated with or without AS605240, PI3Kγ-KD VSMCs and PI3Kγ$^{-/-}$ VSMCs were treated with 10% FBS for 48 hours. The wound gaps were recorded using bright-field microscope and the migration rate was analyzed as the ratio of the migrated area relative to the initial wound area.

For Transwell migration assay, quiescent VSMCs pretreated with or without AS605240, PI3Kγ-KD VSMCs and PI3Kγ$^{-/-}$ VSMCs were treated with 10%FBS for 48h. Then, the cells were seeded into the upper chambers with a filter membrane (8-μm pore size) at a density of 10×10^4 cells/well. 2% FBS as a dynamic factor was added into the lower chambers. After incubation for 6 hours, migrated cells on the filter bottom were stained with 0.1% crystal violet solution and imaged using bright-field microscope. Migration rate was represented by the cellular number quantified in five different fields.

Immunostaining

Immunohistochemistry was performed on paraffin-embedded sections using the avidin-biotin-peroxidase complex method (Vector Laboratories). Tissue sections were deparaffinized with xylene and rehydrated in sequential gradients of ethanol. Antigen retrieval was performed using an antigen unmasking solution and nonspecific binding was blocked in PBS containing species-appropriate serum. Then, the tissue sections were incubated with the following primary antibodies: p110γ and phospho-Akt (Ser473). Isotype-matched antibodies served as negative controls. Subsequently, the sections were incubated with biotinylated secondary antibodies and visualized by 3,3'- diaminobenzidine (DAB), followed by counterstaining with hematoxylin. For quantitative comparison of the expression of indicated molecules, the percent of the positively stained cells to the total medial cells was calculated.

Immunofluorescence staining was performed on paraffin-embedded sections using the following antibodies: SM22α and Calponin. Immunoreactions were visualized using Alexa Fluor 488-conjugated secondary antibodies. The sections were mounted in Hoechst-containing medium and imaged using a fluorescence microscope. For quantitative comparison of the expression of indicated molecules, the percent of the positively stained area to the total traced medial area was determined.

Supplemental References

1. Li J, Liu S, Li W, Hu S, Xiong J, Shu X, Hu Q, Zheng Q, Song Z. Vascular smooth muscle cell apoptosis promotes transplant arteriosclerosis through inducing the production of SDF-1alpha. *Am J Transplant.* 2012;12:2029-2043.
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4. Wang K, Li W, Yu Q, Guo B, Yang B, Zhang C, Li M, Li J, Hu S, Zheng Q, Song Z. High Mobility Group Box 1 Mediates Interferon-gamma-Induced Phenotypic Modulation of Vascular Smooth Muscle Cells. *J Cell Biochem.* 2017;118:518-529

Supplemental Figures

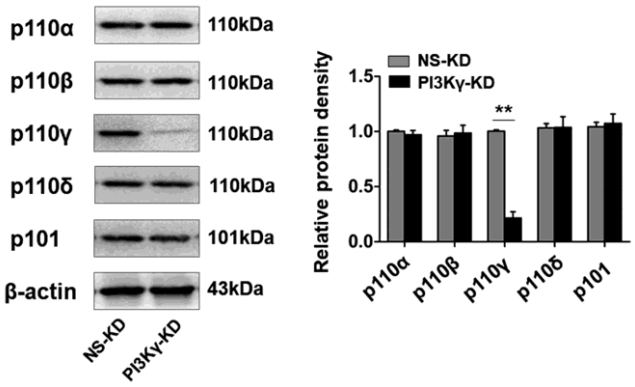

Figure Ⅰ: The effect of Pik3cg-targeting shRNA on the expression of PI3Ks catalytic subunit and regulatory subunit. VSMCs were transfected with recombinant lentiviruses carrying Pik3cg-targeting shRNA (PI3Kγ-KD) and corresponding control (NS-KD). p110α, p110β, p110γ, p110δ, and p101 expression were analyzed by Western analysis (Left panel). Bar graph (right panel) shows the densitometric analysis. Data are showed as mean ± SEM of 6 independent experiments. ***P*<0.01.

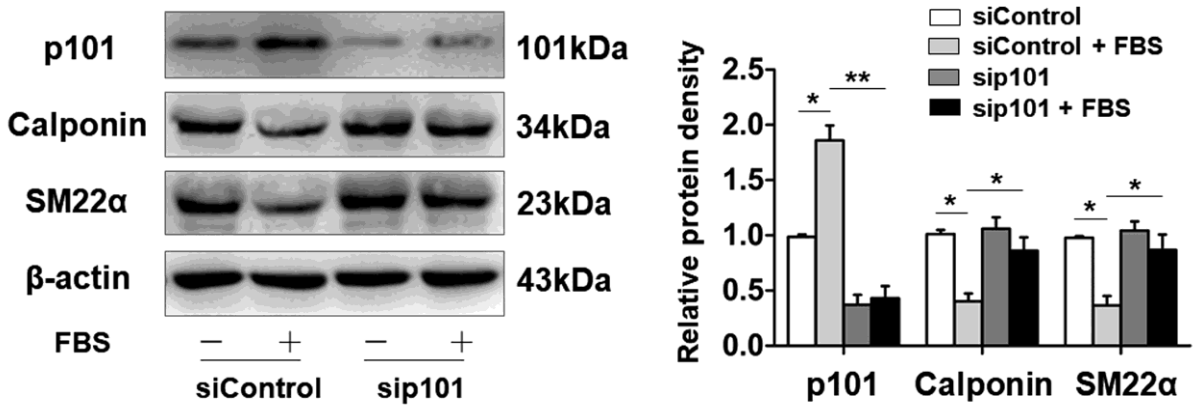

Figure Ⅱ: The effect of p101 knockdown on VSMC-specific genes expression. Western blotting analysis of p101, Calponin and SM22α expression in sip101 VSMCs (left panel). Bar graph (right panel) shows the densitometric analysis. Data are showed as mean ± SEM of 6 independent experiments. * $P<0.05$, ** $P<0.01$.

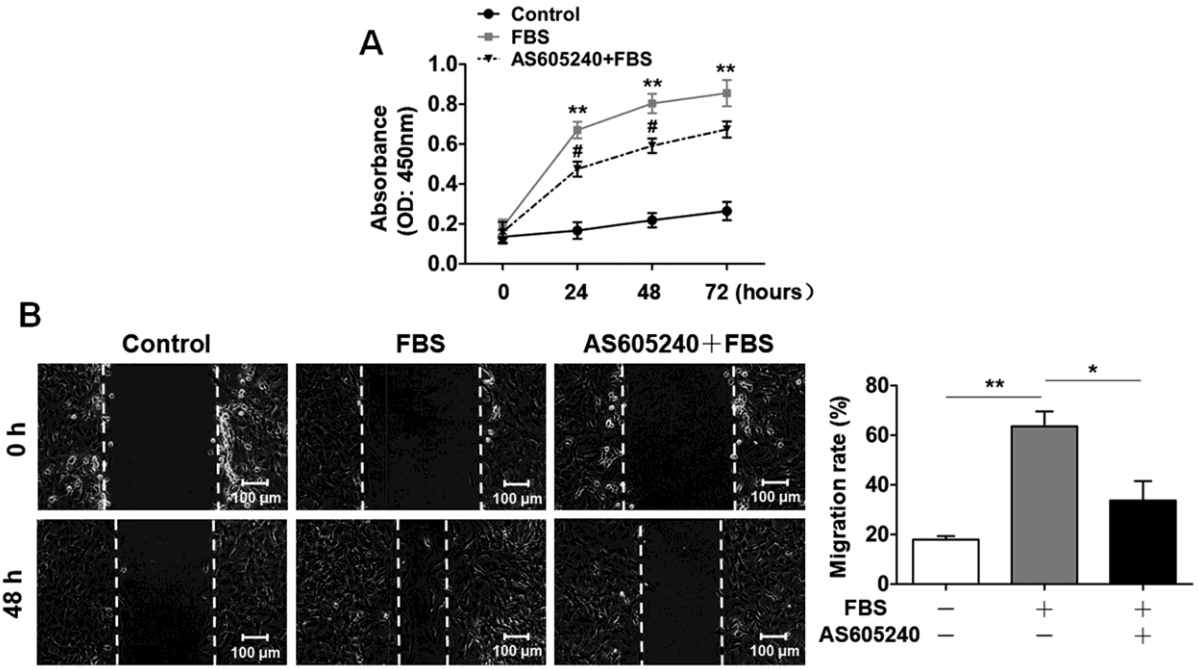

Figure Ⅲ. PI3Kγ signaling is responsible for VSMC proliferation and migration. A, VSMCs pretreated with or without AS605240 (1 μM) were stimulated with 10% FBS for indicated time, cells growth were evaluated by CCK-8. **B,** representative images of scratch wound of VSMCs(left panel). Scale bar: 100 μm. Bar graph (right panel) shows the migration rate analyzed as the ratio of migrated area relative to the initial wound area (right panel). Data are showed as mean ± SEM of at least 4 independent experiments. * $P<0.05$, ** $P<0.01$.

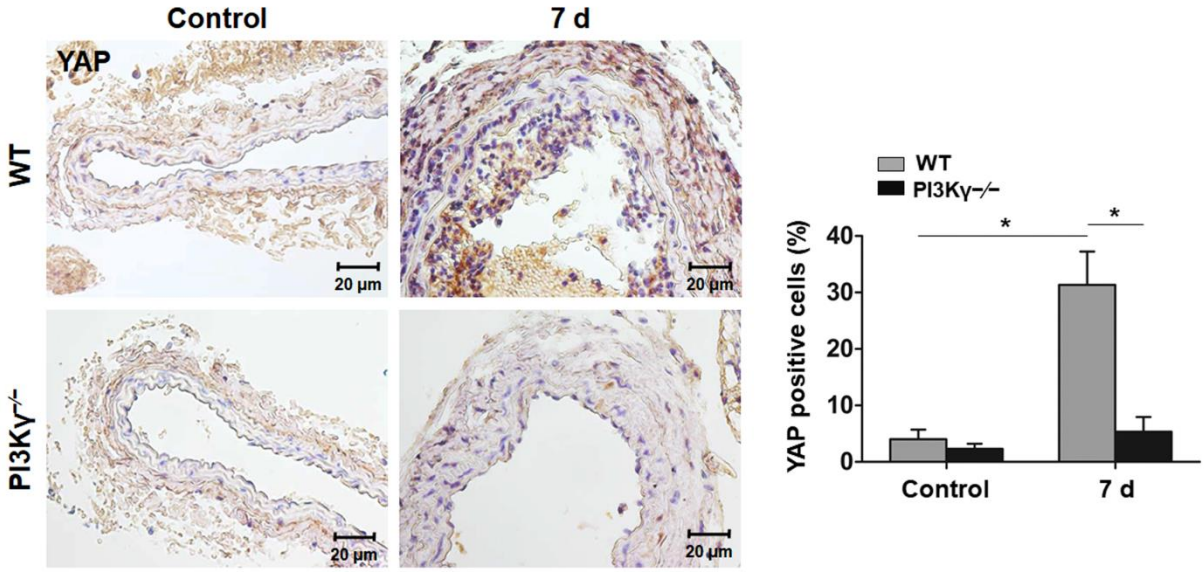

Figure Ⅳ. Injury induced YAP expression in ligated carotid artery. Representative cross sections of ligated carotid arteries from WT and PI3K$\gamma^{-/-}$ mice (7days) immunostained for YAP (left panel). Scale bars: 20 μm. Bar graph (right panel) shows the percentage of YAP-stained cells to the medial cells. Data are showed as mean ± SEM. n=8 mice per group. ***** P<0.05.

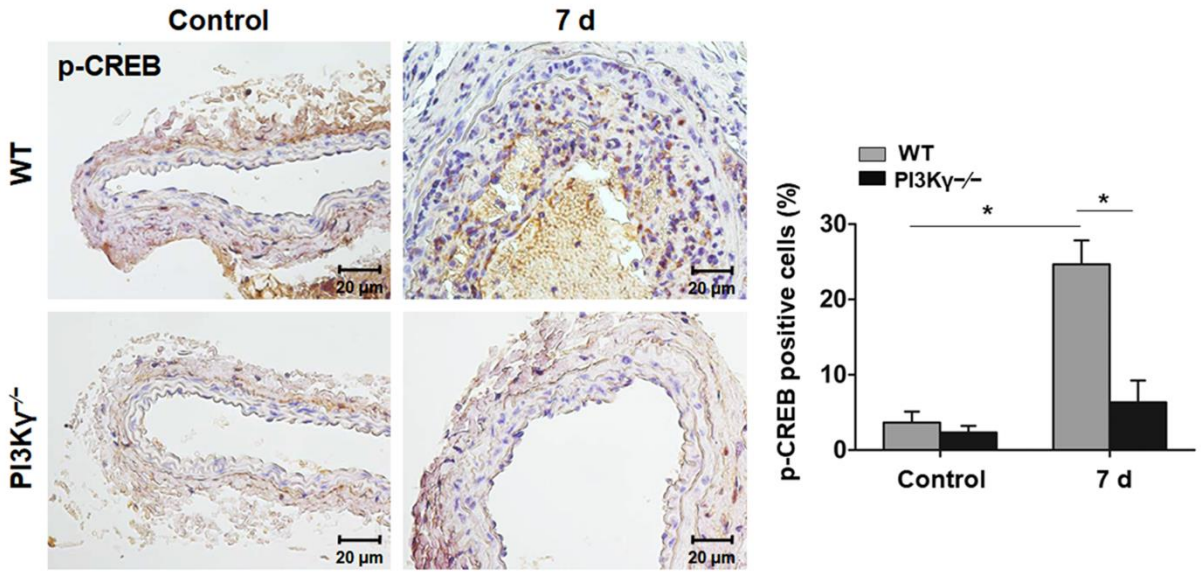

Figure Ⅴ: Injury induced p-CREB expression in ligated carotid artery. Representative cross sections of ligated carotid arteries from WT and PI3Kγ$^{-/-}$ mice (7days) immunostained for p-CREB (ser133) (left panel). Scale bars: 20 μm. Bar graph (right panel) shows the percentage of p-CREB-stained cells to the medial cells. Data are showed as mean ± SEM. n=8 mice per group. ***** *P*<0.05.