



## Research paper

# PI3K $\gamma$ promotes vascular smooth muscle cell phenotypic modulation and transplant arteriosclerosis via a SOX9-dependent mechanism



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## ABSTRACT

**Background:** Transplant arteriosclerosis (TA) remains the major cause of chronic graft failure in solid organ transplantation. The phenotypic modulation of vascular smooth muscle cells (VSMCs) is a key event for the initiation and progression of neointimal formation and TA. This study aims to explore the role and underlying mechanism of phosphoinositide 3-kinases  $\gamma$  (PI3K $\gamma$ ) in VSMC phenotypic modulation and TA.

**Methods:** The rat model of aortic transplantation was established to detect PI3K $\gamma$  expression and its role in neointimal formation and vascular remodeling in vivo. PI3K $\gamma$  shRNA transfection was employed to knockdown PI3K $\gamma$  gene. Aortic VSMCs was cultured and treated with TNF- $\alpha$  to explore the role and molecular mechanism of PI3K $\gamma$  in VSMC phenotypic modulation.

**Findings:** Activated PI3K $\gamma$ /p-Akt signaling was observed in aortic allografts and in TNF- $\alpha$ -treated VSMCs. Lentivirus-mediated shRNA transfection effectively inhibited PI3K $\gamma$  expression in medial VSMCs while restoring the expression of VSMC contractile genes, associated with impaired neointimal formation in aortic allografts. In cultured VSMCs, PI3K $\gamma$  blockade with pharmacological inhibitor or genetic knockdown markedly abrogated TNF- $\alpha$ -induced downregulation of VSMC contractile genes and increase in cellular proliferation and migration. Moreover, SOX9 located in nucleus competitively inhibited the interaction of Myocardin and SRF, while PI3K $\gamma$  inhibition robustly reduced SOX9 expression and its nuclear translocation and repaired the Myocardin/SRF association.

**Interpretation:** These results suggest that PI3K $\gamma$  plays a critical role in VSMC phenotypic modulation via a SOX9-dependent mechanism. Therefore, PI3K $\gamma$  in VSMCs may represent a promising therapeutic target for the treatment of TA.

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## 1. Introduction

Over the past few decades, short-term graft survival after transplantation has dramatically improved, mainly due to the declined incidence of acute rejection with the progress of immunosuppressive therapy. However, there has been relatively little improvement in long-term graft survival that remains hampered by the development of chronic graft failure [1,2]. Transplant arteriosclerosis (TA) has generally been recognized as the major cause of chronic graft failure in solid organ

transplantation. It is characterized by diffuse and concentric neointimal formation in intragraft arteries, leading to progressive narrowing of arterial lumen and eventually to ischemic graft failure [3–5]. Histologically, the neointimal lesions in graft vasculature arise as a result of aberrant accumulation of proliferative vascular smooth muscle cells (VSMCs) and deposition of extracellular matrix [3,4]. Emerging evidence from both animal models and human studies suggests that medial VSMCs within the graft are the major contributor to the development of neointimal formation, although host-derived cells represent a potential source of neointimal VSMCs [6–10].

Indeed, under homeostatic conditions, VSMCs within mature vessels are highly differentiated cells with a low rate of proliferation and maintain vascular tone. Wissler RW first observed the multifunctional mesenchyme nature of VSMCs and identified these characteristics as the basis for accelerated proliferation and migration of these cells toward

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## Research in context

### Evidence before this study

Chronic rejection is the predominant threat to long-term graft survival after organ transplantation. The common histopathological feature of chronic rejection in all transplanted organs are generalized transplant arteriosclerosis (TA) characterized by concentric neointimal formation and diffuse luminal narrowing, leading to ischemic tissue damage and graft dysfunction. It has recently become evident that phenotypic modulation of vascular smooth muscle cells (VSMCs) plays a central role in the development of TA following solid organ transplantation. Therefore, much of work delving the change of cellular biology and elucidating the complex mechanism of VSMC phenotypic modulation appears to be required. PI3K $\gamma$ , a lipid kinase with signaling function to regulate diverse cellular activities, has been reported to actively contribute to neointimal formation and vascular remodeling through regulating inflammatory response. However, few studies have evaluated the potential role of PI3K $\gamma$  in phenotypic modulation of VSMCs in aortic allografts.

### Added value of this study

We identified PI3K $\gamma$  as a critical regulator of VSMC phenotypic modulation and neointimal formation in aortic allografts. In the study, we initially observed PI3K $\gamma$  expression and activation are upregulated and noted a strong inverse relationship between the levels of PI3K $\gamma$  and VSMC differentiation markers in aortic allografts. Selectively PI3K $\gamma$  knockdown in VSMCs resulted in attenuated neointimal formation and vascular stenosis in aortic allografts. In addition, we indicated that PI3K $\gamma$  was responsible for TNF- $\alpha$ -induced VSMC phenotypic modulation and cellular proliferation and migration by modulating SOX9 expression and nuclear translocation.

### Implications of all the available evidence

Our findings define a crucial role for PI3K $\gamma$  in the pathogenesis of TA and implicate PI3K $\gamma$  in VSMCs as a potential therapeutic target to prevent chronic allograft failure after solid organ transplantation. In particular, further studies of the molecular mechanisms by which PI3K $\gamma$  regulates SOX9 expression and nuclear translocation in VSMCs are crucial for better understanding the pathogenesis of TA and for developing more effective therapeutic approaches.

the intima lesion upon pathological stimulus that constitute the main cellular sources of neointimal formation [11]. During repair or regeneration in vivo, VSMCs may undergo reversible and profound changes in phenotype. Ultrastructurally, VSMC exhibits a high degree of alterations in shape and contraction involving in the loss of myofilaments, gained synthetic organelles and decreased contractile genes expression that are the prerequisite for VSMC proliferation [12,13]. The term “phenotypic modulation”, first described by Campbell GR et al. about four decades ago in term of cell morphological structure and functional property, is used to define VSMC phenotypic change from a contractile, differentiated phenotype to a synthetic, dedifferentiated state, and to show that VSMCs are capable of changing their structure and function in response to damaged environment cues [12]. Likewise, the pathogenesis of transplant arteriosclerosis involves the interaction between immune and nonimmune factors, which provokes endothelial injury and vascular inflammation, resulting in VSMC phenotypic modulation that

plays a central role in the initiation and perpetuation of neointimal formation in graft vessels [3,7,10,14]. Specifically, the pleiotropic cytokine TNF- $\alpha$  has also been shown to be a critical modulator of VSMC phenotypic modulation in the progression of neointimal formation [15–17]. However, the molecular mechanisms underlying VSMCs phenotypic modulation has not been fully understood.

Phosphoinositide 3-kinases (PI3Ks) are a family of protein and lipid kinases that generally phosphorylate the 3' end of phospholipids to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), which serves as a second messenger to regulate numerous intracellular signaling pathways involving cellular growth, proliferation and chemotaxis [18]. On the basis of their structural and substrate specificity, PI3Ks are divided into three classes (I, II and III) [19]. Class I PI3Ks are predominant members of signal transduction pathways in response to growth factors, hormones, cytokines and other extracellular stimuli by utilizing cell-surface receptors that regulate various cellular processes [20]. There are two subfamilies of class I PI3Ks. The class IA enzymes are dimers composed of 110 kDa catalytic subunits (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ ) and regulatory subunits (p85 and p55) that are essential for the activation of protein tyrosine kinases. PI3K $\gamma$ , the only member of class IB, consists of a p110 $\gamma$  catalytic subunit associated with a p101 or p84 regulatory subunit and is mainly activated by G-protein-coupled receptors (GPCRs) [18,20]. Furthermore, PI3K $\gamma$  can be activated by proinflammatory factor TNF- $\alpha$ , and TNF- $\alpha$  deficiency may result in reduced PI3K $\gamma$  activity [21,22]. PI3K $\gamma$  is abundantly expressed in inflammatory cells and engages in the mobility and recruitment of various leukocytes into inflammatory sites [23]. Recently, PI3K $\gamma$  has also been shown to be expressed in the major cells of the cardiovascular system, particularly VSMCs and endothelial cells (ECs). Several studies have addressed the importance of PI3K $\gamma$  in regulating many of the functions of vascular cells, such as VSMC contraction and migration [24,25]. However, the potential role of PI3K $\gamma$  in VSMCs phenotypic modulation remains unclear.

Here, using a rat aortic transplant model, we demonstrate that selective knockdown of PI3K $\gamma$  by shRNA in medial VSMCs leads to a dramatic reduction in neointimal formation and lumen stenosis in aortic allografts. In vitro studies reveal that PI3K $\gamma$  is responsible for the induction of SOX9 expression and activation and that elicits phenotypic modulation of VSMCs. These data define a critical role for PI3K $\gamma$  in the pathogenesis of TA and implicate PI3K $\gamma$  in VSMCs as a potential therapeutic target to prevent TA and chronic allograft failure after solid organ transplantation.

## 2. Materials and methods

### 2.1. Animals

Male Brown Norway (BN) (100–120 g) and Lewis (100–120 g) rats were purchased from HFK Bioscience Co. (Beijing, China), and housed in a specific pathogen-free environment. All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of National Institutes of Health (NIH), and all animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji Medical College.

### 2.2. Reagents and antibodies

DMEM/F12 and Fetal Bovine Serum (FBS) were purchased from Life Technology (Grand Island, USA). Recombinant TNF- $\alpha$  was obtained from R&D (Minneapolis, USA). AS605240 (a selective PI3K $\gamma$  inhibitor) was purchased from Sigma-Aldrich (St. Louis, USA). PrimeScript RT Master Mix kit and SYBR Premix Ex Taq kit were purchased from Takara (Dalian, China). BrdU cell proliferation assay kit was obtained from Biovision (California, USA). PI3K activity ELISA kit was acquired from Echelon Biosciences (Utah, USA). Primary antibodies against SOX9 (Cat# ab185230, RRID: AB\_2715497), SM22 $\alpha$  (Cat# ab14016, RRID:

AB\_443021) and Calponin (Cat# ab46794, RRID: AB\_2291941) were purchased from Abcam (Cambridge, UK). Primary antibody against myocardin (Cat#sc-21559; RRID: AB\_2251111) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibody against Flag (Cat#F3165; RRID: AB\_259529) was purchased from Sigma-Aldrich (St. Louis, USA). Primary antibodies against PI3Kinase p110 $\alpha$  (Cat#4249; RRID: AB\_2165248), p110 $\beta$  (Cat#3011; RRID: AB\_2165246), p110 $\gamma$  (Cat#5405; RRID: AB\_1904087), p110 $\delta$  (Cat#34050), p85 (Cat#4257; RRID: AB\_10695255), phospho-Akt (ser473) (Cat#4060, RRID: AB\_2315049), Akt (Cat#4685; RRID: AB\_2225340), SRF (Cat#5147; RRID: AB\_10694554), Myc-tag (Cat#2276, RRID: AB\_331783) and proliferating cell nuclear antigen (PCNA) (Cat#13110, RRID: AB\_2636979) were purchased from Cell Signaling Technology (Danvers, USA). Histone H3 (Cat#BM4715),  $\beta$ -actin (Cat#BM0627) and HRP-conjugated secondary antibodies were obtained from Boster Biological Technology (Wuhan, China).

### 2.3. Construction of lentiviral vector

Recombinant lentiviral vectors carrying a specific SM22 $\alpha$  promoter and rat targeted PI3K $\gamma$  or SOX9 genes were generated as described previously [26]. The recombinant plasmid pMD19-SM22 $\alpha$  containing SM22 $\alpha$  promoter (−441 to +41, Genbank accession no. U36589) has been prepared previously [26]. The mouse SM22 $\alpha$  promoter was subcloned into the EcoRI/EcoRI restriction sites of lentiviral vector CV026 using a gene-recombinant method, in which the target gene was expressed under the promotion of specific SM22 $\alpha$  promoter. Previously tested siRNAs leading to specific and efficient knockdown of PI3K $\gamma$  gene (GenBank accession no. XM\_006240005) or SOX9 gene (GenBank accession no. NM\_08043) were used as templates for the generation of corresponding shRNAs. The 5′-3′ sequence for PI3K $\gamma$ -shRNA is: sense, CGGCCGCGACTCTAGAATTCTGTTGAATGAGGCTTCAG TAC; and anti-sense, GATAAGCTTGATATCGAATTAAGTGATTTAATTTA TACCATTTAATTC. The 5′-3′ sequence for SOX9-shRNA is: sense, CGGCCGCGACTCTAGAATTCTGTTGAATGAGGCTTCAGTAC; and anti-sense, ATAAGCTTGATATCGAATTCAAAGTGATTTAATTTATACCATTTA ATTC. These shRNAs were cloned into the EcoRI/EcoRI restriction sites of lentiviral vector CV026 respectively.

The recombinant lentiviral vector was cotransfected with packaging plasmids pHelper 1.0 and pHelper 2.0 into 293 T cells using Lipofectamine 2000. After 48 h, packaged lentivirus in the supernatant was collected and purified using ultracentrifugation. Concentration and purity of lentiviral particles were determined using affinity chromatography. Real-time PCR was used to analyze functional viral titers.

### 2.4. Aortic transplantation and tissue preparation

Abdominal aortic transplantation was performed as described previously [26]. Male Lewis rats were used as donors, syngeneic recipients and allogeneic recipients. Male BN rats served as donors. A segment of abdominal aorta approximately 1 cm long was harvested from the donors, and infected with lentiviruses expressing pik3cg-targeting shRNA (PI3K $\gamma$ -KD) with a specific SM22 $\alpha$  promoter or corresponding negative control (NS-KD) by incubating into Opti-MEM medium with corresponding lentiviral vector ( $2 \times 10^7$  TU/ml) for 60 min at 37 °C. Subsequently, the aortic segment was implanted into the heterotopic position below the renal artery and above the bifurcation. A proximal end-to-end anastomosis was performed with 11–0 single interrupted nylon suture. No immunosuppressive was used in this study.

The recipient rats were sacrificed by an overdose of pentobarbital (180 mg/kg) 2 and 8 weeks after aortic transplantation. For mRNA and protein analysis, rats were perfused in situ with ice-cold sterile saline into hearts. The adventitia of aortic grafts was removed and the endangium was destroyed by advancing a 0.35 mm-diameter angioplasty guide wire into aortic lumen and pulling back three times, and the harvested medial components of aortic grafts at 2 weeks and the

aortic grafts with the adventitia removal at 8 weeks were collected into lysis buffer and TRIzol reagent. For histology and immunohistochemistry analysis, rats were situ perfused with ice-cold sterile saline, followed by perfusion fixation with 4% paraformaldehyde.

### 2.5. Histology and morphometry

The aortic grafts were harvested and prepared as described previously [26]. Serial cross-sections (5  $\mu$ m thick) were cut and transferred onto gelatin-coated glass slides. Cross-sections from each artery were selected at 250  $\mu$ m intervals for hematoxylin-eosin (H&E) and elastic tissue fibers-Verhoeff's Van Gieson (EVG) staining. Morphometric analysis of digital images of stained sections was performed by two independent investigators blinded to the experimental design using Image-Pro Plus (Media Cybernetics) and the mean of their results was calculated. Measurement was made of the areas surrounded by the luminal surface, internal elastic lamina (IEL), and external elastic lamina (EEL) of each vessel. Lumen stenosis was calculated by dividing the intimal area by the total area within IEL. Mean neointimal area, intima/media ratio and lumen stenosis ratio were calculated.

### 2.6. Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on paraffin-embedded sections using the avidin-biotin-peroxidase complex method as described previously [26]. After antigen retrieval and blocking, the tissue sections were incubated with primary antibodies p-Akt (1:100), PCNA (1:100) and SOX9 (1:50) overnight at 4 °C. 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 nuclear counterstaining using hematoxylin.

Immunofluorescence staining were performed on paraffin-embedded sections of aortic grafts and cultured VSMCs using the primary antibodies including SM22 $\alpha$  (1:100), Calponin (1:100) and SOX9 (1:50). Immunoreactions were visualized using Cy3-conjugated and FITC-conjugated secondary antibodies (Boster Biological Technology). Nucleus was stained with hoechst (Invitrogen). Images were acquired using fluorescence microscope (Olympus).

### 2.7. Cell culture and treatment

Primary thoracic aorta smooth muscle cells were isolated from the BN rats using an explant method as previously described [26]. Briefly, VSMCs were cultured with DMEM/F12 containing 10% FBS and 100  $\mu$ g/ml streptomycin at 37 °C and 5% CO<sub>2</sub>. Cells at passages 3 to 6 were used for all experiments. Before reagents treatment, VSMCs were deprived of serum for 48 h to achieve quiescence. Quiescent cells were pretreated with AS605240 (1  $\mu$ M) for 1 h prior to TNF- $\alpha$  (10 ng/ml) stimulation.

### 2.8. Lentivirus infection and plasmid infection

Recombinant lentiviruses expressing pik3cg-targeting shRNA (PI3K $\gamma$ -KD) with a specific SM22 $\alpha$  promoter and recombinant lentiviruses expressing the short hairpin RNA (shRNA) targeting SOX9 (SOX9-KD) were prepared and used to infect VSMCs as previously described [27]. 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). Transfection efficiency was identified by observing fluorescence using fluorescence microscope three days later.

Plasmid pcDNA-SOX9 (pcSOX9) containing rat SOX9 gene or its control plasmid pcDNA was transfected into cultured VSMCs using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. Total RNA and protein were extracted 48 h post-transfection.

## 2.9. Co-immunoprecipitation and western blotting

Constructed plasmids encoding pcDNA-myocardin-Flag, pcDNA-SOX9-Myc, pcDNA-SRF were cotransfected into HEK293T cell line (ATCC Cat#CRL\_3216, RRID: CVCL\_0063) or PI3K $\gamma$ -KD VSMCs. Cells were lysed 48 h post-transfection, nuclear protein fractions extracted by utilizing a cytoplasmic and nuclear protein extraction kit according to the manufacturer's protocol (Boster Biological Technology, China) were incubated with anti-Myc or anti-Flag or anti-SRF antibody overnight at 4 °C. Normal IgG was acted as a negative control. Protein A + G Agarose beads were added and slowly swung for 2 h at 4 °C. The immunoprecipitates were washed five times with pre-cooling PBS and then mixed with SDS-PAGE buffer, followed by western blotting analysis. For western blotting, whole cell lysates were prepared with lysis buffer containing protease inhibitors and quantified using the Bradford Protein Assay (Bio-Rad). Equal amounts of denatured whole cell lysates of VSMCs were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking, proteins were detected with 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 against  $\beta$ -actin.

## 2.10. Quantitative RT-PCR

Quantitative RT-PCR was performed with corresponding primers as described previously [26]. Briefly, total RNA was extracted from VSMCs and aortic grafts using TRIzol Reagent and reverse-transcribed into cDNA using PrimeScript RT Master Mix. Quantitative PCR was carried out using the SYBR Premix Ex Taq kit in an iCycler Real-Time PCR Detection System (Bio-Rad). RNA expression levels were analyzed by normalizing to GAPDH, and the value was counted by the 2<sup>- $\Delta\Delta$ Ct</sup> method. The primer sequences were used as follows: SM22 $\alpha$ , 5'-ATCCTATGGCATGAGCCGTG-3' (forward) and 5'-CAGGCTGTTACCAACTTGC-3' (reverse); Calponin, 5'-AATACGACCACCAGCGTGAG-3' (forward) and 5'-GGGGTTTACCCCATACTCG-3' (reverse); SOX9, 5'-TCCCGCAACAGATCTCCTA-3' (forward) and 5'-AGCTGTGTAGACGGGTTG-3' (reverse); PI3K $\gamma$ , 5'-GCAGCAGTGGAAAGGTTTCGT-3' (forward) and 5'-GTTGTGCTGTACCTATCCC-3' (reverse); GAPDH, 5'-GTTACCAGGGCTGCCTTCTC-3' (forward) and 5'-GATGGTATGGGTTTCCCGT-3' (reverse).

## 2.11. PI3K activity assay

PI3K activity was measured by detecting phosphatidyl inositol-3,4,5-triphosphate (PIP3) levels and phosphorylation of Akt (p-Akt). p-Akt and total Akt were evaluated by Western blotting using specific antibodies, and PIP3 production was determined using a PIP3 Mass ELISA kit according to the manufacturer's protocol. In brief, cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaF, 100 mM NaCl, 10 mM iodoacetamide, 1 mM sodium orthovanadate, 1% NP-40 and 1 mM PMSF). After centrifugation at 10,000 rpm for 15 min, supernatant was subjected to immunoprecipitation using a monoclonal antibody to PI3K p110 $\gamma$  (1:50), followed by incubation with a PI3K reaction mixture containing PI(4,5)P2 substrate and ATP for 3 h at 37 °C. After adding kinase stop solution, the absorbance was measured at 450 nm in a microplate reader.

## 2.12. BrdU incorporation assay

BrdU incorporation assay was performed to assess cell proliferation as previously described [28]. Suspended cells were seeded into 96-well plates at a density of  $5 \times 10^3$  cells per well, allowed to attach and grow overnight. After serum-starvation for 48 h, quiescent VSMCs pretreated with AS605240 for 1 h and VSMCs infected with lentivirus as mentioned above were stimulated by TNF- $\alpha$ . Then, the cells were

incubated with BrdU-labeling solution for 6 h. After fixation and denaturation, a specific BrdU mAb was added to detect the incorporated BrdU in the synthesized DNA of proliferating cells. Cells proliferation was evaluated by the absorbance at 450 nm that was measured using a microplate reader.

## 2.13. Cell migration assay

VSMC migration was evaluated by wound-healing and Transwell migration assays as previously desired [28]. About ~90% confluence, cells were starved for 48 h prior to scratch wounding. The cells were stimulated by TNF- $\alpha$  with or without AS605240 pretreatment for 48 h, 10% FBS incubation as a positive control. 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, VSMCs were pretreated with AS605240 for 1 h or infected with lentiviruses expressing pik3cg-targeting shRNA (PI3K $\gamma$ -KD), followed by TNF- $\alpha$  stimulation for 24 h. Then, the treated cells were seeded into the upper chambers with a filter membrane (8- $\mu$ m pore size) at a density of  $10 \times 10^4$  cells/well. 2% FBS as a dynamic factor was added to the lower chambers. After incubation at 37 °C for further 6 h, 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.

## 2.14. Statistical analysis

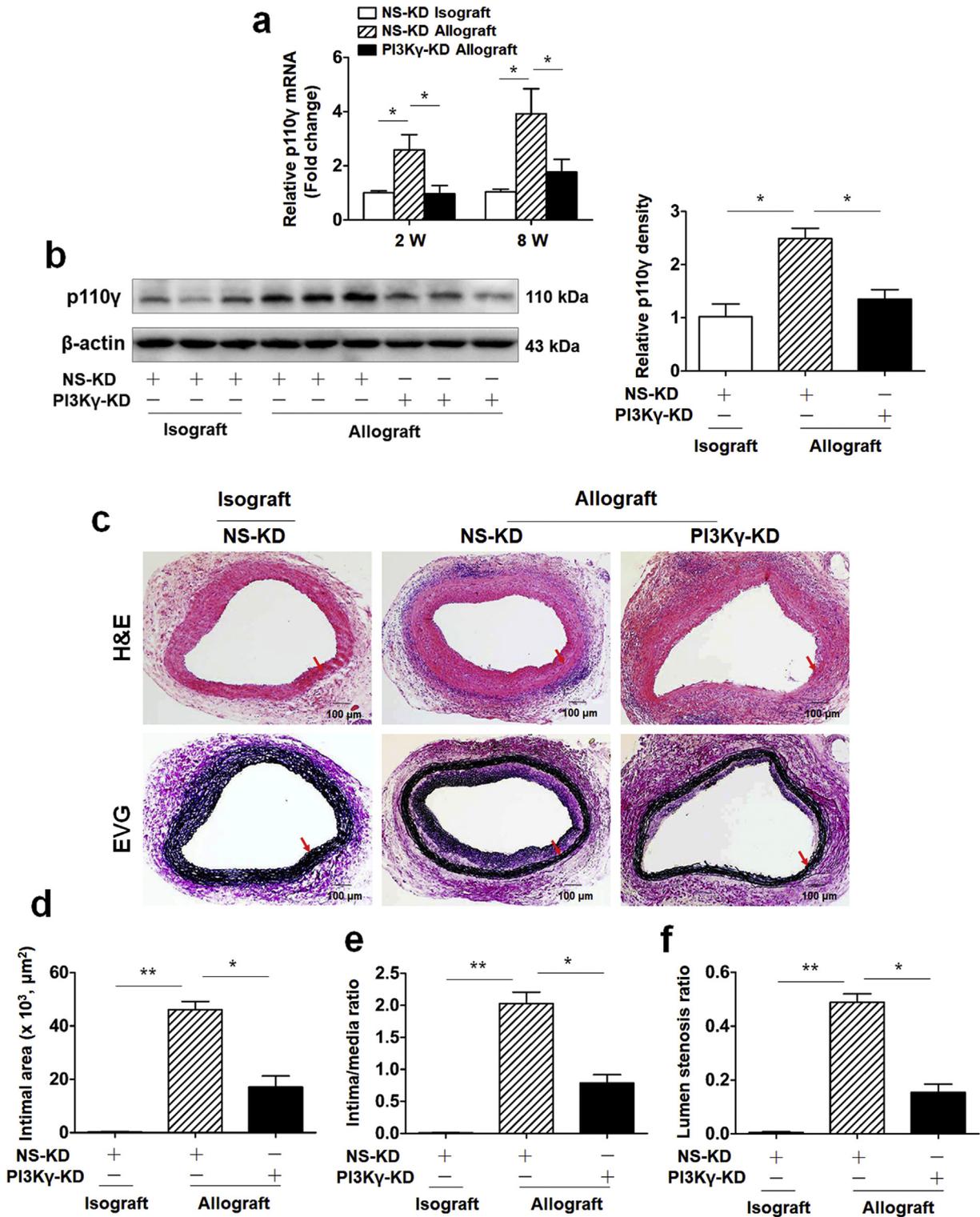
All results are presented as the mean  $\pm$  SEM. Statistical data analysis were performed by unpaired 2-tailed Student's *t*-test or analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A *P* value <0.05 was considered statistically significant.

## 3. Results

### 3.1. Selective knockdown of PI3K $\gamma$ in medial VSMCs prevents the development of TA in aortic allografts

To explore the role of VSMC-derived PI3K $\gamma$  in the development of TA, we employed lentivirus-mediated gene transfer of short hairpin RNA targeting/against p110 $\gamma$  catalytic subunit of PI3K $\gamma$  in rat aortic allografts, whose selective knockdown in medial VSMCs is modulated by a minimal SM22 $\alpha$  promoter. We measured local vascular expression of p110 $\gamma$  using real-time PCR and Western blotting 2 and 8 weeks after aortic transplantation. We observed a significant increase in p110 $\gamma$  expression within the media of aortic allografts at 2 weeks and within the overall neointimal lesions and media of aortic allografts at 8 weeks compared with aortic isografts. In contrast, the alloimmune injury-induced the p110 $\gamma$  upregulation in the aortic media was almost completely abolished/abrogated in the PI3K $\gamma$ -KD allografts at 2 weeks, and the persistent knockdown of p110 $\gamma$  still presented at 8 weeks (Fig. 1a, b and Supplemental Fig. 1), suggesting that transfection of lentiviral-based PI3K $\gamma$ -shRNA is highly effective to knock down the expression of PI3K $\gamma$  in vivo.

Next, we explored whether PI3K $\gamma$  modulates neointimal formation and vascular remodeling. We harvested the aortic grafts 8 weeks after transplantation and performed histological analysis using H&E and EVG staining. Neointimal lesions were evident in the aortic allografts, whereas very small or no lesions were observed in the aortic isografts. Morphometric analysis revealed that PI3K $\gamma$ -KD aortic allografts had a significant reduction in neointima formation and lumen stenosis (Fig. 1c), reflected by a dramatic decrease in intima area (Fig. 1d), intima/media ratio (Fig. 1e), and lumen stenosis ratio (Fig. 1f) than those observed in NS-KD aortic allografts. These findings indicate that VSMC-derived PI3K $\gamma$  plays a crucial role of in the development of TA following transplantation.

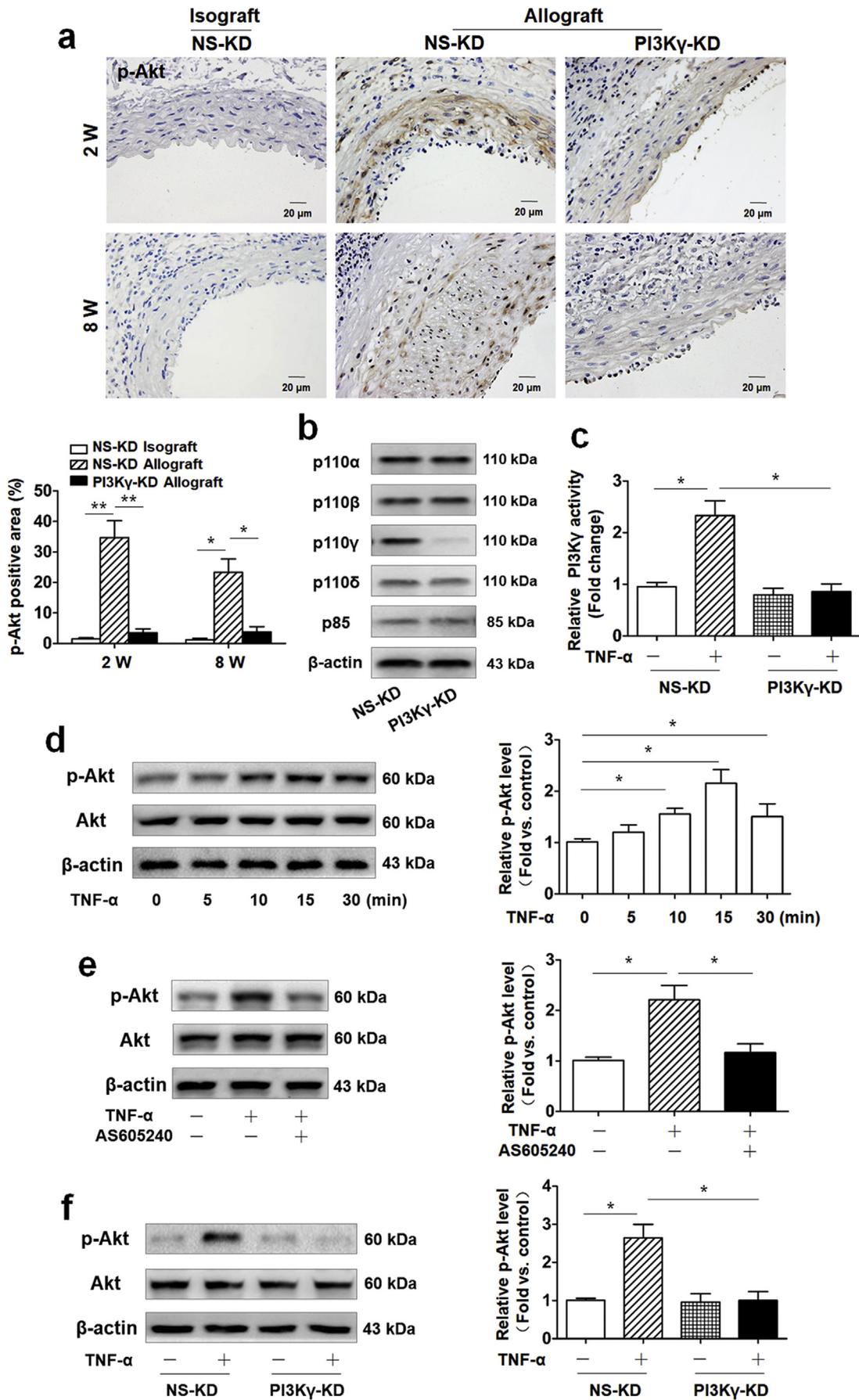


**Fig. 1.** Selective knockdown of PI3K $\gamma$  in medial VSMCs prevents the development of TA in aortic allografts. (a) qRT-PCR analysis of p110 $\gamma$  mRNA expression within the media of aortic grafts at 2 weeks and within the media and neointimal lesions at 8 weeks after aortic transplantation. mRNA expression was normalized to GAPDH. (b) Western blotting analysis of p110 $\gamma$  protein expression within the media of aortic grafts at 2 weeks after aortic transplantation (left panel).  $\beta$ -actin acted as loading control. Densitometric analysis (right panel) is shown as the relative ratio of p110 $\gamma$  protein to  $\beta$ -actin. (c) Representative H&E (upper panel) and EVG (lower panel) staining of cross sections from aortic grafts 8 weeks after aortic transplantation. Red arrows denote the internal elastic lamina. Scale bars: 100  $\mu$ m. Bar graphs show the quantitative analysis of intimal area (d), intima/media ratio (e) and lumen stenosis ratio (f). Data are represented as Mean  $\pm$  SEM of four independent experiments for a and b ( $n = 6$  rats per group), of three independent experiments for c, d, e and f.  $n = 8$  rats per group. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

### 3.2. TNF- $\alpha$ induces activation of PI3K $\gamma$ /Akt pathway in VSMCs

A major downstream effector of PI3K is Akt that functions as a key regulator of multiple cellular processes such as cell proliferation,

survival or migration [29]. Thus, we performed immunohistochemical staining for p-Akt to determine the activation of Akt in aortic grafts 2 and 8 weeks after transplantation. We detected robust activation of Akt in the aortic allografts, mainly within the medial layer at 2 weeks



and within neointimal lesions at 8 week, but not in the isografts. Compared with NS-KD aortic allografts, the p-Akt immunoreactivity was significantly reduced in the medial cells of PI3K $\gamma$ -KD aortic allografts, which is in accordance with the change in p110 $\gamma$  expression (Fig. 2a). To confirm these *in vivo* findings, we examined the activation of PI3K $\gamma$ /Akt signaling pathway in cultured VSMCs in response to proinflammatory cytokine TNF- $\alpha$ . Accordingly, we confirmed the expression of catalytic subunits of class I PI3Ks. Transfection of pik3cg-targeting shRNA into VSMCs robustly knocked down p110 $\gamma$  catalytic subunits of PI3K $\gamma$  but failed to influence the expression of other catalytic subunits (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ ) and regulatory subunits p85 of class IA enzymes (Fig. 2b). Subsequently, we explored the effect of TNF- $\alpha$  on PI3K $\gamma$  activity by measuring PIP3 levels in cultured VSMCs. TNF- $\alpha$  stimulation led to a markedly increase in PI3K $\gamma$  activity, which was abolished by PI3K $\gamma$  knockdown (Fig. 2c). Notably, TNF- $\alpha$  did not influence p110 $\gamma$  protein expression in VSMCs (Supplemental Fig. 2). Consistent with the effect on kinase activity, TNF- $\alpha$  treatment caused a time-dependent increase in phosphorylation of Akt (ser473) (Fig. 2d). However, TNF- $\alpha$  stimulation failed to induce the phosphorylation of Akt in the presence of pharmacological inhibitors AS605240 pretreatment (Fig. 2e). Similarly, PI3K $\gamma$  knockdown also markedly suppressed TNF- $\alpha$ -induced phosphorylation of Akt in VSMCs (Fig. 2f). These results suggest that TNF- $\alpha$  robustly contributes to the activation of PI3K $\gamma$ /Akt signaling pathway in VSMCs following transplantation.

### 3.3. PI3K $\gamma$ represses expression of VSMC-specific marker genes

Distinct PI3K isoforms engage in various physiological and pathological processes in the cardiovascular system [30]. However, the role of PI3K $\gamma$  in VSMCs phenotypic modulation remains unclear. Here, our results revealed that TNF- $\alpha$  exposure extensively repressed the expression of Calponin and SM22 $\alpha$  at both the mRNA and protein levels, whereas this effect was significantly abolished by the pretreatment of AS605240 (Fig. 3a and b). Similarly, TNF- $\alpha$  stimulation failed to diminish the expression of Calponin and SM22 $\alpha$  in PI3K $\gamma$ -KD VSMCs (Fig. 3c and d), suggesting a crucial role for PI3K $\gamma$  in the phenotypic modulation of cultured VSMCs. Furthermore, we measure the expression levels of Calponin and SM22 $\alpha$  in aortic grafts 2 weeks after transplantation. Western blotting demonstrated that alloimmune injury significantly diminished the protein expression of both Calponin and SM22 $\alpha$  in the media of aortic allografts (Fig. 3e). We also observed substantial reduction of immunoreactivity of both Calponin and SM22 $\alpha$  in the medial cells of aortic allografts versus isografts. Importantly, the downregulation of Calponin and SM22 $\alpha$  by alloimmune injury was recovered by PI3K $\gamma$  knockdown (Fig. 3f and g), suggesting that PI3K $\gamma$  controls VSMCs phenotypic modulation *in vivo* and *in vitro*.

### 3.4. PI3K $\gamma$ promotes VSMC proliferation and migration

VSMCs phenotypic modulation toward dedifferentiation is accompanied by functional alterations, including increased cell proliferation and migration, both of which critically contribute to the development of neointimal formation [31,32]. Thus, we explored the potential roles of PI3K $\gamma$  in VSMCs proliferation and migration. BrdU assays indicated that TNF- $\alpha$  stimulation led to a substantial increase in VSMCs proliferation, whereas the effect is somewhat less pronounced than that of FBS stimulation (Fig. 4a). Pretreatment with AS605240 markedly abrogated

TNF- $\alpha$ -induced proliferation of VSMCs (Fig. 4b). Furthermore, genetic knockdown of PI3K $\gamma$  is sufficient to abolish VSMC proliferation even in the presence of TNF- $\alpha$  (Fig. 4c). Additionally, we conducted immunostaining for proliferating cell nuclear antigen (PCNA) in aortic grafts 2 and 8 weeks after transplantation. As expected, a marked increase in the number of PCNA-positive cells within the media of aortic allografts at 2 weeks and neointimal lesions at 8 weeks was significantly reduced by selective PI3K $\gamma$  knockdown, suggesting a pivotal role of PI3K $\gamma$  signaling in the proliferative modulation of VSMCs (Fig. 4d). Consistent with the effect on cell proliferation, TNF- $\alpha$ -treated VSMCs exhibited accelerated migration ability, as measured by wound-healing assay (Supplemental Fig. 3a) and Transwell assay (Supplemental Fig. 3b). In the presence of AS605240 pretreatment, the migration ability conferred by TNF- $\alpha$  was strikingly limited (Fig. 4e), and the number of VSMCs moving through the chamber's polycarbonate membrane was dramatically diminished (Fig. 4f). Correspondingly, the negative influence on VSMC migration was further verified in PI3K $\gamma$ -KD VSMCs (Fig. 4g and h). Taken together, our data indicate a crucial role for PI3K $\gamma$  signaling in both the proliferative and migratory processes of VSMCs.

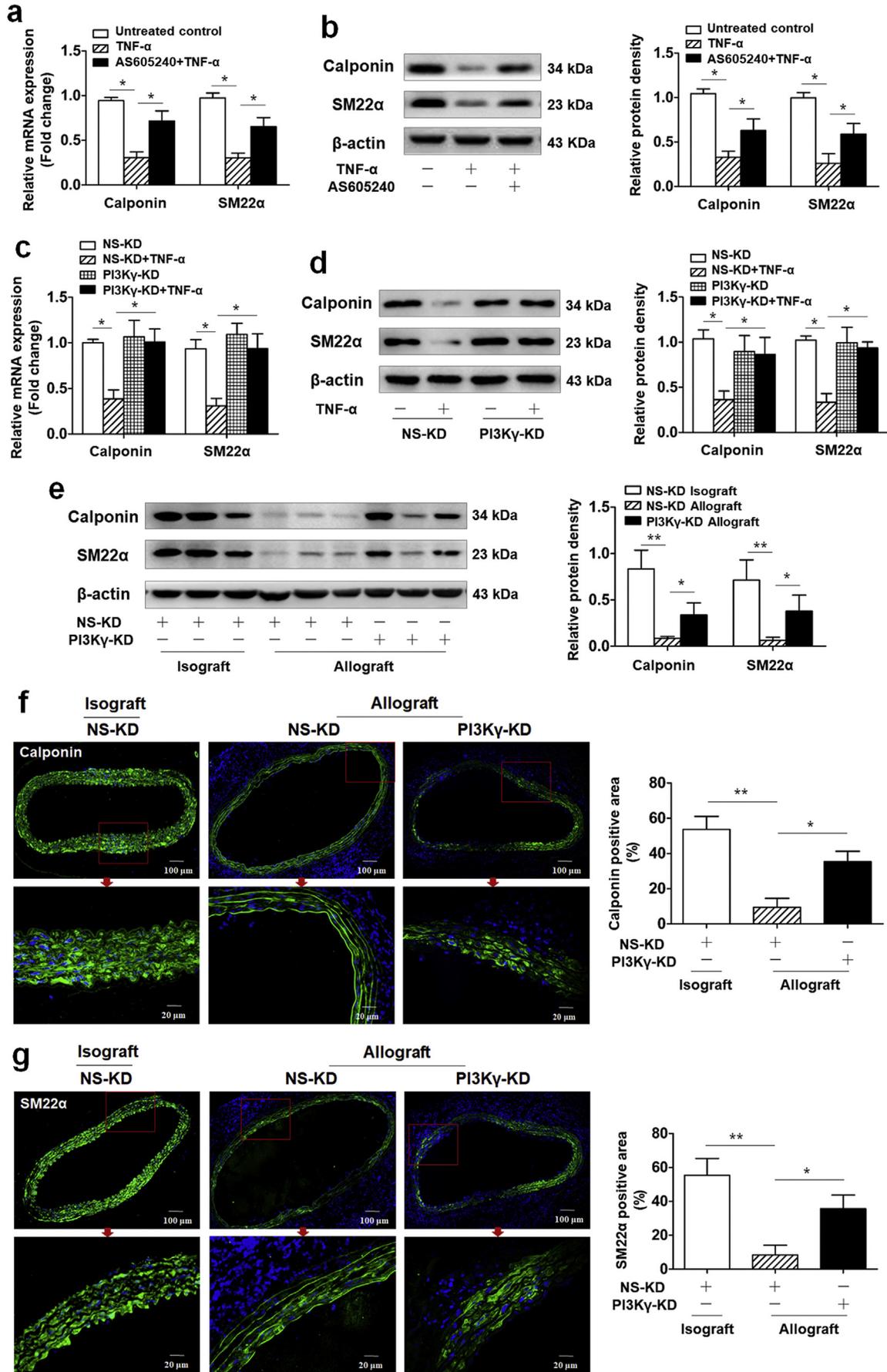
### 3.5. PI3K $\gamma$ modulates SOX9 expression in VSMCs

Transcription factor SOX9 has been identified as an important regulator of VSMCs phenotypic modulation [33]. We wondered whether SOX9 is involved in regulating VSMCs phenotypic modulation induced by PI3K $\gamma$  signaling. Therefore, we firstly explored the potential role of PI3K $\gamma$  on SOX9 expression in VSMCs. As expected, real-time PCR and western blotting analyses of cultured VSMCs highlighted a marked increase in SOX9 expression in response to TNF- $\alpha$  stimulation (Fig. 5a and b). Pretreatment with AS605240 profoundly abrogated the upregulation of SOX9 induced by TNF- $\alpha$  (Fig. 5c and d). Similarly, PI3K $\gamma$  knockdown strikingly reduced the expression of SOX9 (Fig. 5e and f), suggesting an important role for PI3K $\gamma$  signaling in modulating SOX9 expression in VSMCs. To further explore this possibility *in vivo*, we performed immunostaining for SOX9 in aortic grafts. We observed a marked increase in the number of SOX9-positive cells within the aortic media at 2 weeks and within aortic neointima at 8 weeks after transplantation in NS-KD allografts versus isografts. However, the selective PI3K $\gamma$  knockdown in medial VSMCs significantly inhibited SOX9 upregulation both in the medial layer and in the neointimal lesion of aortic allografts (Fig. 5g). Overall, these data confirmed the critical role of PI3K $\gamma$  on the regulation of SOX9 expression in VSMCs.

### 3.6. PI3K $\gamma$ controls SOX9 activation in VSMCs

SOX9 nuclear localization is an essential step for the subsequent onset of its transcriptional activation [34]. To further determine the role of PI3K $\gamma$  in SOX9 activation, cytoplasmic and nuclear proteins were respectively extracted from VSMCs treated with or without TNF- $\alpha$ . Administration of TNF- $\alpha$  resulted in enhanced both protein expression and nuclear localization of SOX9, which could be blocked by either PI3K $\gamma$  pharmacological inhibitors or genetic knockdown as demonstrated by Western blotting (Fig. 6a and b). These findings were further verified with immunofluorescence assays. In untreated VSMCs, SOX9 was distributed in the cytoplasm and nucleus. TNF- $\alpha$  was conducive to the convergence of SOX9 into the nucleus. Once PI3K $\gamma$  was blocked or knocked down, the nuclear localization of SOX9 was strikingly

**Fig. 2.** TNF- $\alpha$  induces activation of PI3K $\gamma$ /Akt pathway in VSMCs. (a) Representative cross sections of aortic grafts 2 and 8 weeks after aortic transplantation immunostained for p-Akt (ser473) (upper panel). Scale bars: 20  $\mu$ m. Bar graph (lower panel) shows the mean percentage of p-Akt positive area to the media area at 2 weeks and to overall medial and neointimal area at 8 weeks. Data are represented as Mean  $\pm$  SEM of at least three independent experiments. n = 8 rats per group. \*  $P < 0.05$ , \*\*  $P < 0.01$ . (b) VSMCs were infected with lentivirus expressing pik3cg-targeting shRNA (PI3K $\gamma$ -KD) and corresponding negative control (NS-KD), p110 ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) and p85 protein expression were measured by Western blotting. (c) PI3K $\gamma$  activity measured by enzyme-linked immunosorbent assay in PI3K $\gamma$ -KD and NS-KD VSMCs followed by TNF- $\alpha$  (10 ng/ml) stimulation for 15 min. (d) VSMCs were treated with TNF- $\alpha$  (10 ng/ml) for indicated time; (e) VSMCs were treated with TNF- $\alpha$  (10 ng/ml) for 15 min in the presence of AS605240 (1  $\mu$ M) pretreatment; (f) VSMCs were transfected with PI3K $\gamma$  shRNA (PI3K $\gamma$ -KD) followed by TNF- $\alpha$  (10 ng/ml) stimulation for 15 min, p-Akt and total Akt protein expression were measured by Western blotting (left panel). Corresponding bar graph (right panel) shows the quantification of phosphorylation of Akt (ser473) presented as the ratio of p-Akt to total Akt. Each cell experiment in one rat was repeated at least three times. Data are represented as Mean  $\pm$  SEM of six independent experiments. \*  $P < 0.05$ .



impaired, accompanied by increased cytoplasmic distribution (Fig. 6c and Supplemental Fig. 4). These data highlight PI3K $\gamma$  as an important signaling regulator that promotes SOX9 expression and activation.

### 3.7. SOX9 is responsible for VSMCs phenotypic modulation induced by PI3K $\gamma$ signaling

Myocardin/SRF (serum response factor) complex formation is a critical molecular program mediating VSMCs contractile genes transcription and maintaining cells differentiation through binding to CA $\gamma$ G boxes located in the promoter region of most VSMCs contractile genes [35,36]. Thus we sought to investigate whether PI3K $\gamma$  signaling affected VSMCs phenotype through directly regulating myocardin or its coactivator SRF expression or not, while the result indicated that neither PI3K $\gamma$  nor SOX9 knockdown could alter their transcriptional expression (Supplemental Fig. 5a and 5b). As previously reported [37,38], direct interaction between myocardin and SOX9 in nucleus was observed by co-immunoprecipitation (co-IP) using anti-Flag and anti-Myc antibodies in HEK293T cells transiently cotransfected with plasmids expressing SOX9-Myc and myocardin-Flag (Fig. 7a). However, co-IP of SOX9 with anti-Myc antibody failed to pull down any SRF in HEK293T cells cotransfected with SOX9-Myc and SRF plasmids, demonstrating that SOX9 in nucleus is unlikely to directly interact with SRF (Supplemental Fig. 5c). Moreover, increased SOX9 expression was negatively associated with the amount of SRF pulled down in myocardin immunoprecipitates (Fig. 7b). In contrast, decreased expression and nuclear localization of endogenous SOX9 in PI3K $\gamma$ -KD VSMCs maintained the myocardin/SRF association as indicated by the increased SRF/myocardin in the complex respectively pulled down with anti-myocardin and anti-SRF (Fig. 7c), suggesting that SOX9 displaces myocardin from SRF mediated by PI3K $\gamma$  signaling.

Next, we explored the involvement of SOX9 in PI3K $\gamma$ -mediated phenotypic modulation of VSMCs. Consistent with the robust influence of PI3K $\gamma$  knockdown on the expression of VSMC-specific marker genes, SOX9 knockdown by using SOX9-targeting shRNA (SOX9-KD) also dramatically abolished TNF- $\alpha$ -induced downregulation of calponin and SM22 $\alpha$  (Fig. 7d and e). Conversely, the ectopic expression of SOX9 conferred by transfecting VSMCs with a plasmid carrying SOX9 gene (pcSOX9) led to a marked downregulation of Calponin and SM22 $\alpha$  (Fig. 7f and g). Based on the above findings regarding the role of PI3K $\gamma$  in controlling SOX9 expression and activation, we speculated that PI3K $\gamma$  may via activating SOX9 signaling to stimulate VSMC phenotypic modulation. To test the hypothesis, we co-transfected PI3K $\gamma$  shRNA and pcSOX9 into VSMCs prior to TNF- $\alpha$  stimulation. As predicted, the ectopic expression of SOX9 was able to rescue the downregulation of VSMC marker genes in the PI3K $\gamma$ -KD VSMCs upon TNF- $\alpha$  stimulation (Fig. 7h and i), demonstrating that SOX9 is responsible for VSMCs phenotypic modulation induced by PI3K $\gamma$  signaling. Moreover, consistent with the inhibition of PI3K $\gamma$  knockdown in proliferative and migratory processes of VSMCs, SOX9-KD VSMCs also exhibited slow proliferation (Fig. 7j) and migration abilities (Fig. 7k and l) that enriched the mechanism that SOX9 accounts for PI3K $\gamma$ -mediated VSMCs phenotypic modulation and neointimal formation in aortic allografts.

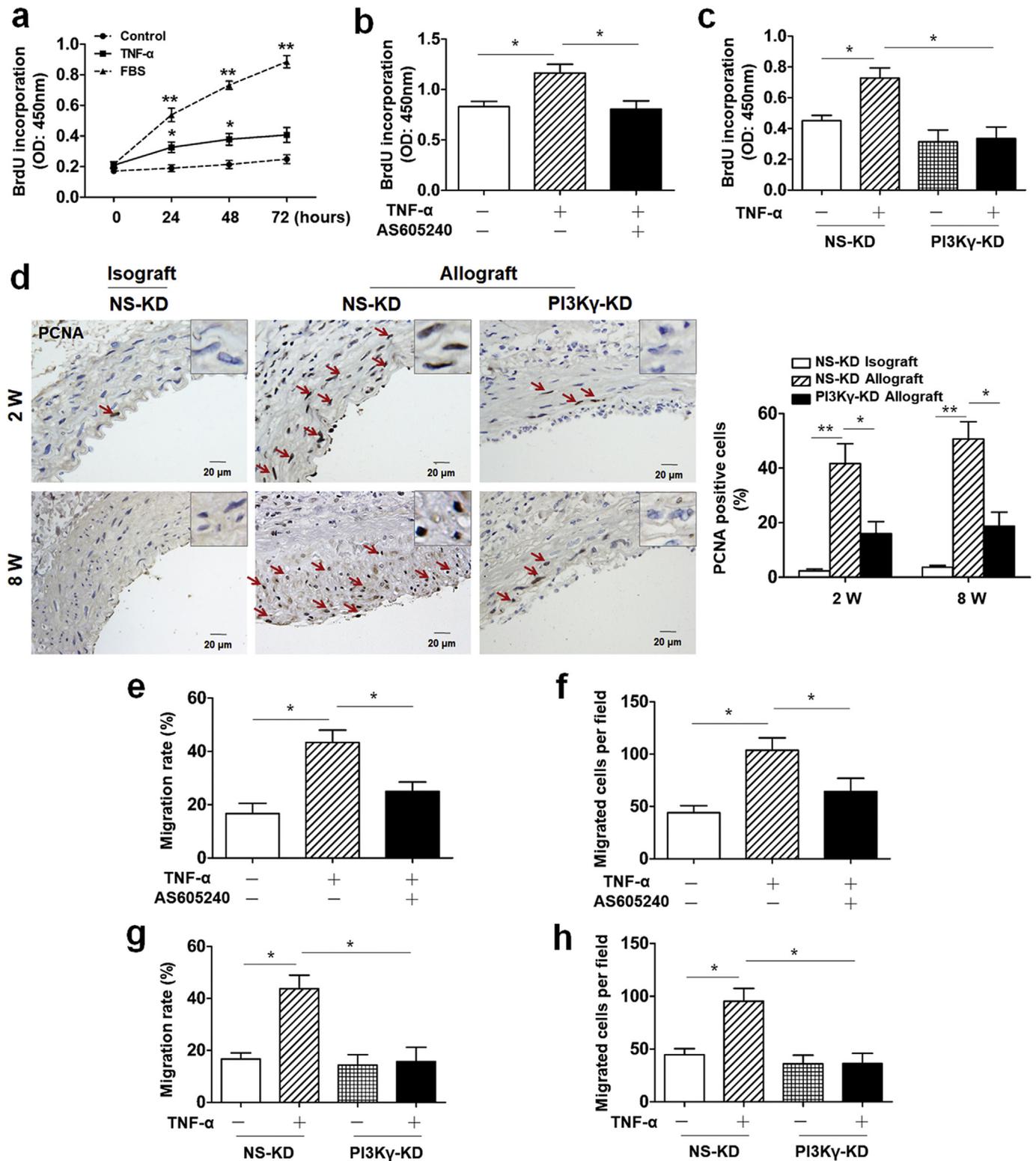
## 4. Discussion

Transplant arteriosclerosis (TA) is the predominant cause of late graft failure after organ transplantation [39]. The common histopathological features of TA are concentric neointimal formation and diffuse luminal narrowing, leading to ischemic tissue damage and graft dysfunction [3,40]. Vascular SMC phenotypic modulation from a contractile state to a synthetic phenotype is a critical step in the pathogenesis of neointimal formation in response to pathological stimuli [31,32]. In this study, we identified PI3K $\gamma$  as a novel regulator for VSMC phenotypic modulation both in vivo and in vitro. Although PI3K $\gamma$  has a low level of expression in normal VSMCs, its expression and activity are markedly upregulated in the aortic allografts following transplantation, suggesting a potential active involvement of PI3K $\gamma$  in VSMC phenotypic modulation in vivo. Importantly, blockade of PI3K $\gamma$  expression or activity strikingly suppresses SOX9 expression and its activation but restores the expression of VSMC-specific contractile genes downregulated by TNF- $\alpha$  in vitro and by alloimmune injury in vivo, indicating that PI3K $\gamma$  promotes VSMC phenotypic modulation by the induction of SOX9. Moreover, the knockdown of endogenous PI3K $\gamma$  in medial VSMCs effectively blocks alloimmune-induced VSMC phenotypic modulation and subsequent neointimal formation, demonstrating that PI3K $\gamma$ -induced VSMC phenotypic modulation is critically important for neointimal formation and transplant arteriosclerosis following transplantation.

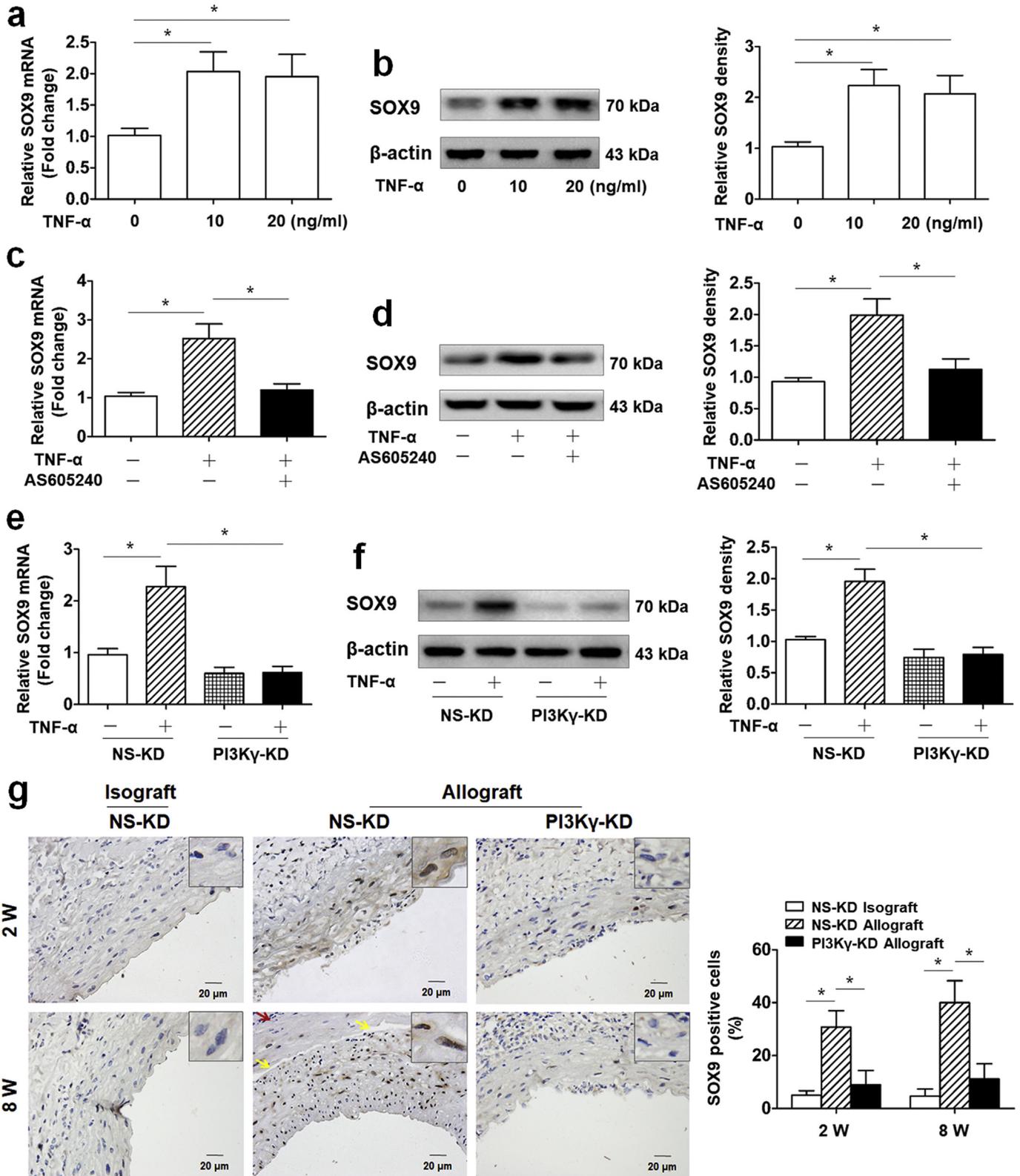
TA generally develops as a consequence of immunologic damage to the graft vasculature, which is boosted by nonimmunologic factors that trigger endothelial injury and inflammatory response [4,41]. The damage of vascular wall cells including vascular ECs and VSMCs results in the overwhelming production of growth factors and cytokines that elicit VSMCs phenotypic modulation and initiate a widespread repair process [7,26,42]. Hence, VSMCs phenotypic modulation in the medial layer of graft vessels is considered to be an initial key step toward the formation of neointimal lesions and TA. Indeed, as early as one week after transplantation, a few of medial VSMCs can be observed to become a synthetic phenotype as indicated by the decrease of myofilaments, the increase in size of endoplasmic reticulum and Golgi complex and in the expression of cellular proliferation markers. After 2–4 weeks, synthetic VSMCs are noted to gradually accumulate in the intimal layer to form a thin neointimal lesion, suggesting that VSMCs phenotypic modulation precedes neointimal formation in allografts after transplantation [10,14]. Consistent with these findings, we found that at 2 weeks after transplantation, the medial VSMCs of aortic allografts shift into synthetic phenotype associated with decreased expression of VSMC-specific contractile genes and increased proliferative activity. More importantly, blocking the initial expression of PI3K $\gamma$  in medial VSMCs accompanied by transplantation strikingly suppresses neointimal formation and lumen stenosis via inhibiting VSMCs phenotypic modulation in aortic allografts. Thus, the present study provides the new direct evidence, to our knowledge, demonstrating that VSMC phenotypic modulation is essential for the development of TA and, accordingly, knocking down the expression of PI3K $\gamma$  in VSMCs is sufficient to prevent alloimmune-induced VSMCs phenotypic modulation and neointimal formation following transplantation.

Recent studies have shown that the PI3K/Akt signaling pathway is critically involved in controlling VSMCs phenotypic alteration [43–45].

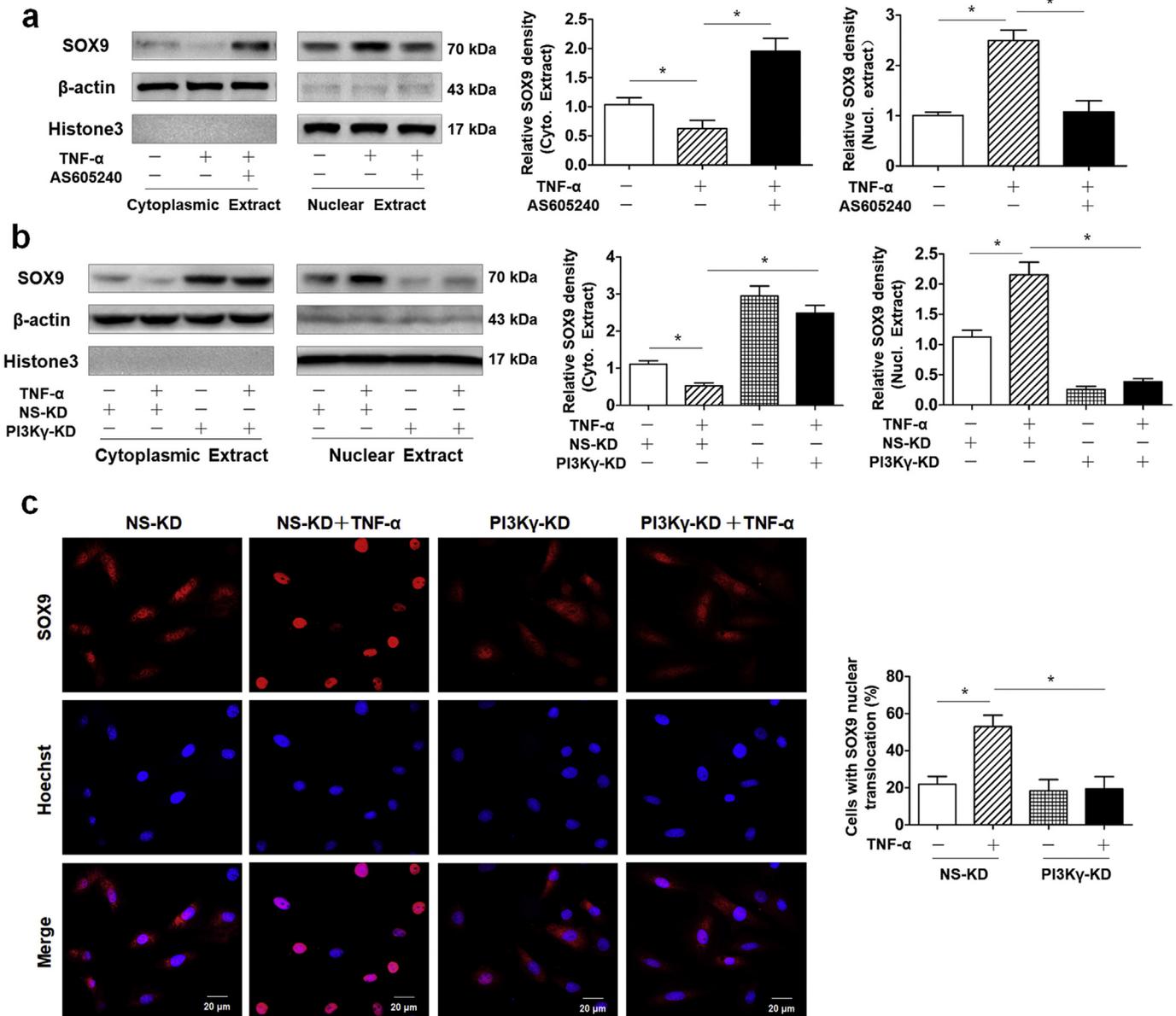
**Fig. 3.** PI3K $\gamma$  represses expression of VSMC-specific marker genes. VSMCs were treated with TNF- $\alpha$  (10 ng/ml) in the presence of AS605240 (1  $\mu$ M) pretreatment. (a) After 12 h, mRNA expression of Calponin and SM22 $\alpha$  was detected by qRT-PCR. (b) After 24 h, protein expression of Calponin and SM22 $\alpha$  was determined by Western blotting (left panel). Bar graph (right panel) shows the densitometric analysis presented as relative ratio of Calponin and SM22 $\alpha$  protein to  $\beta$ -actin. PI3K $\gamma$ -KD and NS-KD VSMCs were stimulated by TNF- $\alpha$  (10 ng/ml) exposure. (c) After 12 h, mRNA expression of Calponin and SM22 $\alpha$  was detected. (d) After 24 h, protein expression of Calponin and SM22 $\alpha$  was determined (left panel). Bar graph (right panel) shows the densitometric analysis. Each cell experiment in one rat was repeated at least three times. Data are represented as Mean  $\pm$  SEM of six independent experiments. \*  $P < 0.05$ . (e) Western blotting analysis of Calponin and SM22 $\alpha$  protein expression in the media of aortic grafts 2 weeks after aortic transplantation (left panel). Bar graph (right panel) shows the densitometric analysis. Representative cross sections of aortic grafts 2 weeks after aortic transplantation immunostained for Calponin (green) (left panel) (f), SM22 $\alpha$  (green) (left panel) (g). Cell nuclei were stained with hoechst (blue). Scale bars: 100  $\mu$ m and 20  $\mu$ m. Bar graphs (right panel) show the mean percentage of positively stained area to the medial area of aortic grafts. Data are represented as Mean  $\pm$  SEM of four independent experiments for e ( $n = 6$  rats per group), of three independent experiments for f and g ( $n = 8$  rats per group). \*  $P < 0.05$ , \*\*  $P < 0.01$ .



**Fig. 4.** PI3K $\gamma$  promotes VSMC proliferation and migration. (a) VSMC proliferation was evaluated by BrdU incorporation assay in response to TNF- $\alpha$  (10 ng/ml) and 10% FBS for indicated time. BrdU incorporation assay was performed to evaluate the proliferation of VSMCs pretreated with AS605240 prior to TNF- $\alpha$  stimulation for 24 h (b), and the proliferation of PI3K $\gamma$ -KD VSMCs followed by TNF- $\alpha$  stimulation for 24 h (c). Proliferation rate is presented as the measured absorbance at 450 nm. (d) Representative cross sections of aortic grafts 2 and 8 weeks after aortic transplantation immunostained for PCNA (left panel). Scale bars: 20  $\mu$ m. Red arrows denote the PCNA positive cells. Bar graph (right panel) shows the mean percentage of PCNA positive cells within the media of aortic grafts at 2 weeks and within the overall media and neointimal lesions of aortic grafts at 8 weeks. VSMCs were treated with TNF- $\alpha$  (10 ng/ml) for 24 h in the presence of AS605240 pretreatment. (e) Scratch wound-healing assay was performed to evaluate the cell migration presented as the percentage of migrated area to the initial wound area. (f) Transwell assay was performed to assess cell migration indicated as the number of migrated VSMCs on the bottom of transwell membrane. Migration ability of NS-KD and PI3K $\gamma$ -KD VSMCs exposed to TNF- $\alpha$  (10 ng/ml) for 24 h was measured by Scratch wound-healing assay (g) and Transwell assay (h). Data are shown as mean  $\pm$  SEM of at least five independent experiments for a, b, c, e, f, g, h, and of three independent experiments for d (n = 8 rats per group). \*  $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 5.** PI3Kγ modulates SOX9 expression in VSMCs. (a) qRT-PCR and (b) western blotting (left panel) analyses of SOX9 expression in VSMCs treated with TNF-α (0, 10, 20 ng/ml). Bar graph (right panel) shows the densitometric analysis of SOX9 protein expression. (c) qRT-PCR and (d) western blotting (left panel) analyses of SOX9 expression in VSMCs pretreated with AS605240 (1 μM) prior to TNF-α (10 ng/ml) stimulation. Bar graph (right panel) shows the densitometric analysis of SOX9 protein expression. (e) qRT-PCR and (f) western blotting (left panel) analyses of SOX9 expression in NS-KD and PI3Kγ-KD VSMCs stimulated by TNF-α. Bar graph (right panel) shows the densitometric analysis of SOX9 protein expression. All data are presented as Mean ± SEM of six independent experiments. (g) Representative cross sections of aortic grafts 2 and 8 weeks after aortic transplantation immunostained for SOX9 (left panel). Scale bars: 20 μm. Red arrow denotes the external elastic lamina and yellow arrows denote the intima region. Bar graph (right panel) shows the mean percentage of SOX9 positive cells within the media of aortic grafts 2 weeks and within the media and neointimal lesions of aortic grafts at 8 weeks. Data are shown as Mean ± SEM of three independent experiments. n = 8 rats per group. \* P < 0.05.

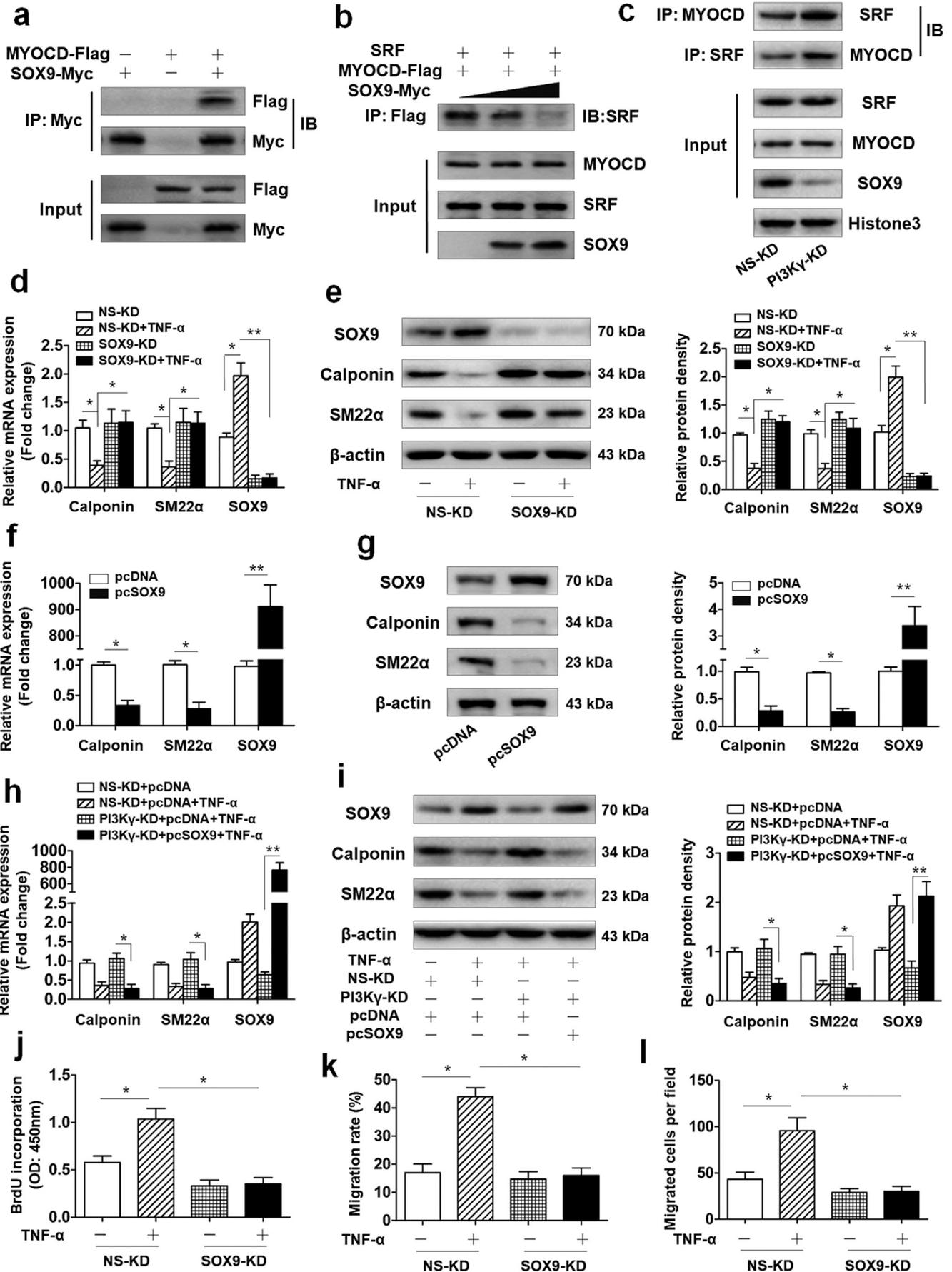


**Fig. 6.** PI3K $\gamma$  controls SOX9 activation in VSMCs. (a) Cytoplasmic and nuclear extracts from VSMCs pretreated with AS605240 (1  $\mu$ M) prior to TNF- $\alpha$  (10 ng/ml) treatment were immunoblotted using anti-SOX9 (left panel). Bar graphs show densitometric analysis of SOX9 expression in cytoplasmic extracts (middle panel) and nuclear extracts (right panel). (b) Cytoplasmic and nuclear extracts from PI3K $\gamma$ -KD and NS-KD VSMCs treated with TNF- $\alpha$  (10 ng/ml) were immunoblotted using anti-SOX9 (left panel). Bar graphs show densitometric analysis of SOX9 expression in cytoplasmic extracts (middle panel) and nuclear extracts (right panel). (c) Representative immunofluorescence staining for SOX9 (red) nuclear location in PI3K $\gamma$ -KD VSMCs exposed to TNF- $\alpha$  (10 ng/ml) (left panel). Cell nuclei were stained with hoechst (blue). Scale bars: 20  $\mu$ m. Bar graph (right panel) shows quantification analysis of SOX9 nuclear translocation indicated as the percentage of SOX9 nuclei-positively stained VSMCs to the total cells (right panel). Data are represented as Mean  $\pm$  SEM of six independent experiments. \*  $P < 0.05$ .

Depending on different pathological stimuli, the PI3K/Akt signaling pathway may function as either a positive or a negative regulator of VSMC differentiation. PI3K/Akt activation induced by IGF-1 or Notch activation maintains the differentiated state of VSMCs and this effect can be prevented by inhibition of PI3K activity [43,46]. Conversely, induction of PI3K/Akt signaling by PDGF, TGF- $\beta$ 1, PTEN deletion, or hypertension leads to acquisition of the dedifferentiated phenotype of VSMCs accompanied with reduced expression of VSMC contractile proteins, whereas blockade of PI3K/Akt signaling pathway can successfully suppress VSMCs phenotypic modulation and restore the expression of VSMC contractile proteins [45,47–49]. Notably, PI3K/Akt signaling has been shown to promote VSMC proliferation and migration in response to TNF- $\alpha$  and PDGF, and blocking PI3K activity or Akt inactivation is sufficient to suppress the ability of VSMCs proliferation and migration and attenuate neointimal formation following vascular injury [50–52]. In

this study, we now additionally show a robust effect of proinflammatory cytokine TNF- $\alpha$  on the activation of PI3K $\gamma$ /Akt and phenotypic modulation of VSMCs. In addition, previous studies have revealed that TNF- $\alpha$  is released early after transplantation, plays a pivotal role in the pathogenesis of TA through inducing the activation of VSMCs [15,53]. These together with our findings suggest that the activation of PI3K $\gamma$ /Akt signaling may constitute a possible mechanism accounting for VSMCs phenotypic modulation and vascular remodeling after transplantation.

PI3Ks are lipid kinases that transduce a variety of cellular signals from growth factors, cytokines, and other environmental stimuli and regulate a broad range of physiological functions and cellular processes, including cell survival, proliferation, migration, and metabolism [18,20]. Class I PI3Ks consist of four isoforms, including PI3K $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , which are expressed in multiple cell types in the cardiovascular system [18,30]. Several lines of evidence have suggested that distinct PI3K isoforms play



different roles in a variety of cellular processes under physiologic and pathologic conditions [18,54]. For example, genetic ablation of PI3K $\alpha$  in VSMCs leads to impaired cell proliferation and migration and attenuated neointimal formation upon vascular injury, whereas pharmacological blockade of PI3K $\beta$  has no effect on VSMCs activation [55,56]. Genetic inactivation of p110 $\delta$  kinase can profoundly prevent the formation of neointimal lesions following vascular injury via suppressing the recruitment of inflammatory cells, but not VSMCs activation [55,57]. In parallel, targeted deletion of p110 $\delta$  in VSMCs fails to prevent neointimal growth and vascular remodeling in injured carotid artery [55]. Consistent with the effect of PI3K $\delta$  on vascular injury-induced neointimal formation, PI3K $\gamma$  is critically implicated in the regulation of atherosclerotic progression and vascular remodeling through modulating the inflammatory response [58–60]. Although previous studies have demonstrated the importance of PI3K $\gamma$  in regulating VSMCs contraction and migration [24,25], the functional role of PI3K $\gamma$  in VSMC phenotypic modulation remains unknown. In this study, VSMC specific-p110 $\gamma$  knockdown in medial VSMCs is effective to prevent the phenotypic modulation of VSMCs in the early stage after transplantation, resulting in impaired neointimal growth and attenuated vascular stenosis in the aortic allografts. Similarly, PI3K $\gamma$  inactivation in vitro restores the expression of VSMC-specific contractile genes downregulated by TNF- $\alpha$ . Our results suggest that PI3K $\gamma$  in VSMCs plays a crucial role in the development of TA through controlling VSMCs phenotypic modulation after transplantation.

Another important finding of this study is the identification of SOX9 as a downstream effector of PI3K $\gamma$  for regulating VSMCs phenotypic modulation. Our results confirm the functional involvement of PI3K $\gamma$  in the induction of SOX9 expression and nuclear translocation in VSMCs. Myocardin is known to be a potent coactivator of serum response factor (SRF) in transactivating the transcription of VSMC-specific genes in a CaRg-dependent manner, which maintains VSMC in the differentiated phenotype [35,36]. Recent evidence indicates that SOX9, a master transcriptional regulator for chondrocyte differentiation, can interact with myocardin to regulate VSMCs phenotypic modulation [37,38]. Consistent with previous studies, our studies demonstrate that SOX9 physically interacts with myocardin and thus competitively disrupts the association of myocardin and SRF, leading to suppression of VSMC-specific genes. PI3K $\gamma$  knockdown in VSMCs reduces the expression and nuclear translocation of SOX9 but restores the association of myocardin/SRF, suggesting that SOX9 acts as a key downstream effector of PI3K $\gamma$  signaling to promote VSMCs phenotypic modulation by suppressing myocardin/SRF-mediated transcription of VSMC-specific genes.

SOX9 is primarily controlled at the transcriptional level. SOX9 transcription is under the control of several transcriptional factors that can directly bind to the SOX9 promoter. NF- $\kappa$ B RelA has been identified as a transcriptional factor for SOX9 induction and chondrogenic differentiation via binding to an NF- $\kappa$ B binding motif in the SOX9 promoter [61]. Indeed, the activation of the NF- $\kappa$ B pathway leads to increased expression of SOX9 and thus promotes chondrogenic conversion of VSMCs [62]. Moreover, it has been shown that both cyclic-AMP response element binding protein (CREB) and Sp1 can interact with specific sites within the SOX9 promoter region to regulate SOX9 transcription in

chondrocytes [63]. Recent data have also shown that the PI3K/Akt signaling is responsible for controlling SOX9 expression in nucleus pulposus cells [64]. However, it is unclear about the molecular mechanism by which PI3K $\gamma$  regulates SOX9 expression in VSMCs. Another question raised by our study is the potential mechanism underlying PI3K $\gamma$ -induced SOX9 nuclear translocation, which is required for the interplay of SOX9 and myocardin to regulate VSMCs phenotype. Therefore, further research should be performed to explore these issues.

Taken together, our study has identified PI3K $\gamma$  as a novel regulator for VSMCs phenotypic modulation and TA after transplantation. This study also provides evidence that SOX9 acts as a major downstream effector of PI3K $\gamma$  signaling to promote VSMCs phenotypic modulation. Therefore, targeting PI3K $\gamma$  in VSMCs may represent a promising therapeutic strategy for the prevention of TA following transplantation.

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## Declaration of interests

The authors have no conflict of interests to declare.

## Author contributions

Zifang Song and Qichang Zheng conceived the study plan and contributed to the revision of the final manuscript. Qihong Yu and Wei Li performed the experiments, analyzed the data and finished the manuscript writing. Dawei Xie, Xichuan Zheng, Tong Huang and Ping Xue participated in data collection and literature search. Bing Guo and Yang Gao contributed to the manuscript writing and data interpretation. Chen Zhang, Ping Sun, Min Li, Guoliang Wang and Xiang Cheng guided the animal experiments.

## Conflict of interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.09.013>.

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**Fig. 7.** SOX9 is responsible for VSMCs phenotypic modulation induced by PI3K $\gamma$  signaling. (a) HEK293T cells were transfected with plasmids expressing pcDNA-myocardin-Flag (MYOCD-Flag) and/or pcDNA-SOX9-Myc, nuclear extracts were precipitated with anti-Myc antibody, followed by immunoblotting with anti-Flag and anti-Myc antibodies. (b) HEK293T cells were co-transfected with pcDNA-myocardin-Flag, pcDNA-SRF and/or pcDNA-SOX9-Myc plasmids (with increased dosage), nuclear extracts was precipitated using anti-Flag antibody and further immunoblotted with anti-SRF antibody. The input cell lysates were immunoblotted with anti-myocardin, anti-SRF and anti-SOX9 antibodies. (c) co-IP of Myocardin and SRF respectively using anti-Myocardin and anti-SRF in the nuclear extracts of PI3K $\gamma$ -KD VSMCs, followed by immunoblotting using indicated antibodies. (d) mRNA and (e) protein expression (left panel) of SOX9, Calponin and SM22 $\alpha$  expression in VSMCs infected with lentivirus expressing SOX9-targeting shRNA (SOX9-KD) and negative control (NS-KD) prior to TNF- $\alpha$  (10 ng/ml). Bar graph (right panel) shows the densitometric analyses of these proteins. (f) mRNA and (g) protein expression (left panel) of SOX9, Calponin and SM22 $\alpha$  in VSMCs transfected with plasmid overexpressing SOX9 (pcSOX9) and negative control (pcDNA). Bar graph (right panel) shows the densitometric analyses of these proteins. PI3K $\gamma$  shRNA (PI3K $\gamma$ -KD), plasmid (pcSOX9) and its corresponding control were co-transfected into VSMCs followed by TNF- $\alpha$  treatment, (h) mRNA and (i) protein expression (left panel) of SOX9, Calponin and SM22 $\alpha$  were detected. Bar graph (right panel) shows the densitometric analyses. (j) BrdU incorporation assay was performed to assess the proliferation of NS-KD and SOX9-KD VSMCs exposed to TNF- $\alpha$  (10 ng/ml) for 24 h. Migration ability of NS-KD and SOX9-KD VSMCs exposed to TNF- $\alpha$  (10 ng/ml) for 24 h was measured by Scratch wound-healing assay (k) and Transwell assay (l). Data are represented as Mean  $\pm$  SEM of six independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

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