Identification of PI3K regulatory subunit p55γ as a novel inhibitor of vascular smooth muscle cell proliferation and neointimal formation

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Aims
Phosphatidylinositol 3 kinases (PI3Ks) play a pivotal role in vascular physiology and pathophysiology. We aimed to investigate the role of p55γ, a regulatory subunit of PI3Ks, in vascular smooth muscle cell (VSMC) proliferation and neointimal formation.

Methods and results

Conclusion
These findings mark p55γ as a novel upstream regulator of the p53-p21 signalling pathway that negatively regulates VSMC proliferation, suggesting that malfunction of p55γ may trigger vascular proliferative disorders.

Keywords
p55γ • Vascular smooth muscle cell proliferation • Neointimal formation • Restenosis

1. Introduction
Proliferative disorders of vascular smooth muscle cells (VSMCs) play pivotal roles in the pathogenesis of various vascular disorders, including restenosis after angioplasty or bypass surgery, transplant vasculopathy, atherosclerosis, and hypertension. Subsequently, these conditions lead to a myriad of cardiovascular diseases, including ischaemic heart disease, myocardial infarction, heart failure, and stroke, constituting the current leading causes of death worldwide. Normally, VSMCs are located in the arterial tunica media and exhibit a low rate of proliferation, even in the presence of mitogenic stimuli. But arterial injury or mechanical stress on arteries evokes transmigration of VSMCs into the intimal layer of the arterial wall and induces VSMC proliferation. In particular, VSMC proliferation plays a crucial role in injury-induced restenosis, highlighting the need for a better understanding of the fundamental mechanisms underlying VSMC proliferation.

Phosphatidylinositol 3 kinases (PI3Ks) are important lipid kinases that regulate diverse cellular activities, including metabolism, proliferation, survival, and polarity. Distinct isoforms of PI3Ks play different roles in vascular physiology and pathophysiology. Class Iα of PI3Ks, the most extensively studied, is a heterodimer consisting of a p110 catalytic subunit and a regulatory subunit. Five regulatory subunits, including...

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17 Previous studies have reported that p55 expression or function may serve as a novel therapeutic approach for the treatment of vascular proliferative diseases. 

Here, we sought to determine biological and potential pathological functions of p55 in atherosclerotic vascular system, in particular, in the regulation of cellular processes such as cell proliferation and cell survival, and that p55 expression or function may serve as a novel therapeutic approach for the treatment of vascular proliferative diseases.

2. Methods

An expanded method section is available in Supplementary material online.

2.1 Animals

Adult male Sprague Dawley (SD) rats were supplied by Vital River Laboratories, Beijing, China, and housed in the Center for Experimental Animals (an Assessment and Accreditation of Laboratory Animal Care-accredited experimental animal facility) at Peking University, Beijing, China. All procedures involving experimental animals were performed in accordance with protocols approved by the Committee for Animal Research of Peking University, China, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH, 2011).

2.2 Primary VSMC culture

VSMCs from thoracic aorta of SD rats (180–200 g) were isolated using a standard enzymatic digestion technique. Rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg) by intra-peritoneal injection. VSMCs were cultured in DMEM with 10% fetal bovine serum (FBS). The growth curves of VSMCs were constructed by cell counting on a FACScan (BD Biosciences) flow cytometer. A total of 10^4 cells were counted for each sample, and the double discriminator module was used to detect single cells. Each experiment was repeated three times.

2.3 Rat carotid artery injury model and morphometric analysis of intimal thickening

Male SD rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). The left carotid artery was injured by a size 2F embolectomy balloon catheter as previously described. Then the injured artery was washed with PBS and incubated with adenoviral vector. Rats were sacrificed with an overdose of pentobarbital sodium (100 mg/kg) by intra-peritoneal injection at the designated time, and arteries were collected for real-time PCR and western blot assays, or embedded in paraffin to prepare cross-sections. Neointimal thickening was assessed using the intima-to-media area ratio (I/M) and neointimal area measured from haematoxylin- and eosin-stained arterial cross-sections with a computer-based Image-Pro Morphometric System in a double-blind manner (see Supplementary material online for details).

2.4 MTT assay and cell counting

Cells first underwent mitogenic quiescence by serum starvation and then viral infection with Ad-GFP or Ad-p55γ at 100 m.o.i. for 48 h. The cells were subsequently stimulated with PDGF-BB (10 ng/mL) or serum (10% FBS). The growth curves of VSMCs were constructed by cell counting on the indicated days after infection. Cell proliferation was also assayed by cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quantified spectrophotometrically at 490 nm with background subtraction at 630 nm (see Supplementary material online for details).

2.5 Proliferating cell nuclear antigen assay in carotid artery

The proliferating cell nuclear antigen (PCNA) immunohistochemical assay was performed 14 days after injury in sections from injured rat carotid arteries transfected with the indicated adenovirus using anti-PCNA antibody and visualized with DAB substrate, see Supplementary material online for details.

2.6 Real-time PCR

Quantitative real-time PCR was performed as described previously. The primers were listed in the Supplementary material online.

2.7 Propidium iodide staining and fluorescence-activated cell sorter analysis

VSMCs were synchronized, infected with adenoviral vectors for 48 h, and then stimulated to proliferate with serum as described above. They were fixed overnight in 75% ethanol at 4°C and stained for 10 min at room temperature with propidium iodide (PI; 50 µg/mL). Cells were analysed with a FACScan (BD Biosciences) flow cytometer. A total of 10^4 cells were counted for each sample, and the double discriminator module was used to detect single cells. Each experiment was repeated three times.

2.8 Immunoprecipitation and ubiquitination assay

To assay immunoprecipitation (IP) and ubiquitination, cells were lysed in IP buffer on ice for 10 min, followed by centrifugation at 13 000 rpm for 10 min. The supernatant was mixed with protein A agarose beads and the antibody, and then incubated at 4°C for 4 h. The immunoprecipitated beads were then washed three times with co-immunoprecipitation (Co-IP) buffer, and the bound proteins were detected by western blot, as further described in the Supplementary material online.
2.9 RNA interference

Small-interfering RNA (siRNA) duplexes for silencing p21, p53, and Bcl-xl were designed using RNA interference (RNAi) Designer and synthesized by GenePharma. The p55γ or the scrambled short hairpin RNAs (shRNAs) were generated using the BLOCK-i-T Adenoviral RNAi expression system (Invitrogen). The sequences of these siRNA were listed in the Supplementary material online. Western blots or functional studies were carried out 72 h after infection.21–23

2.10 Statistical analysis

All data are expressed as the mean ± SEM. Comparisons among groups were analysed by one-way ANOVA, and comparisons of different parameters were made by two-way ANOVA. Student’s t-test was used for differences between two groups. Differences were considered statistically significant at a value of P < 0.05.

3. Results

3.1 Expression of p55γ is decreased in proliferating VSMCs in vitro and in vivo

First, we examined the tissue distribution of p55γ gene expression and found it widely expressed in the cardiovascular system (Supplementary material online, Figure S1A). Relative to the mRNA level of p55γ (a better-studied PI3K regulatory subunit coded by the PIK3R1 gene), p55γ (coded by the PI3KR3 gene) was more abundantly expressed in VSMCs (Supplementary material online, Figure S1B). To define its potential role in vascular biology and disease, we examined the expression profile of p55γ in balloon-injured rat common carotid arteries. Consistent with our previous studies, mild-to-moderate intimal hyperplasia developed at 7 days post-injury and became more severe at 14 days.21 The p55γ protein level was progressively down-regulated in balloon-injured carotid arteries, while its mRNA level was reduced 1 day after injury and maintained at the low level for at least 14 days (Figure 1A and B).

Next, we assessed the responses of p55γ to mitogenic stimuli in primary-cultured rat VSMCs. Treatment of cells with PDGF-BB (10 ng/mL) suppressed the expression of p55γ at the mRNA and protein levels in a time-dependent manner (Figure 1C and D). Similarly, the p55γ mRNA and protein levels were also reduced in response to serum (10% FBS) (Figure 1E and F). These data suggest that reduced expression of p55γ is a generalized VSMC response to proliferative stimuli.

3.2 Overexpression of p55γ suppresses VSMC proliferation

Using adenoviral gene transfer, we manipulated the expression levels of p55γ in VSMCs to further investigate its biological function (Figure 2A). Overexpression of p55γ effectively suppressed the PDGF-BB-evoked increase of VSMC number in a time-dependent manner compared with that of untreated control or a control virus, Ad-GFP (Figure 2B). On average, the PDGF-BB-induced increase in VSMC number was reduced by 50% four days after Ad-p55γ infection (Figure 2B). The growth arrest effect of p55γ overexpression was also confirmed by MTT assay (Figure 2C). Similarly, overexpression of p55γ abrogated serum-induced VSMC proliferation as assayed by cell counting and MTT (Figure 2D and E). These results suggest that up-regulation of p55γ is sufficient to suppress VSMC proliferation.

3.3 p55γ knockdown exacerbates VSMC proliferation

To determine whether down-regulation of p55γ is involved in VSMC hyperproliferation, we performed p55γ gene silencing experiment by adenoviral gene transfer of p55γ shRNA (Figure 2F). Knockdown of p55γ moderately exaggerated the PDGF-BB- or serum-triggered

Figure 1 p55γ expression decreased in proliferating VSMCs in vitro and in vivo. (A) Real-time PCR assays showing that the mRNA level of p55γ was down-regulated in carotid arteries with balloon injury at 1, 7, and 14 days after injury (n = 6; *P < 0.05, **P < 0.01 vs. sham). (B) Representative western blots and averaged data showing down-regulation of p55γ in carotid arteries with balloon injury at 1, 7, and 14 days after injury (n = 5; *P < 0.05, **P < 0.01 vs. sham). (C–F) Real-time PCR assays showing that the mRNA level of p55γ was down-regulated in cultured VSMCs treated with PDGF-BB (10 ng/mL) (C) or serum (10% FBS) (E) at different time points (6, 12, 24, and 48 h; n = 4–6; *P < 0.05, **P < 0.01 vs. control). Representative western blots and averaged data showing down-regulation of p55γ in cultured VSMCs treated with PDGF-BB (10 ng/mL) (D) or serum (10% FBS) (F) at different time points (6, 12, 24, and 48 h; n = 6; *P < 0.05, **P < 0.01 vs. control).
VSMC proliferation in the MTT assay (Figure 2G and H), substantiating the growth arrest effect of p55γ on VSMCs.

3.4 In vivo adenoviral gene transfer of p55γ suppresses injury-induced neointimal formation

To explore potential pathophysiological relevance of the p55γ-induced VSMC growth arrest, we used an in vivo rat carotid artery injury model to test the potential effect of p55γ on neointimal formation. Rat carotid arteries were subjected to balloon injury and simultaneously infected with either Ad-GFP or Ad-p55γ, as described previously. The efficiency of in vivo adenoviral gene transfer of p55γ was confirmed by western blot 4 days after infection (Figure 3A).

The forced expression of p55γ overtly attenuated the balloon injury-induced neointimal formation (Figure 3B). The averaged intima/media ratio with p55γ overexpression decreased to 38% and the neointimal area decreased to 34% of the value in the Ad-GFP group (Figure 3C and D). PCNA immunohistochemical assay showed that overexpression of p55γ markedly suppressed cell proliferation as manifested by a profound reduction in PCNA-positive cells (Figure 3E and F).

3.5 In vivo down-regulation of p55γ enhances injury-induced neointimal thickening

Next, we examined the effect of p55γ knockdown on neointimal thickening in rat carotid artery injury model. Knockdown of p55γ significantly enhanced the balloon injury-induced neointimal formation 2 weeks after injury (Figure 3G and H). Specifically, in vivo p55γ gene silencing increased the averaged intima/media ratio and the neointimal area by 34 and 42% (Figure 3I and J). It also significantly increased the number of PCNA-positive cells (Figure 3K and L).

3.6 p55γ arrests VSMCs in S phase

Moreover, we explored the mechanism underlying the growth arrest effect of p55γ by examining the cell-cycle progression in VSMCs infected with Ad-GFP or Ad-p55γ. Overexpression of p55γ enhanced the accumulation of VSMCs in the S phase. The average percentage of cells in
S phase increased from $21.29 \pm 3.3\%$ in the uninfected control and $24.73 \pm 2.05\%$ in the Ad-GFP control group to $37.06 \pm 2.36\%$ in the Ad-p55 group (Figure 4), suggesting that p55 induces VSMC cell-cycle arrest in S phase, thereby suppressing cell proliferation.

Figure 3 Overexpression of p55γ suppresses VSMC proliferation and inhibits neointimal formation in rat carotid arteries after balloon injury. (A) Representative western blot and averaged data showing expression of p55γ in the rat carotid artery at 4 days after adenovirus Ad-p55γ delivery ($n = 5$; ***$P < 0.01$ vs. Ad-GFP group). (B–D) Representative photomicrographs of haematoxylin and eosin staining (upper panel, 100′; lower panel, 200′; scale bars, 50 mm) (B) and averaged data on the intima/media ratio (I/M) (C) and neointimal area (D) of rat carotid arteries transfected with Ad-GFP or Ad-p55γ 2 weeks after balloon injury. (E and F) Representative photomicrographs of immunohistochemical staining (scale bar, 25 mm) (E) and the averaged data (F) showing the percentage of PCNA-positive cells in rat carotid arteries infected with Ad-GFP or Ad-p55γ 2 weeks after balloon injury. In C, D, and F, $n = 5–7$ for each group; ***$P < 0.01$ vs. Ad-GFP group. (G) Representative western blot showing expression of p55γ in the rat carotid artery at 4 days after adenovirus Ad-p55γ-shRNA1 delivery ($n = 4$; ***$P < 0.01$ vs. Ad-scrambled group). (H–J) Representative photomicrographs of haematoxylin and eosin staining (upper panel, 100′; lower panel, 200′; scale bars, 50 mm) (H) and averaged data on the intima/media ratio (I/M) (I) and neointimal area (J) of rat carotid arteries transfected with Ad-scrambled or Ad-p55γ-shRNA1 2 weeks after balloon injury. (K and L) Representative photomicrographs of immunohistochemistry (scale bar, 25 mm) (K) and the averaged data (L) showing the percentage of PCNA-positive cells in rat carotid arteries infected with Ad-scrambled or Ad-p55γ-shRNA1 2 weeks after balloon injury. In I, J, and L, $n = 7$ for each group; *$P < 0.05$ vs. Ad-scrambled group.

3.7 p55γ inhibits VSMC proliferation through up-regulation of p53

To delineate the molecular mechanism underlying the p55γ-induced suppression of VSMC proliferation, we screened potential targets by identifying its interacting proteins. In HEK293T cells, flag-tagged p55γ was purified in a complex with its associated proteins that were identified by mass spectrometry. Among the constituents, the tumour suppressor p53 was one of the possible interacting proteins (Supplementary material online, Table S1). Furthermore, we conducted Co-IP assays to verify the association of p55γ and p53. A specific p53 band was present in the immunoprecipitated complexes with anti-flag in HEK293T cells when p55γ-flag and p53-myc were co-expressed, and vice versa (Figure 5A), indicating that p55γ specifically binds to p53.

As p53 is a well-known molecule involved in the regulation of cell growth, we explored the possibility of a functional connection between p55γ and p53. Overexpression of p55γ in VSMCs led to an approximately three-fold increase in p53 protein level relative to the control group (Figure 5B). Interestingly, the mRNA level of p53 was not affected by p55γ overexpression in VSMCs (Figure 5C). Moreover, knockdown of p53 fully abolished the p55γ-induced suppression of VSMC proliferation (Figures 5D; Supplementary material online, Figure S2), highlighting that p53 up-regulation is essential for p55γ-induced VSMC growth arrest.

Since p53 is tightly regulated by ubiquitin-dependent degradation,24 we investigated whether p55γ regulates ubiquitin-dependent degradation of p53. Overexpression of p55γ profoundly decreased the amount of ubiquitinated p53 in primary cultured VSMCs (Figure SE) as well as in HCT116 p53+/+ cells with wild-type p53 (Figure Sf). While the protein abundance of p53 was enhanced by inhibiting proteasomes with MG132 (20 μM), p55γ overexpression did not further increase the p53 protein level in HCT116 p53+/+ cells (Supplementary material online, Figure S3), suggesting that p55γ regulates ubiquitination and degradation of p53 via the ubiquitin-proteasome system.
Because the E3 ubiquitin ligase mouse double minute 2 homologue (MDM2) is the most important executor of p53 ubiquitination and degradation,24,25 we then determined whether p55γ-induced ubiquitination of p53 is mediated by MDM2. Indeed, p55γ overexpression significantly abrogated the effect of MDM2 on p53 protein in HCT116 p53+/+ cells (Supplementary material online, Figure S4). In HCT116 p53+/+ cells, Figure 4 Overexpression of p55γ leads to VSMC arrest in S phase. Typical examples and averaged data of the cell-cycle distributions in control VSMCs (A) and VSMCs infected with Ad-GFP (B) or Ad-p55γ (C) (100 m.o.i., 48 h) stimulated with serum for 48 h. Cells were stained with PI and analysed by FACS (n = 6; *P < 0.05 vs. control and Ad-GFP groups).

Figure 5 p55γ interacts with p53 and inhibits MDM2-mediated p53 ubiquitination. (A) Co-IP of p55γ with p53 in HEK293T cells infected with Ad-p55γ-flag or p53-myc-expressing vector as indicated. (B) Representative western blots and averaged data showing enhanced p53 protein in cultured VSMCs infected with Ad-p55γ (100 m.o.i., 48 h) vs. control group (n = 6; **P < 0.01). (C) Real-time PCR assays showing that overexpression of Ad-p55γ did not affect the mRNA level of p53 in cultured VSMCs transfected with Ad-GFP or Ad-p55γ (n = 6). (D) MTT assays showing that the p55γ-induced inhibition of VSMC proliferation was abolished by knockdown of p53 (n = 4; **P < 0.01 vs. Ad-GFP/scrambled; †P < 0.05 vs. Ad-p55γ/scrambled). (E) Overexpression of p55γ inhibited p53 ubiquitination in VSMCs. VSMCs were infected with Ad-GFP or Ad-p55γ, and co-transfected with p53- and ubiquitin (Ub)-expressing vectors as indicated. Cell lysates were harvested after treatment with MG132 (20 μM), immunoprecipitated with anti-p53 antibody, and detected by anti-Ub antibody (n = 4). (F) Overexpression of p55γ blocked MDM2-mediated p53 ubiquitination. Ubiquitination of p53 in HCT116 p53+/+ cells infected with Ad-p55γ (n = 4). (G) Representative western blots showing that the overexpression of p55γ attenuated the MDM2-p53 association. HEK293T cells were infected with Ad-GFP or Ad-p55γ, and co-transfected with MDM2-flag- and p53-myc-expressing vectors as indicated (n = 3).
MDM2 promoted p53 ubiquitination, but the effect was clearly blunted by p55γ overexpression (Figure 5F). As the association between p53 and MDM2 is essential for the regulation of p53 stability by MDM2, we next tested whether p55γ affects the interaction between MDM2 and p53. Overexpression of p55γ profoundly decreased the Co-IP of p53 with MDM2 (Figure 5G), indicating that p55γ stabilizes p53 by attenuating the p53-MDM2 interaction in HEK293T cells. Taken together, these findings demonstrate that p55γ stabilizes p53 by disrupting the p53-MDM2 interaction and inhibiting MDM2-mediated p53 ubiquitination and degradation.

3.8 p55γ suppresses VSMC proliferation via the p53-p21 signalling pathway

Since p21, a primary target gene of p53, is the main cyclin kinase-dependent inhibitor capable of arresting cell-cycle progression, we hypothesized that p21 might be a downstream effector of p55γ since p21, a primary target gene of p53, is the main cyclin kinase-dependent inhibitor capable of arresting cell-cycle progression,26,27 we further investigated whether p55γ affects cell survival, we determined the effect of p55γ overexpression did not affect the viability of VSMCs compared with controls by either the ELISA assay (Figure 7A) or Hoechst staining (Supplementary material online, Figure S5A).

To determine the functional role of p53 in the p55γ-induced up-regulation of p21, we knocked down p53 using siRNA approach (Supplementary material online, Figure S2). Indeed, p53 knockdown significantly abrogated the p55γ-induced p21 up-regulation (Figure 6C). Furthermore, knockdown of p21 completely abolished the p55γ-induced suppression of PDGF-BB- and serum-evoked cell proliferation (Figure 6D–F).

Next we tested the potential interaction between p21 and PCNA, since p21 binds to the DNA polymerase δ processing factor PCNA and blocks DNA synthesis, the key process in S phase. Overexpression of p55γ facilitated the binding of PCNA to p21 as detected by IP (Figure 6G), revealing the mechanism underlying p55γ-induced cell-cycle arrest in S phase. These data indicated that the p55γ-induced inhibition of VSMC proliferation is mediated by activation of the p55γ-p53-p21 signalling pathway.

3.9 p55γ induces Bcl-xl up-regulation

In general, cell growth arrest may be attributable to either increased cell death or reduced cell-cycle progression, or both. To test whether p55γ affects cell survival, we determined the effect of p55γ overexpression on viability by two means, cell death ELISA and Hoechst staining. We found that p55γ overexpression did not affect the viability of VSMCs compared with controls by either the ELISA assay (Figure 7A) or Hoechst staining (Supplementary material online, Figure S5A).

Since Akt is a nodal point of PI3K signalling in regulating cell survival and growth,8,9 we further investigated whether p55γ regulates Akt activation. To our surprise, as a regulatory subunit of PI3K, p55γ did not affect the phosphorylation status of Akt in cultured VSMCs (Supplementary material online, Figure S5B). Furthermore, overexpression of p55γ or knockdown of p55γ did not affect the PI3K activity (Supplementary material online, Figure S5C).

Figure 6 Overexpression of p55γ induces p21 up-regulation. (A) Representative western blots and averaged data showing the p21 protein level in VSMCs infected with Ad-GFP or Ad-p55γ (100 m.o.i., 48 h) (n = 6; ***p < 0.01 vs. control). (B) Real-time PCR assays showing p21 mRNA in VSMCs infected with Ad-GFP or Ad-p55γ (100 m.o.i., 48 h) (n = 5; ***p < 0.01 vs. control). (C) Representative western blots and averaged data showing that the p55γ-induced p21 up-regulation was abolished by p53 knockdown in primary cultured VSMCs (n = 6; ***p < 0.01 vs. Ad-GFP/scrambled; **p < 0.01 vs. Ad-p55γ/scrambled). (D) Representative western blots and averaged data showing the protein level of p21 in VSMCs infected with scrambled or two sets of p21 siRNA (p21 siRNA1 and p21 siRNA2; n = 3; ***p < 0.01 vs. scrambled). (E and F) MTT assays of VSMCs infected with Ad-GFP or Ad-p55γ with or without p21 knockdown upon treatment with PDGF-BB (10 ng/mL) (E) or serum (10% FBS) (F) (n = 4; ***p < 0.01 vs. Ad-GFP/scrambled; **p < 0.01 vs. Ad-p55γ/scrambled). (G) Representative western blots (left) and averaged data (right) showing that overexpression of p55γ enforced endogenous p21-PCNA binding. Co-IP of PCNA with p21 in HEK293T cells infected with Ad-p55γ or Ad-GFP (n = 3; ***p < 0.01 vs. Ad-GFP).
material online, Figure S6), implying that the aforementioned cell biological function of p55γ may be independent of the catalytic subunit of PI3K. Similar to the situation with Akt, the extracellular signal-regulated kinase (ERK) phosphorylation level was not altered by p55γ overexpression in VSMCs (Supplementary material online, Figure SSC).

In addition, we investigated the candidate mechanism underlying the paradoxical up-regulation of p53 and the absence of apoptotic cell death. Surprisingly, p55γ overexpression elevated the protein level of Bcl-xl (Figure 7B), one of the powerful anti-apoptotic proteins. Knockdown of Bcl-xl led to cell death in VSMCs overexpressing p55γ but not GFP (Figure 7C and D), indicating that activation of Bcl-xl may explain the lack of apoptosis in response to p55γ overexpression.

3.10 p55γ regulates p53, p21, and Bcl-xl in balloon-injured rat carotid artery

We next examined the signal of p53, p21, and Bcl-xl in rat balloon-injured arteries in the presence or absence of adenoviral gene transfer of p55γ. The protein levels of p53, p21, and Bcl-xl were significantly increased in rat carotid arteries overexpressing p55γ 1 week after injury (Figure 7E). The p21 mRNA, but not p53 mRNA, was elevated in response to overexpression of p55γ (Figure 7F and G). Thus, similar to the situation of cultured VSMCs, in vivo overexpression of p55γ activates the p55γ-p53-p21 signalling pathway, resulting in suppression of injury-induced VSMCs proliferation and neointimal formation in the balloon injury model.

3.11 p55γ inhibits cell proliferation via p53-p21 in HASMCs

To evaluate the relevance of p55γ in human pathology, we further examined the role of p55γ in HASMCs and found similar results as the case in rat VSMCs. First, in cultured HASMCs, the expression of p55γ declined in response to PDGF-BB stimulation, at both mRNA level and protein level (Figure 8A and B). Second, PDGF-BB-induced cell proliferation of HASMCs was inhibited by overexpression of p55γ compared with control and GFP group, measured by cell count (Figure 8C and D). Furthermore, overexpression of p55γ up-regulated the protein levels of p53, p21, and Bcl-xl in HASMCs (Figure 8E). The p21 mRNA, but not p53 mRNA, was increased by p55γ overexpression (Figure 8F and G). Collectively, consistent with situations in VSMCs from rat, p55γ showed a proliferative inhibitory effect on VSMCs from human, via activating p53-p21 signals.

4. Discussion

4.1 Activation of p55γ-p53-p21 constitutes a novel pathway blocking VSMC proliferation

In this study, we have provided multiple lines of evidence to define a novel role for p55γ in regulating VSMC proliferation. First, p55γ expression was markedly down-regulated by balloon injury in rat arteries.
that activation of the p53-p21 signalling pathway is required for the robust cell-cycle arrest observed in VSMCs. This opposite functional roles of p55γ for this discrepancy merits future investigation. It is noteworthy that, while most studies show that p21 arrests the cell cycle in G1 by inhibiting the cyclin-dependent kinases required for initiation of the S phase, p53-dependent induction of p21 also results in cell-cycle arrest, as p21 binds to PCNA and directly inhibits PCNA-dependent elongation step in DNA replication. In this regard, p21-dependent cell-cycle arrest in S phase has been reported in several cell lines. Here we have shown that p55γ enhances the association of p21 with PCNA, contributing to p55γ-mediated cell-cycle arrest in S rather than G1. The exact underlying mechanism merits future investigation.

In addition, the phosphorylation status of either Akt or ERK was unaffected by overexpression of p55γ, and VSMC viability was unaffected by up-regulation of p55γ concomitantly, suggesting that p55γ does not primarily inhibit cell proliferation via PI3K-Akt or ERK signalling and may have PI3K-independent biological actions.

Figure 8 Overexpression of p55γ inhibits cell proliferation and up-regulates p53-p21 signals in HASMCs. (A and B) The mRNA level of p55γ assayed by real-time PCR (A) and the protein level of p55γ represented by western blot and averaged data (B) in cultured HASMCs treated with PDGF-BB (10 ng/mL) at different time points (6, 12, 24, and 48 h; n = 6; **P < 0.01 vs. control). (C) Representative western blots showing the p55γ expression in cultured HASMCs infected with Ad-GFP (100 m.o.i.) or Ad-p55γ (50 and 100 m.o.i.) for 48 h. (D) Cell counts of HASMCs infected with Ad-GFP or Ad-p55γ (100 m.o.i., 48 h) upon PDGF-BB stimulation at 2, 3, and 4 days (n = 6; ***P < 0.01 vs. control and Ad-GFP groups). (E) Representative western blots and averaged data showing the p53, p21, and Bcl-xl protein level in HASMCs transfected with Ad-GFP or Ad-p55γ for 48 h (n = 6; **P < 0.01 vs. Ad-GFP). (F and G) Real-time PCR assays showing p53 (F) and p21 (G) mRNA in HASMCs transfected with Ad-GFP or Ad-p55γ for 48 h (n = 5–6; ***P < 0.01 vs. Ad-GFP).

and by proliferative stimulation with PDGF-BB and serum in VSMCs. Second, overexpression of p55γ inhibited VSMC proliferation and limited balloon injury-induced neointimal thickening, while in vivo deliver of p55γ shRNA promoted neointimal formation. Finally, p55γ binds and stabilizes p53, subsequently up-regulates p21 expression, thus arresting VSMC cell cycle.

In contrast, up-regulation of p55γ promotes tumour cell growth. This opposite functional roles of p55γ in VSMCs vs. that in cancer cells may reflect cell-host effects as cancer cells have multiple genetic mutations and often behave differently from normal cells. These studies also indicate that p55γ may exhibit distinct functions in different cells and tissues by activating distinct signalling pathways. The underlying mechanism for this discrepancy merits future investigation.

In principle, p55γ-induced growth arrest could be mediated by changes in cell fate, such as cell survival, cell death, or cell-cycle arrest. In terms of cell-cycle regulation, we have demonstrated that p55γ overexpression induces cell-cycle arrest in the S phases by activating the p53-p21 signalling pathway. As a transcription factor, p53 plays a crucial role in cell fate regulation in response to a wide variety of extrinsic and intrinsic stress signals. In particular, p53 serves as a critical regulator of VSMC proliferation. In this study, we have shown that p53 is a downstream signalling event of p55γ. Specifically, p55γ increases p53 protein stability and subsequently enhances its transcriptional activity, as manifested by the increased expression of its target gene p21. Indeed, knockdown of either p53 or p21 blocks the p55γ-induced growth arrest, suggesting that activation of the p53-p21 signalling pathway is required for the p55γ-dependent maintenance of VSMC quiescence. Mechanistically, we have shown that p55γ elevates the p53 protein level via post-translational modification without altering its mRNA level. Since p53 abundance is tightly controlled by MDM2, the major E3 ligase targeting p53 for ubiquitination and degradation, we have investigated the potential relationships of p55γ, p53, and MDM2, and found that p55γ not only binds to p53, but also attenuates its interaction with MDM2, leading to reduced ubiquitination and degradation followed by p53 accumulation. These findings shed new light on our understanding of the fundamental biological functions of p55γ, in addition to its previously reported function in targeting the PI3K catalytic subunit to subcellular locations.

It is noteworthy that, while most studies show that p21 arrests the cell cycle in G1 by inhibiting the cyclin-dependent kinases required for initiation of the S phase, p53-dependent induction of p21 also results in S-phase cell-cycle arrest, as p21 binds to PCNA and directly inhibits the PCNA-dependent elongation step in DNA replication. In this regard, p21-dependent cell-cycle arrest in S phase has been reported in several cell lines. Here we have shown that p55γ enhances the association of p21 with PCNA, contributing to p55γ-mediated cell-cycle arrest in S rather than G1. The exact underlying mechanism merits future investigation.

In addition, the phosphorylation status of either Akt or ERK was unaffected by overexpression of p55γ, and VSMC viability was unaffected by up-regulation of p55γ concomitantly, suggesting that p55γ does not primarily inhibit cell proliferation via PI3K-Akt or ERK signalling and may have PI3K-independent biological actions.
4.3 Potential clinical relevance of p55γ malfunction

In this study, we have demonstrated a causal relationship between p55γ malfunction and vascular proliferative disorder: a reduction in the expression of p55γ promotes, while overexpression of p55γ attenuates, VSMC proliferation and neointimal formation in response to arterial injury or mitogenic stimuli. Because enhanced VSMC proliferation contributes to the development of many proliferative disorders, in particular, in-stent coronary artery restenosis, the failure of coronary or peripheral arterial bypass grafts, and the diffuse narrowing of coronary arteries after cardiac transplantation,1,2,39 anti-proliferative therapy, for instance, stents coated with anti-proliferative agents,40,41 is beneficial in patients with vascular proliferative diseases. In this study, we have shown, for the first time, that up-regulation of p55γ suppresses VSMC growth without triggering apoptotic cell death in vitro and in vivo. Since VSMC apoptosis promotes inflammation, calcification, thrombosis, and plaque rupture, and since neointimal VSMCs show enhanced apoptosis compared with medial VSMCs, limiting VSMC apoptosis is of therapeutic importance in improving plaque stability and in preventing restenosis. Thus, non-apoptotic, anti-proliferative effect of p55γ should provide a potential novel therapy with fewer side effects for treatment of proliferative vascular diseases. What is more, p55γ also inhibits growth of VSMCs from human, increasing its possibility as a therapeutic target in cardiovascular diseases. Taken together, targeting p55γ may provide non-apoptotic approach to inhibit VSMC proliferation and neointimal formation.

In summary, we have defined p55γ, a PI3K regulatory subunit, as an important negative regulator of VSMC proliferation. Up-regulation of p55γ suppresses VSMC proliferation in vitro and in vivo and ameliorates injury-induced neointimal formation in vivo, whereas down-regulation of p55γ exaggerates injury-evoked vascular proliferative growth and neointimal formation. Mechanistically, up-regulation of p55γ leads to concurrent activation of the p55γ-p53-p21 and the p55γ-Bcl-xl signalling pathways with the former of anti-proliferative and the later of anti-apoptotic (Figure 9). Thus, the normal expression and function of p55γ may be responsible for the maintenance of VSMC quiescence. The present findings not only reveal previously unappreciated novel functions of p55γ, but also bear important therapeutic implications in the setting of restenosis and perhaps other vascular proliferative disorders as well.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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4.2 Counterbalance of p55γ-p53 apoptotic signalling by concurrent activation of p55γ-Bcl-xl survival signalling

We have demonstrated that p55γ has a non-apoptotic effect on VSMCs while inhibiting their proliferation, likely due to the concurrent up-regulation of Bcl-xl. It is well-established that members of Bcl-2 family, including Bcl-xl, are critical regulators of apoptosis in VSMCs, and up-regulation of Bcl-xl inhibits VSMC apoptosis.35 Meanwhile, p53 expression is associated with increased VSMC apoptosis in vivo and in vitro.32 Bcl-xl has been reported to inhibit p53-induced apoptosis in diverse cell lines, including MEFs36 and cancer cells,37,38 by preventing the mitochondrial release of cytochrome c and activating execution caspases in a head and neck squamous cell carcinoma cell line38 or by preventing p38 activation in human bladder carcinoma cells.37 In our study, p55γ simultaneously elevates the pro-apoptotic protein p53 and the anti-apoptotic protein Bcl-xl, thus resulting in a non-apoptotic VSMC growth arrest effect.

**Figure 9** Schematic diagram of the signalling pathway responsible for p55γ-induced suppression of VSMC proliferation. p55γ interacts with p53 and attenuates the binding of p53 to MDM2, thus reducing the MDM2-mediated p53 ubiquitination and degradation, resulting in p53 stabilization and activation. Depending on the transcriptional activity of p53, p55γ up-regulates the cell-cycle inhibitor p21 and leads to cell-cycle arrest in S phase, thus suppressing VSMC proliferation. Besides, p55γ concurrently up-regulates the anti-apoptotic protein, Bcl-xl, and counterbalances p53-mediated apoptotic effect. These two effects finally reach a non-apoptotic cell-growth arrest by p55γ.
References


