TRAF6-Mediated SM22α K21 Ubiquitination Promotes G6PD Activation and NADPH Production, Contributing to GSH Homeostasis and VSMC Survival In Vitro and In Vivo

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ABSTRACT

**Rationale:** Vascular smooth muscle cell (VSMC) survival under stressful conditions is integral to promoting vascular repair, but facilitates plaque stability during the development of atherosclerosis. The cytoskeleton-associated smooth muscle (SM) 22α protein is involved in the regulation of VSMC phenotypes, whereas the pentose phosphate pathway (PPP) plays an essential role in cell proliferation through the production of NADPH.

**Objective:** To identify the relationship between NADPH production and SM22α activity in the development and progression of vascular diseases.

**Methods and Results:** We showed that the expression and activity of G6PD is promoted in platelet-derived growth factor (PDGF)-BB-induced proliferative VSMCs. PDGF-BB induced G6PD membrane translocation and activation in an SM22α K21 ubiquitination-dependent manner. Specifically, the ubiquitinated SM22α interacted with G6PD, and mediated G6PD membrane translocation. Furthermore, we found that TNF receptor associated factor (TRAF) 6 mediated SM22α K21 ubiquitination in a K63-linked manner upon PDGF-BB stimulation. Knockdown of TRAF6 decreased the membrane translocation and activity of G6PD, in parallel with reduced SM22α K21 ubiquitination. Elevated levels of activated G6PD consequent to PDGF-BB induction led to increased NADPH generation through stimulation of the PPP pathway, which enhanced VSMC viability and reduced apoptosis in vivo and in vitro via glutathione homeostasis.

**Conclusions:** We provide evidence that TRAF6-induced SM22α ubiquitination maintains VSMC survival through increased G6PD activity and NADPH production. The TRAF6-SM22α-G6PD pathway is a novel mechanism underlying the association between glucose metabolism and VSMC survival, which is beneficial for vascular repair after injury but facilitates atherosclerotic plaque stability.

**Keywords:**
TRAF6, G6PD, SM22α, survival, vascular smooth muscle

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Nonstandard Abbreviations and Acronyms:

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INTRODUCTION

Stable atherosclerotic plaques are characterized by a thick fibrous cap consisting of proliferative vascular smooth muscle cells (VSMCs) and extracellular matrix, which decrease the risk of plaque rupture. However, the progressive accumulation of inflammatory cells and mediators promotes the generation of vulnerable plaques by reducing their collagen content and VSMC survival. VSMC apoptosis increases plaque instability, resulting in a thinner fibrous cap that is heavily infiltrated with inflammatory cells, and an enlarged necrotic core. A previous study showed that the identifiable apoptotic cells in plaques are mainly macrophages, and that the VSMC apoptotic rates therein are only about 2%. This low level of VSMC apoptosis during pathological situations is required for plaque stability and vascular repair after injury. However, the endogenous regulators that maintain low VSMC apoptosis in vivo have not been identified.

Recently, glucose metabolism has been highlighted as part of a novel mechanism involved in the complex regulation of VSMC proliferation. An increase in the rate of glycolysis and impaired glucose oxidation leads to a resistance to apoptosis in the affected vessels. Another study has suggested that glycolysis involving the pentose phosphate pathway (PPP) plays an essential role in platelet-derived growth factor (PDGF)-induced cell proliferation by providing substrates that enhance the mitochondrial reserve capacity, underscoring the integrated metabolic response required for the proliferation of VSMCs in the diseased vasculature. In addition, both restenosis and atherosclerosis are more common in individuals with diabetes than in the general population, suggesting a potential link between an intrinsic glucose metabolic abnormality and the progression of vascular lesions.

Apoptosis induced by oxidative stress is closely related to metabolic response during vascular remodeling and atherosclerosis. The glutathione system removes excess hydrogen peroxide (H$_2$O$_2$) in a dihydronicotinamide adenine dinucleotide phosphate (NADPH)-dependent manner, which reduces apoptosis. The main source of NADPH is the PPP, which contributes to the maintenance of the cardiovascular cell redox state and its contractility. Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme in PPP, and plays an essential role in the oxidative stress response. The activation of G6PD stimulates PPP to supply cytosolic NADPH to counteract oxidative damage and protect erythrocytes. G6PD-derived NADPH in vascular tissues is also involved in the NADPH oxidase activity induced by protein kinase C and angiotensin II, which contributes to hypertension and aortic smooth muscle hypertrophy. An increase in the activity of G6PD is generally considered to be protective for cardiovascular functions, especially under pathological conditions. However, the mechanisms responsible for regulating G6DP activity and maintaining vascular homeostasis are not fully understood.

In the present study, we elucidated the relationship between the function of SM22α, a cytoskeleton-associated protein and a marker of VSMCs, and changes in glucose metabolism related to oxidative stress in the development and progression of vascular diseases. We showed that SM22α was ubiquitinated by TNF receptor associated factor (TRAF) 6 in proliferative VSMCs. The ubiquitinated SM22α interacted with G6PD, and simultaneously mediated its membrane translocation and activation,
contributing to proliferative VSMC survival in vivo and in vitro. Our findings are important to advance our understanding of SM22α-mediated molecular events that contribute to restenosis and other proliferative vascular diseases.

METHODS

An expanded Materials and Methods section is available in the Online Data Supplement.

The SM22α−/− mouse line (B6.129S6-Taglntm2(cre)Yec/J) which has a Cre-recombinase gene inserted into the endogenous transgelin (SM22α) locus was purchased from The Jackson Laboratory.

The cDNA of SM22α from rat and its ubiquitination site mutant K21R were cloned into Ad/CMV/V5-DEST vectors to obtain replication-defective adenoviruses according to the manufacturer’s instructions; these were named Ad-GFP-SM22α and Ad-GFP-K21R, respectively.

VSMCs were transduced for 24 hours with the respective adenovirus constructs, using a multiplicity of infection of 100. VSMC apoptosis was determined by TUNEL assay. For in vivo analyses using a carotid artery ligation model, 25% pluronic F-127 gel containing the adenovirus at a concentration of 10^10 pfu/mL was spread evenly around the outside of the left carotid arteries of the subject mice. Carotid arteries were harvested 14 days after ligation.

Data are presented as means ± SD. ANOVA and unpaired t tests were used for statistical analysis as appropriate. P<0.05 was considered statistically significant.
RESULTS

G6PD activity and GSH level increases in proliferative/synthetic VSMCs.

It has previously been shown that PDGF increases G6PD activity in rat renal cortical cells. To determine the characteristics of glucose metabolism in neointimal formation, transcriptome profiling of 28-day balloon-injured carotid arteries in rats was performed by RNA-Seq. We found that 25% of PPP enzymes were significantly changed, which was higher than the percentage of glycolysis enzymes (10%) altered between balloon-injured and sham groups. In addition, the expression of most (5 of 6) significantly altered PPP enzymes was increased, including a 1.16-fold increase in G6PD in balloon-injured carotid artery (Table 1), implying that the flux of PPP might be upregulated in the injured artery. To verify this finding, cultured VSMCs in vitro were stimulated with PDGF-BB (10 ng/mL) for different durations (0–48 hours). The results showed that the expression of G6PD increased at 6 hours and peaked at 12 hours upon PDGF-BB stimulation, accompanied by associated degrees of increased G6PD activity (Figure 1A and B). G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate and concomitantly reduces NADP⁺ to NADPH. We further showed that NADPH generation paralleled the change of increased G6PD activity in PDGF-BB-induced VSMCs (Figure 1C). In addition, G6PD activation is characterized by translocation from its cytosolic position to its site of activity on the cellular membrane. This translocation allows for entering glucose to shunt rapidly to the PPP, thereby providing substrate for G6PD. To provide further evidence that PDGF-BB induces the activation of G6PD, the membrane translocation of G6PD was detected using a membrane fractionation assay. The results showed that the level of G6PD in the cell membrane fraction increased coincident with the increase in G6PD enzyme activity following PDGF-BB stimulation (Figure 1D). This increased distribution of G6PD in the cellular membrane region was also observed by immunofluorescence staining and confocal microscopy (Figure 1E). These data suggest that both the expression and activity of G6PD is upregulated in proliferative VSMCs.

SM22α is required for PDGF-BB-induced G6PD activation.

To determine whether the disruption of SM22α promotes PDGF-BB-induced G6PD membrane translocation, VSMCs were first transduced with Ad-GFP-shSM22α to specifically silence SM22α expression, and then stimulated with PDGF-BB. However, knockdown of SM22α resulted in decreased G6PD membrane translocation and activity (Figure 2A and B). To determine whether SM22α is directly related to the membrane translocation of G6PD, we detected its distribution in the cell membrane region. Although the level of total SM22α protein in VSMCs decreased upon PDGF-BB stimulation, the distribution of SM22α in the cellular membrane fractions increased in a similar manner to that of G6PD (Figure 2C). Therefore, to determine whether SM22α directly interacts with G6PD, we performed immunofluorescence staining. The results showed that SM22α colocalized with G6PD in VSMCs, and that the putative SM22α-G6PD complex was recruited to the cellular membrane region upon PDGF-BB stimulation for 12 hours; this recruitment was reduced following knockdown of SM22α (Figure 2D). Coimmunoprecipitation of the membrane extracts using anti-SM22α antibody further supported an
interaction of SM22α with G6PD that increased upon PDGF-BB stimulation (Figure 2E). Next, to verify that SM22α is required for G6PD activation, we detected the membrane translocation and activity of G6PD in VSMCs from SM22α knockout (SM22α−/−) mice. Loss of SM22α resulted in reduced G6PD translocation to the cell membrane and activity (Figure 2F and G), accompanied by decreased NADPH generation (Figure 2H). These results suggest that SM22α is involved in G6PD membrane translocation and activation via its interaction with G6PD. To determine whether this mechanism is unique to VSMCs or has broader applications in other cells, we examined this effect in vascular adventitial fibroblasts (VAFs) that express SM22α. From this, we found that the changes of G6PD distribution and activity in VAFs were similar to those observed in VSMCs under the same conditions (Online Figure IA-D). Furthermore, the knockdown of SM22α with Ad-GFP-shSM22α in VAFs resulted in a decrease in G6PD activity and NADPH generation (Online Figure IE-G). These findings suggest that SM22α mediation of G6PD activation might represent a general mechanism shared among vascular cells.

**SM22α K21 ubiquitination is associated with PDGF-BB-induced activation of G6PD.**

Previous studies have demonstrated that SM22α expression is regulated primarily at the transcriptional level, and is inhibited following PDGF-BB stimulation.16-18 Here, we further identified that the expression of SM22α at both the mRNA and protein level was decreased in VSMCs after PDGF-BB stimulation for 3 and 12 hours (Online Figure II and Figure 2C). Furthermore, we recently found that SM22α was polyubiquitinated after 6 hours of PDGF-BB stimulation.19 It has been shown that K48-linked polyubiquitination marks its target protein for proteasomal degradation, whereas K63-linked polyubiquitination have emerged as important mechanisms to control the localization or function of the protein.20,21 To identify the ubiquitinated form of SM22α, 293 cells were transfected with HA-SM22α along with His-Ub, His-Ub K6R, His-Ub K11R, His-Ub K48R, or His-Ub K63R constructs, and treated with the proteasome inhibitor MG132 for 4 hours before harvesting. The ubiquitination of SM22α was mainly found to occur via the K63-linked ubiquitin chain (Figure 3A and Online Figure III). To further verify the K63-linked ubiquitination of SM22α, cultured VSMCs were stimulated with PDGF-BB for different times and the ubiquitination profile of SM22α was tested using K63 polyubiquitin-specific antibodies.22 This demonstrated that the level of K63-polyubiquitinated SM22α was increased following PDGF-BB stimulation in a time-dependent fashion with a peak at 6 hours, similar to the pattern obtained for total ubiquitinated SM22α (Figure 3B). These results indicate that ubiquitination might regulate the activity of SM22α rather than its degradation in PDGF-BB-stimulated VSMCs.

Sequence analysis revealed 5 potential ubiquitination sites in the CH domain (1–151) of SM22α. To determine the potential region for the ubiquitination of SM22α, 293 cells were transfected with His-Ub along with HA-SM22α WT, SM22α1–151, or SM22α152–201 mutants before harvesting. The SM22α152–201 mutant devoid of the actin-binding calponin homology (CH) domain failed to be ubiquitinated (Figure 3C). Of the five lysine (K) residues within the CH domain of SM22α, the mutation of K21 to arginine (R) reduced SM22α ubiquitination to the greatest degree (Figure 3D). The K21 residue is well conserved from drosophila to humans (Online Figure IV). We speculated that K21 might be a key and evolutionarily preserved site for regulating SM22α activity in a ubiquitin-dependent manner.

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To confirm our hypothesis, we transduced the SM22α K21 site-mutant adenovirus Ad-GFP-SM22α K21R into VSMCs and VAFs, respectively, followed by PDGF-BB stimulation. The results showed that the PDGF-BB-induced membrane translocation of G6PD was abolished by overexpression of the SM22α K21R mutant (Figure 3E and Online Figure VA), accompanied by a reduction of G6PD activity in both VSMCs and VAFs (Figure 3F and Online Figure VB). Subsequently, in order to eliminate the effects of endogenous SM22α, cells from SM22α-/- mice were used. Expression of SM22α WT, but not SM22α K21R, rescued G6PD membrane translocation and activity in the VSMCs of SM22α-/- mice (Figure 3G and H). Furthermore, we found that G6PD interacted with SM22α WT but not SM22α K21R (Figure 3I). These data indicate that the ubiquitination of SM22α at K21 might facilitate G6PD activation.

**TRAF6 mediates K63-linked ubiquitination of SM22α.**

To elucidate the mechanism underlying K63-linked ubiquitination of SM22α, we screened a panel of E3 ubiquitin ligases: Mdm2, TRAF6, and Smurf2, which catalyze K63-linked ubiquitination in signaling molecules induced by growth factor stimulation. We found that knockdown of TRAF6 by specific siRNA, but not Mdm2 or Smurf2 knockdown, resulted in decreased PDGF-BB-induced ubiquitination of SM22α (Figure 4A and B). To further verify this finding, 293 cells were transfected with HA-SM22α along with Flag-TRAF6 or the Flag-TRAF6 C70A mutant, which has lost E3 ligase activity. SM22α K63-linked ubiquitination was increased in TRAF6-expressing cells, but not in the cells transfected with the TRAF6 C70A mutant (Figure 4C). In addition, we found that PDGF-BB induced the interaction of TRAF6 with SM22α, with a peak at 6 hours (Figure 4D), consistent with the observed increase in K63-linked ubiquitination (Figure 4C). This interaction was independently verified by a GST pull-down assay in vitro (Figure 4E), or in 293 cells co-transfected with HA-SM22α along with Flag-TRAF6 or the Flag-TRAF6 C70A mutant (Figure 4F). Taken together, these results suggest that TRAF6 mediates the ubiquitination of SM22α K21 via a K63-linked ubiquitin chain.

To further determine whether TRAF6-mediated ubiquitination of SM22α is involved in the activation of G6PD, VSMCs were transfected with TRAF6 siRNA, and then stimulated with PDGF-BB. Knockdown of TRAF6 decreased the membrane translocation of G6PD induced by PDGF-BB (Figure 4G). The distribution of G6PD and the SM22α-G6PD complex at the cell membrane decreased in siTRAF6-transfected VSMCs, which was verified by immunofluorescence staining (Figure 4H). Furthermore, knockdown of TRAF6 resulted in a decrease in G6PD activity and NADPH production (Figure 4I and J). To demonstrate whether the ubiquitinated form of SM22α directly promotes G6PD membrane binding and activation, we analyzed the membrane extracts by co-immunoprecipitation with an anti-G6PD antibody followed by immunoblotting with anti-Ub and anti-SM22α antibodies. The results showed that the G6PD-bound SM22α protein represented the ubiquitinated form, which was decreased in the membrane extracts of siTRAF6-transfected VSMCs (Online Figure VI), consistent with the decreased membrane translocation and activity of G6PD observed in these cells (Figure 4G-I). These data suggest that TRAF6-mediated ubiquitination of SM22α is required for the activation of G6PD.
SM22α ubiquitination facilitates GSH homeostasis and VSMC survival.

As previously described, G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate and concomitantly reduces NADP⁺ to NADPH, which is involved in the conversion of oxidized glutathione to its reduced form (GSH). We showed that GSH generation increased at 12 hours, and peaked at 24 hours (Figure 5A), in parallel with the increased G6PD activity and NADPH level in PDGF-BB-induced VSMCs (Figure 1B and C). In addition, the expression of glutathione reductase increased in VSMCs upon PDGF-BB stimulation (Online Figure VII). To determine whether the ubiquitination of SM22α is associated with PDGF-BB-induced GSH production, Ad-GFP-SM22α WT or K21R mutant constructs were transduced into the VSMCs of SM22α-/- mice. Compared with SM22α WT-transduced cells, the generation of GSH was decreased in SM22α K21R-expressing SM22α-/- VSMCs (Figure 5B). Furthermore, knockdown of TRAF6 decreased the GSH generation induced by PDGF-BB (Figure 5C). These results indicate the TRAF6-mediated SM22α ubiquitination facilitates GSH homeostasis. The lower GSH level was consistent with the higher ROS production observed in SM22α-/- cells subjected to PDGF-BB (Online Figure VIII A and B). Notably, re-expression of SM22α WT, but not the K21R mutant, suppressed ROS production in SM22α-/- cells upon PDGF-BB stimulation (Online Figure VIIIB). However, although NADPH can also serve as a substrate for the NADPH oxidase (Nox) family, and VSMCs express both Nox1 and Nox4,27 SM22α ubiquitination-facilitated NADPH generation did not affect the expression and activity of Nox1 and Nox4 in either SM22α WT or SM22α-/- VSMCs (Online Figure VIIIC and D).

GSH homeostasis is required for cell survival and proliferation.28 In the present study, we identified that BrdU incorporation in VSMCs from SM22α-/- mice increased after PDGF-BB stimulation, and decreased following re-expression of SM22α WT but not K21R mutant protein (Online Figure VIIIIE). However, TUNEL staining showed that the number of cells undergoing apoptosis were markedly increased in VSMCs from SM22α-/- mice compared with those from WT mice (1.8 ± 0.8% versus 8.2 ± 2.7%), and reduced following the expression of exogenous SM22α WT but not K21R mutant protein (Figure 5D and E). These results suggest that SM22α ubiquitination is beneficial to cell survival, which might be related to the increase in GSH production.

To demonstrate that the effect of SM22α ubiquitination on cell survival is mediated directly through G6PD activity, we used a competitive G6PD inhibitor, 6-aminonicotinamide (6AN) to treat VSMCs. Inhibiting G6PD by 6AN enhanced the inhibitory effect of SM22α on cell proliferation, and led to a further decrease of BrdU incorporation in SM22α WT-expressing SM22α-/- VSMCs (Figure 5F and Online Figure IXA-B). However, the latter cells also exhibited increased levels of TUNEL-staining (Figure 5G). These results indicated that G6PD mediates the positive effect of SM22α ubiquitination on cell survival.
SM22α ubiquitination inhibits apoptosis via G6PD activation and GSH production during neointimal formation in SM22α−/− mice.

To determine the relationship between SM22α ubiquitination and the expression and activity of G6PD during neointimal formation, a mouse carotid artery ligation model was prepared. In both WT and SM22α−/− mice, ligation induced upregulated G6PD and TRAF6 expression in the carotid artery at 14 days (Online Figure XA). Restoration of SM22α WT but not K21R expression in SM22α−/− carotid arteries further enhanced injury-induced G6PD activity (Figure 6A), accompanied by increased generation of NADPH and GSH (Figure 6B and C). The disruption of SM22α increased ROS production in the ligated carotid arteries (Online Figure XB); ROS production was inhibited by the re-expression of SM22α WT but not K21R mutant protein in SM22α−/− carotid arteries, whereas Nox1 and Nox4 expression and activity remained unchanged (Online Figure XC and D).

We next examined the effects of SM22α ubiquitination on the vascular hypertrophy concomitant to ligation, and found that the re-expression of SM22α WT but not K21R mutant protein inhibited injury-induced neointimal formation in SM22α−/− carotid arteries (Online Figure XE). To elucidate the mechanism by which ubiquitinated SM22α-induced G6PD activation improves VSMC survival in vivo, we performed BrdU incorporation and TUNEL staining analyses. The increased numbers of BrdU-positive cells observed in the carotid arteries of SM22α−/− mice following ligation were decreased by re-expression of SM22α WT but not K21R mutant protein (Online Figure XF), although the PPP provides nucleotide precursors for cell proliferation, consistent with our previous findings.16 Similarly, the percentage of TUNEL-stained cells also decreased under the same conditions (Figure 6D). Furthermore, G6PD inhibition by 6AN enhanced the inhibitory effect of SM22α on BrdU incorporation in carotid arteries from SM22α−/− mice after ligation (Figure 6E and Online Figure XG-H). However, increased numbers of TUNEL-stained cells were observed in 6AN-treated SM22α−/− mice upon re-expression of SM22α WT (Figure 6F). Taken together, these findings suggest that the ubiquitination of SM22α inhibits VSMC apoptosis during neointimal formation in vivo.
DISCUSSION

Our previous studies have demonstrated that SM22α, a marker of VSMCs, is involved in the regulation of inflammation, proliferation, and oxidative stress responses in addition to actin dynamics through alteration of its protein expression or protein modification in VSMCs. These findings suggest a functional link between SM22α and VSMC phenotypes. The current study uncovers a biochemical mechanism by which G6PD is activated subsequent to TRAF6-mediated SM22α ubiquitination, which promotes G6PD membrane translocation and glucose metabolism via the PPP. The increased generation of NADPH by the PPP contributes to the maintenance of cytosolic glutathione stores and subsequent cellular survival in proliferative VSMCs (Figure 7).

In the present study, we first analyzed the transcriptome profiling by RNA-Seq of balloon-injured carotid arteries in rats, and found that 25% of PPP enzymes were significantly changed between the balloon-injured and sham groups. The majority of these significantly altered enzymes (83%) including G6PD were upregulated, suggesting that PPP is activated in the injured artery. Glucose metabolism is vital for cell survival and function, as it provides substrates and energy for numerous enzymatic reactions. In unstressed cells, PPP is greatly inhibited whereas glycolysis represents the major carbon metabolic pathway. After stimulation, e.g., by stress response pathways, the glycolysis pathway is blocked, driving glucose flux into the PPP. G6PD, as the first rate-limiting enzyme in PPP, is a target for regulating glycolysis and PPP flux. Here, we demonstrated that G6PD is activated in response to PDGF-BB stimulation in VSMCs, accompanied by the increased translocation of G6PD from the cytosol to the cell membrane. This translocation allows glucose to be rapidly shunted to the PPP, thereby facilitating the generation of NADPH.

A previous study has demonstrated that the inhibition of G6PD activity prevented hypoxia-induced downregulation of SM22α expression, implying a negative correlation between G6PD activity and SM22α expression. In the present study, we determined that G6PD activity is regulated by SM22α, and showed that the membrane translocation and activation of G6PD is dependent on SM22α in proliferative VSMCs. Although SM22α expression is reduced in PDGF-BB-stimulated VSMCs, even low levels of endogenous SM22α can function as a adaptor to promote G6PD activation. Previously, we have reported that SM22α is ubiquitinated in PDGF-BB-stimulated VSMCs. Protein ubiquitination is an important posttranslational modification that regulates various biological functions. Although ubiquitination such as through K48 of the ubiquitin chain generally results in protein degradation, other types of ubiquitination including through K63 are important for signaling activation and protein trafficking. Here, we demonstrated that the ubiquitination of SM22α in a K63-linked manner promotes the activation of G6PD as well as slightly increasing in its expression, thus contributing to VSMC survival in vitro and in vivo. Furthermore, we identified K21 as an important regulatory ubiquitination site within the SM22α protein, as PDGF-BB-induced membrane translocation of G6PD was abolished by overexpression of the SM22α K21R mutant. Thus, we propose that ubiquitin modification at this site enables SM22α to acquire the ability of facilitating G6PD activation. The effect of SM22α might also be amplified by its ubiquitination, in which even low SM22α level could increase in the PPP flux to supply NADPH.
study reveals an intriguing mechanism wherein the residual SM22α regulates G6PD activity in an ubiquitination-dependent manner, directly accounting for the positive effect of SM22α ubiquitination on VSMC survival through G6PD-mediated NADPH production.

TRAF6, which induces Lys63-linked polyubiquitination of its substrates, is an important regulator of VSMC physiology.35,36 Previous studies have demonstrated that TRAF6 deficiency reduces neointimal formation and remodeling after carotid artery ligation by inhibiting inflammatory cell infiltration and matrix degrading protease activity.37 TRAF6 knockdown suppressed the CD40L-induced proinflammatory phenotype of SMCs by inhibiting NF-κB activation.37 In addition, both in vivo and in vitro data showed that TRAF6 deficiency prevented injury-induced SMC apoptosis but did not affect SMC proliferation and migration.38 These findings suggest that TRAF6 might be a negative regulator of VSMC survival and vascular remodeling. However, another study found that TRAF6 silencing inhibited IL-1β-induced angiogenesis in mice in vivo,39 implying a protective role of TRAF6 in vascular cell survival. Here, we showed that TRAF6-mediated SM22α K63-linked ubiquitination facilitates the membrane translocation and activation of G6PD in VSMCs induced by PDGF-BB. These contradictory findings can be explained by the varied effects of TRAF6 substrates and signaling pathways, which have opposite effects on cell survival during vascular remodeling. In the present study, we demonstrated that SM22α is an endogenous substrate of TRAF6, which couples glucose metabolism to VSMC survival. Although the expression of SM22α is significantly downregulated in proliferative VSMCs, the residual protein is still able to effectively exert a role in metabolism regulation that is different from its intrinsic function in stabilizing the actin cytoskeleton. Thus, TRAF6-mediated K63-linked ubiquitination contributes to the precise regulation of glucose metabolism homeostasis during vascular repair after injury.

NADPH is primarily generated from the PPP, aside from several other lesser-known mechanisms dependent upon the presence of mitochondria. NADPH provides the reducing equivalents for biosynthetic reactions and the oxidation-reduction necessary for protection against reactive oxygen species (ROS) toxicity, allowing the regeneration of GSH. It has been suggested that NADPH might be involved in mediating angiotensin II-induced hypertension and smooth muscle hypertrophy.13 In the present study, we found that SM22α ubiquitination inhibited ROS production through increased G6PD activity and NADPH generation in both SM22α WT and SM22α−/− VSMCs in vivo and in vitro. These increases might inhibit mitochondrial oxidative stress because of the unchanged activities of Nox1 and Nox4. In addition, a recent study has shown that NADPH has a protective effect on human vascular endothelial cells under the condition of high-glucose-induced apoptosis by upregulating GSH production.40 Consistent with this, our findings support the notion NADPH production inhibits VSMC apoptosis during neointimal formation. Furthermore, both in vivo and in vitro data showed that ubiquitination of SM22α inhibited proliferation but increased the number of VSMCs, which was related to a decreased level of apoptosis through increased generation of NADPH and GSH. These results indicate that the promotion of NADPH generation through SM22α ubiquitination is required to maintain the low level of VSMC apoptosis during neointimal formation.
In summary, we provide evidence that TRAF6-mediated SM22α ubiquitination maintains VSMC survival through increasing G6PD expression and activity and thus increasing NADPH production, which enhances the glutathione system (Figure 7). We demonstrate the important role of SM22α in suppressing VSMC apoptosis, and identify SM22α as a link between glucose metabolism and VSMC survival. The TRAF6-SM22α-G6PD pathway is beneficial for vascular repair after injury as well as for plaque stability during the development of atherosclerosis. Our study also identifies potential therapeutic targets in the pathway activated by SM22α. Further studies are needed to determine whether the SM22α-dependent induction of G6PD expression is a universal mechanism involved in the phenotypic modulation of SMCs in vascular diseases such as experimental and naturally occurring atherosclerosis.

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DISCLOSURES
None.
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Table 1. Fold change of gene expression ratios that were obtained by RNA-seq.

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FIGURE LEGENDS

**Figure 1.** PDGF-BB induces G6PD activation and translocation to the cellular membrane. VSMCs were treated with PDGF-BB for the indicated time points. A, G6PD expression. *P*<0.05 vs. 0 h. B, G6PD enzyme activity. *P*<0.05 vs. 0 h. C, NADPH levels. *P*<0.05 vs. 0 h. D, Subcellular localization of endogenous G6PD. E, Subcellular localization of endogenous G6PD visualized by immunofluorescence. Scale bar, 50 μm. Data are expressed as means ± SD from 3 independent experiments (n = 3).

**Figure 2.** SM22α is required for PDGF-BB-induced G6PD activation. A and B, The expression (A) and activity (B) of G6PD in the membrane fractions of untreated VSMCs and those in which endogenous SM22α was silenced by Ad-GFP-shRNA, with (+) or without (-) PDGF-BB treatment. WCE, whole-cell extracts. C, The expression of G6PD and SM22α in membrane fractions of VSMCs treated with PDGF-BB for the indicated time periods. D, Colocalization of G6PD and SM22α was visualized by immunofluorescence. Scale bar, 50 μm. E, Coimmunoprecipitation for the interaction of SM22α with G6PD in the membrane proteins of VSMCs. F, Expression of G6PD and SM22α in the membrane fractions from WT and SM22α−/− VSMCs. G, G6PD activity of VSMCs treated as in panel F. H, NADPH levels of VSMCs treated as in panel F. Bar graphs show the means ± SD from 3 independent experiments (n = 3). *P*<0.05.

**Figure 3.** SM22α K21 ubiquitination is associated with PDGF-BB-induced activation of G6PD. A, In vivo ubiquitination was analyzed in HEK293 cells transiently transfected with His-tagged WT, K63R, and K48R ubiquitin plasmids in the presence of HA-SM22α, and incubated with MG132 for 4 hours before harvesting. B, Complexes immunoprecipitated (IP) with anti-SM22α antibodies were immunoblotted (IB) with a Lys63 (K63)-linked polyubiquitin-specific antibody. C, HEK293 cells were transfected with His-Ub plasmid in the presence of HA-SM22α WT or mutant proteins 1–151 or 152–201. Ub-conjugated proteins were analyzed by IB with an anti-HA antibody. D, HEK293 cells were transfected with His-Ub along with HA-SM22α or various HA-SM22α mutants, and subjected to ubiquitination assay. E, Expression of G6PD in the membrane fraction of VSMCs transduced with Ad-GFP-SM22α WT or K21R, with or without PDGF-BB treatment. F, G6PD activity of VSMCs treated as in panel E. *P*<0.05. G, Expression of G6PD in the membrane fraction of SM22α−/− mice VSMCs transduced with Ad-GFP-SM22α WT or K21R, and then treated with PDGF-BB. H, G6PD activity of VSMCs from SM22α−/− mice treated as in panel G. *P*<0.05. I, Coimmunoprecipitation for the interaction of SM22α with G6PD in VSMCs from SM22α−/− mice treated as in panel G. Bar graphs show the means ± SD from 3 independent experiments (n = 3). WCE, whole-cell extracts.

**Figure 4.** TRAF6 mediates K63-linked ubiquitination of SM22α, contributing to G6PD activation. A, The ubiquitination of SM22α in VSMCs transfected with siRNAs of various E3 ligases (Mdm2, TRAF6, or Smurf2). WCE, whole-cell extracts. B, SM22α ubiquitination. C, HEK293 cells were transfected with His-Ub and HA-SM22α plasmids in the presence of Flag-TRAF6 WT or C70A. Ub-conjugated proteins were analyzed by immunoblotting with anti-HA (top) antibody. SM22α and TRAF6 expression in WCEs

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was determined with anti-HA and anti-Flag antibodies, respectively (bottom). D, Coimmunoprecipitation for the interaction of SM22α with TRAF6 in VSMCs. E, GST pull-down assays for the interaction of TRAF6 with SM22α. GST fusion proteins were analyzed by Coomassie staining. F, HEK293 cells were transfected with HA-SM22α and Flag-TRAF6 or Flag-TRAF6 C70A, and used for immunoprecipitation with a Flag antibody, followed by immunoblot analysis. G, G6PD and SM22α expression in the membrane fraction of VSMCs following knockdown of TRAF6. H, Colocalization of SM22α with G6PD and TRAF6 in VSMCs with or without TRAF6 knockdown. Scale bar, 50 μm. I, G6PD activity of VSMCs treated as in panel G. *P<0.05. Bar graphs show the means ± SD from 3 independent experiments (n = 3).

**Figure 5. SM22α ubiquitination facilitates GSH homeostasis and VSMC survival.** A, GSH levels in VSMCs treated with PDGF-BB for the indicated time points. *P<0.05 (vs. 0 h). B, GSH levels in SM22α−/− VSMCs transduced with Ad-GFP-SM22α WT or K21R. C, GSH level in PDGF-BB-treated VSMCs following knockdown of TRAF6. D and E, SM22α−/− VSMCs were transduced with Ad-GFP-SM22α WT or K21R, double-stained with TUNEL and DAPI, and observed under a confocal microscope (D). Scale bar, 50 μm. Five fields were randomly selected in each group, and the numbers of TUNEL (red) and DAPI (blue) double-positive cells were counted (E). F, G6PD activity in SM22α−/− VSMCs after 6AN treatment (1 mM, 12 hours). G, TUNEL-Red positive cells in VSMCs from SM22α−/− mice transduced with Ad-GFP-SM22α WT. Data are expressed as means ± SD from 3 independent experiments (n = 3). *P<0.05

**Figure 6. SM22α ubiquitination inhibits apoptosis via G6PD activation and GSH production in neointima of SM22α−/− mice.** Left common carotid arteries of SM22α WT and SM22α−/− mice were ligated for 14 days, and used for the following analysis. A, G6PD activity. B and C, Levels of NADPH (B) and GSH (C). D, TUNEL assay. Red inserts show medial apoptotic VSMCs. Scale bar, 50 μm. E, G6PD activity in ligated carotid arteries after 6AN treatment (100 mg/kg, 12 hours). F, TUNEL assay in the ligated carotid arteries after 6AN treatment. Red inserts show medial apoptotic VSMCs. Scale bar, 50 μm. All data are expressed as means ± SD (n=18). *P<0.05.

**Figure 7. A working model for G6PD activation in proliferative VSMCs.**
Novelty and Significance

What Is Known?

- Apoptosis induced by oxidative stress is closely related to metabolic responses during vascular remodeling and atherosclerosis.
- Smooth muscle (SM) 22α, a marker of vascular smooth muscle cells (VSMCs), is involved in the regulation of inflammation, proliferation, and oxidative stress response in addition to actin dynamics through alteration of its protein expression or protein modification in VSMCs.

What New Information Does This Article Contribute?

- Platelet-derived growth factor (PDGF)-BB induces SM22α ubiquitination in VSMCs; ubiquitinated SM22α interacts with glucose-6-phosphate dehydrogenase (G6PD) and facilitates its membrane translocation and activation.
- Tumor necrosis factor receptor associated factor 6 (TRAF6) mediates SM22α K21 ubiquitination in a K63-linked manner.
- SM22α ubiquitination maintains VSMC survival through increased G6PD activity and NADPH production in vivo and in vitro, whereas VSMCs from SM22α−/− mice display increased apoptosis.

VSMC apoptosis increases the instability of plaques during the development of atherosclerosis; an intrinsic abnormality in glucose metabolism is also important for the progression of vascular lesions. Our present work provides evidence that the TRAF6-SM22α-G6PD pathway is a novel mechanism in which SM22α provides a link between glucose metabolism and VSMC survival. We found that activated TRAF6 triggers ubiquitination of SM22α, which is prerequisite to membrane recruitment of G6PD. This translocation allows for entering glucose to shunt rapidly to the pentose phosphate pathway, thereby providing substrate for G6PD and subsequent NADPH generation. This finding expands our knowledge about the functions of SM22α, which, in addition to its role in stabilizing the actin cytoskeleton, acts to inhibit VSMC hypertrophy and proliferation. The ubiquitination of SM22α represents a potential novel therapeutic avenue for facilitating vascular repair after injury and modulating plaque stability during the development of atherosclerosis.
TRAF6-Mediated SM22α K21 Ubiquitination Promotes G6PD Activation and NADPH Production, Contributing to GSH Homeostasis and VSMC Survival In Vitro and In Vivo

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Supplemental Material

TRAF6-mediated SM22α K21 ubiquitination promotes G6PD activation and NADPH production, contributing to GSH homeostasis and VSMC survival in vitro and in vivo

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Detailed Methods

Reagents

Recombinant human PDGF-BB was obtained from R&D Systems Inc. (Minneapolis, MN). 6AN (A68203) and MG132 (M7449) were purchased from Sigma-Aldrich. Antibodies were purchased from suppliers as follows: anti-SM22α (ab14106), anti-Nox1 (ab131088), anti-Nox4 (ab133303) and anti-GR (ab84963) were from Abcam; anti-His (SAB4600113), anti-HA (H3663), anti-Flag (F1804), anti-GFP (G1546), anti-GST (G7781) were from Sigma-Aldrich; anti-K63Ub (PW0600) was from Enzo Life Sciences; anti-Mdm2 (T0175), anti-Smurf2 (2078-1), anti-Na⁺-K⁺-ATP (2047-1) were from Epitomics; anti-G6PD (sc46971), anti-TRAF6 (sc7221), anti-Ub (sc9133), anti-β-actin (sc47778) and horseradish peroxidase (HRP)-linked anti-rabbit (sc-2030) or anti-mouse (sc-2031) antibodies were from Santa Cruz Biotechnology. TRITC (03-15-06)- or FITC (02-13-06)-conjugated secondary antibodies were from KPL (Kirkegaard & Perry Laboratories, Inc). DyLight 649 AffiniPure goat anti-mouse antibody (E032610) was from EarthOx. Flag-TRAF6 and Flag-TRAF6C70A were gifts from Dr. Hui-Kuan Lin. His-Ub was gift from Dr. Jaewhan Song. His-Ub K6R, K11R, K48R and K63R constructs were generated using a site-directed mutagenesis kit (Stratagene, US) according to the manufacturer’s standard procedures with pcDNA3-His-Ub as the template.

Cell culture and treatment

VSMCs were isolated from the thoracic aorta of 80-100 g male Sprague-Dawley rats as previously described.¹ VSMCs were grown in low glucose Dulbecco’s-modified Eagle’s medium (DMEM) (Invitrogen, US) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Mouse VSMCs were isolated from the thoracic aorta of
SM22α−/− mouse, and grown in low glucose DMEM with 20% FBS. The VSMCs were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and only passages 3 to 5 cells at 70-80% confluence were used in the experiments, except if stated otherwise. HEK293 cells were cultured in high glucose DMEM containing 10% FBS. To reduce the degradation of ubiquitin, VSMCs were pretreated with MG132 (10 µmol/L, Sigma-Aldrich) for 2 hours before stimulated by PDGF-BB. This study was performed via a protocol approved by the Institutional Animal Care and Use Committee of Hebei Medical University, in accordance with the Guide for the Care and Use of Laboratory Animals, and the Hebei Medical University Clinical Research Ethics Committee.

Site-directed mutagenesis of SM22α

Full-length cDNA of rat SM22α was subcloned into the pCMV-HA vector to generate HA-SM22α, which is the template of site-directed mutagenesis. Site-directed mutation of SM22α was carried out by PCR using oligonucleotide primers that coded for the appropriate point substitutions of amino acids. The reactions were carried out using a QuikChange site-directed mutagenesis kit (Stratagene, US). Each mutation was verified by DNA sequence analysis. PCR primers used in the site-directed mutagenesis: HA-K17R (17K to R): 5’-ATGAGCCGTGAAGTGCAGTCCAGGATTGAGAAGTATGATGAG-3’ and 5’-CTCATCATACTTCTTCTCAATCTCTGACTGCATCTCAGCTCAT-3’; HA-K20R (20K to R): 5’-GAAGTGCAGTCCAAGATTGAGAAGTATGATGAGGAGCTGGAG-3’ and 5’-CTCCAGCTCCTCATCATACTTCTTCTCAATCTCTTGAGACTGCACCTTC-3’; HA-K21R (21K to R): 5’-GTGCAGTCCAAGATTGAGAAGTATGATGAGGAGCTGGAGGAG-3’ and 5’-CTCCTCCAGCTCCTCATCATACTTCTTCTCATACTCTTGAGACTGCAC-3’; HA-K89R (89K to R): 5’-AACCCGCCCTCCATGGTCTTCAGGCAGATGGAACAGGTGGCTCAA-3’ and 5’-TTGAGCCACCTGTCCATCTGCTGACTGACATGGAGGCGGCATT-3’; HA-K121R (121K to R): 5’-ACTGTTGACCTCTTTGAAGGAAGATATGGCAGCAGTGCAGAGG-3’ and 5’-CTCCTGCACTGCTGCCATATCTTTTCCTTCAAAGAGGTCAACAGT-3’.

Adenovirus packaging and infection

Full-length cDNA of rat SM22α was cloned into pEGFP-C2, a mammalian expression vector and encoding a red-shifted variant of wild-type GFP, to generate pEGFP-SM22α, so SM22α was expressed as fusions to the C terminus of GFP. Then the fusions of SM22α plus GFP were subcloned into pAd/CMV/V5-DEST Gateway Vector (Invitrogen) to make the GFP tagged SM22α adenovirus Ad-GFP-SM22α, according to the manufacturer’s protocol.
Ad-GFP and Ad-GFP-K21R were obtained the same to the above. All of these clones were verified by sequencing. The VSMCs were infected with the above adenovirus (5×10^9 pfu/mL) for 24 h, washed and incubated in serum-free medium without adenovirus for 24 h, then stimulated by PDGF-BB.

**siRNA transfection**

The cultured VSMCs were grown to 50-60% confluence, and then transfected with specific duplex siRNA, siSM22α (5'-GCUAGUGGAGUGGAUUGUATT-3' and 5'-UAACAUCACUCCACUAGCTT-3'), siTRAF6 (5'-AUCGCAUGGCGCAAGAGGGUA-5' and 5'-UAACCUCUCUUUGCGCCAUUGCAU-3'), siMdm2 (5'-GACCGAUGAUUCCUGUGAU-3' and 5'-AATCAGCGAGAAAATCCGAAC-3'), siSmurf2 (5'-CAAAGUGGAAGCAGCAAU-3' and 5'-AAUAAUGCUGAUUCCUCUUG-3') or non-specific scrambled siRNA, siCon (5'-GUC AGA GAU GCG GUG AAU UCG TT-3' and 5'-GCU AGA GUA GCG GUG AAU UCG TT-3') using Lipofectamine RNAiMIX reagent (Invitrogen) according to the manufacturer’s protocol. At 6-12 hours after transfection, VSMCs were treated with PDGF-BB as mentioned.

**Membrane fractionation**

VSMCs were serum-starved for 24 hours in DMEM following PDGF-BB stimulation, and cytosolic and membrane fractions were prepared using the ProteoExtract kit (Calbiochem) according to the manufacturers’ standard procedures.

**Western blot analysis**

Lysates from VSMCs were prepared with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na3VO4, 0.2 mM PMSF, and 0.5% NP-40). Equal amounts of protein (60–100 μg) were separated by 10% SDS-PAGE, and electrotransferred to a PVDF membrane. Membranes were blocked with 5% BSA (bovine serum albumin) for 2 hours at room temperature, and incubated with specific antibodies as described above overnight, and then with the HRP-conjugated secondary antibody (1:10000) for 2 hours. The blots were evaluated with the ECL (enhanced chemiluminescence) detection system. These experiments were replicated three times.

**Quantitative real-time PCR**

Total RNA was extracted from cell cultures using TRizol Reagent (Invitrogen) and treated
with DNase I to remove genomic DNA. A quantitative real-time reverse-transcriptase (RT)-PCR was performed with a Bio-Rad thermocycler and an SYBR green kit (Invitrogen) following manufacturer’s instructions. The relative mRNA expression was normalized to β-actin. Sequence-specific primers used were presented as following: SM22α (5’-AGGTGTGGCTGAAGAATGGCG-3’ and 5’-TCTTCGTGACTCCATAATCCTC-3’) and β-actin (5’-CACCCCGCGAGTACAACCTTC-3’ and 5’-CCCATAACCACCACACACACC-3’).

**Immunofluorescent**

The VSMCs were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature for 20 minutes. Thereafter, cells were incubated with anti-SM22α, anti-G6PD and anti-TRAF6 antibody and further stained with appropriate DyLight 649-, TRITC- or FITC-conjugated secondary antibodies. Confocal microscopy was performed with the Confocal Laser Scanning Microscope Systems (Leica).

**Co-immunoprecipitation assay**

Cells stimulated with PDGF-BB were lysed in a buffer composed of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 10 mM sodium phosphate, 10 mM NaF, 1mM sodium orthovanadate, 2 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin. After centrifugation, 50 μg of the clarified cell lysate was incubated with 15 μL protein G/A-agarose (Santa Cruz, CA) and 1 μg of anti-SM22α or anti-G6PD antibodies. After 24 hours incubation, the immune complexes were centrifuged, and washed four times with ice-cold lysis buffer. The immunoprecipitated protein was further analyzed by Western blot as described above.

**Glutathione S-transferase (GST) pull-down assay**

GST, GST-SM22α fusion proteins were produced by BL21 E.coli under induction by IPTG at 37 °C. Proteins were purified by affinity absorption using glutathione-Sepharose 4B beads (Amerham Pharmacia Biotech). The recombinant GST, GST-SM22α proteins on the glutathione beads were incubated with total cell lysates of VSMCs at 4 °C overnight followed by extensive washing. Proteins on the beads were resolved on 10% SDS-PAGE and visualized by immunoblotting with anti-SM22α and anti-TRAF6 antibodies.

**Nickel-agarose chromatography**

Cells were transfected with the indicated plasmids and lysed in Ni-agarose lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 5 mM imidazole, 0.05% Tween-20, 10 mM N-ethylmaleimide, complete protease inhibitor). Ub-conjugated proteins were purified by nickel chromatography
(Ni-nitrolotriacetic acid-agarose; Invitrogen) and detected by immunoblotting with anti-HA or anti-His antibody.²

**G6PD activity assay**

G6PD catalyzes the conversion of glucose-6-phosphate and NADP⁺ to 6-phosphogluconolactone and NADPH. G6PD activity was measured using a specific kit according to the manufacturer’s instructions (BioVision, Milpitas, CA, USA).

**Intracellular NADPH content analysis**

The intracellular redox state of VSMC was analyzed spectrophotometrically by quantification of NADPH content using a commercial kit (NADP⁺/NADPH Quantification Assay Kit; BioVision, Milpitas, CA, USA) according to the manufacturer’s instructions. Protein content of each sample was adjusted to 500 μg/mL to ensure that all measurements were performed within the linear range of the NADPH standard curve. The assay specifically quantified NADPH by an enzyme cycling reaction in which NADP⁺ reacts to NADPH. To selectively quantify NADPH content, samples were heated to 60 °C for 30 minutes to eliminate NADP⁺.

**Measurement of glutathione/glutathione disulfide (GSH/GSSG) activity**

The contents or activities of GSH/GSSG in cells were determined by spectrophotometric methods according to the kit manufacturer's protocols (Biovision, Milpitas, CA, USA).

**TUNEL assay**

Apoptosis was determined by ApopTag Red In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer’s instructions. Cells were grown on cover slips and fixed with 1% paraformaldehyde in PBS for 20 minutes and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate for 10 minutes at room temperature. After that cells were incubated in Terminal deoxynucleotidyl Transferase (TdT) enzyme reaction mixture for 60 minutes at 37°C, then stained with anti-digoxigenin conjugate for 30 minutes at room temperature. Coverslips were mounted on microscope glass slide and analyzed using a Confocal Laser Scanning Microscope Systems (Leica).

**Cell Proliferation assays**

VSMC proliferation was performed with the BrdU Cell Proliferation kit (Millipore cat. no. 2750) according to manufacturer’s recommendations. Cells were cultured for 12 hours and then labeled for 12-24 hours with BrdU. OD readings were done at 450 nm.
Measurement of ROS levels

Superoxide in VSMCs on chamber slides was detected using 2 μmol/L dihydroethidium (DHE) for 30 minutes at 37°C, then visualized using laser scanning confocal microscope or fluorescence microscope. Semi-quantitative analysis of DHE relative fluorescence intensity was performed on 3 independent images (100-300 cells/image) using integrative optical density (IOD) in the Image-Pro Plus 6.0 software.

NADPH oxidase (Nox) activity assay

The activities of Nox were determined by chemiluminescence methods according to the kit manufacturer's protocols (Genmed Scientific Inc, USA).

Adventitial gene transfer and carotid artery ligation injury

Briefly, male SM22α knockout mice (20–25 g) were anesthetized with an inhalation of isoflurane. The left common carotid artery was ligated with a 6-0 silk suture so that the common carotid artery blood flow was completely disrupted. The common carotid artery was dissected free of the surrounding connective tissue and Ad-GFP-SM22α, Ad-GFP-SM22α K21R or Ad-GFP (10^10 pfu/mL) was suspended together in 50 μL pluronic F127 gel (Sigma-Aldrich; 25% wt/vol) and applied around the carotid artery. Carotid arteries were harvested 14 days after ligation. Frozen arterial segments were sectioned at 5 μm and stained with hematoxylin and eosin (HE) and was examined by a light microscope (Nikon). This study was reviewed and approved by the Institutional Animal Care and Use Committee at Hebei Medical University.

Data analysis

Data are presented as mean ± SD. ANOVA and the unpaired t test was used for statistical analysis as appropriate. p<0.05 was considered statistically significant.

Supplemental References


Online Figure Ⅰ. SM22α is required for PDGF-BB-induced G6PD activation in VAFs.
A, The expression of G6PD in VAFs treated with PDGF-BB. B, The activity of G6PD in VAFs. * p<0.05. C, NADPH level of VAFs. * p<0.05. D, The expression of G6PD and SM22α in the membrane fraction of VAFs. E, The expression of G6PD and SM22α in the membrane fraction of VAFs treated with PDGF-BB following knockdown of SM22α by Ad-GFP-shRNA. WCE, whole-cell extracts. F, G6PD activity. * p<0.05. G, NADPH level. * p<0.05. All data are expressed as mean ± SD from 3 independent experiments (n=3).
**Online Figure II.** PDGF-BB inhibits SM22α expression. Total RNAs were extracted from VSMCs treated with PDGF-BB for indicated time points, and used to examine the mRNA expression of SM22α using quantitative real-time PCR. β-actin served as controls. *p<0.05 vs. 0 h. Data are expressed as mean ± SD from 3 independent experiments (n=3).

**Online Figure III.** The ubiquitination of SM22α is mainly via K63-linked ubiquitin chain. In vivo ubiquitination assays was performed using HEK293 cells transiently transfected with His-tagged WT, K6R, K11R, K63R and K48R ubiquitin plasmids in the presence of HA-SM22α, and incubated with MG132 for 4 hours before harvesting. Ub-conjugated proteins were purified by nickel chromatography, and analyzed by immunoblotting with anti-HA antibody. WCE, whole-cell extracts.
Online Figure IV. Conservation of K21 residues within the SM22α CH domain between the species.

Online Figure V. The ubiquitination of SM22α K21 is associated with PDGF-BB-induced activation of G6PD in VAFs. A, The expression of G6PD in the membrane fraction of VAFs transduced with Ad-GFP-SM22α WT or K21R, and then treated with PDGF-BB. WCE, whole-cell extracts. B, G6PD activity. * p<0.05. Data are expressed as mean ± SD from 3 independent experiments (n=3).
Online Figure VI. The relationship between ubiquitinated form of SM22α and G6PD in membrane extracts of VSMCs transfected with TRAF6 siRNA or not.

Online Figure VII. The expression of GR of VSMCs treated with PDGF-BB for indicated time points.
Online Figure VIII. The ubiquitination of SM22α inhibits oxidative stress in PDGF-BB-induced VSMCs. A, ROS levels were measured using DHE staining and fluorescence-activated cell sorting analysis in VSMCs treated with PDGF-BB. B, ROS levels were measured using DHE staining. VSMCs from SM22α−/− and WT mice were transduced with Ad-GFP-SM22α WT or K21R mutant.* p<0.05. C, The expression of Nox1 and Nox4 in VSMCs from SM22α−/− and WT mice following PDGF-BB treatment. D, Nox enzyme activity of VSMCs. * p<0.05. E, Proliferation of VSMCs was determined by BrdU incorporation. * p<0.05. All data are expressed as mean ± SD from 3 independent experiments (n=3).
Online Figure IX. G6PD activation is involved in SM22α-mediated VSMC survival. A, The activity of G6PD in VSMCs after 6AN treatment (1mM, 12 hours). *\( p<0.05 \). B, The BrdU incorporation in VSMCs from SM22α\(^{-/-}\) mice transduced with Ad-GFP-SM22α WT. *\( p<0.05 \). All data are expressed as mean ± SD from 3 independent experiments (n=3).
Online Figure X. Ubiquitination of SM22α is involved in ROS generation and vascular hyperplasia in vivo. Left common carotid arteries of SM22α WT and SM22α−/− mice were ligated for 14 days, and used for following analysis. A, The expression of G6PD, TRAF6 and SM22α by immunofluorescence. * p<0.05. B, ROS levels were measured using DHE staining. * p<0.05. C, The expression of Nox1 and Nox4 by immunofluorescence. D, The activity of Nox. E, Representative histological sections and the intima to media (I/M) area ratio in the carotid arteries plus adenovirus. * p<0.05. F, BrdU incorporation in ligated carotid arteries plus adenovirus. * p<0.05. G, The activity of G6PD in ligated carotid arteries after 6AN treatment. H, BrdU assay after 6AN treatment. * p<0.05. All data are expressed as mean ± SD (n=18).