Homocysteine-induced proliferation of vascular smooth muscle cells occurs via PTEN hypermethylation and is mitigated by Resveratrol

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Abstract. Vascular smooth muscle cell (VSMC) proliferation is a primary pathological event in the development of atherosclerosis (AS), and the presence of homocysteine (Hcy) acts as an independent risk factor for AS. However, the underlying mechanisms remain to be elucidated. Phosphatase and tensin homologue on chromosome 10 (PTEN), is endogenously expressed in VSMCs and induces multiple signaling networks involved in cell proliferation, survival and inflammation, however, the specific role of PTEN is still unknown. The present study detected the proliferation ratio of VSMCs following treatment with Hcy and Resveratrol (RSV). In the 100 μM Hcy group, the proliferation ratio increased, and treatment with RSV decreased the proliferation ratio induced by Hcy. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to analyze PTEN expression, RSV treatment was associated with decreased PTEN expression levels in VSMCs. PTEN levels were decreased in Hcy treated cells, and the proliferation ratio of VSMCs were increased following treated with Hcy. To study the mechanism of regulation of PTEN by Hcy, the present study detected PTEN methylation levels in VSMCs, and PTEN DNA methylation levels were demonstrated to be increased in the 100 μM Hcy group, whereas treatment with RSV decreased the methylation status. DNA methyltransferase 1 is important role in the regulation of PTEN methylation. Overall, Hcy impacts the methylation status of PTEN, which is involved in cell proliferation, and induces the proliferation of VSMCs. This effect is alleviated by treatment with RSV, which exhibits an antagonistic mechanism against Hcy.

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Introduction

Epidemiologic and case control studies have consistently indicated that moderate and mild elevation of plasma homocysteine (Hcy), an intermediate metabolite of methionine, is an independent risk factor in the development of atherosclerosis (AS) (1). Previous studies have intensively focused on the involvement of Hcy in the dysfunction and injury of vascular cells, including vascular smooth muscle cells (VSMCs) (2,3). The authors previously demonstrated that Hcy induces VSMC proliferation, however the underlying mechanisms remain to be elucidated (4).

Hcy is a non-protein, sulfur containing amino acid, which is a metabolic intermediate of the methionine cycle. One of the precursors is S-adenosylmethionine (SAM), the unique methyl group donor for DNA methylation (5). Following the transfer of the methyl group, SAM is transformed into S-adenosylhomocysteine (SAH), which is hydrolyzed to form Hcy (6). DNA methylation refers to the addition of a methyl group to the 5 position of cytosine in the context of a CpG dinucleotide. Increasing evidence indicates that human diseases, including AS, are either caused or impacted by abnormal methylation (7). The authors previously demonstrated that abnormal DNA methylation of genes including peroxisome proliferator activated receptor-α, apolipoprotein E and genomic DNA contribute to the development of AS induced by Hcy (8,9). Phosphatase and tensin homologue on chromosome 10 (PTEN), is a dual-specificity protein and lipid phosphatase that suppresses multiple signaling networks involved in cell proliferation, survival and inflammation (10). PTEN, specifically expressed in VSMCs, is additionally involved in regulation of a variety of physiological and pathological processes, including cell proliferation, differentiation, apoptosis, adhesion and migration (11). PTEN is expressed endogenously in VSMCs, and overexpression of PTEN significantly inhibits both basal and platelet derived growth factor-mediated VSMC proliferation

and migration (12). PTEN overexpression in VSMCs using adenovirus transfection results in inhibition of cell proliferation and migration induced by angiotensin II (13). However, the direct mechanisms responsible for the PTEN influence on Hcy-induced VSMC proliferation have not been explored.

Resveratrol (RSV), naturally occurring in various plant foods, and particularly in grapes and red wine, is the most investigated and well-known member of this class of compounds (14). It exhibits a protective role against cardiovascular diseases, and is involved in the 'French paradox', characterized by the low incidence of cardiovascular diseases in the French population, despite a high intake of saturated fats, due to their moderate red wine consumption (15). RSV is an effective therapeutic candidate, due to its protective action in vascular walls towards oxidation, inflammation, platelet oxidation and thrombus formation (16). However, the association between Hcy and RSV on PTEN regulation in VSMC proliferation remains to be elucidated.

Therefore, the present study sought to address: i) The role of PTEN DNA methylation in VSMC proliferation induced by Hcy; ii) whether the mechanism of methylation is regulated by DNA methyltransferase (DNMT)1.

Materials and methods

Cell culture. The T/G HA-VSM cell line was used, which was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium-Han's F12 media (DMEM-F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FCS at 37˚C in an incubator with an environment containing 5% CO_2. Hcy was applied at concentrations of 50, 100, 200 and 500 μM in addition to 30 μM folate and vitamin B12 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 72 h.

Bromodeoxyuridine (BrdU) assay. VSMCs at $5x10^3$ cells per well were cultured in triplicate in 96-well plates in 0, 50, 100, 200 and 500 μM concentrations of Hcy and antagonist (30 μM folate and 30 μM vitamin B12 were added to 100 μM Hcy) then incubated for 72 h. Cell proliferation was measured by BrdU incorporation assay using a commercial ELISA kit according to the manufacturer's protocol (cat. no. 11647229001; Roche Diagnostics GmbH, Mannheim, Germany).

Cell viability assessment. MTT (Sigma-Aldrich; Merck KGaA) was used for the evaluation of cell viability. The cells were grown in 96-well microtiter plates at a density of $1x10^4$ cells in 200 μl per well. Subsequently, cells were treated with treated with a range of 10-100 μM RSV to determine an optimal concentration. Following incubation for 10 min, the plates were read on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of DNMT1 and PTEN. RNA was then reverse transcribed by using the Revert Aid first strand cDNA synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.); the SYBR-Green kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) was then used for RT-qPCR analysis. The primer sequences of the DNMT1 and PTEN genes were as follows: Forward, 5'-AGCTCTTACCTTACCATC-3' and reverse, 5'-CCATCTGATACTCTGAA-3' for DNMT1; and forward, 5'-CAGCATCTTATCCGAGTG-3' and reverse, 5'-GATGGTGGTACAGTCAGA-3' for PTEN. GAPDH was applied as an internal control: Forward, 5'-AGAAGGCTG GGGCTCATTT-3' and reverse, 5'-AGGGGCCACAGTCTT CG-3'. The thermal cycler (Funglyn Biotech, Inc., Toronto, ON, Canada) conditions comprised an initial activation step at 95˚C for 5 min, followed by a 2-step PCR program of 95˚C for 15 sec, annealing temperatures for 15 sec and at 72˚C for 30 sec for 30 cycles. Subsequently, the relative alterations in the mRNA expression levels of DNMT1 and PTEN were determined by fold-change analysis, in which the degree of change was calculated as $2^{-\Delta\Delta Cq}$, where $Cq=(Cq_{gene}-Cq_{GAPDH})$ treatment-$(Cq_{gene}-Cq_{GAPDH})$ control (17).

Western blotting for DNMT1 and PTEN. Total proteins were isolated from the cells using cell lysis buffer (Keygen Biotech Co., Ltd., Nanjing, China), then protein concentration was determined via a bicinchoninic acid protein assay kit (KeyGen Biotech, Nanjing, China). Equal amounts of protein (~80 μg) and known molecular weight marker were separated by 12% SDS-PAGE and were transferred to polyvinylidene fluoride membrane by electrophoresis at 300 mA for 50 min at 4˚C, the membrane was then blocked in 10 ml 5% skimmed milk for 2 h at room temperature with gentle agitation on a platform shaker. The DNMT1 and PTEN proteins were detected using DNMT1 (cat. no. sc-271729) and PTEN (cat. no. sc-65604) antibodies were obtained from Santa Cruz Biotechnology, Inc, (Dallas, TX, US) diluted 1:500, and β-actin protein was detected using a rabbit anti-human β-actin antibody (cat. no. sc-70319, Santa Cruz Biotechnology, Inc.) diluted 1:2,000; all primary antibodies were incubated at 4˚C. The secondary antibody (goat anti-mouse immunoglobulin G-horseradish peroxidase, cat. no. sc-2031, 1:2,000; Santa Cruz Biotechnology, Inc.) was added for 2 h at room temperature. The protein bands were visualized and analyzed by the Gel Documentation and Analysis System ChemiDoc XRS system with Image Lab software, version 4.1 (Bio-Rad Laboratories, Inc.) and calculated by the gray value of the bands.

Nested methylation-specific (nMS)-PCR for PTEN methylation assay. DNA denaturation and bisulfite conversion processes were integrated into one step by using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp., Irvine, CA, USA). nMS-PCR consists of two-step PCR amplifications following a standard sodium bisulfite DNA modification. The first step uses an outer primer pair set that does not contain any CpGs. The second-step PCR was conducted with the conventional PCR primers. The primers of the nMS-PCR assays were as follows: i) PTEN-outer primers: Forward, 5'-GTTTTGGTTTGAAGGATAGTAGT-3' and reverse, 5'-AAAAACCCTAAAACTTAATAAAAAC-3'; ii) PTEN-methylated primers: Forward, 5'-TTAGTTTTAGTT AGGATGGTTTCGA-3' and reverse, 5'-GAAAAATAAACC GAAATCCCG-3'; iii) PTEN-unmethylated primers: Forward, 5'-ATTAGTTTTAGTAGGATGGTTTTGA-3' and reverse, 5'-CAAAAAATAAACCAAAATCCCAC-3'. DNA bands were visualized by ultraviolet illumination, and the percentage

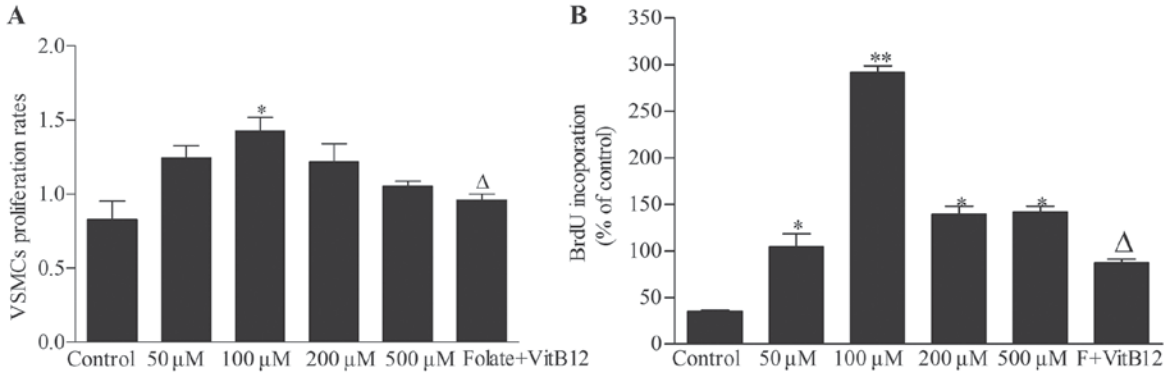

Figure 1. Hcy increases the proliferation ratio of VSMCs. (A) Following treatment with different concentrations of Hcy, MTT was used to detect the proliferation of VSMCs. (B) VSMC proliferation was analyzed using a BrdU assay, following cell pre-treatment with various concentrations of Hcy. Folate and vitamin B12 group: Cells were treated with 100 μM Hcy, 30 μM folate and 30 μM vitamin B12. *P<0.05, **P<0.01 vs. control; $^{\Delta}$P<0.05 vs. 100 μM Hcy group. Hcy, homocysteine; VSMC, vascular smooth muscle cells; BrdU, bromodeoxyuridine.

of methylation was calculated by using the following formula: Methylation %=methylation/(methylation+unmethylation) x100%.

Cell transfection. The recombinant plasmids EGFP-N1-PTEN/DNMT1 were established obtain from Hanbio Biotechnology Co., Ltd., (Shanghai, China). Cells were plated to 70-90% confluence at the time of transfection; 100 ng plasmid and 0.5 μl for DNA-lipid complexes were prepared and incubated for 30 min. The DNA-lipid complexed were then applied to cells. Then, the VSMCs were transfected with 3-5 μg/μl recombinant plasmids: EGFP-N1-PTEN/DNMT1. EGFP-N1-PTEN/DNMT1 molecule-Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) complexes were established and then added to the plate wells, where the VSMCs had grown to 80-90% confluence. The transfection efficiency was determined by detecting the fluorescence intensity via an inverted fluorescence phase contrast microscope (Olympus IX71, Olympus Corporation, Tokyo, Japan). The transfected VSMCs were treated with Hcy for 24 h and mRNA expression of PTEN/DNMT1 was detected with RT-qPCR (17). Western blotting was carried out to examine the protein expression of PTEN/DNMT1 (17).

Statistical analysis. Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for data processing. Data are expressed as the mean ± standard deviation. Each experiment was repeated three times. Statistical comparison of each parameter between two groups was performed using the paired Student's t-test. One-way analysis of variance was used to compare the means of multiple groups, followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hcy promotes the proliferation of VSMCs. In order to investigate the effect of Hcy on VSMCs, the present study detected the cell proliferation ratio following cell treatment with Hcy for 72 h, using an MTT assay. As presented in Fig. 1A, the proliferation of VSMCs was increased in the cells which were co-incubated with different concentrations of Hcy, compared with control group, and in 100 μM Hcy it was increased 1.72-fold (P<0.05). The proliferation ratio in the folate and vitamin B12 group treated with 100 μM Hcy was decreased by 35.46% (P<0.05). Furthermore, the effect of Hcy on VSMC proliferation was evaluated with a BrdU incorporation assay to determine the effects of Hcy on DNA synthesis, and it was demonstrated that 100 μM Hcy treatment significantly promoted DNA synthesis (Fig. 1B), whereas in the folate and vitamin B12 group, DNA synthesis was decreased (P<0.05). These data indicated that Hcy promoted the proliferation ratio, and this effect was counteracted with folate and vitamin B12.

Hcy decreases PTEN expression in VSMCs. PTEN is ranked the most mutated tumor suppressor gene, which regulates numerous cellular processes, including cell cycle, adhesion, migration, proliferation and cell survival (18). To study whether Hcy regulates PTEN expression, and if this was responsible for the VSMC proliferation, the present study detected PTEN expression levels in VSMCs following treatment with Hcy for 72 h. As presented in Fig. 2, PTEN mRNA was decreased by 27.5, 34.7 and 46.7% in 100, 200 and 500 μM Hcy groups respectively, compared with control group (P<0.05, P<0.01). PTEN protein levels in VSMCs decreased by 28.6, 54.3 and 71.4% in 100, 200 and 500 μM Hcy groups, compared with control group (P<0.05, P<0.01), whereas in folate and vitamin B12 group, PTEN protein levels were increased 1.44-fold, compared with 100 μM Hcy group (P<0.05). Therefore, PTEN may have an important role in VSMCs proliferation induced by Hcy.

RSV antagonizes proliferation of VSMCs induced by Hcy. RSV has biological activities, and similar to estrogen, is known to have several effects on cardiovascular disease and malignancies (19). The present study co-incubated VSMCs with different concentrations of RSV and 100 μM Hcy for 72 h at 37˚C, then detected the VSMC proliferation ratio by MTT, as presented in Fig. 3. Compared with 100 μM Hcy group, the proliferation ratio of VSMCs was decreased by 45.16, 53.76 and 63.44% in 20, 50 and 100 μM RSV groups (P<0.05, P<0.01), respectively. Compared with 100 μM RSV group, the proliferation ratio in the folate and vitamin B12 group was increased by 1.62-fold

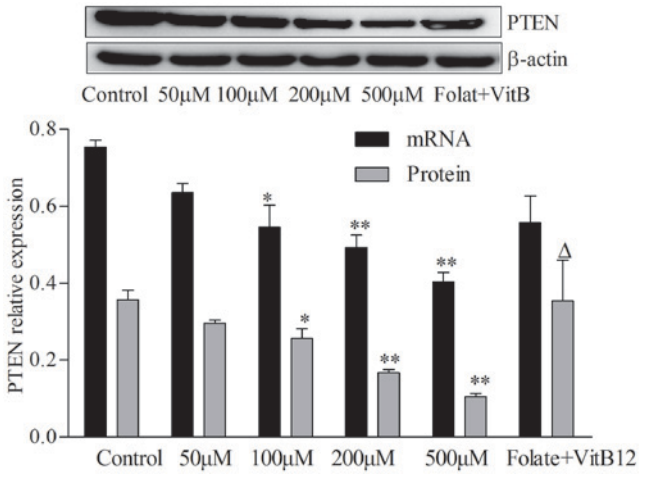

Figure 2. PTEN expression is downregulated in VSMCs treated with Hcy. Following incubation with different concentrations of Hcy for 72 h, the PTEN mRNA level was detected by reverse transcription-quantitative polymerase chain reaction, and the expression of PTEN protein analyzed by western blotting. Folate and vitamin B12 group: Cells were treated with 100 μM Hcy, 30 μM folate and 30 μM vitamin B12. *P<0.05, **P<0.01 vs. control; $^{\Delta}$P<0.05 vs. 100 μM Hcy groups. Hcy, homocysteine; VSMC, vascular smooth muscle cells; PTEN, phosphatase and tensin homologue on chromosome 10.

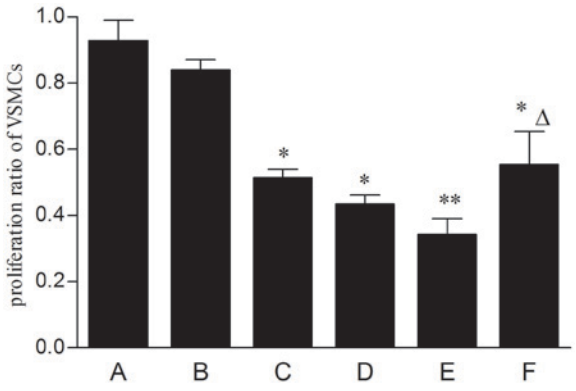

Figure 3. Resveratrol antagonistic effects on proliferation of VSMCs induced by Hcy. VSMCs were treated with Hcy and different concentrations of Resveratrol for 72 h, then the proliferation ratio of VSMCs was detected by MTT. A, 100 μM Hcy group, as control group; B, 100 μM Hcy+10 μM Resveratrol; C, 100 μM Hcy+20 μM Resveratrol; D, 100 μM Hcy+50 μM Resveratrol; E, 100 μM Hcy+100 μM Resveratrol; F, cells were treated with 100 μM Hcy, 30 μM folate and 30 μM vitamin B12. *P<0.05, **P<0.01, compared with 100 μM Hcy group; $^{\Delta}$P<0.05 vs. 100 μM Hcy+100 μM Resveratrol group. Hcy, homocysteine; VSMC, vascular smooth muscle cells.

(P<0.05). Notably, compared with 100 μM Hcy group, the folate and vitamin B12 group proliferation ratio of VSMCs was decreased by 40.86% (P<0.05). These data indicated that RSV acted as an antagonist which decreased the proliferation ratio of VSMCs, which was previously increased when induced by Hcy. The inhibitory effect of RSV was more potent compared with folate and vitamin B12.

Methylation status of PTEN in VSMCs. DNA methylation is important in the development of AS induced by Hcy (20). To investigate whether PTEN DNA methylation alterations occur in VSMC proliferation induced by Hcy, the present study detected PTEN DNA methylation by nMS-PCR, following cell treatment with Hcy and various antagonistic compounds for 72 h. As presented in Fig. 4A, compared with control group, PTEN DNA methylation levels were increased 8.56-fold in 100 μM Hcy group (P<0.01), and 2.56-fold in folate and vitamin B12 group (P<0.05). Compared with 100 μM Hcy group, PTEN DNA methylation levels decreased by 79.2 and 70.1% in RSV (P<0.01) and folate and vitamin B12 groups (P<0.01), respectively. These data suggested that PTEN DNA methylation was affected by Hcy in VSMCs and this may act as the primary reason for proliferation of VSMCs induced by Hcy.

DNMT1 catalyzes the transfer of methyl groups to DNA from SAM. In mammals, DNMT1 is the maintenance methyltransferase, which preferentially methylates unmethylated or hemi-methylated double-stranded DNA. To further study the regulation mechanism of PTEN DNA methylation, the expression of DNMT1 in VSMCs was analyzed by RT-qPCR and western blotting. As presented in Fig. 4B, DNMT1 mRNA levels were detected in VSMCs. Compared with control group, DNMT1 mRNA increased 3.28-fold in 100 μM Hcy group (P<0.01), whereas compared with 100 μM Hcy group, DNMT1 mRNA levels decreased 47.4 and 57.9% in RSV (P<0.01) and folate and vitamin B12 groups (P<0.01), respectively. DNMT1 protein expression in VSMCs exhibited the same trends as the DNMT1 mRNA levels. Compared with control group, DNMT1 protein expression increased 2.81-fold in the100 μM Hcy group (P<0.01), whereas compared with 100 μM Hcy group, DNMT1 protein levels decreased by 47.4 and 43.4% in RSV (P<0.01) and folate and vitamin B12 groups (P<0.01), respectively.

To identify whether DNMT1 was a key molecule in regulation of PTEN DNA methylation in VSMC proliferation induced by Hcy, the levels of DNMT1 were overexpressed. The recombination vector of DNMT1 was transfected into untreated VSMCs, and the observed green fluorescence verified that the recombined vector had been successfully transfected into VSMCs (Fig. 4C). The expression levels of DNMT1 were detected with RT-qPCR and western blotting. As presented in Fig. 4D, DNMT1 mRNA and protein expression levels were increased in the overexpression group by 3.57-fold and 2.03-fold (P<0.01), compared with control group, and these data indicated that the transfection of the overexpression recombination vector DNMT1 had been successful. Following this, PTEN DNA methylation was analyzed with nMS-PCR. As presented in Fig. 4E, compared with control group, PTEN DNA methylation was increased 2-fold (P<0.05) in DNMT1 overexpression group and 2.59-fold (P<0.01) in DNMT1 overexpression and Hcy group. Overall, these data suggested that DNMT1 has an important role in regulating PTEN DNA methylation in VSMCs, induced by Hcy.

PTEN exhibits a primary role in the proliferation of VSMCs induced by Hcy. To further verify the role of PTEN in VSMC proliferation resulting from Hcy, a recombination PTEN overexpression vector was transfected into untreated VSMCs and proliferation of VSMCs induced by Hcy was analyzed, as presented in Fig. 5. Following transfection with recombination vector of PTEN, green fluorescence in VSMCs was observed, and the levels of PTEN mRNA and protein were increased 1.91-fold (P<0.01) and 2.27-fold (P<0.01) in the PTEN recombination group (Fig. 5A and B).

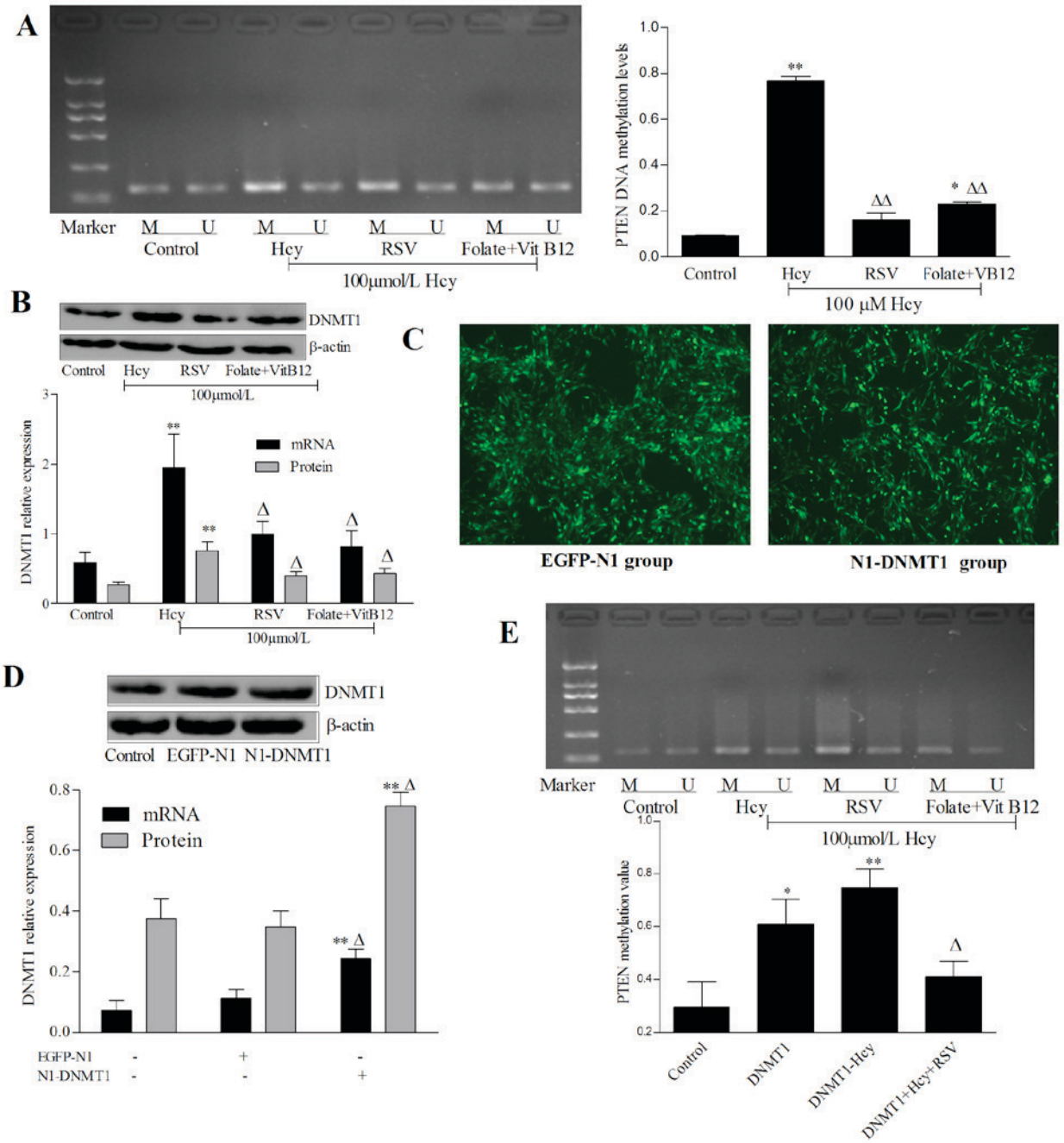

Figure 4. PTEN DNA methylation and its regulation mechanism in VSMCs induced by Hcy. (A) PTEN DNA methylation levels in VSMCs detected by nested methylation-specific PCR, following cell treatment with Hcy and other antagonists for 72 h. (B) DNMT1 expression levels in VSMCs detected by RT-qPCR and western blotting, following cell treatment with Hcy and other antagonists for 72 h. (C) pEGFP-N1-DNMT1 was transfected into VSMCs using Lipofectamine® 2000, and detected with fluorescence microscopy (magnification, x1,000). (D) DNMT1 mRNA and protein levels in VSMCs analyzed by RT-qPCR and western blotting, following cell transfection with DNMT1 overexpression vector. (E) Levels of PTEN DNA methylation in VSMCs, following cell transfection with recombination vector of DNMT1, then co-incubation with Hcy for 72 h. Control group, untreated cells; Hcy group: Cells treated with 100 μM Hcy for 72 h; RSV group, cells treated with 100 μM Hcy and 100 μM Resveratrol for 72 h; Folate and vitamin B12 group, cells treated with 100 μM Hcy, 30 μM folate and 30 μM vitamin B12; EGFP-N1, pEGFP-N1 control vector; DNMT1-N1, recombination vector of DNMT1; DNMT1 group, cells transfected with recombination vector of DNMT1; DNMT1+Hcy group, cells transfected with recombination vector of DNMT1, then treated with 100 μM Hcy for 72 h; DNMT1+Hcy+RSV group, cells transfected with recombination vector of DNMT1, then treated with 100 μM Hcy and 100 μM Resveratrol for 72 h; *P<0.05, **P<0.01, vs. control group; $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 vs. 100 μM Hcy group; $^{\#}$P<0.05 vs. pEGFP-N1 vector group. M, methylation; U, unmethylated; Marker, DNA marker (top to bottom, 2,000, 1,000, 750, 500, 250 and 100 bp). Hcy, homocysteine; VSMC, vascular smooth muscle cells; PTEN, phosphatase and tensin homologue on chromosome 10; DNMT1, DNA methyltransferase; RSV, Resveratrol; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Following this, the proliferation ratio of VSMCs was detected following cell transfection with the PTEN recombination vector. As presented in Fig. 5C, the proliferation ratio was increased 4.75-fold (P<0.01), 3.25-fold (P<0.01) and 2.1-fold (P<0.01) in Hcy, PTEN overexpression cells treated with Hcy and PTEN overexpression cells treated with Hcy and RSV groups, respectively. Compared with 100 μM Hcy group, in PTEN overexpression cells treated with Hcy and PTEN overexpression cells treated with Hcy and RSV, the proliferation ratio was decreased by 33.3% (P<0.05) and 56.6% (P<0.05).

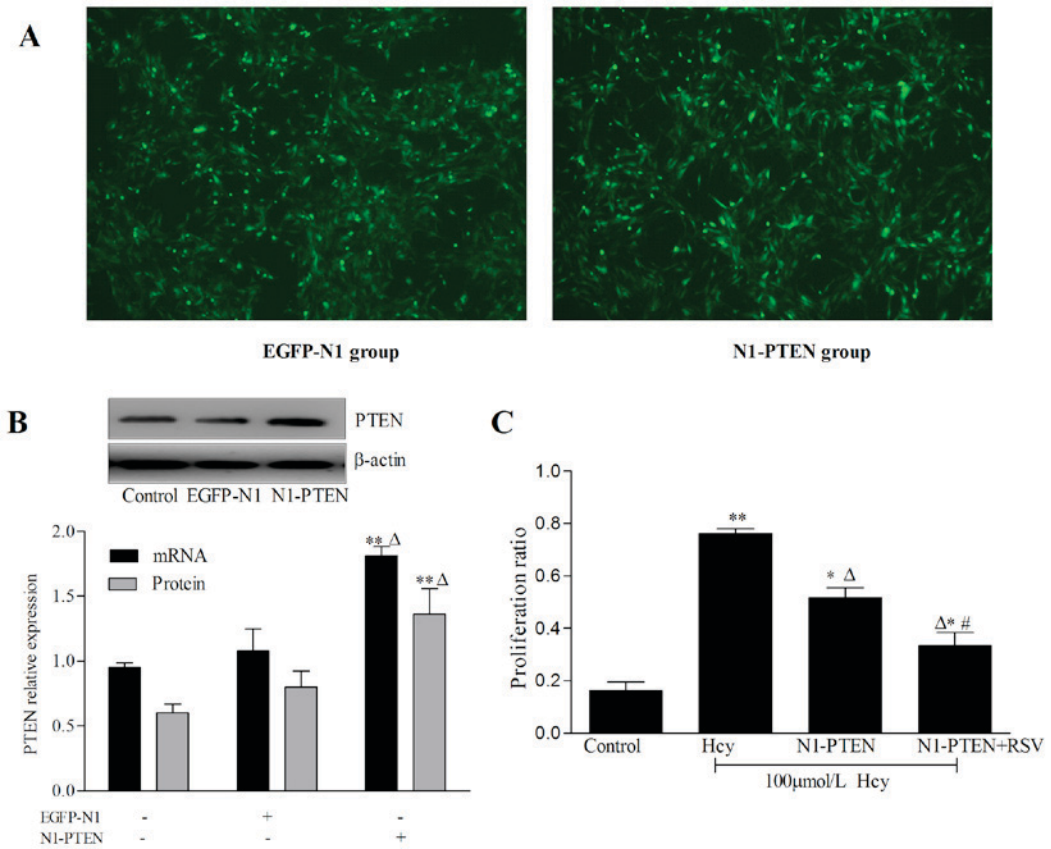

Figure 5. PTEN is a pivotal molecule in proliferation of VSMCs induced by Hcy. (A) pEGFP-N1 and pEGFP-N1-PTEN were transfected into VSMC using Lipofectamine™ 2000, and detected with fluorescence microscopy (magnification, x100). (B) Expression levels of PTEN in VSMCs, following cell transfection with recombination vector. mRNA and protein levels were analyzed with reverse transcription-quantitative polymerase chain reaction and western blotting. (C) Proliferation ratio of VSMCs was detected by MTT, following cell transfection with recombination vector PTEN, and then co-incubation with Hcy and other antagonistic compounds. EGFP-N1, pEGFP-N1 control vector; PTEN-N1, recombination vector of PTEN; Hcy group, cells treated with 100 μM Hcy for 72 h; N1-PTEN+Hcy group, cells transfected with recombination vector of PTEN, then treated with 100 μM Hcy for 72 h; N-PTEN+Hcy+RSV group, cells transfected with recombination vector of PTEN, then treated with 100 μM Hcy and 100 μM Resveratrol for 72 h; *P<0.05, **P<0.01 vs. control group; $^{\Delta}$P<0.05 vs. pEGFP-N1 vector group; $^{\#}$P<0.05, vs. 100 μM Hcy group. Hcy, homocysteine; VSMC, vascular smooth muscle cells; PTEN, phosphatase and tensin homologue on chromosome 10.

These results suggested that PTEN is important in proliferation of VSMCs which is induced by Hcy, and RSV may relieve this nocuous effect of Hcy.

Discussion

It has previously been demonstrated that elevated Hcy levels are a diagnosed cause of VSMC proliferation and further promote the formation of AS (21). The present study demonstrated that Hcy induced the proliferation of VSMCs, which may contribute to development of AS. Clinical studies regarding treatment with RSV are not as promising as the preclinical findings, with regards to the beneficial effects of RSV on AS (22,23). The present study demonstrated that Hcy promoted VSMC proliferation, and decreased PTEN expression via an increase in PTEN DNA methylation levels. DNMT1 was important in this process, and RSV relieved the pathological process via a promotion of PTEN expression levels.

As an important independent risk factor of AS, Hcy stimulates VSMC proliferation, which has been demonstrated in previous studies (24,25). To investigate the effect of Hcy on VSMCs, the present study detected the cell proliferation ratio following cell treatment with Hcy for 72 h using an MTT assay, and the proliferation of VSMCs was observed to be increased with different concentrations of Hcy. PTEN is a tumor suppressor gene, which regulates various cellular processes, including cell cycle, adhesion, migration, proliferation and cell survival (26). PTEN has recently been demonstrated to have an important role in cells of the cardiovascular system (27). Schwartzbauer and Robbins (28) demonstrated that PTEN regulates cardiac myocyte hypertrophy and survival. The present study demonstrated that PTEN expression levels were decreased in Hcy groups, at the mRNA and protein level.

RSV is a naturally occurring polyphenolic phytoalexin derived from plants such as *Polygonum cuspidatum* and grapes, it has anti-aging, -apoptotic, -tumorigenic, -oxidant, and -inflammatory properties (29). Guo (30) reported that RSV inhibits high glucose-induced oxidative stress and VSMC proliferation by suppressing reactive oxygen species generation, nicotinamide adenine dinucleotide phosphate oxidase, AKT Serine/Threonine Kinase phosphorylation, p38 mitogen activated protein kinase/c-Jun N-terminal kinase/extracellular signal-regulated kinase phosphorylation, and IκB-α and nuclear factor-11κB activities, however the effects of RSV in anti-proliferation of VSMCs still remain unclear. The present

study co-incubated VSMCs with different concentrations of RSV and 100 μM Hcy for 72 h, and demonstrated that the VSMC proliferation ratio was decreased in different concentrations of RSV groups. These data indicated that RSV acted as an antagonist which decreased the proliferation ratio of VSMCs induced by Hcy.

DNA methylation serves as an important mechanism that controls gene expression in AS (31). As previously demonstrated, PTEN DNA methylation levels are significantly decreased in Hcy groups. In Hcy metabolism, SAM is a metabolic intermediate which is synthesized from methionine catalysed by methionine adenosyltransferase, providing methyl group moieties in several dozens of transmethylation reactions (32). SAM is converted into SAH that is the sole metabolic precursor of Hcy in a reversible reaction catalyzed by SAH hydrolase, and inhibits DNMT1 (33). The present study demonstrated that PTEN DNA methylation levels were increased in 100 μM Hcy group and decreased in RSV group. The role of DNMT1 in the regulation of PTEN was also observed. DNMT1, 3a, 3b have all previously been detected, however only DNMT1 exhibits an important role in this process (34). To test whether DNMT1 had an effect on PTEN promoter hypermethylation, overexpression of DNMT1 and PTEN occurred. Following transfection with pDNMT1, the methylation levels of PTEN significantly increased.

In conclusion, Hcy impacted the methylation status of PTEN involved in cell proliferation, and induced the proliferation of VSMCs, whereas RSV alleviated VSMC proliferation by antagonism of Hcy. The data from the present study provided evidence for the mechanisms of VSMC proliferation in AS induced by Hcy, and may act as a potential diagnostic marker for AS induced by Hcy.

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