

# Troglitazone upregulates PTEN expression and induces the apoptosis of pulmonary artery smooth muscle cells under hypoxic conditions

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**Abstract.** The increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells (PASMCs) are the main causes of hypoxic pulmonary hypertension. In this study, we investigated the role of troglitazone [peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonist] in the regulation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression and the apoptosis of PASMCs under hypoxic conditions. Normal human PASMCs were cultured in growth medium (GM) and treated with troglitazone (0.5-80  $\mu$ M) under hypoxic conditions (5% CO<sub>2</sub>+94% N<sub>2</sub>+1% O<sub>2</sub>) for 72 h. The gene expression of PTEN, AKT-1 and AKT-2 was determined by quantitative reverse transcription PCR (qRT-PCR). The protein expression level of PTEN, AKT and phosphorylated AKT (p-AKT) was determined by western blot analysis. The apoptosis of PASMCs was determined by measuring the activities of caspase-3, -8 and -9 and by TUNEL assay. The proliferation rate of the PASMCs was altered in a concentration-dependent manner by troglitazone. A significantly reduced proliferation rate was observed at troglitazone concentrations starting from 20  $\mu$ M under hypoxic conditions (72 $\pm$ 5.8%). Although the gene expression levels of PTEN were increased, the gene expression levels of AKT-1 and AKT-2 remained unaltered. Consistent with this, PTEN protein expression was also altered in a concentration-dependent manner by troglitazone. Although AKT expression was unaltered in all the cell samples, reduced p-AKT expression was observed in the troglitazone-treated PASMCs. Troglitazone increased the

activities of caspase-3, -8 and -9 in the PASMCs. bpV(HOpic) (PTEN inhibitor) and GW9662 (PPAR $\gamma$  inhibitor) inhibited PTEN protein expression and recovered the proliferation rate of the PASMCs. TUNEL assay demonstrated that troglitazone significantly increased the apoptosis of PASMCs under hypoxic conditions. In conclusion, troglitazone increases PTEN expression under hypoxic conditions in a concentration-dependent manner. Troglitazone increases the apoptosis of PASMCs under hypoxic conditions. The increase in PTEN expression is mediated through the PPAR $\gamma$  signaling pathway.

## Introduction

Hypoxic pulmonary hypertension (HPH) is an important pathophysiological process and plays a key role in the development of a variety of pulmonary diseases, including chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and pulmonary heart disease. Multiple pathogenic pathways have been implicated in the development of HPH, including those at the molecular and genetic levels of smooth muscle and endothelial cells and adventitia. The HPH 'phenotype' is characterized by a decreased ratio of apoptosis/proliferation in pulmonary artery smooth muscle cells (PASMCs) and a thickened, disordered adventitia (1). The increased proliferation and reduced apoptosis of PASMCs is a component of pulmonary hypertension, increased pulmonary vascular resistance and pulmonary arterial pressure, which is associated with COPD and right ventricular failure (2). Since the proliferation of SMCs is an essential feature of vascular proliferative disorders, considerable efforts have been made to develop a therapeutic strategy that effectively suppresses SMC proliferation.

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) agonists act as negative regulators of SMC growth (3). They are potent inhibitors of vascular SMC proliferation *in vitro* and *in vivo* (3). PPAR $\gamma$  agonists can stimulate the production and secretion of apolipoprotein E (ApoE), which has anti-proliferative effects on human PASMCs (4). Nisbet *et al* (5) found that rosiglitazone (a PPAR $\gamma$  agonist) attenuated hypoxia-induced pulmonary vascular remodelling and hypertension by suppressing oxidative and proliferative signals. These studies provide the mechanisms underlying the therapeutic effects of PPAR $\gamma$  activation in pulmonary hypertension.

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Studies have also suggested that PPAR $\gamma$  agonists upregulate phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression in allergen-induced asthmatic lungs (6). Zhang *et al* (7) demonstrated that rosiglitazone inhibits the migration of human hepatocellular BEL-7404 cells by upregulating PTEN. The tumor suppressor gene, PTEN, encodes a dual-specificity phosphatase that recognizes protein and phosphatidylinositol substrates and modulates cellular functions, such as migration and proliferation. However, the role of PPAR $\gamma$  in regulating PTEN expression in PSMCs under hypoxic conditions has not yet been elucidated. Therefore, the present study aimed to examine the molecular mechanisms through which PPAR $\gamma$  activation modulates PTEN expression in PSMCs under hypoxic conditions, since hypoxia is a proliferative stimulator of PSMCs (5).

In the present study, we investigated the effects of troglitazone (a PPAR $\gamma$  agonist) on PTEN gene expression and the apoptosis of PSMCs under hypoxic conditions. We found that troglitazone increased PTEN expression in PSMCs in a concentration-dependent manner and increased the apoptosis of PSMCs under hypoxic conditions.

## Materials and methods

**Cell culture of PSMCs.** Human PSMCs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The PSMCs were cultured and expanded in SMC growth medium (GM): smooth muscle cell basal medium (BM; ScienCell Research Laboratories) supplemented with SMC growth supplement (ScienCell Research Laboratories), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator, and the cell culture medium was changed every 2 days. Cells were regularly passaged every 4-5 days.

**Hypoxia treatment.** The PSMCs were cultured under hypoxic conditions in order to determine the anti-proliferative effects of troglitazone on PSMCs. A hypoxic environment for PSMC culture was created in an incubator: 5% CO<sub>2</sub>+94% N<sub>2</sub>+1% O<sub>2</sub>. The cells were collected for gene and protein expression experiments after 72 h. Generally, the PSMCs were seeded in plates in BM overnight before being used in the experiments.

**PASMC culture with PTEN inhibitor or PPAR $\gamma$  antagonist.** The PSMCs were seeded at 1x10<sup>4</sup> cells/well in a 12-well plate and cultured in GM supplemented with 2.5  $\mu$ M dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (V) [bpV(HOpic)], a PTEN inhibitor, or 1  $\mu$ M 2-chloro-5-nitro-N-phenylbenzamide (GW9662), a PPAR $\gamma$  antagonist (both were purchased from EMD4Biosciences, Darmstadt, Germany). bpV(HOpic) and GW9662 were added to the cell culture medium 1 h prior to the addition of troglitazone. The PSMCs were cultured in an incubator under hypoxic conditions for 72 h. Cell proliferation was determined using the CyQUANT<sup>®</sup> Cell Proliferation assay kit (Invitrogen, Carlsbad, CA, USA). Proteins were extracted from the treated cells using PhosphoSafe<sup>™</sup> Extraction Reagent (EMD Millipore, Billerica, MA, USA). Following quantification, the proteins were used for western blot analysis.

**Quantitative reverse transcription PCR (qRT-PCR) analysis.** PSMCs were analyzed by qRT-PCR to determine gene expression at 0.5, 1, 2, 4, 8 and 24 h after treatment with troglitazone. Total RNA was isolated as previously described (8). DNase I was used to remove DNA contamination. cDNA was synthesized using the Maxima<sup>®</sup> First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA).

To quantify gene expression levels, Maxima<sup>®</sup> SYBR-Green qPCR Master Mix (2X) (Fermentas) was used. The qPCR thermal cycling protocol was programmed for 40 cycles: 1 cycle of initial denaturation for 10 min, then denaturation at 95°C for 15 sec, annealing for 30 sec and extension at 72°C for 30 sec. The primers used are listed in Table I.

**Cell proliferation.** PASMC proliferation was determined using the CyQUANT<sup>®</sup> Cell Proliferation assay kit (Invitrogen). Briefly, 1x10<sup>4</sup> PSMCs/well were seeded into 24-well plates and cultured with BM for 24 h. The cell culture medium was changed to GM supplemented with various concentrations of troglitazone (stock solution 20 mM in 100% DMSO) for 72 h in an incubator under hypoxic conditions at 37°C. Subsequently, the cell culture supernatant was removed. The cells were washed with PBS and frozen at -80°C for 1 h. Each well was then incubated with 200  $\mu$ l CyQUANT<sup>®</sup> cell-lysis buffer containing DNase-free RNase (1.35 U/ml) for 1 h at room temperature to eliminate the RNA component of the fluorescence signal. Subsequently, 200  $\mu$ l cell-lysis buffer containing 2X solution of CyQUANT<sup>®</sup> GR dye were added to each well for 10 min. The fluorescence intensity was measured using a Tecan fluorescence microplate reader (Tecan Infinite M200 microplate reader; LabX, Canada) with 480 nm excitation and 520 nm emission.

**Lactate dehydrogenase (LDH) release for cytotoxic determination.** The cytotoxicity of troglitazone towards PSMCs was determined using CytoTox-ONE<sup>™</sup> Homogeneous Membrane Integrity assay (Promega, Madison, WI, USA). Briefly, 5x10<sup>4</sup> PSMCs/well were cultured in a 12-well plate with GM supplemented with troglitazone for 72 h under normoxic conditions at 37°C. Subsequently, cell culture supernatant was collected and mixed with CytoTox-ONE<sup>™</sup> Reagent for 20 min. After the addition of stop solution, the fluorescence signal was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

**Western blot analysis of PSMCs.** Protein expression in the treated and non-treated PSMCs was determined by western blot analysis as previously described (9). Cells were lysed with PhosphoSafe<sup>™</sup> Extraction Reagent (EMD Millipore) and the protein concentration was determined using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Proteins were separated and electrophoretically blotted onto a nitrocellulose membrane. After washing with 10 mM Tris/HCl wash buffer (pH 7.6) containing 0.05% Tween-20, the membrane was incubated in blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 3 h at room temperature. Subsequently, the blots were incubated with diluted primary antibodies: AKT (1:1,000), phosphorylated (Ser 473) AKT (p-AKT) (1:500), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000) and

Table I. Primers used for RT-PCR.

| Gene  | Primer sequence                       | Annealing temp. (°C) | Size (bp) |
|-------|---------------------------------------|----------------------|-----------|
| GAPDH | Forward 5'-AGCCACATCGCTCAGACAC-3'     | 60                   | 90        |
|       | Reverse 5'-TAAAAGCAGCCCTGGTGAC-3'     |                      |           |
| PTEN  | Forward 5'-TCACCAACTGAAGTGGCTAAAGA-3' | 60                   | 155       |
|       | Reverse 5'-CTCCATTCCCCTAACCCGA-3'     |                      |           |
| AKT-1 | Forward 5'-TAACCTTTCCGCTGTGCGC-3'     | 58                   | 125       |
|       | Reverse 5'-ATGTTGTAAAAAACGCCG-3'      |                      |           |
| AKT-2 | Forward 5'-GGTCGCCAACAGCCTCAA-3'      | 58                   | 127       |
|       | Reverse 5'-CACTTTAGCCCGTGCCTTG-3'     |                      |           |

RT-PCR, reverse transcription PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

PTEN (1:200) (all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Subsequently, anti-rabbit IgG conjugated with HRP (dilution, 1:3,000-1:8,000) was used to detect the binding of antibodies. The binding of the specific antibody was visualized using the SuperSignal Chemiluminescent Substrate kit and exposed to X-ray film (both from Pierce Biotechnology, Inc., Rockford, IL, USA).

**Apoptosis assay.** Proteins from the treated and non-treated PSMCs were used to determine the apoptosis of PSMCs by determining the activities of caspase-3, -8 and -9.

Caspase-3 and -8 activities were determined using caspase-3 and -8 assay kits, Fluorimetric (Sigma-Aldrich, Sigma, St. Louis, MO, USA). The fluorescence intensity of caspase-3 was recorded at a wavelength of 360 nm for excitation and a wavelength of 460 nm for emission, and that of caspase-8 was recorded at an excitation of 360 nm and an emission of 440 nm. The activity of caspase was calculated as fluorescence intensity (FI)/min/ml =  $\Delta FI / (t \times v)$ , where  $\Delta FI$  is the difference in fluorescence intensity between time zero and time t minutes; t is the reaction time in min; and v is the volume of the sample in ml.

Similarly, caspase-9 activity was determined using the caspase-9 assay kit, Fluorimetric (QIA72; EMD4Biosciences). The fluorescence intensity was recorded at a wavelength of 400 nm for excitation and a wavelength of 505 nm for emission. The same formula as the one used for the calculation of caspase-3 activity was used to calculate caspase-9 activity.

**TUNEL assay.** To detect the apoptosis of PSMCs following treatment with troglitazone, the PSMCs were cultured in BM and incubated for 72 h under hypoxic conditions. TUNEL assay was performed using the *In situ* Cell Death Detection kit (Roche) as per the manufacturer's instructions. In brief, the cells were fixed with 4% paraformaldehyde for 20 min at 25°C. After washing with PBS for 30 min, the cells were incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 10 min at 4°C. After washing, the cells were incubated with rabbit anti-calponin antibody overnight at 4°C. On the second day, reaction mixture from the kit supplemented with goat anti-rabbit IgG conjugated with FITC was applied to the cells for 1 h at 37°C in the dark. The samples were counterstained with DAPI to visualize the nuclei after TUNEL

staining. The apoptotic index was calculated as TUNEL<sup>+</sup> cells/total cells/low magnification field.

**Statistical analysis.** All statistical analyses were performed using SPSS software (version 10.0). All experiments were performed at least 3 times. The data are presented as the means  $\pm$  standard error means (SEM) and analyzed with the method of analysis of variance (ANOVA) using the Bonferroni test. A Student's t-test was performed to determine statistical difference. All tests were performed with a significance level of 5%.

## Results

**Toxicity of troglitazone towards PSMCs.** The toxicity of troglitazone towards the cells was determined in order to exclude the possibility that the decrease in PSMC proliferation was due to the toxicity of troglitazone towards PSMCs. PSMCs were cultured in GM supplemented with troglitazone for 48 h in an incubator under normal conditions. The supernatant was collected to measure LDH concentration (an index of cell injury). Significant cell injury was only observed when the troglitazone concentration was increased up to 60 and 80  $\mu$ M (Fig. 1).

The percentage of LDH in the supernatant was 1.2 $\pm$ 0.5% when the cells were cultured in GM without troglitazone, while it was 1.5 $\pm$ 0.5% with 0.5  $\mu$ M troglitazone, 1.8 $\pm$ 0.4% with 1  $\mu$ M, 2 $\pm$ 0.4% with 2  $\mu$ M, 1.8 $\pm$ 0.6% with 5  $\mu$ M, 2.1 $\pm$ 0.5% with 10  $\mu$ M, 1.8 $\pm$ 0.3% with 20  $\mu$ M, 2.1 $\pm$ 0.5% with 40  $\mu$ M, 3 $\pm$ 0.8% with 60  $\mu$ M and 3.7 $\pm$ 0.6% with 80  $\mu$ M troglitazone (Fig. 1). These results demonstrated that troglitazone was well tolerated by PSMCs.

**Concentration-dependent effects of troglitazone on PSMC proliferation.** The proliferation rate of the PSMCs was altered in a concentration-dependent manner by troglitazone in the cells cultured in GM under hypoxic conditions (Fig. 2A). The cell number of PSMCs treated with 0.5  $\mu$ M troglitazone was reduced to 93.3 $\pm$ 1.2% compared with that of the PSMCs not treated with troglitazone (considered as 100%). The percentage cell number of PSMCs cultured with troglitazone was 91 $\pm$ 2.7% with 1  $\mu$ M of troglitazone, 91.1 $\pm$ 3.5% with 2  $\mu$ M, 93.5 $\pm$ 2.8% with 5  $\mu$ M, 84.3 $\pm$ 4.2 with 10  $\mu$ M, 72 $\pm$ 5.8% with

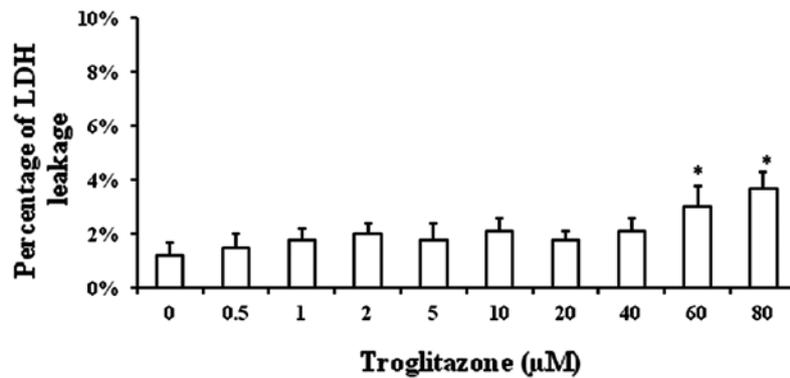


Figure 1. Cytotoxicity of various concentrations of troglitazone towards pulmonary artery smooth muscle cells (PASMCS). PASMCS were cultured in growth medium (GM) supplemented with troglitazone at 37°C in an incubator under normoxic conditions for 48 h. The toxicity of troglitazone significantly increased when its concentration was >60 µM. (\*P<0.05 vs. GM with 0 µM troglitazone). The experiment was performed in triplicate.

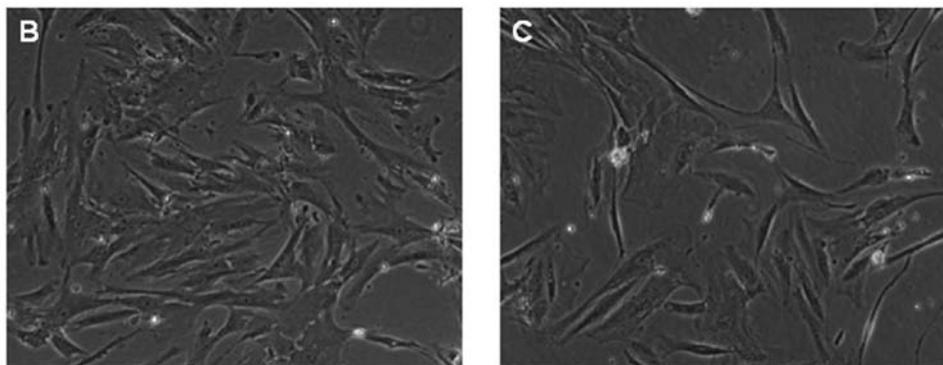
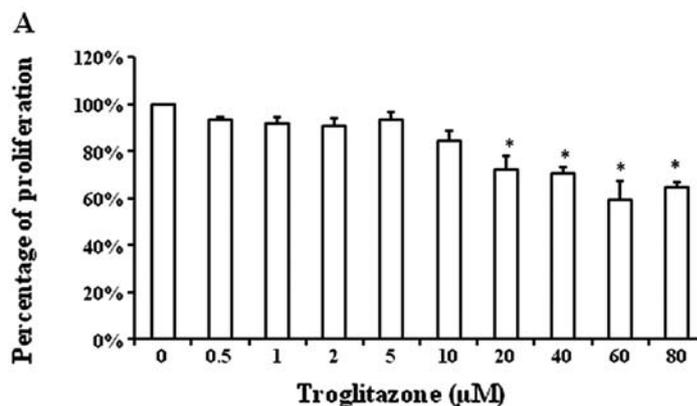


Figure 2. (A) Effects of various concentrations of troglitazone on the proliferation rate of pulmonary artery smooth muscle cells (PASMCS). PASMCS were cultured in growth medium (GM) supplemented with troglitazone under hypoxic conditions for 72 h. (B) Typical images of PASMCS cultured in GM only; (C) PASMCS cultured in GM supplemented with 20 µM troglitazone; (magnification, x100). (\*P<0.05 vs. GM with 0 µM troglitazone). The experiment was performed at least in triplicate.

20 µM, 70.4±3% with 40 µM, 59.3±8.3% with 60 µM and 64.6±2.1% with 80 µM troglitazone. Starting at the concentration of 20 µM, troglitazone significantly reduced the proliferation of PASMCS under hypoxic conditions (P<0.05).

Typical images of PASMCS density following culture in GM with or without troglitazone are shown in Fig. 2B and C. Starting at the concentration of 20 µM, troglitazone significantly reduced the PASMCS proliferation rate. Thus, we selected the concentration of 20 µM troglitazone for the remaining experiments.

*Troglitazone increases PTEN gene expression.* Troglitazone significantly increased PTEN gene expression by 2.4±0.1-fold at 2 h as compared with the baseline levels (P<0.05) under hypoxic conditions. Upregulated PTEN gene expression was maintained up to 8 h (1.7±0.02-fold, P<0.05) (Fig. 3A).

*Troglitazone does not alter gene expression of AKT-1 and AKT-2.* Troglitazone did not significantly alter the gene expression of AKT-1 and AKT-2 in the PASMCS between any 2 time points (Fig. 3B and C).

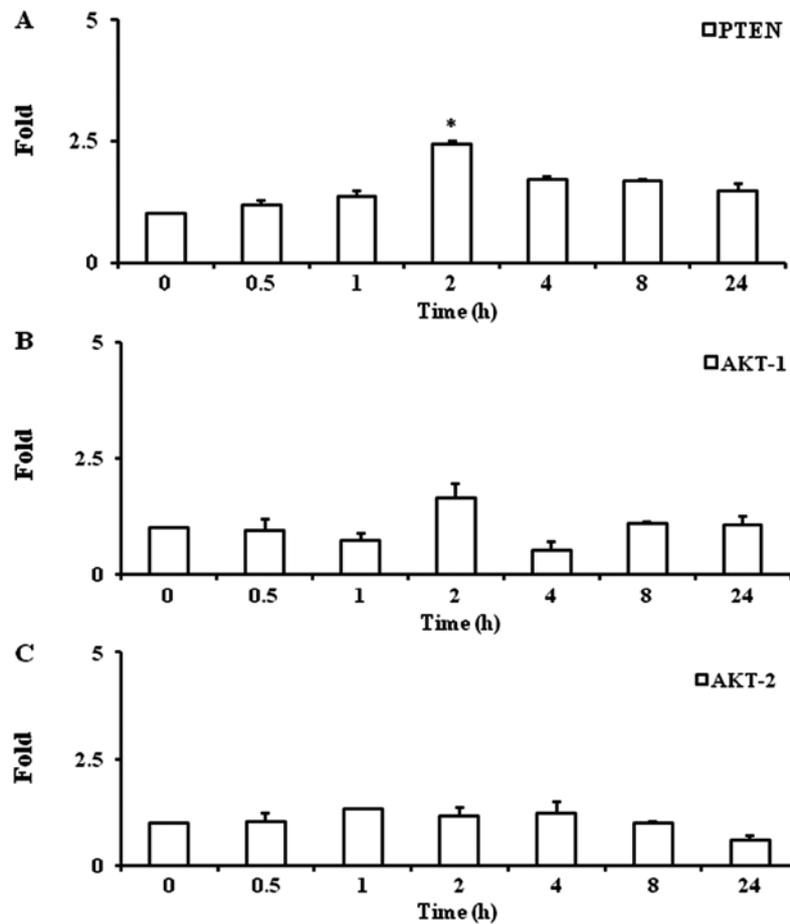


Figure 3. Effects of troglitazone on the expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), AKT-1 and AKT-2. (A) Troglitazone significantly increased the gene expression of PTEN in pulmonary artery smooth muscle cells (PASMCS) cultured in growth medium (GM) supplemented with troglitazone under hypoxic conditions starting at 2 h; this effect lasted up to 8 h (\* $P < 0.05$  vs. any other time point). Troglitazone did not significantly alter (B) AKT-1 and (C) AKT-2 gene expression in PASMCS cultured in GM under hypoxic conditions. The experiment was performed in triplicate.

*Western blot analysis of cultured PASMCS supplemented with troglitazone.* Troglitazone increased PTEN protein expression in PASMCS cultured under hypoxic conditions for 2 h. A significantly increased PTEN protein expression level was observed when the cells were treated with troglitazone at concentrations between 20-80  $\mu\text{M}$ , while no significant changes in p-AKT and AKT expression were observed (Fig. 4A). The PASMCS were then cultured with 20  $\mu\text{M}$  troglitazone for 24 h and DMSO-treated cells (without troglitazone) were used as controls, since troglitazone was diluted in DMSO. In the cells treated with troglitazone, a significantly increased PTEN protein expression was observed within 2 h compared with the cells treated with DMSO alone. The highest PTEN protein expression level was observed within 4 h after the addition of troglitazone, which was significantly higher than that at 0.5 h (Fig. 4B). Troglitazone also progressively reduced p-AKT expression within 2 h; these levels were significantly lower than those from the cells treated with DMSO alone.

To determine whether the upregulated PTEN protein expression induced by troglitazone was mediated by the PPAR $\gamma$  signaling pathway and inhibited by the PTEN inhibitor, PASMCS were pre-treated with 2.5  $\mu\text{M}$  bpV(HOpic) (a PTEN inhibitor) or 1  $\mu\text{M}$  GW9662 (PPAR $\gamma$  antagonist) for 1 h prior to

the addition of troglitazone. It was found that the upregulated protein expression of PTEN was reduced by pre-treatment with bpV(HOpic) and GW9662 for up to 24 h (Fig. 4C and D).

*Effects of bpV(HOpic) and GW9662 on proliferation of PASMCS treated with troglitazone.* Typical images of the PASMCS growth profile are shown in Fig. 5A-G. Troglitazone significantly reduced the proliferation rate of the PASMCS under hypoxic conditions. bpV(HOpic) was used to determine whether the PTEN inhibitor reversed the inhibitory effects of troglitazone on PASMCS proliferation. It was found that troglitazone did not reduce the proliferation rate of the PASMCS that were pre-treated with bpV(HOpic). These results suggest that troglitazone inhibits PASMCS proliferation by upregulating PTEN expression, which may be reversed by the PTEN inhibitor (Fig. 5H).

GW9662 was also used to determine whether the upregulation in PTEN expression stimulated by troglitazone was mediated by the PPAR $\gamma$  signaling pathway. It was found that GW9662 reversed the inhibitory effects of troglitazone on PASMCS proliferation. These results suggest that troglitazone upregulates PTEN expression through the PPAR $\gamma$  signaling pathway (Fig. 5).

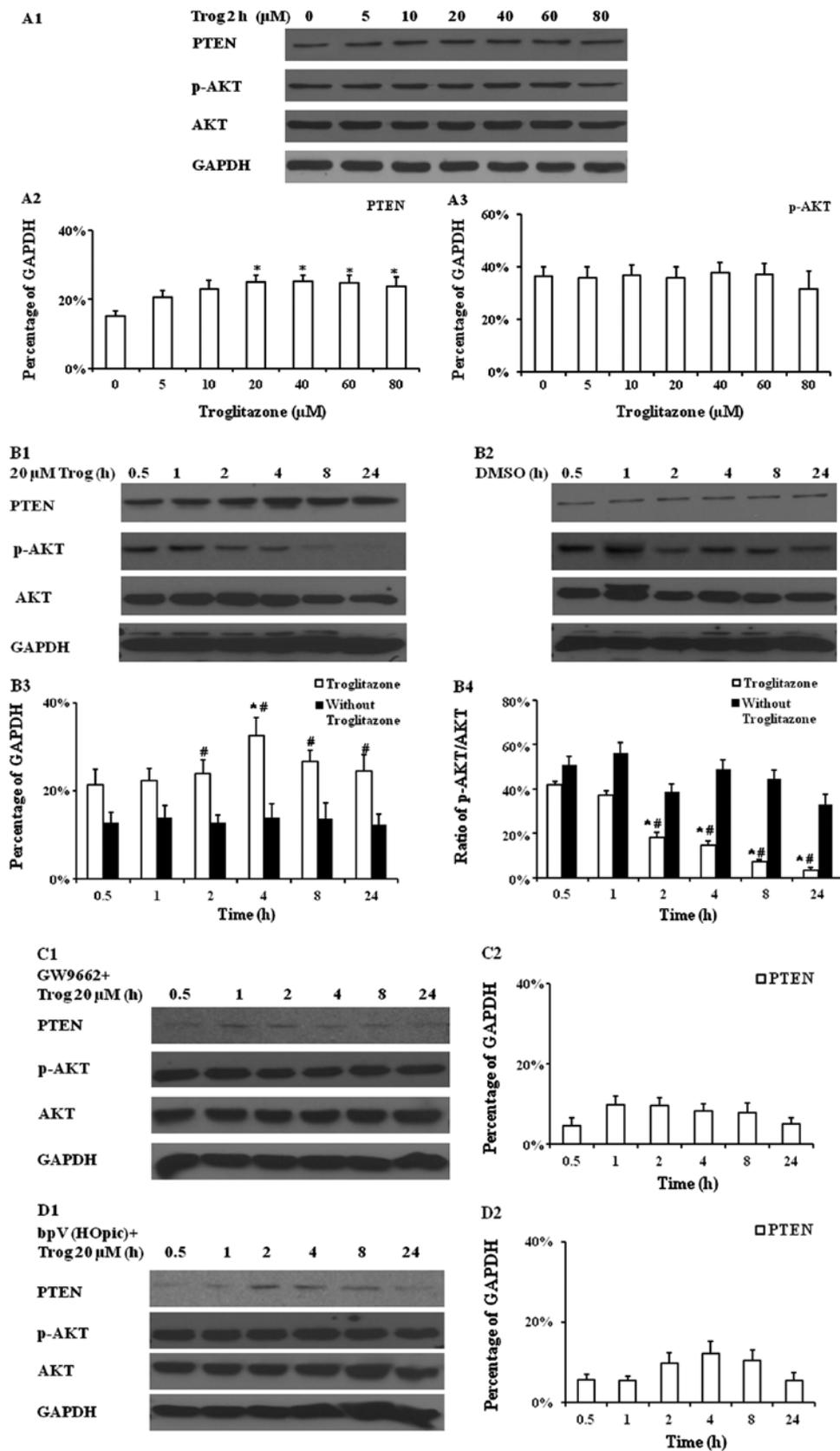


Figure 4. Western blot analysis of pulmonary artery smooth muscle cells (PASMCs) cultured in GM supplemented with troglitazone for 2 h under hypoxic conditions. (A1-3) Effects of various concentrations of troglitazone on the protein expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and p-AKT in PASMCs cultured under hypoxic conditions. A significantly increased PTEN protein expression was observed with concentrations of troglitazone starting from 20  $\mu$ M, while no significant change in phospho-AKT (p-AKT) expression was observed. (\* $P$ <0.05 vs. 0  $\mu$ M troglitazone). (B1-4) Effects of troglitazone on PTEN protein expression and p-AKT expression in PASMCs cultured with 20  $\mu$ M troglitazone under hypoxic conditions for various periods of time. PASMCs treated with DMSO (without troglitazone) were used as controls. Troglitazone significantly increased PTEN protein expression starting from 2 h as compared with DMSO treatment only, while troglitazone progressively reduced p-AKT expression. (\* $P$ <0.05 vs. 0.5 h troglitazone; # $P$ <0.05 vs. without troglitazone at 2, 4, 8 and 24 h). (C1-2) GW9662 (PPAR $\gamma$  antagonist) inhibited PTEN protein expression in PASMCs. GW9662 was added 1 h prior to the addition of 20  $\mu$ M troglitazone. (D1-2) bpV(HOpic) (PTEN inhibitor) inhibited PTEN protein expression in PASMCs. Trog, troglitazone. The experiment was performed in duplicate.

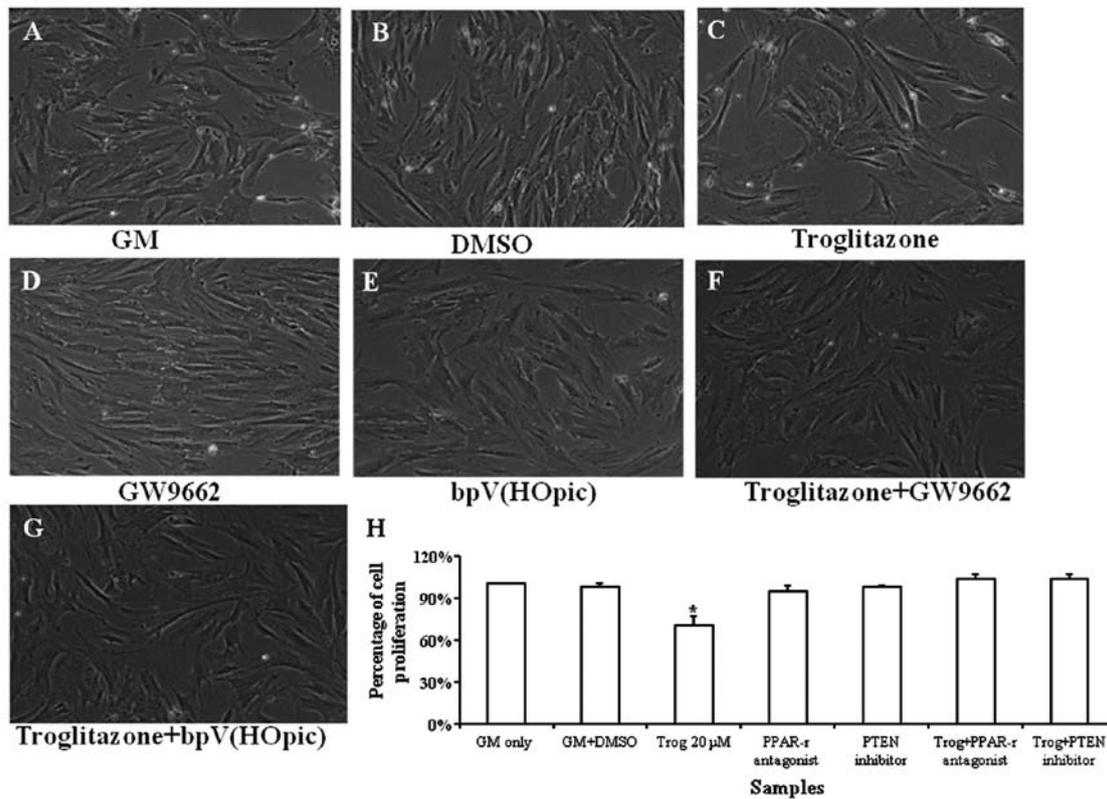


Figure 5. Growth profile of pulmonary artery smooth muscle cells (PASMCs) cultured in growth medium (GM) supplemented with troglitazone, GW9662 and bpV(HOPic) for 72 h under hypoxic conditions. Phase contrast images of (A) PASMCs cultured in GM only, (B) GM supplemented with DMSO, (C) GM supplemented with troglitazone, (D) GW9662, (E) bpV(HOPic), (F) troglitazone + GW9662, (G) troglitazone + bpV(HOPic). (H) Quantification of PASMCs treated with troglitazone, GW9662 and bpV(HOPic). The cell number of PASMCs cultured in GM was regarded as 100%. \*P<0.05 vs. any other samples. Magnification, x100; Troglitazone. The experiment was performed in triplicate.

*Troglitazone increases caspase-3, -8 and -9 activities.* A significantly increased caspase-3 activity was observed when the PASMCs were cultured in GM ( $6.5 \pm 0.15$  FI/min/ml) supplemented with troglitazone for 8 h (Fig. 6A). Similarly, a significant increase in caspase-8 and -9 activities was observed when the PASMCs were cultured in GM ( $5.3 \pm 0.15$  and  $6.1 \pm 0.27$  FI/min/ml) supplemented with troglitazone for 8 h (Fig. 6B and C).

*Troglitazone increases the apoptosis of PASMCs under hypoxic conditions.* TUNEL staining revealed that the apoptosis of PASMCs cultured in BM increased after 72 h under hypoxic conditions (Fig. 6D and E). Quantification of TUNEL<sup>+</sup> cells suggested that troglitazone significantly increased the apoptotic PASMC number compared with the cells cultured in BM alone (P<0.05).

## Discussion

The present study demonstrates that troglitazone, a high-affinity synthetic ligand of PPAR $\gamma$  upregulates PTEN gene and protein expression levels in PASMCs under hypoxic conditions in a concentration-dependent manner. In addition, we demonstrate that troglitazone increases the apoptosis of PASMCs under hypoxic conditions.

Pulmonary vascular remodelling in pulmonary arterial hypertension is characterized by changes in pulmonary

vascular structure (1,2). This is partially caused by an imbalance in PASM proliferation and apoptosis (10). The increased proliferation and decreased apoptosis of PASMCs result in the thickening of the pulmonary vasculature, which subsequently increases pulmonary vascular resistance and pulmonary arterial pressure (11).

Our results demonstrated that troglitazone at the concentration of 20-80  $\mu$ M effectively inhibited PASM proliferation under hypoxic conditions. The anti-proliferative effects of troglitazone may be related to its role in the upregulation of PTEN and the induction of the apoptosis of PASMCs under hypoxic conditions. PPAR $\gamma$  expression has been found in pulmonary vascular endothelial cells and SMCs (12,13). However its expression is reduced in lung vascular cells of rats with pulmonary hypertension and in patients with advanced primary or secondary pulmonary hypertension (12). The targeted depletion of PPAR $\gamma$  from SMCs has been shown to result in spontaneous pulmonary hypertension in mice (4). However, the activation of PPAR $\gamma$  with thiazolidinediones attenuates vascular remodeling and pulmonary hypertension caused by monocrotaline (14) or hypobaric hypoxia (15) in rats. Thiazolidinediones also reduce pulmonary hypertension in ApoE-deficient mice fed high-fat diets (16) and attenuate hypoxia-induced pulmonary hypertension and Nox4 expression and activity in mice (5).

However, the role of PPAR $\gamma$  in the regulation of PTEN expression of PASMCs under hypoxic conditions has not

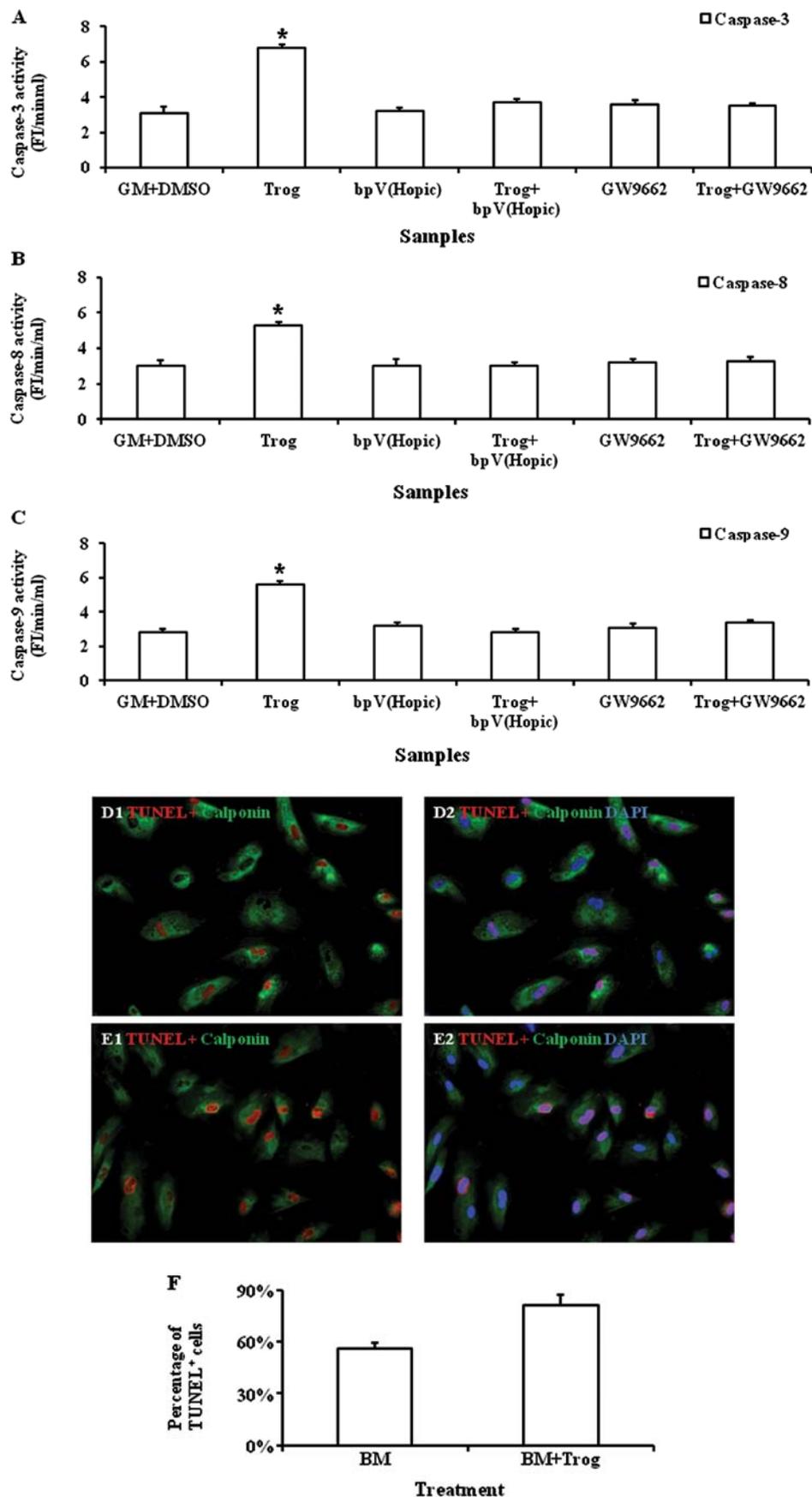


Figure 6. Troglitazone increases caspase, -3, -8 and -9 activity. Troglitazone increased (A) caspase-3, (B) caspase-8 and (C) caspase-9 activity in pulmonary artery smooth muscle cells (PASMCs) cultured under hypoxic conditions after 72 h ( $P < 0.05$  vs. all other samples). TUNEL staining to detect the apoptosis of PASMCs cultured in basal medium (BM) under hypoxic conditions for 72 h. (D1 and E1) The apoptotic nuclei of the PASMCs are shown in red colour. (D2 and E2) The cells were counterstained with DAPI to show cell nuclei. Magnification,  $\times 100$ . (F) Quantification of TUNEL<sup>+</sup> cells suggested that troglitazone significantly increased the apoptotic PASMC number as compared with those cultured in BM only. ( $P < 0.05$  vs. BM). Trog, troglitazone. The experiment was performed in duplicate.

been examined yet. Our results demonstrate that PPAR $\gamma$  upregulates PTEN gene and protein expression levels. Studies have shown that PPAR $\gamma$  agonists upregulate the transcription of PTEN in cancer cells. PPAR $\gamma$  agonists have been used as tumor suppressors in breast cancer by upregulating PTEN gene expression (17), while cells lacking PTEN or PPAR $\gamma$  are unable to induce PTEN-mediated cellular events in the presence of lovastatin or rosiglitazone. Zhang *et al* (7) showed that PTEN is required for the rosiglitazone-induced inhibition of BEL-7404 cells. Rosiglitazone upregulated PTEN expression in a concentration- and time-dependent manner, which was mediated by PPAR $\gamma$ . Furthermore, PTEN overexpression resulted in the inhibition of BEL-7404 cell migration, and PTEN knockdown blocked the effects of rosiglitazone on cell migration.

It is known that PPAR $\gamma$  agonists can stimulate the production and secretion of ApoE, which has anti-proliferative effects on human PSMCs (4). Nisbet *et al* (5) found that rosiglitazone attenuated hypoxia-induced pulmonary vascular remodelling and hypertension by suppressing oxidative and proliferative signals. They found that rosiglitazone attenuated the reduction in PTEN expression. However, the present study demonstrates that troglitazone upregulates PTEN gene and protein expression levels in human PSMCs under hypoxic conditions. This may provide new insight into the mechanisms behind the anti-proliferative effects of PPAR $\gamma$  on PSMCs under hypoxic conditions.

The data from the present study also suggest that troglitazone increases the apoptosis of PSMCs under hypoxic conditions. It is known that chronic hypoxia can prolong the growth of human vascular SMCs by inducing telomerase activity and telomere stabilization (18). The upregulation of PTEN negatively regulates the AKT/PKB signaling pathway and leads to a decrease in p-AKT levels and, consequently, to a decrease in AKT-mediated proliferation (19). Thus, a reduced p-AKT expression in PSMCs was found after the addition of troglitazone in this study. By reducing the cellular levels of p-AKT, PTEN downregulates cell division and increases apoptosis. This may result in increased activities of caspase-3, -8 and -9, thus inducing the apoptosis of PSMCs under hypoxic conditions.

In conclusion, the present study highlights that troglitazone increases PTEN gene expression at the transcriptional level under hypoxic conditions in a concentration-dependent manner. Troglitazone increases the apoptosis of PSMCs under hypoxic conditions. The increased PTEN expression and apoptosis of PSMCs provide new insight into the mechanisms behind the anti-proliferative effects of troglitazone on PSMCs.

## References

- McLaughlin VV, Archer SL, Badesch DB, *et al*: ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. *Circulation* 119: 2250-2294, 2009.
- Pietra GG, Edwards WD, Kay JM, *et al*: Histopathology of primary pulmonary hypertension. A qualitative and quantitative study of pulmonary blood vessels from 58 patients in the National Heart, Lung, and Blood Institute, Primary Pulmonary Hypertension Registry. *Circulation* 80: 1198-1206, 1989.
- Nakaoka T, Gonda K, Ogita T, *et al*: Inhibition of rat vascular smooth muscle proliferation in vitro and in vivo by bone morphogenetic protein-2. *J Clin Invest* 100: 2824-2832, 1997.
- Hansmann G, de Jesus Perez VA, Alastalo TP, *et al*: An anti-proliferative BMP-2/PPAR $\gamma$ /apoE axis in human and murine SMCs and its role in pulmonary hypertension. *J Clin Invest* 118: 1846-1857, 2008.
- Nisbet RE, Bland JM, Kleinhenz DJ, *et al*: Rosiglitazone attenuates chronic hypoxia-induced pulmonary hypertension in a mouse model. *Am J Respir Cell Mol Biol* 42: 482-490, 2010.
- Lee KS, Park SJ, Hwang PH, Yi HK, Song CH, Chai OH, Kim JS, Lee MK and Lee YC: PPAR- $\gamma$  modulates allergic inflammation through up-regulation of PTEN. *FASEB J* 19: 1033-1035, 2005.
- Zhang W, Wu N, Li ZX, Wang LY, Jin JW and Zha XL: PPAR $\gamma$  activator rosiglitazone inhibits cell migration via upregulation of PTEN in human hepatocarcinoma cell line BEL-7404. *Cancer Biol Ther* 5: 1008-1014, 2006.
- Ye L, Haider KhH, Esa WB, *et al*: Liposome-based vascular endothelial growth factor-165 transfection with skeletal myoblast for treatment of ischaemic limb disease. *J Cell Mol Med* 14: 323-336, 2010.
- Ye L, Lee KO, Su LP, *et al*: Skeletal myoblast transplantation for attenuation of hyperglycaemia, hyperinsulinaemia and glucose intolerance in a mouse model of type 2 diabetes mellitus. *Diabetologia* 52: 1925-1934, 2009.
- Zhang S, Fantozzi I, Tigno DD, *et al*: Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 285: L740-L754, 2003.
- Barst RJ, McGoon M, Torbicki A, Sitbon O, Krowka MJ, Olschewski H and Gaine S: Diagnosis and differential assessment of pulmonary arterial hypertension. *J Am Coll Cardiol* 43: 40S-47S, 2004.
- Ameshima S, Golpon H, Cool CD, *et al*: Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) expression is decreased in pulmonary hypertension and affects endothelial cell growth. *Circ Res* 92: 1162-1169, 2003.
- Michalik L, Auwerx J, Berger JP, *et al*: International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* 58: 726-741, 2006.
- Matsuda Y, Hoshikawa Y, Ameshima S, *et al*: Effects of peroxisome proliferator-activated receptor gamma ligands on monocrotaline-induced pulmonary hypertension in rats. *Nihon Kokyuki Gakkai Zasshi* 43: 283-288, 2005 (In Japanese).
- Crossno JT Jr, Garat CV, Reusch JE, *et al*: Rosiglitazone attenuates hypoxia-induced pulmonary arterial remodeling. *Am J Physiol Lung Cell Mol Physiol* 292: L885-L897, 2007.
- Hansmann G, Wagner RA, Schellong S, *et al*: Pulmonary arterial hypertension is linked to insulin resistance and reversed by peroxisome proliferator-activated receptor- $\gamma$  activation. *Circulation* 115: 1275-1284, 2007.
- Teresi RE, Shaiu C, Chen C, Chatterjee K, Waite KA and Eng C: Increased PTEN expression due to transcriptional activation of PPAR $\gamma$  by Lovastatin and Rosiglitazone. *Int J Cancer* 118: 2390-2398, 2006.
- Minamino T, Mitsialis SA and Kourembanas S: Hypoxia extends the life span of vascular smooth muscle cells through telomerase activation. *Mol Cell Biol* 21: 3336-3342, 2001.
- Maehama T and Dixon JE: The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273: 13375-13378, 1998.