Mol Cell Biochem (2010) 344:81–89
DOI 10.1007/s11010-010-0531-5

# Exploring the molecular mechanisms of OSU-03012 on vascular smooth muscle cell proliferation

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Received: 12 April 2010 / Accepted: 22 June 2010 / Published online: 13 July 2010


**Abstract** Restenosis is resulted from the proliferation and migration of vascular smooth muscle cells (VSMCs) from the arterial media into the intima within the vessel lumen following percutaneous transluminal coronary angioplasty (PTCA). OSU-03012, a synthetic compound (2-amino-*N*-{4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-phenyl} acetamide) acting as a PDK-1 inhibitor, is used as an apoptosis-promoting anticancer drug. However, whether OSU-03012 can inhibit VSMC proliferation and migration following PTCA remains unclear. In this study, we used A10 smooth muscle cells cultured in 10% FBS for stimulating proliferation and evaluated the inhibitory effects of OSU-03012 on cell proliferation and migration. The data demonstrated that OSU-03012 dose-dependently inhibited A10 cell proliferation examined by Trypan blue, MTT and morphological alteration assays, and inhibited the levels of proliferation-related proteins, proliferating cell nuclear antigen (PCNA), phosphorylated ERK examined by western blotting. Additionally, 10 μM OSU-03012 also enhanced apoptosis examined using DAPI assay by regulating apoptosis-related proteins. Furthermore, compared with the control group, A10 cells treated with 10 μM OSU-03012 showed a lower number of migrating cells examined by Boyden Chamber assay, and a dose-dependently reduced NF*k*B-dependent and interferon-stimulated response element (ISRE) promoter luciferase activities, implying the anti-migration and anti-inflammation effects of OSU03012. Taken together, this study provides insights into the pharmacological mechanisms of OSU-03012 in preventing smooth muscle cell proliferation, migration, and inflammation supporting the novel discovery of OSU-03012 as an adjuvant therapy for balloon injury-induced restenosis.

**Keywords** Restenosis · OSU-03012 · Percutaneous transluminal coronary angioplasty (PTCA) · Migration · Vascular smooth muscle cell (VSMC) proliferation

**Abbreviations**

| | |
|---|---|
| VSMC | Vascular smooth muscle cell |
| PTCA | Ercutaneous transluminal coronary angioplasty |

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PCNA Proliferating cell nuclear antigen
ISRE Interferon-stimulated response element

## Introduction

Percutaneous transluminal coronary angioplasty (PTCA), a balloon catheter-based interventional procedure, is a standard procedure for treating coronary artery stenosis. PTCA has been routinely used in patients with stable and unstable angina and acute myocardial infarction. However, the reoccurrence of restenosis in 30% of the patients within 6 months following the angioplasty procedure is the major disadvantage of PTCA [1, 2]. The arterial remodeling resulting from balloon injury is manifested as neointimal formation with significant loss of luminal patency. However, the systemic side effects of this application were inevitable. Stents are used to decrease restenosis. However, 20 to 30% of the patients are still affected by restenosis after coronary stenting [3]. The main cause of restenosis has been referred to as abnormal vascular smooth muscle cell (VSMC) proliferation into the intimal layer. Therefore, blocking VSMC proliferation becomes an important therapeutic targete to inhibit restenosis following PTCA.

OSU-03012, 2-amino-*N*-{4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] -phenyl} acetamide, is derived from Celecoxib, which is a cyclooxygenase-2 inhibitor. Celecoxib has been shown to inhibit cancer cell growth in vitro via induction of apoptosis [4]. It is reported that OSU-03012 acts as a 3-phosphoinositide-dependent kinase-1 (PDK-1) inhibitor to inhibit cancer cell growth and possesses greater inhibitory ability than Celecoxib [4]. OSU-03012 is also used in killing primary human glioma and other transformed cells [5]. Based on the inhibitory effects on cell growth by OSU-03012, we hypothesized that OSU-03012 could be treated for inhibiting abnormal vascular smooth cell proliferation to improve restenosis following PTCA.

Accordingly, we aimed in the present study at exploring a pharmacological remedy of OSU-03012 in inhibiting restenosis. To test whether OSU-03012 can be an effective therapeutic intervention for restenosis, the effects and related molecular mechanisms of OSU-03012 in inhibiting abnormal cell proliferation were examined. The component protein levels of cell proliferation- and apoptosis-related pathways were detected to explore its inhibitory mechanism on neointimal formation. These findings may provide an insight into the therapeutic strategy of OSU-03012 on restenosis following PTCA.

## Methods

### OSU-03012

OSU-03012 was synthesized in Dr. Jing-Ru Weng's laboratory with purity greater than 99% according to published procedures, and the identity and purity of these agents were confirmed by nuclear magnetic resonance and mass spectrometry.

### Cell culture

A10 VSMCs derived from rat thoracic aorta were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in 10-cm dishes containing DMEM supplemented with 10% FBS, 3.7 g/l $NaHCO_3$, 1.028 g/l N-acetyl L-alanyl-L-glutamine, 1% Na-pyruvate, 4.5 g/l D-glucose, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfates. The culture medium was replaced every 2 days and the cells were passaged every week. The cells that became at least 80% confluent were starved for 24 h in DMEM containing 0.5% FBS followed by a treatment with 1,5 and 10 μM of OSU-03012 in DMEM containing 10% FBS for 24 h.

### Cell proliferation assay

We used 2 different methods, cell counting assay and MTT assay. For cell counting assay, cells were seeded in 24-well plates with $1 \times 10^5$ cells/well in DMEM supplemented with 10% FBS. After 24 h, cells were washed with phosphate-buffered saline (PBS) and then exposed to either DMSO alone or serial dilutions (5, 10, and 15 μM) of OSU-03012. After incubated with 24, 48, and 72 h, cells and Trypan Blue dye were mixed. A hemacytometer was used to analyze the mixture under a microscope. The number of blue cells was counted, and fractional viability was calculated by dividing the number of clear cells by the total number of cells. MTT assay was performed to measure the cytotoxicity of OSU-03012 on VSMCs. Cells were seeded in 96-well plates with $8 \times 10^3$ cells/well in DMEM supplemented with 10% FBS. After 24 h, cells were washed with phosphate-buffered saline (PBS) and then exposed to either DMSO alone or serial dilutions (1, 2, 4, 8, 16, and 32 μM) of OSU-03012. After 24 h, the number of viable cells was determined [6]. Briefly, MTT (3 mg/ml in PBS) was added to each well (25 μl per 200 ml medium), and the plate was incubated at 37°C for 4 h. Cells were then spun at 300×*g* for 5 min, and the medium was carefully aspirated. A 50 μl aliquot of DMSO was added, and the absorbance at 595 nm was measured for each well on a ELISA reader (Anthos, 2001, Anthos Labtech. Austria).

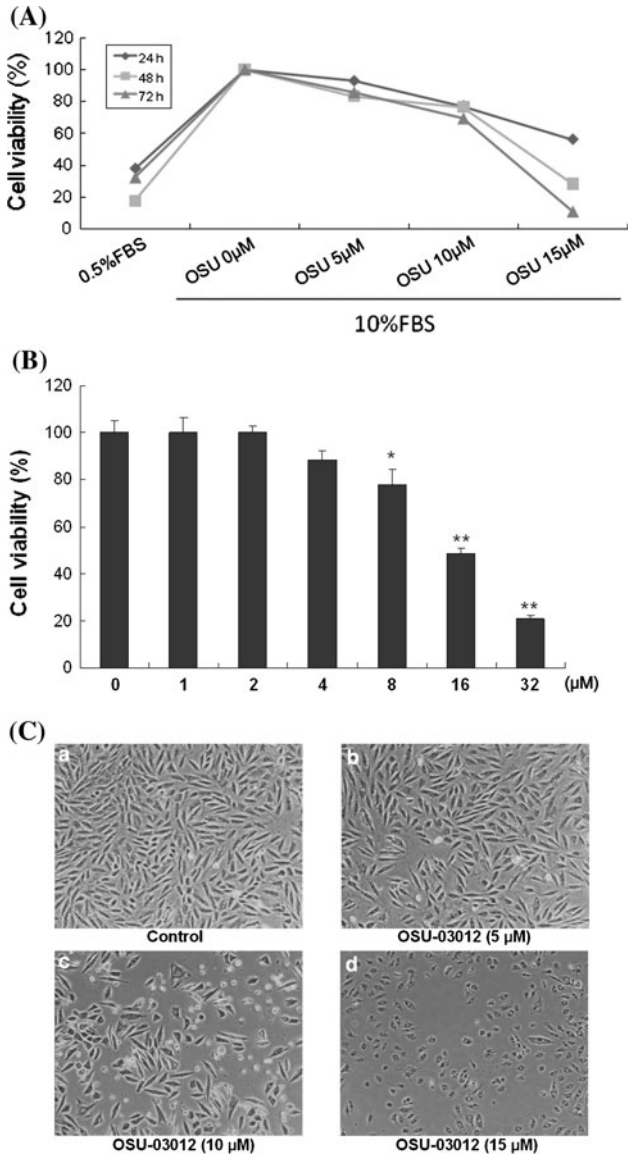



**Fig. 1** **A** Inhibitory effects of OSU-03012 on A10 cell growth examined by cell counting assay. The cells on 24-well plate were stimulated with 10% FBS with different concentrations of OSU-03012 (5, 10, and 15 μM) for 24, 48, 72 h. Cells treated with 0.5% FBS were used as a control. After incubation, cell viability was determined by trypan blue staining. **B** Inhibitory effects of OSU-03012 on A10 cell growth evaluated by MTT assay. The cells on 96-well plate were stimulated with 10% FBS with different concentrations of OSU-03012 (0, 2, 4, 8, 16, and 32 μM) for 24 h. The culture medium was then replaced with MTT and the plate was incubated at 37°C for 4 h. A 50 μl aliquot of DMSO was added after 4 h, and the absorbance at 595 nm was measured for each well on an ELISA reader. Values are expressed as a percentage of the control group. The average result ± SE of three independent experiments is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to the cells stimulated with 10% FBS only. **C** Morphology alteration of A10 cells stimulated with 10% FBS and treated with different doses of OSU-03012, **a** Control, **b** 5 μM, **c** 10 μM, **d** 15 μM, and then observed using microscope

### Western blot

A10 cells cultured in petri dishes were incubated with 1, 5, and 10 μM of OSU-03012 in DMEM containing 10% FBS in time course. The cells were then lysed in protein extraction buffer (iNtRON Biotechnology Inc.), followed by incubation at 95°C for 5 min. Samples were separated using SDS–PAGE, transferred to PVDF membranes, blocked with 5% nonfat dry milk in PBS-Tween for 1 h, and then probed with the desired antibodies (anti-PCNA, anti-Raf, anti-Erk-2, anti-p-Erk-2, anti-Bcl-2, anti-AIF, anti-caspase-3, anti-caspase-8, and anti-cyt.c, which were purchased from Santa Cruz Biotechnology Inc. Santa Cruz (California, USA)) overnight at 4°C. The blots were then incubated with horseradish peroxidase-linked secondary antibody for 1 h followed by development with the ECL reagent and exposure to Hyperfilm (Amersham, Arlington Height, IL, USA).

### DAPI staining

Seeding A10 cells in 6-wells plate were incubated with 10 μM of OSU-03012 in DMEM containing 10% FBS in time course. Wash cells three times with PBS. Add 1 ml 3% Formaldehyde for 15–20 min. Wash cells two times with PBS. Add 1 ml 0.1% Triton X-100 for 15 min. Add DAPI to 1 μg/ml. Stain 1 min. Wash cells two times with PBS. Cells were observed by using fluorescence microscope, and then all pictures were taken at 400×.

### Boyden chamber assay

A10 cells were plated into 6-well plates at $1.5 \times 10^5$ cells/well, cultured in 10 μM of OSU-03012 or 10% FBS only, and then harvested. Cells were placed in the upper compartment and allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents were present. After 6 h, the membrane between the two compartments were fixed and stained by using methanol and Giemsa stain solution. The number of cells that have migrated to the lower side of the membrane was determined.

### Reporter assay

A10 cells were plated into 24-well plates at $1 \times 10^5$ cells/well 24 h before transfection. 0.8 μg pGL3-Basic (Promega) reporter plasmid each is containing the NF-κB, ISRE and 0.2 μg pRL-CMV vector (Promega) were cotransfected into each well by using the Arrest-In™ transfection reagent (Open Biosystems) in triplicates. After transfection, the cells

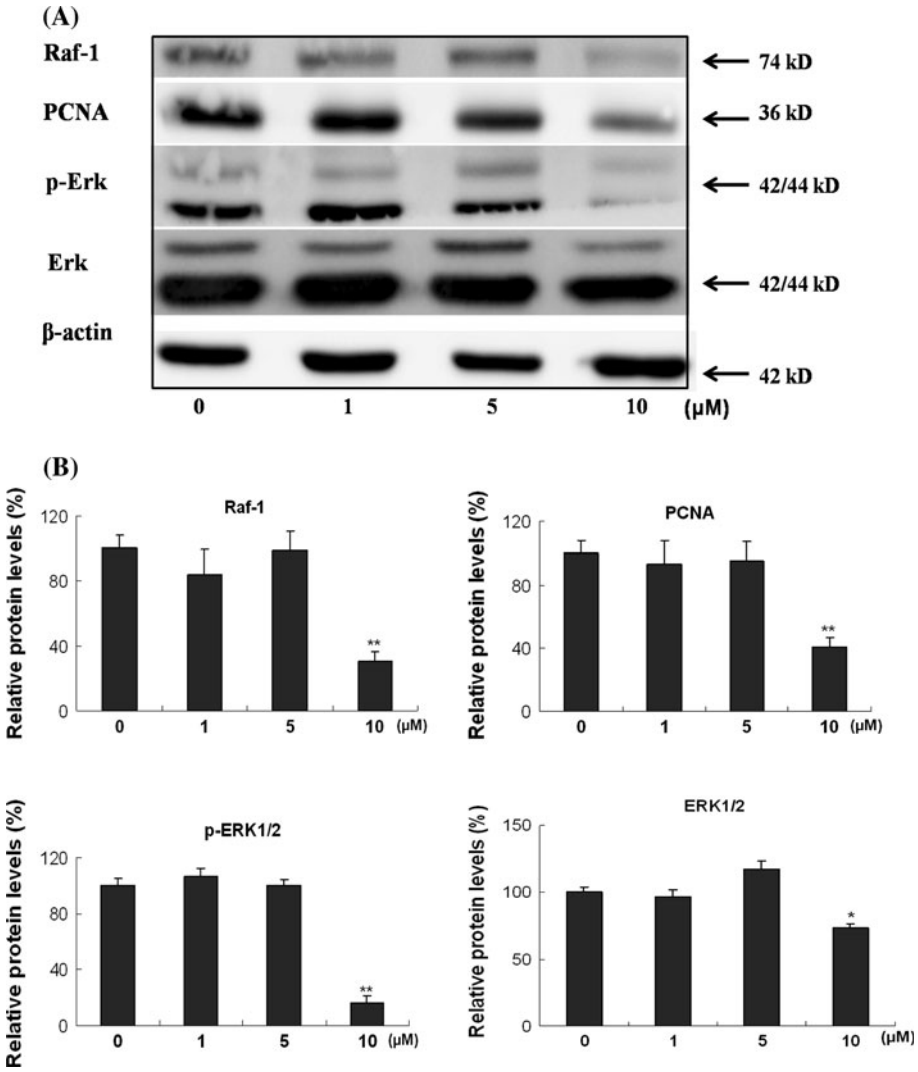



**Fig. 2** OSU-03012 decreased proliferative protein levels of Raf-1, PCNA, p-Erk and Erk in rat smooth muscle cells. **a** A10 cells were cultured in 10% FBS medium with various concentrations of OSU-03012 (1, 5, and 10 μM) for 24 h. The cells were then lysed, and 50 μg protein underwent SDS–PAGE followed by Western blotting. **b** Signal intensity was quantitated using a PhosphoImager. $\beta$-actin was used as a loading control. Values are expressed as a percentage of the control group. The average result ± SE of three independent experiments is shown. * $P < 0.05$, ** $P < 0.01$ as compared with control

were treated with different concentrations of OSU-03012 (1, 5, and 10 μM) for 24 h. Luciferase assays were performed 24 h after the treatment by using the Dual-Luciferase Reporter Assay System (Promega) on a luminometer.

### Statistics

Data are expressed as mean ± S.E.M. Statistical analysis was conducted using unpaired-Student's $t$ test. A $P$ value $\leqq 0.05$ was considered significant.

## Results

### Inhibitory effects of OSU-03012 on A10 cell proliferation

Although OSU-03012 is identified to be a competitive compound for cytotoxicity in several cancer cells, its role for preventions of cardiovascular diseases remains to be elucidated. We first performed Trypan blue assay to measure the viability of smooth muscle cell cultured in 10% FBS and treated with different doses of OSU-03012 (5, 10, and 15 μM) for different time periods (24, 48, and 72 h). Figure 1A shows that the cell viability dose-dependently decreased with each time period.

To determine whether OSU-03012 possesses inhibitory effects on A10 cell proliferation, the MTT assay was performed. Figure 1B shows that OSU-03012 (1, 2, 4, 8, 16, or 32 μM) inhibited the proliferation of A10 smooth muscle cells in a dose-dependent manner. Figure 1C shows that OSU-03012 (5, 10, and 15 μM) affected the morphology of cells cultured in 10% FBS after treatment for 24 h. Compared with the control group, the cells shrank as the dose increased. These results indicate that OSU-03012 significantly inhibited smooth muscle cell proliferation.

To further confirm how OSU-03012 affected cell proliferation, 10% FBS-treated A10 cells were administered

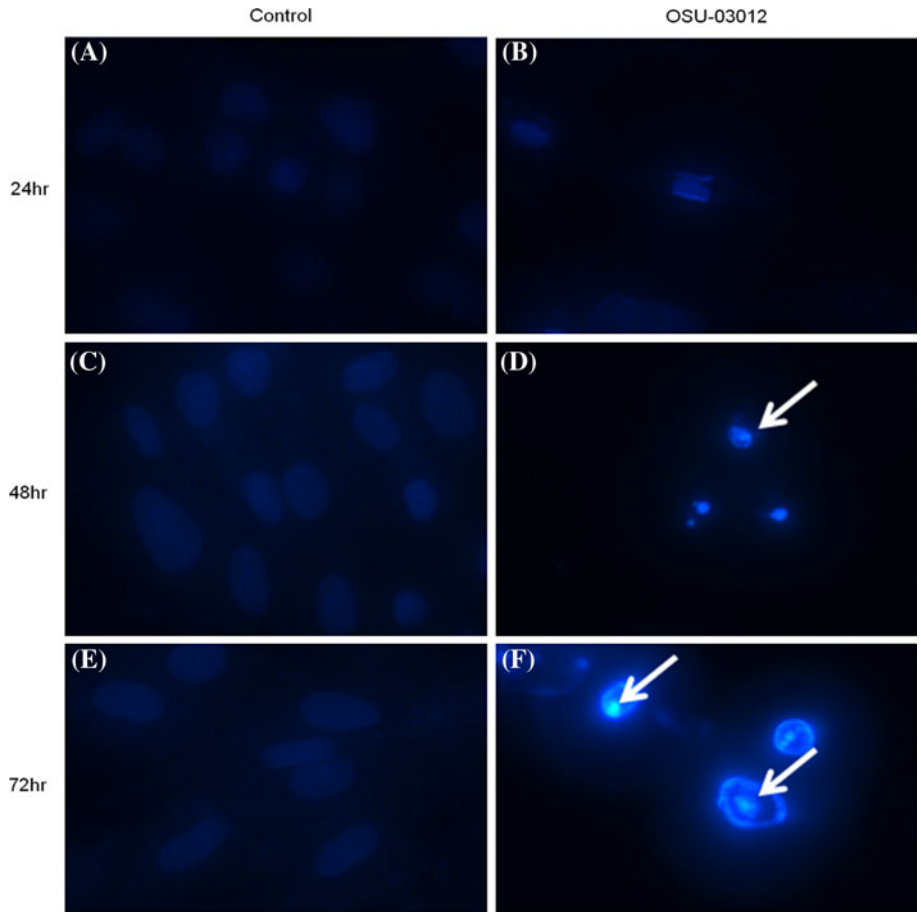



**Fig. 3** The DAPI staining demonstrates the apoptotic effects of OSU-03012 on smooth muscle cells. The cells were cultured in 10% FBS with or without 10 μM of OSU-03012 for 24, 48, and 72 h. After incubated with time periods, the cells were fixed and incubated with DAPI for 1 min, and observed using a fluorescent microscope. *White arrows* indicate the apoptotic nucleus. All graphs were taken at ×400

with 1, 5, and 10 μM of OSU-03012 for 24 h. Total proteins were extracted from cells and subjected to Western blot analysis with antibodies against PCNA, Raf-1, Erk-2, p-Erk-2. Figure 2 shows that 10 μM of OSU-03012 significantly reduced the protein levels of PCNA, Raf-1 and p-Erk, by 41.04, 30.72, and 16.57%, respectively, of control group. This data is similar to the Trypan blue assay results, in which 10 μM, but not 1 or 5 μM, OSU-03012 reduced cell proliferation. The results demonstrate that OSU-03012 inhibits cell proliferation through inhibiting MAP kinase pathway activity as well as the PCNA protein level.

## Enhancing effects of OSU-03012 on A10 cell apoptosis are through affecting apoptosis-related proteins

It is reported that OSU-03012 can enhance death in some cancer cells [5]. To confirm that the inhibitory effects of OSU-03012 on cell proliferation were mediated by inducing cell apoptosis, we performed DAPI staining assays. The results are shown in Fig. 3. We found that 10 μM OSU-03012 induced cell apoptosis in VSMCs with increasing brightness with increasing time periods, suggesting that OSU-03012-induced VSMC apoptosis.

To test whether OSU-03012 could act as the agent that induced proliferated smooth muscle cell death through the apoptosis induction, 10% FBS-treated A10 cells were administered with 1, 5, and 10 μM of OSU-03012 for 24 h. Total proteins were extracted and analyzed by Western blotting with antibodies against Bcl-2, AIF, Caspase-3, Caspase-8, and PARP. Figure 4a shows that 10 μM of OSU-03012 significantly reduced the protein levels of Bcl-2 (47.79%) and significantly increased the protein levels of AIF (163.74%), Caspase-3 (242.92%), and Caspase-8 (380.80%) compared with the control group (100%). In addition, we treated cells with 10 μM of OSU-03012 and collected cells at different time points (6, 12, 24, and 48 h). Figure 4b shows that the protein level of Bcl-2 significantly decreased (71.35%) after treatment with OSU-03012 for 48 h. The caspase-3 and Caspase-8 protein levels were increased about 105.48 and 137.19%, respectively, whereas cytochrome *c* slightly increased about 140.44% compared with the control (100%) after 24-h treatment. The results demonstrate that OSU-03012

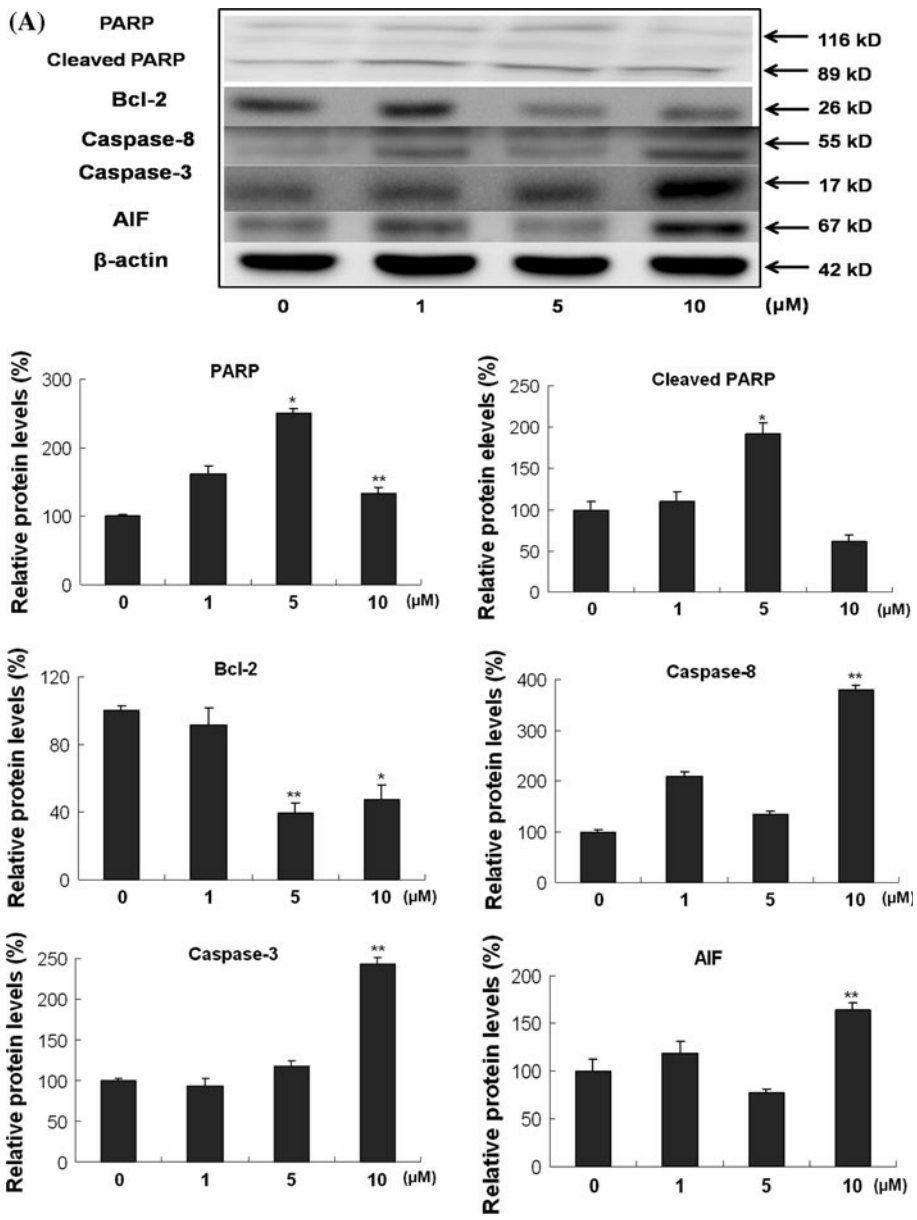



**Fig. 4** OSU-03012 enhanced cell apoptosis through upregulating apoptotic proteins and downregulating anti-apoptotic proteins in rat smooth muscle cell. **a** A10 cells were cultured in 10% FBS medium with various concentrations of OSU-03012 (0, 1, 5, and 10 μM) for 24 h. The cells were then lysed, and 50 μg protein underwent SDS–PAGE followed by Western blotting. **b** A10 cells were treated with 10 μM of OSU-03012 for 0, 6, 12, 24, and 48 h. After incubation for the indicated time periods, the cells were lysed and 50 μg of the protein underwent SDS–PAGE followed by Western blotting. The signal intensity was quantitated using a PhosphoImager. $\beta$-actin was used as a loading control. Values are expressed as a percentage of the control group. The average result ± SE of three independent experiments is shown. * $P < 0.05$, ** $P < 0.01$ as compared with control

may induce smooth muscle cell apoptosis through affecting apoptosis-related proteins.

## Inhibitory effects of OSU-03012 on A10 cell migration

Restenosis is the result of smooth muscle cells migrating from the arterial media into the intima, where they change into a synthetic phenotype, produce extracellular matrix and proliferate, resulting in a stenosis within the vessel lumen [7, 8]. To test whether OSU-03012 could inhibit the migration of smooth muscle cells, 10% FBS-treated A10 cells were administered with or without 10 μM OSU-03012 for 24 h. We performed the Boyden chamber assay to examine whether OSU-03012 treatment could decrease the number of cells passing through the membrane between two compartments. Figure 5 shows that OSU-03012 significantly inhibited VSMC migration compared with the control. These data indicate that OSU-03012 possesses the potential to inhibit VSMC migration.

## Inhibition of NF-$\kappa$B-dependent and interferon-stimulated response element (ISRE) transcription activities by OSU-03012 in 10% FBS-treated rat vascular smooth muscle cells

The transcription activities of NF-$\kappa$B-dependent and ISRE promoter were evaluated in the smooth muscle cells transfected with a plasmid construct containing NF-$\kappa$B

**Fig. 4** continued

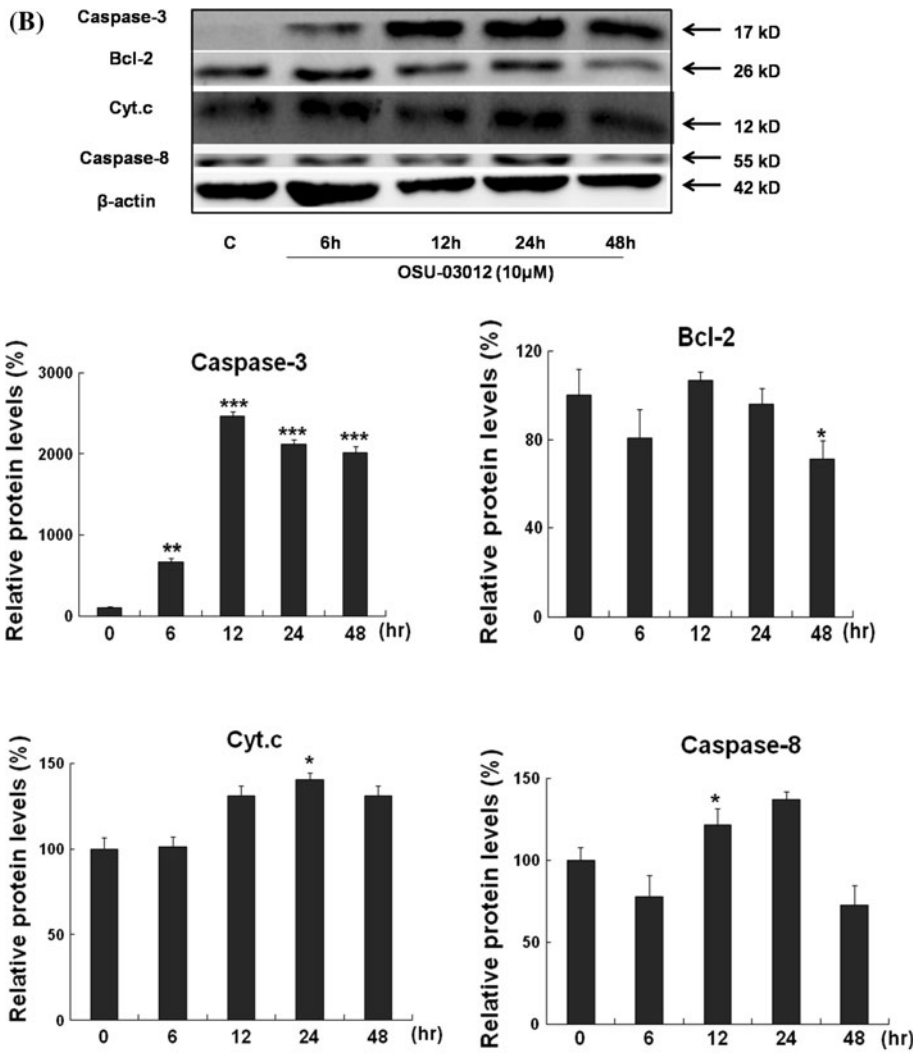
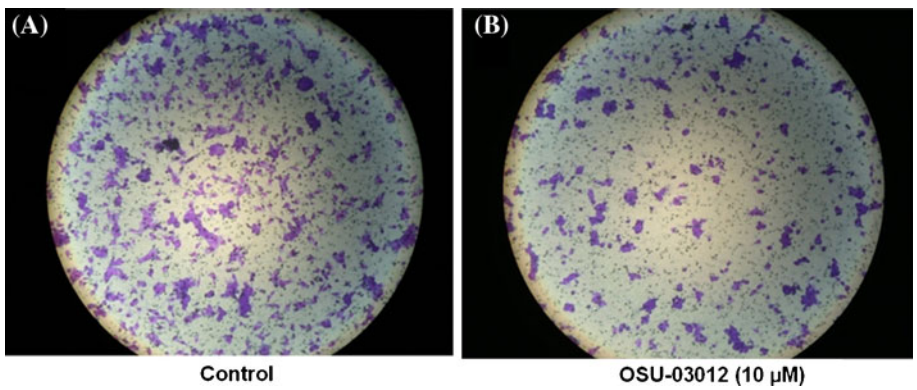



**Fig. 5** The decreases in VSMC migration following the treatment of OSU-03012. VSMCs cultured in 10% FBS were treated with or without 10 μM OSU-03012 for 24 h. Cell migration was analyzed by Boyden chamber assay. Cells receiving **a** 10% FBS only served as a control and **b** plus 10 μM of OSU-03012 were evaluated. All pictures were taken at ×100




binding site or ISRE promoter conjugated with luciferase reporter. These transfected cells were treated with 1, 5, and 10 μM of OSU-03012 in the presence of 10% FBS. Figure 6 shows that the 10% FBS-stimulated transcription activation of ISRE promoter and NF-κB-dependent promoter measured as luciferase activities were dose-dependently reduced by OSU-03012, especially the activity of ISRE promoter reached a significant level.

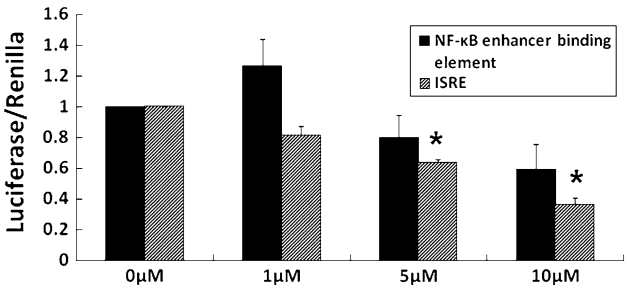



**Fig. 6** Inhibitory effects of OSU-03012 on NF-$\kappa$B-dependent and interferon-stimulated response element (ISRE) luciferase activities in rat smooth muscle cells. A10 cells transfected with a plasmid construct containing NF-$\kappa$B-dependent or ISRE promoter conjugated with luciferase reporter. Cells were co-transfected with the Renilla luciferase gene for normalizing the luciferase activity to decrease the bias from different transfection efficiency. These transfected cells were treated with 1, 5, and 10 μM of OSU-03012 in the presence of 10% FBS for 24 h. Luciferase assays were performed 24 h after the treatment using the Dual-Luciferase Reporter Assay System (Promega) on a luminometer. The average result ± SE of three independent experiments is shown. * $P < 0.05$, as compared to the control

## Discussion

We evaluated whether the anti-proliferative effects of OSU-03012 on cancer therapy can be extended to inhibit the growth of VSMCs for the treatment of cardiovascular diseases. Our results first demonstrated that OSU-03012 exerted potent inhibitory effects on the growth of VSMCs analyzed using Trypan blue, MTT, and cell morphology assays. Our previous reports show that the *ras* gene is involved in the underlying mechanisms for neointimal formation by balloon injury [9, 10]. The mitogen-activated protein kinase (MAPK) pathways play an important role in promoting VSMC proliferation [11, 12]. In addition, proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase delta [13, 14], is required for DNA synthesis and combines with other key cell-cycle control proteins, such as the cyclins and cyclin-dependent kinase to enhance cell proliferation [15]. Wei and his colleagues showed that PCNA was markedly induced after balloon injury using a rat carotid-injury model [16]. Therefore, several proteins involved in these pathways were examined. In our results, the findings demonstrate that the increased levels of PCNA, Raf-1 and phosphorylated Erk were suppressed by OSU03012, indicating the anti-proliferation effects of OSU03012 is mediated through downregulating MAPK signaling.

So far, all previous studies on OSU-03012 are focused on cancer cells [4, 5, 17]. There are no reports that identify OSU-03012 as a treatment in preventing vascular smooth cell proliferation. Our study demonstrates the novel finding that OSU-03012 can inhibit VSMC proliferation as a therapeutic target for improving the development of restinosis following PCTA. OSU-03012 acts as 3-phosphoinositide-dependent kinase-1 (PDK-1) inhibitor and its anticancer effect on cancer cells is through phosphoinositide-3-kinase/Akt pathway inhibition to cause cell apoptosis [5, 18]. We found that the protein levels of those apoptosis-associated proteins, caspase-3, caspase-8, AIF and PARP as well as apoptosis examined by DAPI staining were all increased following OSU-03012 treatment. These results may identify that OSU-03012 not only exerts inhibiting effects on VSMC proliferation, but also exerts promoting effects on VSMC apoptosis.

One of the major processes in retenosis is the VSMC migration from the arterial media to the intima within the vessel lumen following PTCA, and our results demostrating decreased migration cell number evaluated by Boyden chamber assay, agree with the anti-migration effect of OSU-03012 on VSMCs at dose 10 μM. NF-$\kappa$B is a key transcription element closely related to cell proliferation. Studies showed that NF-$\kappa$B is responsible for smooth muscle cell proliferation induced by serum and tumor necrosis factor-alpha [19, 20]. In addition, the NF-$\kappa$B expression was considerably increased in aortic smooth muscle cells following balloon injury [21]. Therefore, NF-$\kappa$B down regulation can be expected to result in cell proliferation arrest. This is supported by our finding that the NF-$\kappa$B-dependent transcription activity was inhibited by OSU-03012 in a dose-dependent manner, indicating the anti-proliferative effect of OSU-03012.

The process of neointimal formation, one of the major processes of restenosis cascades, is the combination of multiple complex mechanisms. Evidence suggests that activation of inflammatory mechanisms plays a key role in the development of neointima [22]. Therefore, interference with the actions of inflammatory mechanisms provides a wealth of potential therapeutic targets. Among the inflammatory factors, interferons (IFNs) impact the cellular mechanisms implicated in the development of vascular proliferative diseases [1, 2]. The common interferon signallings involve several steps: (a) IFN induces dimerization of the receptor on the cellular surface, (b) causes the initiation of intracellular tyrosine phosphorylation, further leads to (c) the dimerization of phosphorylated STATs, (d) activates them for nuclear translocation, and (e) bindsto specific DNA sequences, a conserved ISRE [23], to stimulate transcription for prokiferation. Therefore, the evaluation of ISRE transcription activity would imply the development of vascular proliferative diseases regulated by INF. In our finding, the transcription activity of ISRE, which is the downstream target of IFNs, was also inhibited by OSU-03012 in a dose-dependent manner. Therefore, both decreased NF-$\kappa$B-dependent and ISRE promoter transcription activities imply the inhibitory effects of OSU-03012 on VSMC proliferation and inflammation.

Taken together, our study demonstrates that VSMC proliferation could be reduced by OSU-03012. Its pharmacological mechanism may be associated with the down regulation of PCNA, ERK phosphorylation, and Raf protein levels. Our results unmask the molecular mechanisms of OSU-03012 in inhibiting smooth muscle cell proliferation, migration and inflammation in vitro to shed light into the conjunctive roles of OSU-03012 with some other pharmacological agents in preventing restenosis. Further studies using animal models or clinical evaluations need to be conducted to confirm the proposed theory in this aspect.

**Acknowledgement** This study was supported by a grant from China Medical University (CMU94 -032, CMU95-028, CMU95-030, and CMU95-029) and from Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004) and in part by Taiwan Department of Health Cancer Research Center of Excellence (DOH99-TDC-111-005).

**Conflict of interest statement** The authors have declared no conflict of interest statement.

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