Full-length article

Onychin inhibits proliferation of vascular smooth muscle cells by regulating cell cycle[1]

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Key words

onychin; vascular smooth muscle; cell cycle; retinoblastoma; cyclin D_1; cyclin E; tyrosine kinase; mitogen-activated protein kinase

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Abstract

Aim: To investigate the effects of onychin on the proliferation of cultured rat artery vascular smooth muscle cells (VSMCs) in the presence of 10% new-born calf serum (NCS). **Methods:** Rat VSMCs were incubated with onychin 1–50 μmol/L or genistein 10 μmol/L in the presence of 10% NCS for 24 h. The proliferation of VSMCs was measured by cell counting and MTS/PMS colorimetric assays. Cell cycle progression was evaluated by flow cytometry. Retinoblastoma (Rb) phosphorylation, and expression of cyclin D_1 and cyclin E were measured by Western blot assays. The tyrosine phosphorylation of ERK1/2 was examined by immunoprecipitation techniques using anti-phospho-tyrosine antibodies. **Results:** The proliferation of VSMCs was accelerated significantly in the presence of 10% NCS. Onychin reduced the metabolic rate of MTS and the cell number of VSMCs in the presence of 10% NCS in a dose-dependent manner. Flow cytometry analysis revealed that the G_1-phase fraction ratio in the onychin group was higher than that in the 10% NCS group (85.2% *vs* 70.0%, $P<0.01$), while the S-phase fraction ratio in the onychin group was lower than that in 10% NCS group (4.3% *vs* 16.4%, $P<0.01$). Western blot analysis showed that onychin inhibited Rb phosphorylation and reduced the expression of cyclin D_1 and cyclin E. The effects of onychin on proliferation, the cell cycle and the expression of cyclins in VSMCs were similar to those of genistein, an inhibitor of tyrosine kinase. Furthermore immunoprecipitation studies showed that both onychin and genistein markedly inhibited the tyrosine phosphorylation of ERK1/2 induced by 10% NCS. **Conclusion:** Onychin inhibits the proliferation of VSMCs through G_1 phase cell cycle arrest by decreasing the tyrosine phosphorylation of ERK1/2, and the expression of cyclin D_1 and cyclin E, and sequentially inhibiting Rb phosphorylation.

Introduction

Alterations in vascular smooth muscle cell (VSMC) proliferation plays an important role in the development of the pathological process that underlies restenosis, atherosclerosis, and vascular graft occlusion[1]. Despite intense efforts, no widely effective therapy exits for the prevention of such vascular diseases. Recently some agents have been shown to have potential value by virtue of their abilities to attenuate the proliferation of VSMCs[2–4]. Among them, genistein (4,5,7-trihydroxyisoflavone), a soybean-derived isoflavone, has been shown to inhibit platelet derived growth factor (PDGF)-induced proliferation and DNA synthesis of aortic smooth muscle cells in stroke-prone spontaneously hypertensive rats[5]. Moreover, epidemiological studies indicate that genistein has beneficial effects on cardiovascular disease[6,7], such as lowering total and low density lipoprotein (LDL)-cholesterol levels. More recently, onychin, a new compenent extracted from *Onychin lucidum* of sinopteridaceae, has attracted our interest because its structure is similar to genistein[8]. Interestingly, our previous results have shown that onychin protects the endothelium-dependent relaxation in rabbit aortic rings and inhibits oxidative stress-

induced apoptosis of endothelial cells, which is mediated by increasing NO release, regulating expression of caveolin-1 and activation of mitogen-activated protein kinases (MAPK)[9–13]. In the present study, we extend our investigation to the effects of onychin on the proliferation of rat VSMCs induced by 10% NCS.

Materials and methods

Materials Male Sprague-Dawley rats were obtained from the Experimental Animal Center of Nanhua University (Hengyang, China). Genistein, elastase, and monoclonal antibodies for α-actins of smooth muscle cells were obtained from Sigma (St Louis, USA). Dulbecco's modified Eagle's medium (DMEM) and NCS were from GibcoBRL (Maryland, USA). The Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (MTS/PMS) was purchased from Promega (WI, USA). Monoclonal mouse anti-ERK1/2, -pTyr, -cyclin D_1, -cyclin E, and polyclonal mouse anti-Rb were from Santa Cruz Biotechnology (Santa Cruz, USA).

Cell culture The thoracic aortas were isolated from the 150–200 g male Sprague-Dawley rats and cultured by using a novel enzymatic dispersion method[14]. Briefly, rat aortas were in sterile conditions removed from left subclavian origin to the point of diaphragmatic insertion. The vascular media was digested, and the cells were centrifuged and plated down in prepared flasks. VSMCs were cultivated in DMEM (containing 10% NCS) and in a 5% CO_2 humidified-atmosphere incubator until they displayed as a typical "hill and valley" morphology. The immunohistochemistry staining with a monoclonal antibody against α-actins showed that there were no co-cultured fibroblast cells. VSMCs from 5 to 15 passages were used for experimentation. Before mitogenic stimulation, subconfluent cells were arrested in a quiescent state with DMEM containing 0.1% NCS for 48 h.

Cell count VSMCs (2.5×10^4) were plated into a 24-well plate and grown in DMEM supplemented with 10% NCS for 24 h. In some experiments cells were growth-arrested by incubation in DMEM containing 0.1% NCS for 48 h and then exposed to different concentrations of onychin before treatment with 10% NCS for 24 h or 48 h. Genistein (10 μmol/L) was used as the positive control. The cells were then washed with phosphate-buffered saline (PBS), trypsinized, and diluted with isotonic solution. Cells were stained with Trypane blue and the viable cells were counted using a coulter counter.

MTS assay Cell growth was measured by using MTS assay according to the manufacture's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) and phenazine methosulfate (PMS) were mixed at a ratio of 20:1 immediately before being added to the samples. The MTS/PMS solution (20 μL) was added to each well of the 96-well plate and and the plates were incubated at 37 ºC in a humidified 5% CO_2 atmosphere for 90 min. Absorbance was read at 490 nm using Bio-Tek's Power Wave X reader-assay system (BIO-TEK Instruments, USA). Each sample was read in triplicate.

Flow cytometry Cells were suspended in phosphate-buffered saline (PBS, pH 7.4), and fixed with 70% (v/v) ethanol at -20 ºC for 30 min. After the ethanol was removed, the cells were incubated with PBS containing RNase (172 MU/L) at 37 ºC for 30 min and then stained for 30 min with 0.005% propidium iodide. Fluorescence was measured by using Epics Altra Flow Cytometer (Beckman Coulter Co, USA).

Western blot analysis After treatment, VSMCs were washed twice with cold (4 ºC) PBS (pH 7.4) and harvested on ice in buffer A containing HEPES 20 mmol/L (pH 7.4), EDTA 2 mmol/L, glycerophosphate 50 mmol/L, dithiothreitol 1 mmol/L, Na_3VO_4 1 mmol/L, 1% Triton, 10% glycerol, leupeptin 1 mg/L, aprotinin 1 mg/L, and phenylmethylsulfonyl fluoride 100 μmol/L. The suspension was incubated on ice for 20 min and vortexed every 5 min. Cell lysates were then centrifuged at 13 000 r/min (Eppendorf centrifuge, Osterode, Germany) for 30 min at 4 ºC. The supernatants were collected, and protein concentration was measured by using the Bio-Rad assay. Forty micrograms of protein (40 μg) weas electrophoresed onto a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 1 h with primary antibodies followed by incubation with second antibodies (horseradish peroxidase-conjugated). Immunoactive bands were visualized using chemiluminescence (Amersham-Pharmacia Biotech, Piscataway, USA) and densitometric analysis was carried out using an imager and densitometer[15].

Immunoprecipitation Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin, or 10% NCS+10 μmol/L genistein for 20 min. Cell lysates were prepared for immunoprecipitation as described previously[16]. Briefly, equal amounts of protein (500 μg) were immunoprecipitated with anti-ERK1/2 polyclonal antibodies overnight at 4 ºC. Immune complexes were collected by incubation with protein A-Sepharose beads for 2 h at 4 ºC. Immunoprecipitates were separated by using SDS-PAGE, and proteins were detected by immunoblotting as described above using anti-pTyr monoclonal antibodies.

Statistical analysis All values are expressed as mean±SD. Statistical significance was determined by one-way ANOVA. $P<0.05$ was considered significant.

Results

Effect of onychin on the proliferation of VSMCs Both cell counting and the MTS assay showed that onychin treatment for 24 h or 48 h significantly inhibited VSMC proliferation induced by 10% NCS. The inhibition was concentration-dependent with peak at 10 μmol/L (Table 1). The efficiency of 10 μmol/L onychin treatment for 24 h was similar to that of 10 μmol/L genistein. Therefore we chose 10 μmol/L onychin treating cells for 24 h in the following experiments.

Changes in the VSMC cycle induced by onychin treatment The percentages of G_0/G_1 or S phase cells in the 10% NCS-stimulated group were 70.0% and 16.4% respectively. In contrast, 10 μmol/L onychin and 10 μmol/L genistein treatments for 24 h led to a significant inhibition of DNA synthesis as evidenced by the fact that the percentages of G_0/G_1 phase cells increased to 85.2% and S phase cells decreased to 4.3% in the onychin group (Figure 1).

Decreased Rb phosphorylation induced by onychin treatment The cell cycle progression from G_1 to S phase is usually accompanied by Rb phosphorylation in the late G_1 phase. We therefore examined Rb phosphorylation in VSMCs by using Western blotting with anti-phospho Rb antibody. NCS (10%) stimulated Rb phosphorylation markedly and onychin and genistein inhibited Rb phosphorylation by 70.1% and 58.8%, respectively (Figure 2).

Onychin lowers the expression of cyclin D_1 and cyclin E To further explore the mechanism by which onychin inhibited Rb, we examined the expression of cyclin D_1 and cyclin E by Western blotting. Results showed that 10% NCS treatment significantly increased both expressions of cyclin D_1 and cyclin E and that pretreatment with onychin 10 μmol/L and genistein 10 μmol/L decreased the NCS-induced expression of these two proteins (Figure 3, 4).

Effect of onychin on the tyrosine phosphorylation of ERK1/2 ERK1/2 activation is at upstream of cyclins. It has been reported that the protein tyrosine inhibitor genistein is able to inactivate ERK1/2. To investigate whether the inhibitory effect of onychin on ERK1/2 is mediated by tyrosine kinase inhibition, we observed the phosphorylation of the tyrosine residue in ERK1/2. When growth-arrested VSMCs were treated with 10% NCS, a marked time-dependent tyrosine phosphorylation of ERK1/2 was observed, with peak at 20 min (data not shown). Pretreatment with genistein, as well as onychin, significantly inhibited the 10% NCS-induced

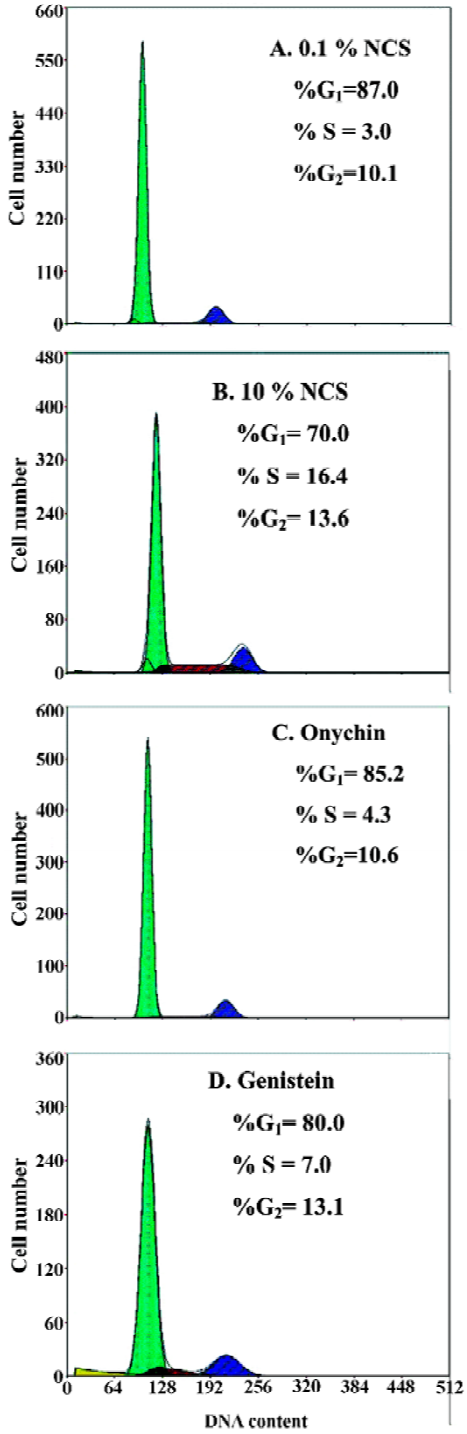

Figure 1. Effect of onychin 10 μmol/L on cell cycle progression induced by 10% NCS for 24 h in cultured vascular smooth muscle cells (VSMCs). Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin or 10% NCS+10 μmol/L genistein for 24 h. Then cells were fixed with ethanol and incubated with PBS containing Rnase followed by staining with propidium iodide. Fluorescence was determined by using a flow cytometer.

Table 1. Inhibitory effect of onychin on the proliferation of rat VSMCs induced by 10% NCS. $n=8$. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* 0.1 % NCS group. $^eP<0.05$ *vs* 10% NCS group.

		$10^{-7}\times$Cell counts/mL^{-1}		MTS measurement (*OD*)	
		24 h	48 h	24 h	48 h
Control	0.1% NCS	2.50±0.12	2.50±0.05	0.44±0.04	0.44±0.04
	10% NCS	7.74±0.57^c	13.68±0.96^c	1.04±0.06^c	1.94±0.06^c
Onychin	1 μmol/L	7.32±0.24	11.03±0.26	0.93±0.04	1.79±0.07
	5 μmol/L	6.36±0.20^e	9.88±0.13^e	0.85±0.07^e	1.47±0.07^e
	10 μmol/L	5.46±0.51^e	8.58±0.56^e	0.64±0.05^e	1.21±0.05^e
	50 μmol/L	5.74±0.21^e	8.75±0.23^e	0.69±0.03^e	1.34±0.04^e
Genistein	10 μmol/L	5.58±0.75^b	8.71±0.45^b	0.67±0.06^b	1.27±0.06^b

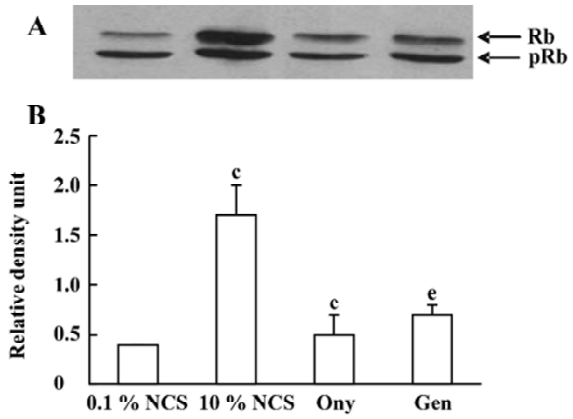

Figure 2. Inhibitory effect of onychin 10 μmol/L on phosphorylation of Rb induced by 10% NCS (24 h) in cultured vascular smooth muscle cells (VSMCs). A) Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin or 10% NCS+10 μmol/L genistein for 24 h. Cells were harvested, and lysates were analyzed for Rb phosphorylation by Western blotting using anti-phosphor Rb antibodies. B) The relative expression of phosphorylated Rb over un-phosphorylated Rb was measured by using densitometry of autoradiograms in the linear range of film development. n=3 experiments. Mean±SD. $^cP<0.01$ *vs* 0.1% NCS group. $^eP<0.05$ *vs* 10% NCS group.

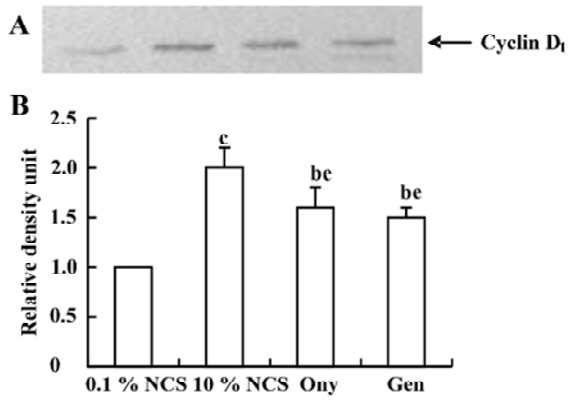

Figure 3. Inhibitory effect of onychin on cyclin D_1 expression in cultured VSMCs. A) Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin, or 10% NCS+10 μmol/L genistein for 24 h. Cells were harvested, and lysates were analyzed for cyclin D_1 by using Western blotting. B) The relative expression of cyclin D_1 was measured by using densitometry of autoradiograms in the linear range of film development. The results of each experiment were normalized to the density of the control (0.1% NCS) sample, which was arbitrarily adjusted to 1.0. The response to 10% NCS, and 10% NCS+10 μmol/L onychin, 10% NCS+10 μmol/L genistein was then determined on a relative basis. n=3 experiments. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* 0.1% NCS group. $^eP<0.05$ *vs* 10% NCS

tyrosine phosphorylation of ERK1/2, suggesting that onychin might be a potential inhibitor of tyrosine kinase (Figure 5).

Discussion

VSMC proliferation contributes to the remodeling of blood vessels and has been implicated in the pathogenesis of atherosclerosis. Phytoestrogens such as genistein can inhibit the proliferation of VSMC and have been implicated in the suppression of VSMC proliferation in neo-intima formation *in vivo*. Onychin, which has a similar structure to genistein, has been considered as a candidate for providing several cardiovascular protective effects[9–13]. To further investigate its actions in vascular biology, in the present study we studied the effect of onychin on the proliferation of rat VSMC induced by 10% NCS. The results showed that 1–50 μmol/L onychin inhibited the proliferation of VSMCs in a concentration-dependent manner, with a peak at 10 μmol/L.

Proliferating cells pass through several cell cycle checkpoints, mainly the G_1 to S and G_2 to M transitions. The former checkpoint is considered to be the most important one in the replication of DNA and mitosis. We thus

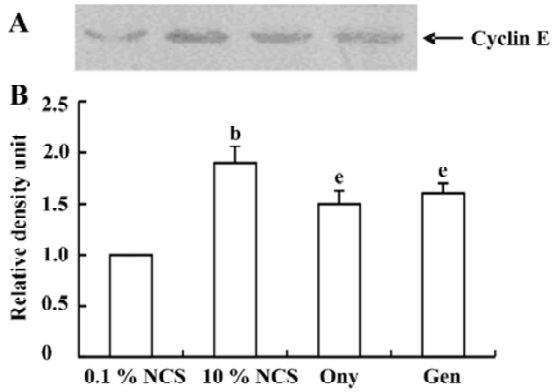

Figure 4. Inhibitory effect of onychin on cyclin E expression in cultured VSMCs. A. Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin, or 10% NCS+10 μmol/L genistein for 24 h. Cells were harvested, and lysates were analyzed for cyclin E by Western blotting. B. The relative expression of cyclin E. The results of each experiment were normalized to the density of the control (0.1% NCS) sample, which was arbitrarily adjusted to 1.0. The response to 10% NCS, and 10% NCS+10 μmol/L onychin, 10% NCS+10 μmol/L genistein was then determined on a relative basis. n=3 experiments. Mean±SD. $^bP<0.05$ *vs* 0.1% NCS group. $^eP<0.05$ *vs* 10% NCS group.

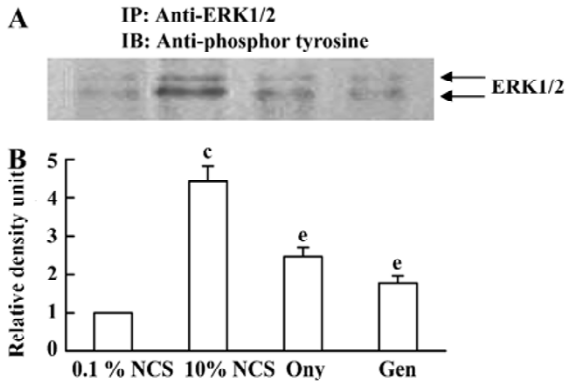

Figure 5. Inhibitory effect of onychin on the tyrosine phosphorylation of ERK1/2. A) Growth-arrested VSMCs were exposed to 0.1% NCS, 10% NCS, 10% NCS+10 μmol/L onychin, or 10% NCS+10 μmol/L genistein for 20 min. Cells lysates were immunoprecipitated with anti-ERK1/2 antibody, and Western blotting was performed with anti-phospho tyrosine antibodies. IP: immunoprecipitation; IB: immunoblot. B) The relative expression of tyrosine phosphorylation in ERK1/2 was measured by using densitometry of autoradiograms in the linear range of film development. The results of each experiment were normalized to the density of the control (0.1% NCS) sample, which was arbitrarily adjusted to 1.0. The response to 10% NCS, 10% NCS+10 μmol/L onychin, and 10% NCS+10 μmol/L genistein was then determined on a relative basis. n=3 experiments. Mean±SD. $^cP<0.01$ *vs* 0.1% NCS group. $^eP<0.05$ *vs* 10% NCS group.

logically speculate that onychin affects VSMC proliferation via alterations in the cell cycle progression. Indeed our data show that onychin blocks cell cycle progression at the G_1 transition and stops VSMCs entering the S phase from G_0/G_1, consequently inhibiting the replication of DNA and the growth of VSMCs.

Rb is a key modulator of cell-cycle transit because of its ability to control the G_1/S-phase transition[17]. Rb activity is related to its ability to modulate the activation of the E_2F family, which are required for S-phase progression[18]. Cell cycle progression from G_1 to S phase is usually accompanied by Rb phosphorylation, which is induced by the cyclin D_1-CDK_4 and the cyclin E-CDK_2 complexes in the late G_1 phase. Rb phosphorylation leads to E_2F release. Conversely, hypophosphorylated Rb sequesters E_2F and prevents transcription of the E_2F target genes. Recent evidence indicates that phosphorylated Rb (pRb) inactivation is a key molecular event leading to S-phase commitment at the G_1 restriction point in the cell cycle[19]. Therefore, deregulating pRb inactivation in the G_1 phase becomes a universal mechanism underlying cellular transformation. Recently, Chang *et al.* showed that a nonphosphorylatable, constitutive active form of murine Rb inhibited VSMC proliferation and reduced neointima formation in injured rat carotid arteries and porcine femoral artery models[20]. Growth factors such as platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor have been shown to induce Rb phosphorylation in quiescent cells entering into the cell cycle[21]. In light of this broad spectrum of activity, Rb is a strong candidate for use as an anti-proliferative agent in a number of disorders that are characterized by inappropriate proliferate responses. To test the possible effects of onychin on Rb phosphorylation, we observed the relative amounts of hypophosphorylated and phosphorylated forms of Rb in VSMCs by using Western blotting. The results showed that onychin decreased the phosphorylation levels of Rb.

Progression through the mammalian mitotic cycle is coordinated by expression and/or activation of multiple holoenzymes composed of a catalytic cyclin-dependent kinase (CDK) and a cyclin-regulatory subunit[22]. Different cyclin/CDK complexes are temporally activated at specific phases of the cell cycle. Progression through the first gap phase (G_1) requires cyclin D-dependent kinase (CDK_4 and CDK_6) and cyclin E/CDK_2 activity[22,23], which results in Rb hyperphosphorylation. The association of CDK_4 or CDK_6 with D-type cyclins is critical for G1 phase progression, whereas the CDK_2-cyclin E complex is important for initiation of the S phase. It has been reported that administration

of genistein in rats with an acute renal injury decreases ERK activation and cyclin D_1 expression[24]. It has been reported that exposure of VSMC to 10% FCS causes a time-dependent increase in cyclin D1 and cyclin E[25]. In the present study, we also found that 10% FCS up-regulated the expression of cyclin D_1 and cyclin E, and onychin treatment inhibited the effects of NCS.

The MAPK family constitutes a major ubiquitous intracellular signaling system involved in the regulation of cell growth, differentiation, and survival[26]. To date, at least three distinct mammalian MAPKs have been characterized: the extracellular-regulated kinases (ERKs), Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) and p38. Among of them, the mammalian ERKs (ERK1/p44 and ERK2/p42) are generally thought to play a role in cell proliferation and differentiation. ERK1/2 is serine/threonine kinase, which is activated by phosphorylation at both threonine and tyrosine residues. Evidence indicates that phosphorylation of the residues Thr 202/Tyr 204 (for human ERK, or Thr 185/Tyr 187 for bovine ERK) in the conserved Thr/Glu/Tyr ERK sequences is necessary and sufficient for full activation of these enzymes and serves as an indicator of their activation status[27]. In our experiments, we observed an obvious tyrosine phosphorylation of ERK1/2 in the 10% NCS group. Onychin, like genistein, markedly declined the phosphorylation level of tyrosine of ERK1/2.

In summary, onychin inhibits the proliferation of VSMCs through G_1 phase cell cycle arrest by decreasing the tyrosine phosphorylation of ERK1/2, decreasing the expression of cyclin D_1 and cyclin E, and sequentially inhibiting Rb phosphorylation.

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