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ORIGINAL ARTICLE

Baicalin inhibits PDGF-BB-stimulated vascular smooth muscle cell proliferation through suppressing PDGFRβ-ERK signaling and increase in p27 accumulation and prevents injury-induced neointimal hyperplasia

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The increased proliferation and migration of vascular smooth muscle cells (VSMCs) are key events in the development of atherosclerotic lesions. Baicalin, an herb-derived flavonoid compound, has been previously shown to induce apoptosis and growth inhibition in cancer cells through multiple pathways. However, the potential role of baicalin in regulation of VSMC proliferation and prevention of cardiovascular diseases remains unexplored. In this study, we show that pretreatment with baicalin has a dose-dependent inhibitory effect on PDGF-BB-stimulated VSMC proliferation, accompanied with the reduction of proliferating cell nuclear antigen (PCNA) expression. We also show that baicalin-induced growth inhibition is associated with a decrease in cyclin E-CDK2 activation and increase in p27 level in PDGF-stimulated VSMCs, which appears to be at least partly mediated by blockade of PDGF receptor β (PDGFRβ)-extracellular signal-regulated kinase 1/2 (ERK1/2) signaling. In addition, baicalin was also found to inhibit adhesion molecule expression and cell migration induced by PDGF-BB in VSMCs. Furthermore, using an animal carotid arterial balloon-injury model, we found that baicalin significantly inhibited neointimal hyperplasia. Taken together, our results reveal a novel function of baicalin in inducing growth arrest of PDGF-stimulated VSMCs and suppressing neointimal hyperplasia after balloon injury, and suggest that the underlying mechanism involves the inhibition of cyclin E-CDK2 activation and the increase in p27 accumulation via blockade of the PDGFRβ-ERK1/2 signaling cascade.

***Keywords*:** baicalin; vascular smooth muscle cells; proliferation; cyclin E; neointimal hyperplasia

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Introduction

The increased proliferation and migration of vascular smooth muscle cells (VSMCs) are key events in the development of atherosclerosis and restenosis. Both of these events can be induced by cytokines and growth factors, such as platelet-derived growth factor (PDGF) [1]. PDGF initiates a multitude of biological effects through the activation of intracellular signal transduction pathways that contribute to VSMC proliferation, migration, and collagen synthesis [2]. The cell cycle is a common convergent point for the mitogenic signaling cascades. Progression through several major checkpoints in the cell cycle is controlled by multiple protein kinases, each of which contains a regulatory cyclin component and a catalytic cyclin-dependent kinase (CDK) [3]. The expression

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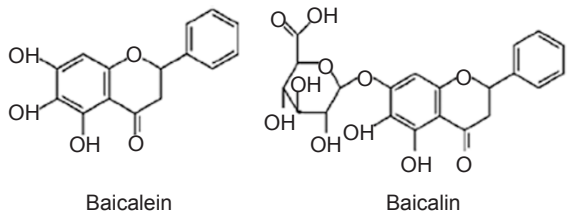

Figure 1 Chemical structure of baicalin and baicalein.

levels of each component, their phosphorylation status, and the presence of specific CDK inhibitors regulate the activity of these kinases. $p27^{kip1}$ (p27) is a negative regulator of the protein kinase CDK2/cyclin E, and can block the cell cycle at G0/G1 phase [4]. The levels of p27 are high in the G0/G1 phases of cell cycle. Upon mitogenic stimulation, p27 is rapidly degraded, thus allowing the action of CDK2/cyclin E to promote cell proliferation [5]. ERK1/2 signaling is necessary for the degradation or downregulation of p27, and is also crucial in mediating VSMC proliferation in response to a multitude of mitogenic stimuli and vascular injury [6, 7]. Therefore, agents that can regulate the cell cycle processes in VSMCs may have a role in the prevention and treatment of atherosclerosis and restenosis after angioplasty.

Scutellaria baicalensis has been shown to harbor a broad spectrum of biological activities, including antiinflammatory, antioxidant and antiproliferation activities, based on its long history in clinical applications. Baic-

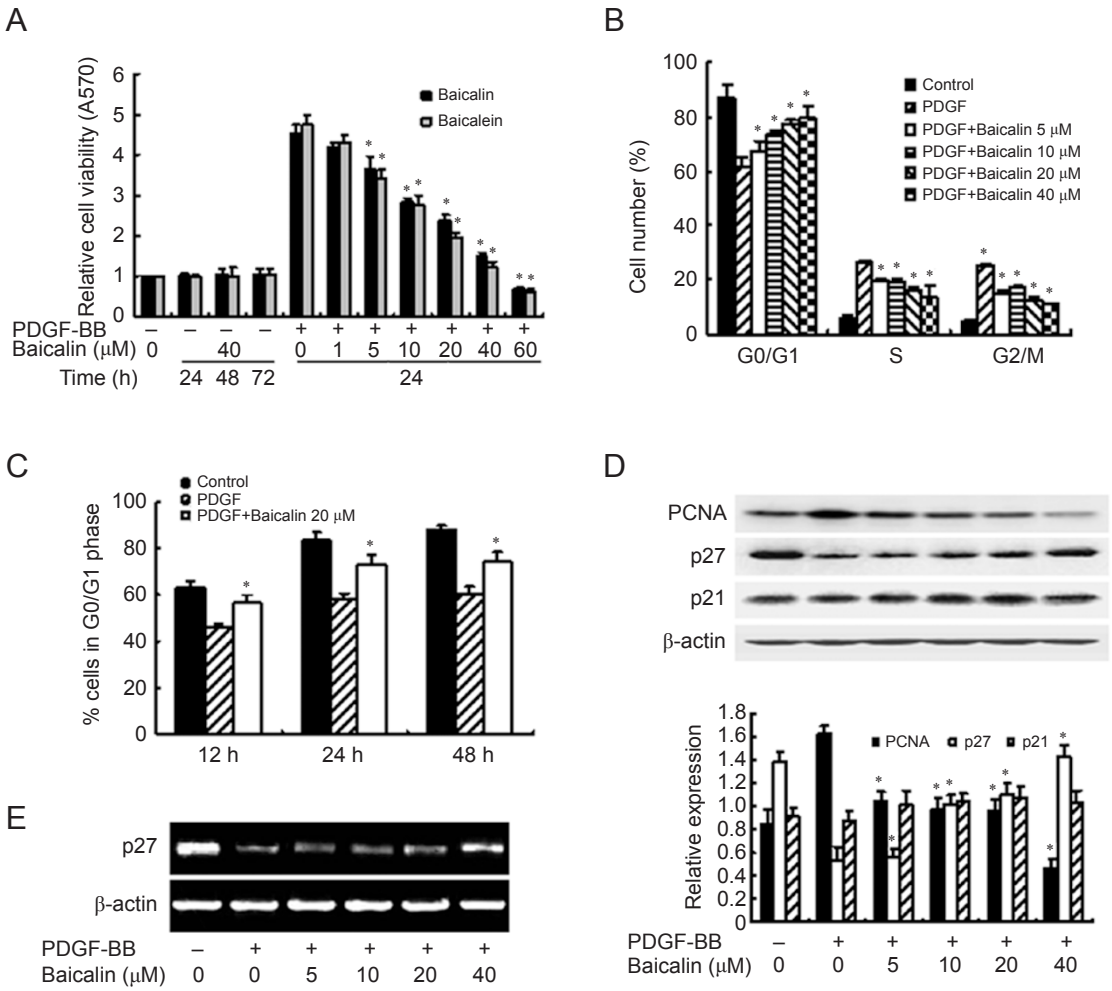

Figure 2 Baicalin inhibits proliferation of VSMCs induced by PDGF-BB. **(A)** MTT assays. VSMCs were pretreated by various concentrations of baicalin or baicalein for indicated time periods, and then stimulated with PDGF-BB for 24 h (n = 5). **(B**, **C)** Flow cytometric analysis (n = 5). **(D)** Western blotting. The graphs represent the relative level of the proteins in four independent experiments. **(E)** RT-PCR. β-Actin was used as an internal control. VSMCs were pretreated by indicated concentrations of baicalin for 24 h followed by 24 h of PDGF-BB stimulation. **(B-E)** *P < 0.05, compared with PDGF-BB.

alin and baicalein are flavonoids derived from the root of *Scutellaria baicalensis Georgi* (Figure 1). It has been reported that baicalein, which has the same backbone structure as baicalin, shows a stronger cardioprotective effect and can inhibit the proliferation of cancer cells via induction of apoptosis [8-11]. A recent study showed that baicalin pretreatment reduced the hyperthermia, intracranial hypertension, and increase of nitric oxide metabolite levels during heatstroke; baicalin also suppressed the heatstroke-induced increase in IL-1β and TNF-α levels [12]. Baicalin may thus protect against cerebrovascular dysfunction and brain inflammation in heatstroke [13]. Although baicalin has been also found to induce apoptosis and to inhibit inflammation through multiple pathways, little is known about the role of baicalin in regulating VSMC proliferation and prevention of restenosis. In this study, we show that baicalin has an inhibitory effect on PDGF-BB-stimulated proliferation and migration of VSMCs, and prevents neointimal hyperplasia induced by balloon injury. We also show that baicalin-induced growth inhibition is associated with the inhibition of cyclin E-CDK2 activation and an increase of p27 levels in PDGF-stimulated VSMCs. This effect of baicalin is mediated, at least partly, through blockade of the PDGF receptor β (PDGFRβ)-extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade.

Results

Baicalin inhibits PDGF-BB-induced proliferation of VSMCs

Baicalin has been demonstrated as an active antitumor compound that leads to cell cycle arrest and growth suppression in cancer cells [8, 9]. We first examined the effect of baicalin on PDGF-BB-induced proliferation of VSMCs using the MTT assay. VSMCs were pretreated with different concentrations (1-60 μM) of baicalin followed by stimulation with PDGF-BB (10 ng/ml) for 24 h. Baicalin inhibited the PDGF-BB-induced proliferation of VSMCs in a concentration-dependent manner (Figure 2A). The IC50 value (concentration needed for 50% growth inhibition) of baicalin was about 16.39 μM under the present experimental conditions. Higher concentrations of baicalin (40 and 60 μM) almost completely blocked the cell proliferation induced by PDGF-BB (Figure 2A). When quiescent cells were treated with baicalin (40 μM) for 24-72 h in the absence of PDGF-BB, no significant difference was observed in the viability of VSMCs as compared with the untreated cells, suggesting that baicalin is not cytotoxic at the concentrations tested (Figure 2A). To determine the effect of baicalin on cell cycle progression, VSMCs were pretreated with 5-40 μM baicalin before PDGF-BB stimulation for 24 h, and then subjected to flow cytometry analysis. Baicalin (10-40 μM) significantly increased the fraction of G0-G1 phase cells, but decreased the numbers of G2-M and S phase cells in VSMCs ($P < 0.05$) (Figure 2B). Treatment with 20 μM baicalin for 24 h increased the fraction of G0-G1 phase cells by ~15% compared with PDGF-BB alone (Figure 2C). Inhibition of cell proliferation by baicalin was verified by its ability to reduce proliferating cell nuclear antigen (PCNA) expression and to elevate p27 levels in a concentration-dependent manner, coinciding with cell cycle arrest (Figure 2D). Treatment with 40 μM baicalin resulted in an ~twofold increase in the p27 protein level relative to that of PDGF-BB alone, whereas a slight induction of p27 mRNA level by baicalin was also observed (Figure 2D and 2E). However, the expression of p21 protein was not markedly altered by baicalin. Together, these data show that baicalin inhibits PDGF-BB-induced proliferation of VSMCs, which is accompanied by increased p27 expression.

Baicalin inhibits cyclin E-CDK2 activation and p27 phosphorylation in VSMCs

Cell cycle progression is controlled by cyclins and CDKs. To determine the expression of cyclins and CDKs in baicalin-treated VSMCs, the lysates of VSMCs were analyzed by western blotting. Treatment with baicalin (5-40 μM) reduced PDGF-BB-induced expression of cyclin E and CDK2 in a concentration-dependent manner with complete inhibition at 20 μM (Figure 3A). However, the levels of cyclin D1, CDK4 and CDK6 proteins were not affected by baicalin treatment. Cyclin E is known to play a role in the G0/G1 transition. Coinciding with the onset of cell cycle arrest, the increased cyclin E level after 24-48 h of PDGF-BB stimulation was reduced in baicalin-pretreated VSMCs (Figure 3B). Cyclins and CDKs regulate phosphorylation of several substrates, including p27. Specifically, the active cyclin E-CDK2 complex has been shown to regulate p27 protein level through phosphorylation on Thr187 [14]. This phosphorylation is a prerequisite for the proteasome-dependent degradation of p27 [15]. Therefore, the observed baicalin-induced increase in p27 protein levels may have resulted from the downregulation of cyclin E-CDK2 activation. To confirm this, we performed co-immunoprecipitation using anti-cyclin E and anti-CDK2 antibodies. Cyclin E-CDK2 complex formation induced by PDGF-BB was concentration-dependently reduced by pretreatment of baicalin in VSMCs (Figure 3C). To address whether there is a causal relationship between the reduction of cyclin E-CDK2 complexes and the upregulation of p27, the phosphorylation of p27 was detected

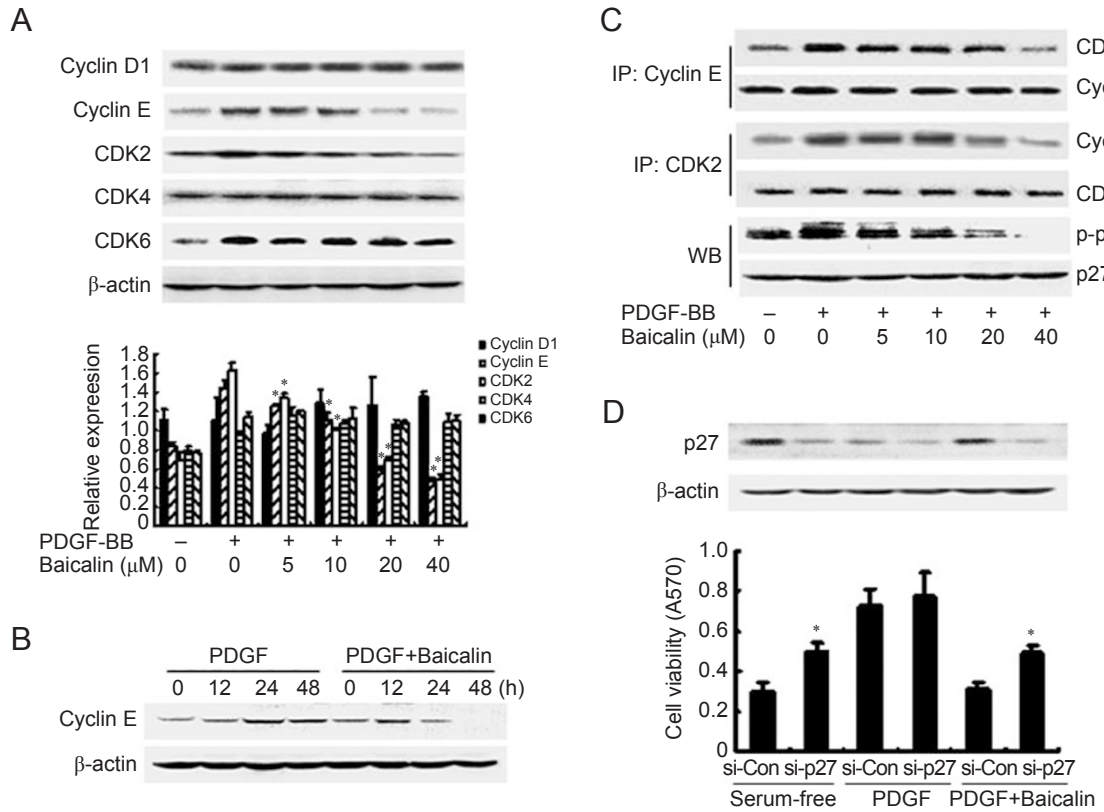

Figure 3 Baicalin inhibits expression and activation of cyclin E and CDK2 in VSMCs. **(A)** Western blotting. VSMCs were pretreated by baicalin at indicated concentration for 24 h, and then stimulated with PDGF-BB for additional 24 h. β-Actin was used as an internal control. The graphs represent the relative level of these proteins for four independent experiments. **P* < 0.05, compared with PDGF-BB. **(B)** Western blotting. VSMCs were stimulated with PDGF-BB for indicated time periods with or without baicalin (20 μM) pretreatment. **(C)** Cross co-immunoprecipitation (IP) and western blotting (WB). VSMCs were stimulated with PDGF-BB for 3 h following baicalin pretreatment. **(D)** Western blotting (upper) and MTT assay (below). VSMCs were transfected with siRNA for 20 h, and then stimulated with PDGF-BB for 24 h with or without baicalin pretreatment. Serum-free was used as positive control. **P* < 0.05, compared with si-Control.

using an anti-phospho-p27 antibody under the same conditions. As expected, the reduction of phosphorylated p27, as a result of baicalin treatment, was linked to the downregulation of cyclin E-CDK2 complex formation (Figure 3C). Thus, baicalin may increase p27 protein stability by inhibiting its phosphorylation. To test the involvement of p27 in baicalin-induced growth arrest, we examined the effects of p27 knockdown by specific small interfering RNA (siRNA). Our results showed that p27 knockdown impaired the growth suppression induced by baicalin (Figure 3D). Taken together, pretreatment of baicalin inhibits cyclin E-CDK2 activation and upregulates p27 protein levels in PDGF-BB-stimulated VSMCs.

Baicalin inhibits PDGFRβ-ERK signaling cascade activated by PDGF-BB in VSMCs

To further delineate the cellular and molecular mechanisms underlying baicalin-induced VSMC growth inhibition, we evaluated the effect of baicalin on the mitogen-activated protein kinase (MAPK) signaling cascades. After pretreatment with baicalin, VSMCs were stimulated with PDGF-BB for 30 min, and the phosphorylation status of ERK, c-Jun NH2-terminal kinase (JNK), p38 MAPK, and Akt was measured by western blot analysis using antibodies that identify the active (phosphorylated) forms of these kinases. Baicalin markedly inhibited MEK-ERK1/2 activation by PDGF-BB in a concentration-dependent manner in VSMCs, which was associated with a reduction of PDGFRβ phosphorylation, while there were no changes in the phosphorylated forms of JNK, p38 and Akt (Figure 4A). To elucidate the mechanism by which baicalin inhibits PDGFRβ phosphorylation induced by PDGF-BB, cells were treated simultaneously with both PDGF-BB (10 ng/ml) and baicalin (20 μM) or with a pre-mixture of both at the same concentrations. Under such conditions, no changes

were observed in PDGFRβ phosphorylation compared to PDGF-BB-stimulated cells (Figure 4B). We thus speculate that baicalin pretreatment may result in the impaired response capacity of PDGFRβ to stimuli. To confirm this possibility, the phosphorylation of ERK1/2 and p27, as well as cyclin E-CDK2 complex formation was exam-

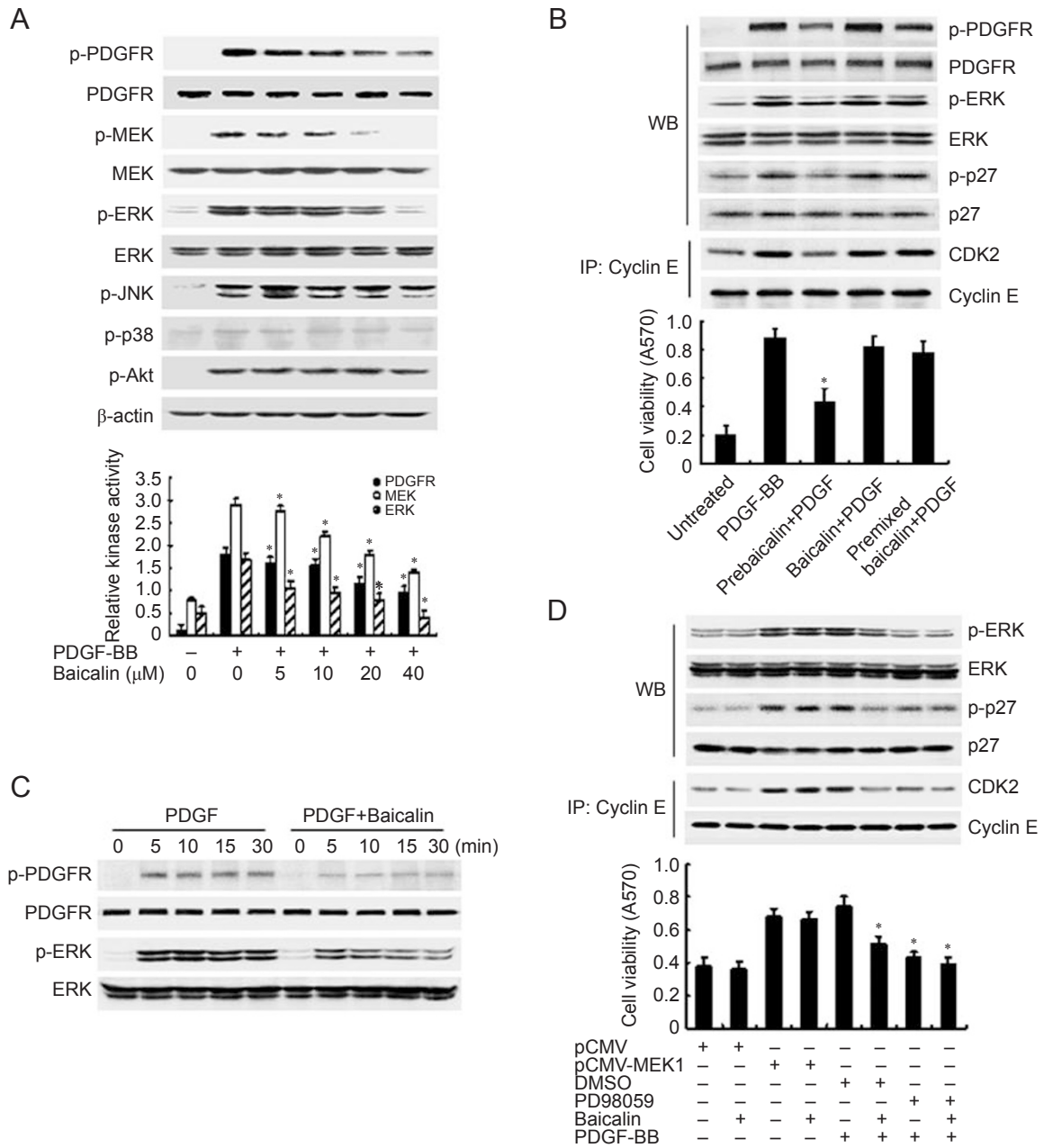

Figure 4 Baicalin inhibits PDGFRβ-ERK1/2 signaling cascade activated by PDGF-BB in VSMCs. **(A)** Western blotting. VSMCs were stimulated with PDGF-BB for 30 min following pretreatment with baicalin at the indicated concentration. β-Actin was used as an internal control. The graphs represent the relative activity of these kinases for four independent experiments. **P* < 0.05, compared with PDGF-BB. **(B)** Western blotting (WB), co-immunoprecipitation (IP) and MTT assay. VSMCs were stimulated with PDGF-BB for 30 min (for PDGFR and ERK1/2), 3 h (for p27 and IP), and 24 h (for cell viability) with treatment by baicalin in indicated manners. **P* < 0.05, compared with PDGF-BB. **(C)** Western blotting. VSMCs were stimulated with PDGF-BB for indicated time periods with or without pretreatment by baicalin (20 μM). **(D)** Western blotting (WB), co-immunoprecipitation (IP), and MTT assay. VSMCs were stimulated with PDGF-BB as in **B** following treatment by PD98059 (20 μM) or transfection by pCMV-MEK1 for 24 h, with or without baicalin pretreatment. **P* < 0.05, compared with PDGF-BB.

ined under the same experimental conditions. The reduction of phosphorylated p27 and cyclin E-CDK2 complex formation was only found in baicalin-pretreated VSMCs following 3 h of PDGF-BB stimulation, paralleled with the inhibition of PDGFRβ-ERK1/2 signaling cascade and cell proliferation (Figure 4B). In addition, the inhibitory effect of baicalin on ERK1/2 activation downstream of PDGFR signaling was further supported by parallel changes in phosphorylation levels of PDGFRβ and ERK1/2 after PDGF-BB stimulation with or without baicalin pretreatment. The phosphorylated forms of PDGFRβ and ERK1/2 were decreased in a time-dependent manner, starting from 5 min of PDGF-BB treatment, in baicalin-pretreated VSMCs, with no changes of the total proteins, demonstrating an actual decrease in phosphorylation levels, indicative of reduced activity (Figure 4C). Because treatment with PD98059, a specific inhibitor of ERK1/2, reduced p27 phosphorylation and cell proliferation, which was similar to the effects of baicalin (Figure 4D), the inhibition of ERK activation may be an alternative mechanism for suppression of p27 phosphorylation by baicalin. To test this, VSMCs were transfected with a constitutively active MEK expression plasmid (pCMV-MEK1), and then treated with baicalin and PDGF-BB. We found that increase of ERK1/2 activity impaired the inhibitory effect of baicalin on p27 phosphorylation in VSMCs, and the growth inhibition by baicalin was abolished under such conditions (Figure 4D). Further, the relationship between ERK1/2 activation and cyclin E-CDK2 complex formation was examined by co-immunoprecipitation under the same conditions. Treatment of baicalin and/or PD98059 resulted in reduction of cyclin E-CDK2 complex formation along with inhibition of ERK1/2 activation, while the opposite change was found upon ERK1/2 activation by the overexpression of a constitutively active MEK (Figure 4D). These results suggest that reduced cyclin E-CDK2 activity is likely an event downstream of the blockade of ERK1/2 pathway by baicalin.

Baicalin inhibits adhesion molecule expression and VSMC migration induced by PDGF-BB

PDGF initiates a multitude of biological effects through the activation of intracellular signal transduction pathways that contribute to both VSMC proliferation and migration. We examined the effect of baicalin on PDGF-BB-induced VSMC migration by the cell monolayer wounding assay that represents chemokinesis. Treatment with PDGF-BB for 12 h increased the basal migration of VSMCs by ~fourfold. Compared with PDGF-BB alone, baicalin pretreatment caused a 60% reduction of cell migration, but no change was found in VSMCs treated simultaneously with both PDGF-BB and baicalin or with a pre-mixture of PDGF-BB and baicalin (Figure 5A). Then, we examined the effect of baicalin on several migration regulatory proteins, including osteopontin (OPN), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and matrix metalloprotease 2 (MMP-2). Baicalin pretreatment inhibited the upregulation of OPN, ICAM-1, VCAM-1 and MMP-2 proteins by PDGF-BB in a concentration-dependent manner (Figure 5B). A measure of 20 μM baicalin resulted in an ~40% decrease in the levels of these proteins compared to that in PDGF-BB-treated cells, and a 60%

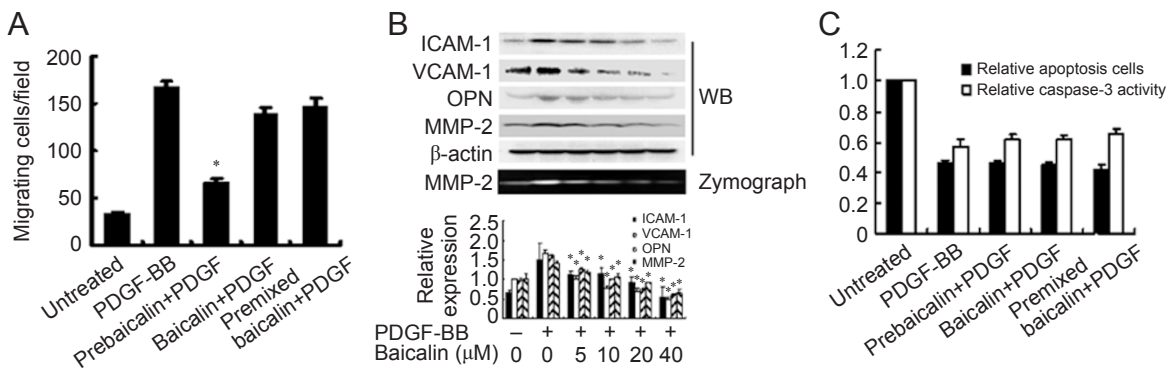

Figure 5 Baicalin inhibits adhesion molecule expression and migration induced by PDGF-BB in VSMCs. **(A)** Migration assay. VSMCs were stimulated with PDGF-BB for 12 h with or without treatment by baicalin in indicated manners. **P* < 0.05, compared with PDGF-BB. **(B)** Western blotting (WB) and Zymograph. β-Actin was used as an internal control. VSMCs were stimulated with PDGF-BB for 24 h following baicalin pretreatment at indicated concentration. The graphs represent the relative level of these proteins for four independent experiments. **P* < 0.05, compared with PDGF-BB. **(C)** Apoptosis analysis by flow cytometry (black) and caspase-3 activity assay (white). VSMCs were stimulated with PDGF-BB for 24 h with or without treatment by baicalin in indicated manners.

decrease was observed with 40 μM baicalin (Figure 5B). Consistent with the decrease in MMP-2 protein levels, zymography assay showed that baicalin (20 μM) decreased MMP-2 activity by 70%, compared with that induced by PDGF-BB alone (Figure 5B). These findings suggest that baicalin inhibits the migration of VSMCs induced by PDGF-BB via suppressing the expression of migration-related proteins in these cells. Flow cytometry analysis showed no effect of baicalin on apoptosis of PDGF-BB-stimulated VSMCs, which was verified by no change in caspase-3 activity (Figure 5C).

Baicalin inhibits neointimal hyperplasia

To investigate the role of baicalin in regulating VSMC proliferation *in vivo*, rat carotid arteries were harvested on day 28 after balloon injury, and an increased intima-to-media (I/M) thickness ratio of carotid arteries was observed (Figure 6A). Administration with baicalin (70 mg/kg/day) significantly reduced I/M thickness ratio by over 55% compared with injured controls (Figure 6A), and inhibited the injury-induced increase in PCNA expression in neointima of carotid arteries, which was accompanied with an increase in p27 expression and attenuation of neointimal hyperplasia (Figure 6B). However, lower doses of baicalin (≤ 35 mg/kg/day) showed only a slight inhibitory effect on neointimal hyperplasia, which did not reach statistic significance (data not shown). These results suggest that baicalin exhibits an inhibitory effect on cell proliferation *in vivo*, and may be an effective agent for prevention of restenosis after angioplasty.

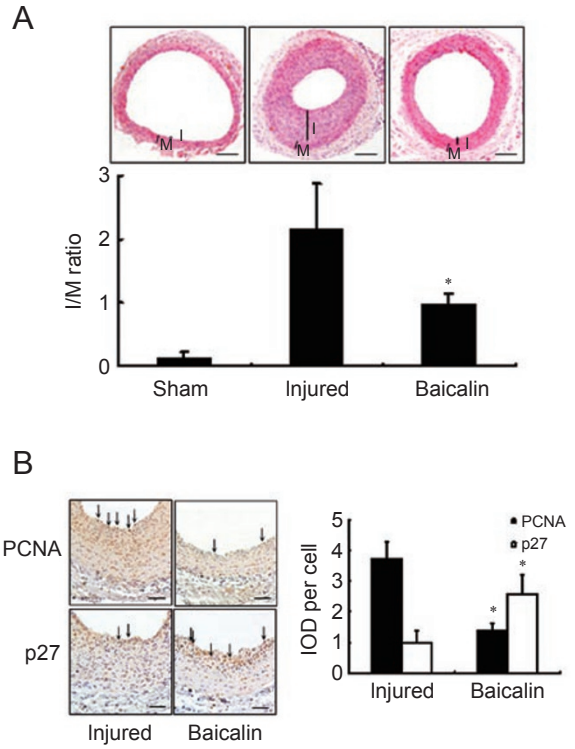

Figure 6 Baicalin inhibits neointimal hyperplasia. The sections of rat carotid arteries were prepared on day 28 after balloon injury. **(A)** H and E staining (upper) and I/M thickness ratio analysis (below). The bar indicates 200 μm. *P < 0.05, compared with injured group (n = 6). **(B)** Immunohistochemistry analysis. The graphs represent the integrated optical density per cell of intima. Representative positive signals (brown) are indicated by arrows. The bar indicates 50 μm. *P < 0.05, compared with injured group (n = 6).

Discussion

PDGF-BB plays an important role in vascular remodeling during cellular and extracellular responses to injury [16, 17]. It has been suggested that different signaling pathways mediated by PDGFRβ regulate the proliferative and the migratory responses to PDGF-BB, with MAPK regulating proliferation and Akt regulating migration [18]. In this study, we show that baicalin has potent and concentration-dependent inhibitory effects on PDGF-BB-stimulated VSMC proliferation and migration without inducing apoptosis (IC50 of 16.39 μM for the induction of G0-G1-phase arrest), and that it does not affect the viability of quiescent VSMCs. Baicalin pretreatment inhibits PDGFRβ phosphorylation in a concentration-dependent manner and this inhibitory effect is associated with the inhibition of downstream ERK1/2 signaling events triggered by the receptor, including the reduction of p27 phosphorylation and cyclin E-CDK2 complex formation. As treatment with the specific ERK1/2 inhibitor, PD98059, reveals a similar effect as baicalin, the inhibition of ERK1/2 activation is likely a major mechanism of growth suppression by baicalin. Nevertheless, the inhibition of tyrosine phosphorylation of PDGFRβ by baicalin did not occur when VSMCs were treated simultaneously with both PDGF-BB and baicalin or with a pre-mixture of the two agents, suggesting that pretreatment of baicalin likely acts by impairing the response capacity of PDGFRβ to PDGF-BB stimulation. Baicalin belongs to a class of polyphenols. Previous studies have shown that polyphenols inhibit PDGFRβ autophosphorylation [19-22]. Although the mechanisms underlying the inhibitory effect of these polyphenols remain to be established, previous studies suggested that some of these molecules may inhibit the activation of receptor tyrosine kinase by competing with adenosine triphosphate for binding to the kinase domain of the receptor, resulting in impaired activation of key signaling intermediates [23, 24]. We speculate that such a mechanism could also be involved in the inhibitory effect of baicalin toward PDGFRβ. In

addition, the involvement of ERK1/2 was further supported by the fact that transient expression of a constitutively active mutant of MEK abolished the inhibitory effect of baicalin on cell proliferation, which was accompanied with an increase in cyclin E-CDK2 activation and reduction of p27 level. However, baicalin failed to attenuate PDGF-stimulated phosphorylation of JNK, p38 MAPK, and Akt. Our results suggest that blockade of the PDGFRβ-ERK1/2 pathway is involved in baicalin-induced proliferation suppression in PDGF-BB-stimulated VSMCs. It is currently unknown how baicalin enters the cell and/or reacts with the compartment where the kinases are localized. Also, baicalin may have no effect on kinase activities in quiescent cells and only interfere with the ATP-binding site when the enzyme translocates upon activation [25].

Using RNAi-mediated knockdown, we have found that the increase in p27 accumulation is involved in suppression of proliferation induced by baicalin in VSMCs. Recent studies have suggested a physiological role of p27 as a regulator of VSMC growth during the pathogenesis of cardiovascular diseases [26]. We focused on the regulatory mechanism of p27 induction by baicalin and found that baicalin-induced upregulation of p27 is associated not only with the blockade of PDGFRβ-ERK1/2 signaling but also with the reduction of cyclin E-CDK2 activation in VSMCs. Treatment of baicalin and/or PD98059 results in reduction of p27 phosphorylation along with inhibition of ERK1/2 activation, while the opposite change is found upon ERK1/2 activation by the overexpression of a constitutively active MEK. In addition, we show that the expression and activation of CDK2 and cyclin E markedly decrease in baicalin-pretreated VSMCs, which is associated with p27 accumulation and cell cycle arrest. The reduction of phosphorylated p27 as a result of baicalin treatment is linked to the downregulation of cyclin E-CDK2 complex formation. Cyclin E-CDK2 may phosphorylate p27 and result in its elimination [27]. It is now clear that the level of p27 protein is mainly controlled by its rate of degradation rather than by changes in transcription or translation [28]. Thus, two possibilities could account for the inhibition of p27 phosphorylation by baicalin observed in this study. Suppression of ERK1/2 activation may directly result in reduced p27 phosphorylation, and/or do so indirectly via inhibiting cyclin E-CDK2 activation. Previous study showed that baicalin had antiproliferation effects on hepatocytes via TGF-β1 secretion from Kupffer cells, which was related to the activation of NF-κB and PKC pathways [29]. In cancer cell lines, baicalin induced apoptosis and proliferation retardation, which may be related to the activation of p38 MAPK and Akt [8]. Our data indicate that baicalin may increase p27 protein stability by inhibiting its phosphorylation and degradation, which is required for baicalin-induced growth arrest in VSMCs.

There is much evidence that enhanced proliferation and migration of VSMCs is a fundamental feature of the pathogenesis of atherosclerosis and other vascular proliferative disorders. Thus, prevention of pathological VSMC proliferation still remains as a major clinical challenge, which underscores the need for new therapeutic strategies. In the present study, we have also shown using an animal arterial balloon-injury model characterized by PDGF receptor upregulation [16] that baicalin (70 mg/kg/day) significantly inhibits neointimal formation, accompanied with reduction of cell proliferation after vascular injury in rats. These results substantiate our *in vitro* findings and strongly support the notion that baicalin confers protection against injury-induced pathological vascular remodeling. Lower dose of baicalin (≤ 35 mg/kg/day), however, did not significantly inhibit the injury-induced neointima formation. Pharmacokinetics study showed that the major forms in the serum are their conjugated metabolites after oral administration of the polyphenols, including baicalin and baicalein, indicating a high level of biotransformation [30]. Moreover, the bioavailability of baicalin is relatively low among polyphenols. Our findings point to the possibility that lower inhibitory effect of baicalin on cell proliferation *in vivo* may result from its high level of biotransformation and/or low bioavailability. Because the principle metabolites *in vivo* possess completely different physicochemical properties from the free form [30], it is hard to directly infer the *in vivo* effects of this compound from its *in vitro* activities. The bioavailability and bioactivity of baicalin would be an important issue to consider for any future clinical applications.

In summary, baicalin induces growth arrest in PDGF-BB-stimulated VSMCs, and prevents neointimal hyperplasia after balloon injury. This process is associated with inhibition of cyclin E-CDK2 activation, and subsequent increase in p27 accumulation via blockade of the PDGFRβ-ERK1/2 signaling cascade.

Materials and Methods

Reagents

Recombinant human PDGF-BB was purchased from R&D Systems Inc. Baicalin and baicalein was purchased from Sigma Co and dissolved in DMSO, and the concentration of DMSO was < 0.8% in the control and drug-containing medium.

VSMC culture and treatment

VSMCs were obtained from thoracic aorta of male Sprague-Dawley rats weighing between 100 and 200 g, as described [7]. All

of the experiments were performed using 3-5 passages of VSMCs. Before isolating, VSMCs were pretreated by different concentrations of baicalin for 24 h and then stimulated with PDGF-BB (10 ng/ml) for indicated time periods. This study was performed via a protocol approved by the Institutional Animal Care and Use Committee of Hebei Medical University in accordance with the Guide for the Care and Use of Laboratory Animals.

Cell proliferation assay

VSMCs were seeded onto 96-well plates (2×10^3 cells per well) and pretreated with various concentrations of baicalin or baicalein for 24 h before stimulation with or without PDGF-BB (10 ng/ml). Proliferation was measured using MTT assay [31].

Fluorescence-activated cell sorting analysis

VSMCs were collected after 24 h of treatment with or without of PDGF-BB (10 ng/ml) and various concentrations of baicalin, fixed in 70% ethanol, washed twice with PBS, and stained with a 50 μg/ml propidium iodide solution containing 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml RNase A. Fluorescence was measured and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

Cell migration assay

The cell migration was measured using a monolayer wounding protocol in which cells migrated from a confluent area into an area that was mechanically denuded of cells [31]. Three different fields of migration were photographed with a video camera system using Scion Image software at the intersection of the previously marked line and wound edge before and after treatment with PDGF-BB (10 ng/ml) for 12 h in the presence or absence of baicalin (20 μM). The migration activity was expressed as the number of cells migrating into the wound in each field.

Cell transfection

The constitutively active ERK kinase MEK1 plasmid (pCMV-MEK1) was kindly provided by Kun-Liang Guan (Moore's Cancer Center, CA, USA). VSMCs were transfected with pCMV and pCMV-MEK1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, VSMCs were stimulated with PDGF-BB and then treated with baicalin (20 μM) for 24 h.

siRNA transfection

The siRNA duplex targeting rat p27 mRNA (5′-UUG CCG AGA UAU GGA AGA ATT-3′) was synthesized from TaKaRa. Scrambled siRNA (si-Control) served as a negative control. The siRNA was transiently transfected into VSMCs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. At 20 h after transfection, the VSMCs were treated with baicalin and PDGF-BB as above mentioned.

Western blot analysis

Lysates from VSMCs were prepared as previously described [6]. Equal amounts of protein (60-100 μg) were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. Membranes were blocked, and then incubated with anti-cyclin D1 (1:300), anti-cyclin E (1:400), anti-CDK2 (1:300), anti-CDK4 (1:300), anti-CDK6 (1:300), anti-PCNA (1:300), anti-p21 (1:300), anti-p27 (1:300), anti-phospho-p27 (1:300), anti-phospho-JNK (Tyr183/185; 1:300), anti-p38 MAPK (1:300), anti-phospho-p38 MAPK (Thr180/Tyr182; 1:300), anti-MEK1/2 (1:300), anti-phospho-MEK1/2 (Ser217/221; 1:400), anti-ERK1/2 (1:300), anti-phospho-ERK1/2 (Tyr204; 1:300), anti-phospho-Akt (1:300), anti-PDGFRβ, anti-phospho-PDGFRβ (Tyr751; 1:300), anti-OPN (1:300), anti-ICAM-1 (1:300), anti-VCAM-1 (1:300), anti-MMP-2 (1:300), and anti-β-actin (1:300) antibodies (Santa Cruz Biotechnology) overnight, and then with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) (1:10 000) for 2 h. The blot was detected with the enhanced chemiluminescence detection system (Santa Cruz Biotechnology). The experiments were replicated four times.

Co-immunoprecipitation assay

VSMCs were harvested after PDGF-BB treatment for 3 h with or without baicalin pretreatment. Cells were lysed in a buffer composed of 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 10 mM sodium phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. After centrifugation, 50 μg of the clarified cell lysate was incubated with 15 μl of protein G plus/protein A-agarose (Santa Cruz Biotechnology) and 1 μg of anti-CDK2, anti-cyclin E antibodies. After 24 h incubation, the immune complexes were centrifuged and washed four times with ice-cold lysis buffer. The immunoprecipitated protein was further analyzed by western blot as described above.

Caspase activity assays

Caspase-3 activity was analyzed according to the manufacturer's instructions with the caspase colorimetric assay kit (Promega Co). Cells were harvested, lysed in lysis buffer for 30 min, and vibrated for 10 s at 4 °C. After centrifugation, the supernatant was collected and the protein concentration was determined. Each sample (50 μl) was incubated with caspase-3 substrate (5 μl) and 2× reaction buffer (50 μl) at 37 °C for 4 h and measured by chromatography at 405 nm wavelength.

Balloon injury and morphometric analysis of neointima formation

Balloon denudation of the left common carotid artery of male Sprague-Dawley rats was performed, as described [32]. Baicalin was freshly dissolved in 10% polyglycol-400 and orally administered at doses of 17.5, 35, and 70 mg/kg/day by gastric gavage from 3 days before balloon injury to 28 days after the injury (n = 6 per group). The arteries were collected at day 28 after balloon injury, and embedded in paraffin to prepare cross sections. Neointima thickening was assessed using the I/M thickness ratio measured from haematoxylin- and eosin-stained arterial cross sections with a computer-based Image-Pro Morphometric System in a double-blind manner. Four discontinuous sections from each vessel were measured in a Sprague-Dawley rat, whereas six rats were used in each experimental group.

Immunohistochemistry analysis

Standard immunohistochemical techniques were used with anti-PCNA (1:100) and anti-p27 (1:100) antibodies and a biotinylated anti-mouse secondary antibody (1:100) on perfusion-fixed, paraffin-embedded tissues. Slides were treated with avidin-biotin block

and exposed to diaminobenzidine black chromogen with nuclear fast red counterstain. Slides were coverslipped for analysis under light microscopy. Staining intensities were determined by measurement of the integrated optical density with light microscopy using a computer-based Image-Pro Morphometric System by two independent observers in a double-blind manner.

Zymography

MMP-2 activity in the medium was analyzed by non-reducing SDS-PAGE in 10% gels containing 0.1% gelatin [33]. Samples were denatured at room temperature in an equal volume of 0.25 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, and 10 μg/ml bromophenol blue. After electrophoresis, MMP-2 was renatured by rinsing the gel in 2.5% Triton X-100 at room temperature for 30 min, and then activated in reaction buffer (50 mM Tris-HCl (pH 7.6) containing 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35) at 37 °C for 18 h. The gels were stained for 90 min with 0.5% Coomassie brilliant blue R250 and destained with 10% acetic acid in 40% methanol. MMP-2 activity was evident as a clear band against the blue background of stained gelatin.

Data statistics

Data are presented as means ± SEM. ANOVA and paired or unpaired *t*-test were performed for statistical analysis as appropriate. $P < 0.05$ was considered statistically significant.

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References

1 Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med* 2002; **8**:1249-1256.
2 Huang M, Duhadaway JB, Prendergast GC, Laury-Kleintop LD. RhoB regulates PDGFR-beta trafficking and signaling in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2007; **27**:2597-2605.
3 Sherr CJ. Cancer cell cycles. *Science* 1996; **274**:1672-1677.
4 Egozi D, Shapira M, Paor G, Ben-Izhak O, Skorecki K, Hershko DD. Regulation of the cell cycle inhibitor p27 and its ubiquitin ligase Skp2 in differentiation of human embryonic stem cells. *FASEB J* 2007; **21**:2807-2817.
5 Kossatz U, Malek NP. p27: tumor suppressor and oncogene …? *Cell Res* 2007; **17**:832-833.
6 Hu Y, Cheng L, Hochleitner BW, Xu Q. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. *Arterioscler Thromb Vasc Biol* 1997; **17**:2808-2816.
7 Zheng B, Han M, Bernier M, *et al*. Krüppel-like factor 4 inhibits proliferation by platelet-derived growth factor receptor β-mediated, not by retinoic acid receptor α-mediated, phosphatidylinositol 3-kinase and ERK signaling in vascular smooth muscle cells. *J Biol Chem* 2009; **284**:22773-22785.
8 Chao JI, Su WC, Liu HF. Baicalein induces cancer cell death and proliferation retardation by the inhibition of CDC2 kinase and survivin associated with opposite role of p38 mitogen-activated protein kinase and AKT. *Mol Cancer Ther* 2007; **6**:3039-3048.
9 Ma Z, Otsuyama K, Liu S, *et al.* Baicalein, a component of Scutellaria radix from Huang-Lian-Jie-Du-Tang (HLJDT), leads to suppression of proliferation and induction of apoptosis in human myeloma cells. *Blood* 2005; **105**:3312-3318.
10 Lee HZ, Leung HW, Lai MY, Wu CH. Baicalein induced cell cycle arrest and apoptosis in human lung squamous carcinoma CH27 cells. *Anticancer Res* 2005; **25**:959-964.
11 Woo AY, Cheng CH, Waye MM. Baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via a prooxidant mechanism. *Cardiovasc Res* 2005; **65**:244-253.
12 Chang CP, Huang WT, Cheng BC, Hsu CC, Lin MT. The flavonoid baicalin protects against cerebrovascular dysfunction and brain inflammation in experimental heatstroke. *Neuropharmacology* 2007; **52**:1024-1033.
13 Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: recent advances. *Pharmacol Rev* 2009; **61**:62-97.
14 Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev* 1997; **11**:1464-1478.
15 Vlach J, Hennecke S, Amati B. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. *EMBO J* 1997; **16**:5334-5344.
16 Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM. PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol* 1990; **111**:2149-2158.
17 Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 1999; **79**:1283-1316.
18 Yang X, Thomas DP, Zhang X, *et al*. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neointima formation. *Arterioscler Thromb Vasc Biol* 2006; **26**:85-90.
19 Lamy S, Beaulieu E, Labbe D, *et al*. Delphinidin, a dietary anthocyanidin, inhibits platelet-derived growth factor ligand/receptor (PDGF/PDGFR) signaling. *Carcinogenesis* 2008; **29**:1033-1041.
20 Godichaud S, Si-Tayeb K, Auge N, *et al*. The grape-derived polyphenol resveratrol differentially affects epidermal and platelet-derived growth factor signaling in human liver myofibroblasts. *Int J Biochem Cell Biol* 2006; **38**:629-637.
21 Chen A, Zhang L. The antioxidant (-)-epigallocatechin-3-gallate inhibits rat hepatic stellate cell proliferation in vitro by blocking the tyrosine phosphorylation and reducing the gene expression of platelet-derived growth factor-beta receptor. *J Biol Chem* 2003; **278**:23381-23389.
22 Ahn HY, Hadizadeh KR, Seul C, Yun YP, Vetter H, Sachinidis A. Epigallocathechin-3 gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma

cells (A172). *Mol Biol Cell* 1999; **10**:1093-1104.
23 Li M, He Z, Ermakova S*, et al.* Direct inhibition of insulin-like growth factor-I receptor kinase activity by (-)-epigallocatechin-3-gallate regulates cell transformation. *Cancer Epidemiol Biomarkers Prev* 2007; **16**:598-605.
24 Yang EB, Wei L, Zhang K, Chen YZ, Chen WN. Tannic acid, a potent inhibitor of epidermal growth factor receptor tyrosine kinase. *J Biochem* 2006; **139**:495-502.
25 Middleton E Jr., Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000; **52**:673-751.
26 Sanz-Gonzalez SM, Poch E, Perez-Roger I, Diez-Juan A, Ivorra C, Andres V. Control of vascular smooth muscle cell growth by cyclin-dependent kinase inhibitory proteins and its implication in cardiovascular disease. *Front Biosci* 2000; **5**:D619-628.
27 Swanson C, Ross J, Jackson PK. Nuclear accumulation of cyclin E/Cdk2 triggers a concentration-dependent switch for the destruction of p27Xic1. *Proc Natl Acad Sci USA* 2000; **97**:7796-7801.
28 Hengst L, Reed SI. Translational control of p27Kip1 accumulation during the cell cycle. *Science* 1996; **271**:1861-1864.
29 Wang JY, Chuang HN, Chiu JH*, et al.* Effects of Scutellaria baicalensis Georgi on macrophage-hepatocyte interaction through cytokines related to growth control of murine hepatocytes. *Exp Biol Med (Maywood)* 2006; **231**:444-455.
30 Shia CS, Hou YC, Juang SH, *et al.* Metabolism and pharmacokinetics of San-Huang-Xie-Xin-Tang, a polyphenol-rich chinese medicine formula, in rats and ex-vivo antioxidant activity. *Evid Based Complement Alternat Med* 2009; **10**:1093.
31 Li JJ, Han M, Wen JK, Li AY. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. *Biochem Biophys Res Commun* 2007; **356**:13-19.
32 Andres V, Urena J, Poch E, Chen D, Goukassian D. Role of Sp1 in the induction of p27 gene expression in vascular smooth muscle cells *in vitro* and after balloon angioplasty. *Arterioscler Thromb Vasc Biol* 2001; **21**:342-347.
33 Han M, Wen JK, Zheng B, Cheng Y, Zhang C. Serum deprivation results in redifferentiation of human umbilical vascular smooth muscle cells. *Am J Physiol Cell Physiol* 2006; **291**:C50-C58.