

# Baicalein induces the apoptosis of HCT116 human colon cancer cells via the upregulation of DEPP/Gadd45a and activation of MAPKs

MENG-QI SU<sup>1\*</sup>, YI-RAN ZHOU<sup>2\*</sup>, XUAN RAO<sup>1</sup>, HONG YANG<sup>1</sup>, XIN-HAO ZHUANG<sup>4</sup>, XUE-JIA KE<sup>1</sup>, GUANG-YONG PENG<sup>3</sup>, CHANG-LIN ZHOU<sup>1</sup>, BAI-YONG SHEN<sup>2</sup> and JIE DOU<sup>1</sup>

<sup>1</sup>State Key Laboratory of Natural Medicines, School of Life Science and Technology, China Pharmaceutical University, Nanjing, Jiangsu 210029; <sup>2</sup>Department of General Surgery, Ruijin Hospital, Research Institute of Pancreatic Diseases, School of Medicine, Shanghai JiaoTong University, Shanghai 200025, P.R. China; <sup>3</sup>Division of Infectious Diseases, Allergy and Immunology and Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, MO 63104; <sup>4</sup>Department of Chemistry and Biochemistry, University of Oregon, OR 97401, USA

Received January 22, 2018; Accepted April 24, 2018

DOI: 10.3892/ijo.2018.4402

**Abstract.** Baicalein has efficient antitumor properties and has been reported to promote the apoptosis of several human cancer cell lines. Decidual protein induced by progesterone (DEPP), a transcriptional target of Forkhead Box O, was originally identified from the human endometrial stromal cell cDNA library. However, the expression and physiological functions of DEPP in human colon cancer cells remain to be fully elucidated. In the present study, it was reported that baicalein stimulated apoptosis and morphological changes of HCT116, A549 and Panc-1 cells in a dose-dependent manner. It also upregulated the mRNA and protein levels of DEPP and growth arrest and DNA damage-inducible 45 $\alpha$  (Gadd45a). In addition, the overexpression of DEPP promoted mitogen-activated protein kinase (MAPK) phosphorylation. To further investigate the role of DEPP and Gadd45a in baicalein-induced apoptosis, HCT116 cells were transfected with small interfering RNA against either DEPP or Gadd45a as *in vitro* models. Through an Annexin V/PI double staining assay, it was observed that baicalein-induced apoptosis

was impaired by the inactivation of either DEPP or Gadd45a, which in turn restricted the baicalein-induced activation of caspase-3 and caspase-9 and phosphorylation of MAPKs. In addition, the inhibition of c-Jun N-terminal kinase (JNK)/p38 activity with SP600125/SB203580 decreased the expression of Gadd45a, whereas the inactivation of extracellular signal-regulated kinase with SCH772984 had no effect on the expression of Gadd45a. Taken together, these results demonstrated that baicalein induced the upregulation of DEPP and Gadd45a, which promoted the activation of MAPKs with a positive feedback loop between Gadd45a and JNK/p38, resulting in a marked apoptotic response in human colon cancer cells. These results indicated that baicalein is a potential antitumor drug for the treatment of colon cancer.

## Introduction

Natural products isolated from plants have attracted increased interest due to their potent biological and pharmaceutical activities (1). Baicalein (5,6,7-trihydroxyflavone) extracted from the dry root of *Scutellaria baicalensis* Georgi, also known as *Baikal skullcap*, has a wide range of pharmacological functions and has been reported to possess potential antitumor activities against human liver cancer (2), breast cancer (3-6) and lung cancer cells (7). Multiple mechanisms are associated with its antitumor activity, however, the detailed mechanisms of baicalein-induced apoptosis in the HCT116 human colon cancer cell line remain to be fully elucidated.

Decidual protein induced by progesterone (DEPP) was originally identified from the human endometrial stromal cell cDNA library as a progesterone-induced gene (8). It is expressed in multiple tissues, including the placenta, ovary, kidney, white adipose and liver (9,10). In our previous study, DEPP was markedly upregulated in baicalein-treated HCT116 cells as assessed by microarray. Additionally, previous reports have suggested an association between DEPP and tumor cell death (8). However, there have been no reports on the physiological functions of DEPP in human colon cancer cells.

**Correspondence to:** Professor Jie Dou, State Key Laboratory of Natural Medicines, School of Life Science and Technology, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, Jiangsu 210029, P.R. China  
E-mail: doujie@cpu.edu.cn

Professor Bai-Yong Shen, Department of General Surgery, Ruijin Hospital, Research Institute of Pancreatic Diseases, School of Medicine, Shanghai JiaoTong University, 197 Rui Jin Er Road, Shanghai 200025, P.R. China  
E-mail: shenby@shsmu.edu.cn

\*Contributed equally

**Key words:** baicalein, colon cancer, apoptosis, decidual protein induced by progesterone, growth arrest and DNA damage-inducible 45 $\alpha$ , mitogen-activated protein kinase

Therefore, it was hypothesized that DEPP may be involved in the baicalein-induced cell death of HCT116 cells.

Growth arrest and DNA damage-inducible 45 $\alpha$  (Gadd45a) is a member of the Gadd45 family, the constituents of which can interact with pivotal cell effectors, including p21, p38, proliferating cell nuclear antigen, Cdc2/Cyclin B1 and mitogen-activated protein kinase kinase kinase 4 (11). In addition, Gadd45a is a vital regulator of cell cycle arrest, apoptosis and differentiation, all of which are strictly regulated by p53 (12). Gadd45a has been shown to be crucial in suppressing tumor cell growth (13,14). However, whether baicalein-induced apoptosis is associated with the activity of Gadd45a remains to be elucidated. Therefore, in the present study, experiments were performed to investigate whether Gadd45a and DEPP are involved in the baicalein-stimulated apoptosis of human colon cancer cells and to examine the mechanism involved in this activity.

The results of the present study verified that baicalein significantly stimulated the apoptosis of HCT116, A549 and Panc-1 cells, upregulated the expression of DEPP and Gadd45a, and triggered the phosphorylation of MAPKs. Further experiments revealed that, as DEPP increased the protein and mRNA levels of Gadd45a, the expression of DEPP and Gadd45a contributed to baicalein-induced apoptosis and MAPK activation. Inhibiting c-Jun N-terminal kinase (JNK)/p38 signaling also reduced the expression of Gadd45a, however, there was no such change in the inactivation of extracellular signal-regulated kinase (ERK). Taken together, these findings identified the essential role of DEPP and Gadd45a in baicalein-induced apoptosis and indicated that baicalein may be a promising antitumor agent for the treatment of colon cancer in humans.

## Materials and methods

**Cell cultures and drug treatments.** The HCT116, A549, Panc-1 and FHC (normal human colorectal mucosal cells) cell lines were obtained from American Type Culture Collection (Rockville, MD, USA) and were grown in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (CellMax, Beijing, USA) and penicillin-streptomycin (Beyotime Institute of Biotechnology, Nanjing, China). The cells were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub> and were exposed to the indicated concentrations (0, 10, 20 or 40  $\mu$ M) of baicalein (Jiangsu Institute for Food and Drug Control, Nanjing, China), 1  $\mu$ M gemcitabine (cat. no. G8970, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), SP600125 (cat. no. S1460, Selleck Chemicals, Houston, TX, USA), SB203580 (cat. no. S1076, Selleck Chemicals) or SCH772984 (cat. no. S7101, Selleck Chemicals).

**Western blot analysis.** The cell lysates were prepared with RIPA buffer containing PMSF (Beyotime Institute of Biotechnology). Protein concentration was determined by Enhanced Bicinchoninic Acid Protein assay kit (cat. no. P0010, Beyotime Institute of Biotechnology). Equal quantities (50  $\mu$ g) of cellular protein were loaded onto a 12% SDS-PAGE gel and then transferred onto a PVDF membrane (cat. no. IPVH00010, EMD Millipore, Billerica, MA, USA). The membranes were blocked by 5% non-fat milk in Tris-buffered saline with 0.07% Tween-20 for 2 h and incubated with specific primary antibodies (1:1,000) in 1% bovine serum albumin (Biosharp, Hefei, China)

in Tris-buffered saline with 0.07% Tween-20 at 4°C overnight. The following primary antibodies were used: Rabbit anti-DEPP (cat. no. 25833-1-AP, 1:1,000) from ProteinTech Group, Inc. (Rosemont, IL, USA); mouse anti- $\beta$ -actin (cat. no. 21800, 1:1,000) from SAB Signalway Antibody (Nanjing, China); and rabbit anti-Gadd45a (cat. no. 4632, 1:1,000), rabbit anti-caspase-3 (cat. no. 14220, 1:1,000), rabbit anti-caspase-9 (cat. no. 9502, 1:1,000), rabbit anti-cleaved-caspase-9 (cat. no. 9505, 1:1,000), mouse anti-JNK (cat. no. 3780, 1:1,000), rabbit anti-phosphorylated (p-)JNK (cat. no. 4668, 1:1,000), rabbit anti-ERK (cat. no. 4695, 1:1,000), rabbit anti-p-ERK (cat. no. 4370, 1:1,000), rabbit anti-p38 (cat. no. 9212, 1:1,000) and rabbit anti-p-p38 (cat. no. 4511, 1:1,000) from Cell Signaling Technology, Inc. (Danvers, MA, USA). The secondary antibodies used were goat anti-rabbit IgG (cat. no. L3012-2, 1:5,000) or goat anti-mouse IgG (cat. no. L3032-2, 1:5,000) from SAB Signalway Antibody. The membranes were incubated with diluted secondary antibodies (1:5,000) in 1% bovine serum albumin at room temperature for 1.5 h. The signal was visualized using the Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500, EMD Millipore), using Image Lab 5.2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The quantitative densitometric analysis was performed using ImageJ 1.48v with 32-bit Java 1.6.0\_20 (National Institutes of Health, Bethesda, MD, USA).

**Detection of apoptosis.** Apoptosis induced by baicalein was determined using an Annexin V-FITC/PI Apoptosis Detection kit (cat. no. A211-02, Vazyme, Nanjing, China). The cells were seeded into a 12-well plate at 5 $\times$ 10<sup>4</sup> cells/ml and treated with different concentrations of baicalein for 48 h. The cells were detached with 0.25% trypsin without EDTA, washed twice with cold PBS and resuspended in 100  $\mu$ l binding buffer. The cells were then incubated with 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l PI for 10 min at room temperature in the dark, and analyzed with the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA from the cultured cells was extracted using TRIzol (cat. no. R401-01, Vazyme, Piscataway, NJ, USA) and chloroform/isopropanol, as specified by the manufacturer, and then reverse transcribed using a HiScript Q RT SuperMix for qPCR (+gDNA wiper) kit (cat. no. R223-01, Vazyme). The qPCR was subsequently performed using a ChamQ SYBR qPCR Master Mix kit (cat. no. Q311-02, Vazyme) and each sample [5  $\mu$ l 2X ChamQ SYBR qPCR Master Mix, 0.2  $\mu$ l Primer 1 (10  $\mu$ M), 0.2  $\mu$ l Primer 2 (10  $\mu$ M), 0.2  $\mu$ l 50X ROX Reference Dye1, 2  $\mu$ l template cDNA and 2.4  $\mu$ l ddH<sub>2</sub>O] was analyzed on the StepOne™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Stage 1, 95°C for 3 min; Stage 2, 95°C for 10 sec, 56°C for 30 sec, 72°C for 30 sec; Stage 3, 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec. The following PCR primers were used: GAPDH forward, 5'-GGGAACTG TGGCGTGAT-3' and reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'; DEPP forward, 5'-ATACGTCCTGTGTTGGCATTG-3' and reverse, 5'-CCTGATTCCCGTTCCCTGAT-3'; Gadd45a forward, 5'-TTGCAATATGACTTTGGAGGAA-3' and reverse, 5'-CATC CCCCACCTTATCCAT-3'; p21 forward, 5'-AGCAGAGGAAGACCATGTGGA-3' and reverse, 5'-AAT CTGTCATGCTGGTCT

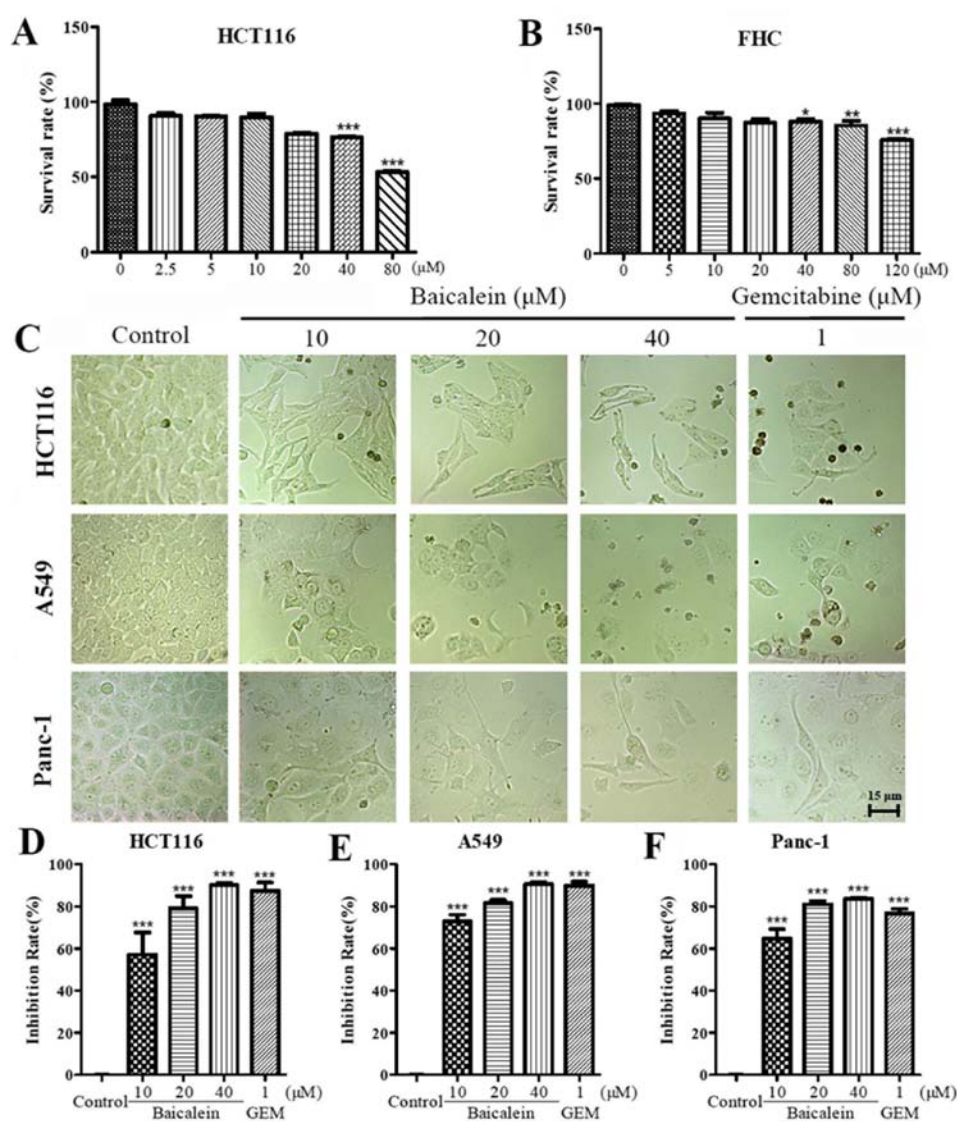


Figure 1. Baicalein alters the morphology and inhibits the proliferation of HCT116, A549 and Panc-1 cells. Effect of baicalein on the proliferation of (A) HCT116 and (B) FHC cells was determined by the MTT assay. The cells were treated with various concentrations of baicalein for 48 h. (C) Morphological changes of HCT116, A549 and Panc-1 cells under light microscopy (magnification, x200) following treatment with 0, 10, 20 or 40  $\mu$ M of baicalein and 1  $\mu$ M of GEM (positive control) for 72 h. Quantification of the number of viable (D) HCT116, (E) A549 and (F) Panc-1 cells. All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the control group). GEM, gemcitabine.

GCC-3'; and p53 forward, 5'-CTCTCCCCAGCCAAAGAA GAA-3' and reverse, 5'-TCCAAGGCCTCATTGAGCTCT-3' (Springen Biotechnology, Nanjing, China). The relative mRNA expression was normalized to that of GAPDH and was determined using the comparative Cq method ( $2^{-\Delta\Delta C_q}$ ) (15).

**Small interfering (si)RNA transfection.** siRNA targeting DEPP and Gadd45a, and non-specific siRNA (NC) were synthesized by GenScript Co., Ltd. (Nanjing, China): DEPP, 5'-GCAGUGUCCUCAGAACACU-3'; Gadd45a 5'-AAAGUCGCUACAUGGAUCAAU-3'; NC 5'-UUCUCCG AACGUGACACGU-3'. The cells were transfected with siDEPP, siGadd45a or non-specific siRNA using Entranster™-R4000 (Engreen Biosystem, Ltd., Beijing, China) according to the manufacturer's protocol.

**Overexpression of DEPP.** The HCT116 cells were transfected with 0.82  $\mu$ g/ $\mu$ l of either empty pcDNA3.1 or pcDNA3.1 (GenScript

Co., Ltd.) containing DEPP cDNA using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.). The cells were harvested 72 h following transfection.

**MTT proliferation assay.** The cells were treated with the indicated concentrations of Baicalein at 37°C for 48 h. Subsequently, MTT was added to reach a final concentration of 5 mg/ml for 4 h. The supernatant was removed, and the purple-colored formazan precipitate was dissolved in 150  $\mu$ l DMSO and measured at 490 nm with a microplate reader (iMark; Bio-Rad Laboratories, Inc.).

**Statistical analysis.** All data are shown as the mean  $\pm$  standard deviation of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA), and data were analyzed using one-way analysis of variance, followed by the Dunnett's multiple comparison test. In certain cases, Student's t-test was

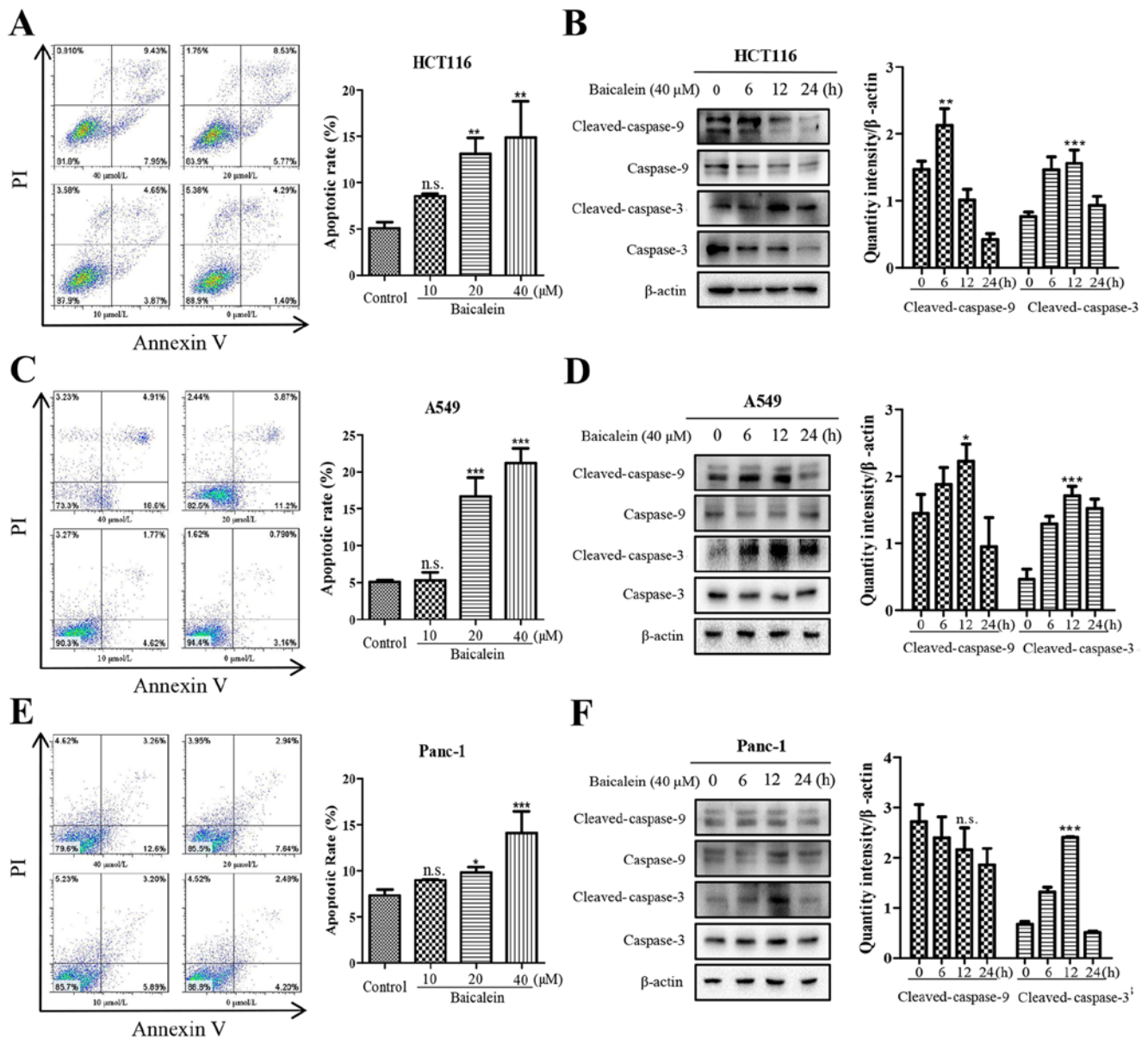


Figure 2. Baicalein induces the apoptosis of HCT116, A549 and Panc-1 cells. (A) Flow cytometric analysis of HCT116 cell apoptosis; (B) Immunoblot assay of cleaved-caspase-9 and caspase-3 in HCT116 cells; (C) Flow cytometric analysis of A549 cell apoptosis; (D) Immunoblot assay of cleaved-caspase-9 and caspase-3 in A549 cells; (E) Flow cytometric analysis of Panc-1 cell apoptosis; (F) Immunoblot assay of cleaved-caspase-9 and caspase-3 in Panc-1 cells. Flow cytometric analysis was performed following treatment with baicalein (0, 10, 20 or 40  $\mu$ M) for 48 h. Representative results are shown and the histogram (right) shows the mean apoptotic rate as detected by flow cytometry. For immunoblotting, cells were treated with 40  $\mu$ M baicalein for 0, 6, 12 or 24 h, and the induced cleavage of caspase-9 and caspase-3 was detected. All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the control group). DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45a; n.s., not significant.

used for comparing two groups.  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**Baicalein induces the apoptosis of HCT116, A549 and Panc-1 cells.** Firstly, an MTT assay was performed to confirm that the appropriate concentration of baicalein was used. The data from the HCT116 (Fig. 1A) and FHC (Fig. 1B) cells demonstrated that 40  $\mu$ M baicalein significantly inhibited the proliferation of HCT116 (~24%) and caused less damage to FHC cells (~10%). Subsequently, to detect the effect of baicalein on human cancer cells, light microscopy was used to observe the morphological

changes of HCT116, A549 and Panc-1 cells following treatment with 0, 10, 20 or 40  $\mu$ M baicalein with 1  $\mu$ M gemcitabine serving as a positive control. As shown in Fig. 1C, cells treated with baicalein (10, 20 or 40  $\mu$ M) or gemcitabine (1  $\mu$ M) were in flattened, blebby and shrunken in appearance, consistent with cell death, whereas the negative control cells presented with an intact and polygonal morphology. Additionally, baicalein markedly inhibited the proliferation of HCT116 (Fig. 1D), A549 (Fig. 1E), and Panc-1 (Fig. 1F) cells in a dose-dependent manner. For example, 40  $\mu$ M baicalein inhibited proliferation by up to 90%. Furthermore, the data indicated that baicalein induced apoptosis of the HCT116 cells in a dose-dependent manner as assessed by the Annexin V/PI double staining assay (Fig. 2A),

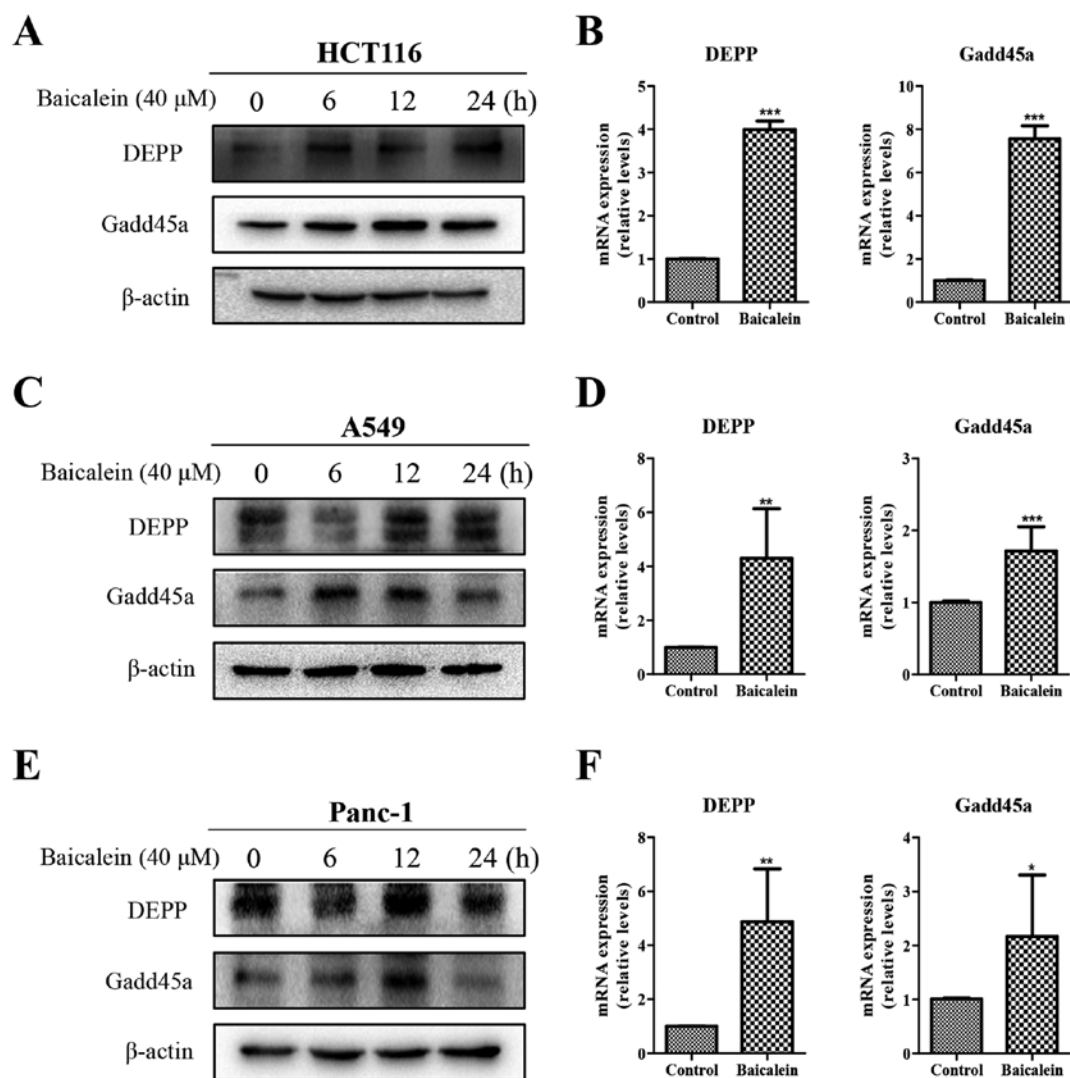


Figure 3. DEPP and Gadd45a are upregulated in HCT116, A549 and Panc-1 cells in response to baicalein. (A) Western blot analysis of the expression of DEPP and Gadd45a in HCT116 cells; (B) mRNA expression of DEPP and Gadd45a in HCT116 cells treated with 40  $\mu$ M baicalein for 24 h; (C) western blot analysis of the expression of DEPP and Gadd45a in A549 cells; (D) mRNA expression of DEPP and Gadd45a in A549 cells treated with 40  $\mu$ M baicalein for 6 h; (E) western blot analysis of the expression of DEPP and Gadd45a in Panc-1 cells; (F) mRNA expression of DEPP and Gadd45a in Panc-1 cells treated with 40  $\mu$ M baicalein for 6 h. Western blot analyses were performed in cells treated with baicalein (40  $\mu$ M) for 0, 6, 12 or 24 h. All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the control group). DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45 $\alpha$ .

and significantly increase the levels of cleaved-caspase-3 and cleaved-caspase-9 (Fig. 2B) following treatment for the indicated times. Similar results were also observed in the A549 (Fig. 2C and D) and Panc-1 (Fig. 2E and F) cells, suggesting that baicalein induced the apoptosis of HCT116, A549 and Panc-1 cells.

*Expression levels of DEPP and Gadd45a are elevated in cancer cells subjected to baicalein-induced apoptosis.* To verify the potential role of DEPP and Gadd45a in baicalein-induced apoptosis, the present study investigated whether the expression levels of DEPP and Gadd45a were altered in baicalein-treated cells. Western blot analysis (Fig. 3A) and RT-qPCR analysis (Fig. 3B) showed that the protein and mRNA expression levels of DEPP and Gadd45a were elevated more than two-fold in the baicalein-treated HCT116 cells. The data for protein levels in the A549 and Panc-1 cells were similar to those for the HCT116 cells. However,

the mRNA expression of DEPP and Gadd45a in the A549 and Panc-1 cells had distinctly increased following 6 h of baicalein treatment, vs. 24 h of treatment (Fig. 3C-F), which may be attributable to the delay between transcription and translation. These results confirmed that baicalein upregulated DEPP and Gadd45a in three distinct human cancer cell lines.

*DEPP and Gadd45a deficiency inhibits baicalein-induced apoptosis of HCT116 cells.* To determine whether DEPP and Gadd45a were necessary for baicalein-induced apoptosis, siRNA against either DEPP or Gadd45a was transfected into HCT116 cells. Western blot analysis showed that the accumulation of baicalein-induced cleaved-caspase-3 and cleaved-caspase-9 were markedly reduced when DEPP and Gadd45a were silenced (Fig. 4A and B). These results were further confirmed by the Annexin V/PI double staining assay. As shown in Fig. 4C and D, there was a decrease of ~10% in



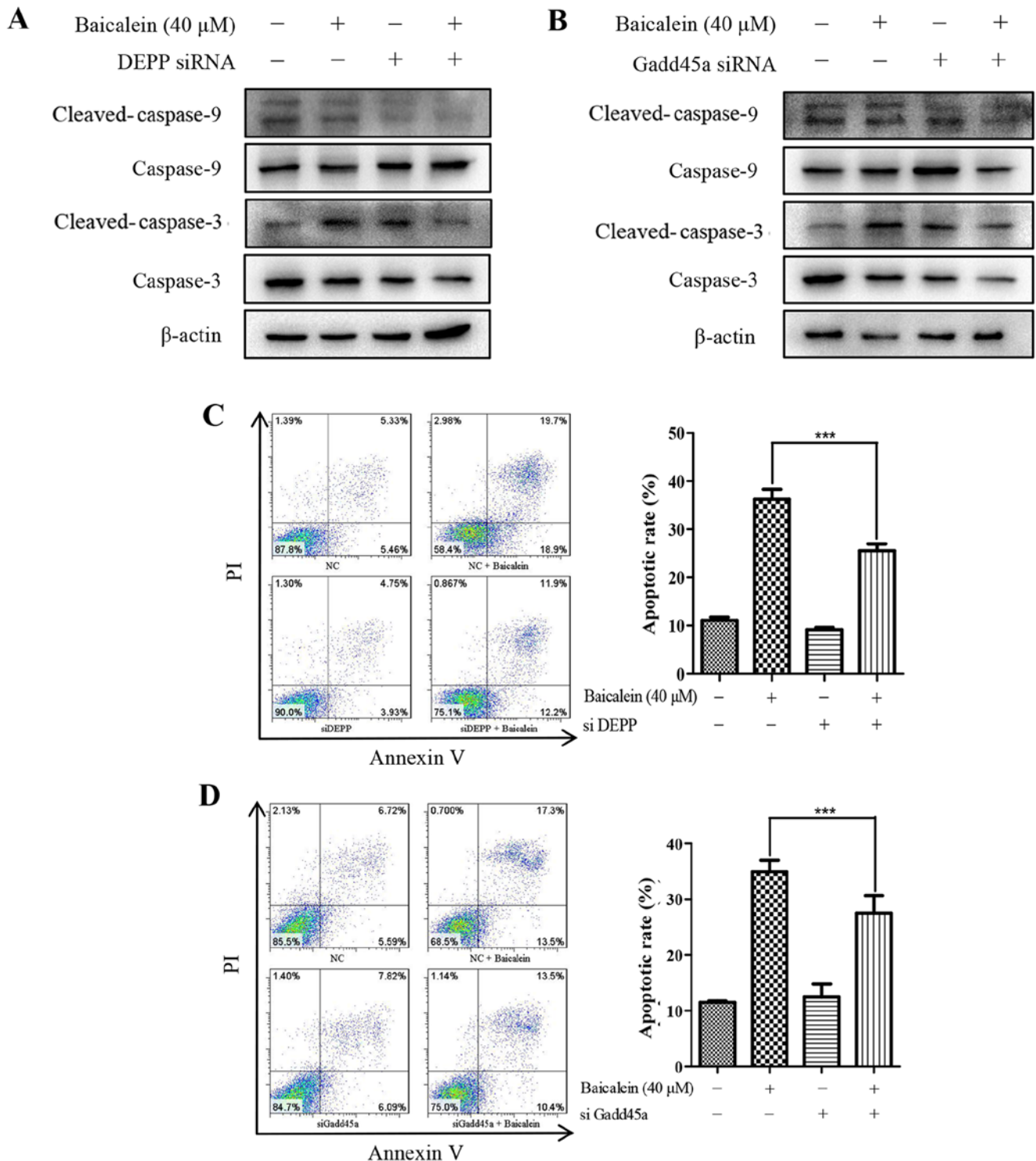


Figure 4. Baicalein-induced apoptosis of HCT116 cells is inhibited by either DEPP or Gadd45a deficiency. HCT116 cells were transfected with (A) siDEPP or (B) siGadd45a for 48 h prior to treatment with baicalein (40  $\mu$ M). The inducible cleavage of caspase-9 and caspase-3 was detected by an immunoblot assay at 24 h. HCT116 cells were transfected with (C) siDEPP or (D) siGadd45a for 48 h prior to treatment with baicalein (40  $\mu$ M). Cells stained with Annexin V-FITC/PI were measured by flow cytometry. Representative results are shown. The histogram shows the mean apoptotic rate (right). All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments ( $^{***}P < 0.001$ , as indicated). DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45a; siRNA/si, small interfering RNA.

the apoptotic rate of cells treated with 40  $\mu$ M baicalein when transfected with either DEPP siRNA or Gadd45a siRNA, compared with cells transfected with non-specific siRNA. Taken together, these data showed that baicalein upregulated the expression of DEPP and Gadd45a, which was involved in

the apoptotic response in HCT116 cells through the activation of caspase-3 and caspase-9.

*Baicalein-induced HCT116 cell apoptosis via the upregulation of DEPP/Gadd45a is mediated by the phosphorylation*

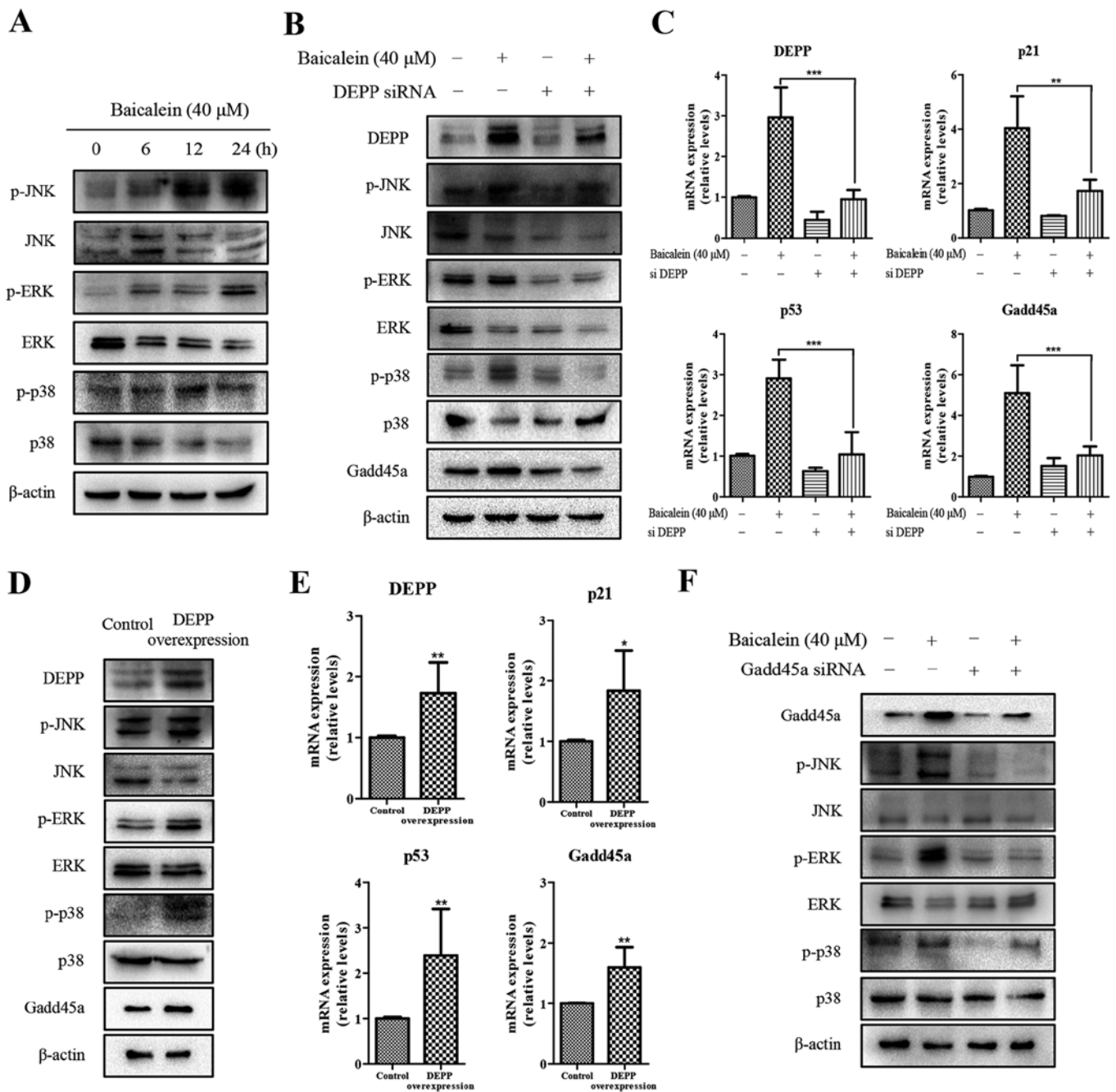


Figure 5. Expression of DEPP and Gadd45a contribute to the baicalein-induced phosphorylation of MAPKs in HCT116 cells. (A) Western blot analysis of the expression of p-JNK, JNK, p-ERK, ERK, p-p38 and p38 in HCT116 cells treated with baicalein (40  $\mu$ M) for 0, 6, 12 or 24 h. (B) Western blot analysis of the expression of DEPP, Gadd45a, p-JNK, JNK, p-ERK, ERK, p-p38, p38 in DEPP siRNA-transfected HCT116 cells treated with baicalein (40  $\mu$ M) for 24 h. (C) RT-qPCR analysis of the mRNA expression of DEPP, Gadd45a, p21 and p53 in DEPP siRNA-transfected HCT116 cells treated with baicalein (40  $\mu$ M) for 24 h. (D) Western blot analysis of the expression of DEPP, Gadd45a, p-JNK, JNK, p-ERK, ERK, p-p38 and p38 in HCT116 cells overexpressing DEPP for 72 h. (E) RT-qPCR analysis of the mRNA expression of DEPP, Gadd45a, p21 and p53 in HCT116 cells overexpressing DEPP for 72 h. (F) Western blot analysis of the expression of Gadd45a, p-JNK, JNK, p-ERK, ERK, p-p38 and p38 in Gadd45a siRNA-transfected HCT116 cells treated with baicalein (40  $\mu$ M) for 24 h. All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the control group, or as indicated). DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45a; siRNA/si, small interfering RNA; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p-, phosphorylated; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

of MAPKs. To further elucidate the mechanism linking baicalein-induced apoptosis and the expression of DEPP and Gadd45a, the activation of MAPK was examined using an immunoblot assay in HCT116 cells at 0, 6, 12 and 24 h post-baicalein treatment. Notably, increases in the phosphorylation of JNK, ERK and p38 were observed in the

western blot analysis (Fig. 5A). The expression of DEPP was then stably inhibited using siRNA to determine whether DEPP is required for the baicalein-induced phosphorylation of MAPKs. Following baicalein treatment, the absence of DEPP (Fig. 5B and C) had a negative effect on the baicalein-mediated protein expression of p-JNK, p-ERK, p-p38 and

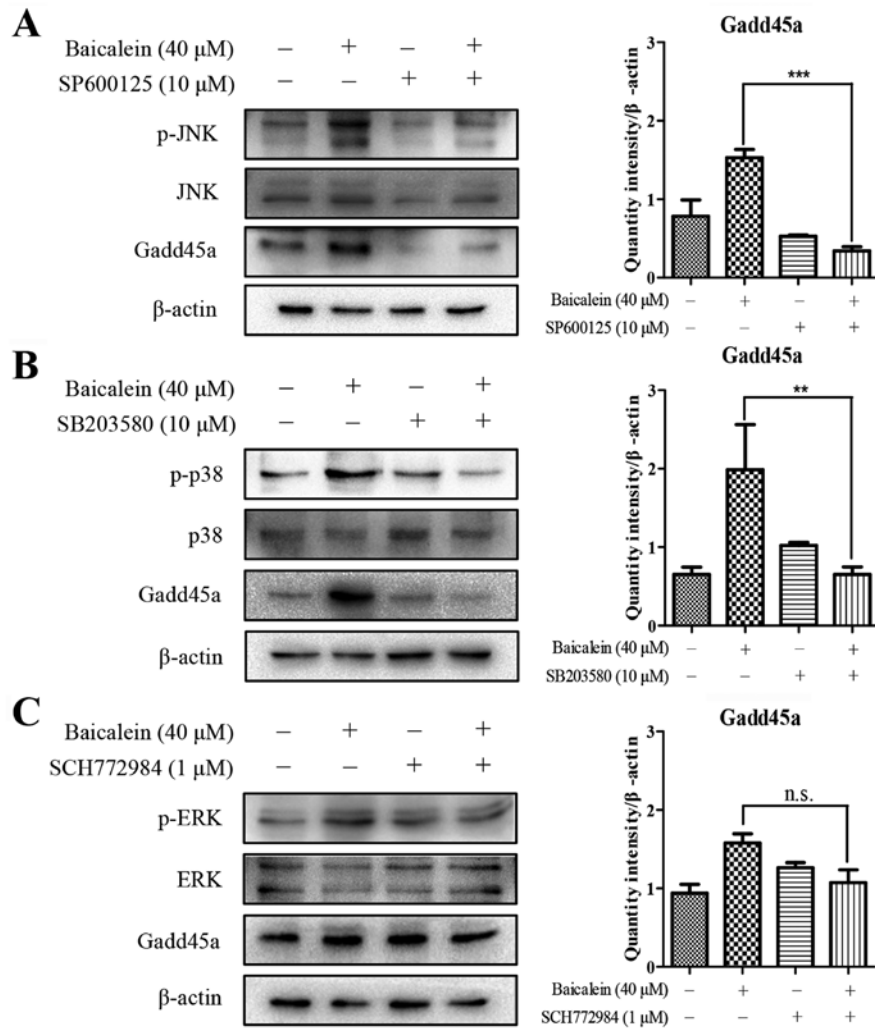


Figure 6. Baicalein-induced expression of Gadd45a is mediated by MAPK inhibitors in HCT116 cells. HCT116 cells were treated with baicalein (40  $\mu$ M) for 24 h following preincubation with (A) 10  $\mu$ M SP600125, (B) 10  $\mu$ M SB203580 or (C) 1  $\mu$ M SCH772984 for 1 h. The protein levels of Gadd45a were determined by western blot analysis. All data shown are presented as the mean  $\pm$  standard deviation from three independent experiments (\* $P$ <0.01 and \*\*\* $P$ <0.001, as indicated). DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45a; siRNA/si, small interfering RNA; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

Gadd45a, or on the mRNA expression of p21, p53 and Gadd45a. In addition, a DEPP-overexpression plasmid was used to further validate these findings (Fig. 5D). Similarly, p-JNK, p-ERK, p-p38, p21, and p53 were induced only when the overexpression of DEPP was present (Fig. 5D and E). Furthermore, the gene expression of Gadd45a was increased by 1.60-fold. Together, these results suggested that DEPP is required for the baicalein-induced phosphorylation of MAPKs and upregulated expression of Gadd45a at the protein and mRNA levels. The present study subsequently examined whether Gadd45a also regulated the baicalein-induced phosphorylation of MAPKs, and similar results were observed in the levels of p-JNK, p-ERK and p-p38 in cells transfected with siRNA against Gadd45a (Fig. 5F). In summary, these data suggested that DEPP and Gadd45a are essential in baicalein-induced apoptosis via MAPK signaling.

*Baicalein-induced upregulation of Gadd45a is attenuated by inhibiting the phosphorylation of MAPK.* The abovementioned

results showed that Gadd45a upregulated the phosphorylation of JNK, ERK and p38 in the baicalein-induced apoptosis of HCT116 cells. A previous report demonstrated that MAPK signaling mediates the expression of Gadd45a (14). Therefore, to determine whether MAPK signaling was involved in the baicalein-mediated upregulation of Gadd45a, the JNK-specific inhibitor SP600125, p38 inhibitor SB203580, and ERK inhibitor SCH772084 were used to prevent the activation of JNK, p38 and ERK, respectively. The inhibition of JNK and p38 was accompanied by a marked decrease in the baicalein-induced expression of Gadd45a (Fig. 6A and B), however, no such change was observed in the presence of the ERK inhibitor SCH772084 (Fig. 6C). These results suggested that there was a positive feedback loop between Gadd45a and JNK/p38 when baicalein triggered the apoptotic response in human colon cancer cells, whereas the suppression of ERK had no effect on the expression of Gadd45a. Therefore, these data revealed the critical role of the activation of MAPK in inducing of the expression of Gadd45a in baicalein-induced apoptosis (Fig. 7).



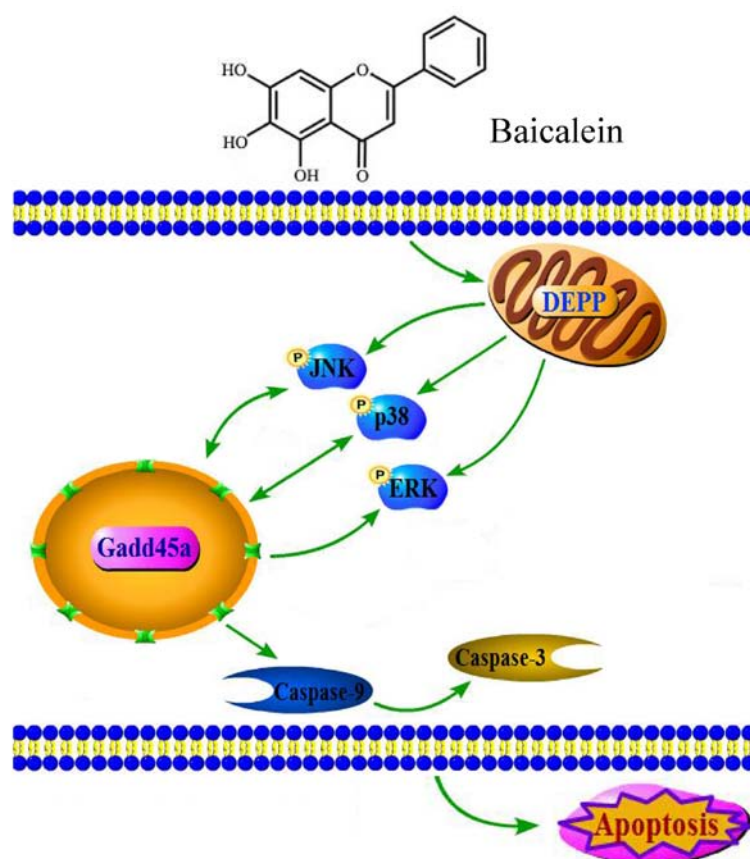


Figure 7. Schematic diagram of the proposed signaling pathways involved in the activities of DEPP and GADD45a in baicalein-induced apoptosis of HCT116 cells. Baicalein induced the expression of DEPP, which upregulated the expression of Gadd45a and JNK/ERK/p38, and activated the Gadd45a-JNK/p38 feed-back loop, resulting in the apoptosis of tumor cells. DEPP, decidual protein induced by progesterone; Gadd45a, growth arrest and DNA damage-inducible 45α; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

## Discussion

In the present study, it was shown that baicalein upregulated DEPP and Gadd45a, which activated caspase-3, caspase-9 and the JNK/ERK/p38 MAPK pathways leading to apoptosis of HCT116 human colon cancer cells. As a natural compound extracted from *Scutellaria baicalensis* Georgi, baicalein has been widely investigated for its potent antitumor properties. The majority of studies have revealed that baicalein exerts its antitumor ability via the induction of apoptosis in human lung cancer (6), breast cancer (7) and osteosarcoma cells (16). In addition, it also has been demonstrated that the MAPK signaling pathway results in the baicalein-induced apoptosis of cancer cells (7). Therefore, the present study was performed to validate these findings and observed not only marked induction of apoptosis by baicalein in HCT116 colon cancer cells, A549 lung carcinoma cells and Panc-1 pancreatic cancer cells, but also the prominent activation of caspase-3, caspase-9 and JNK/ERK/p38 in baicalein-treated HCT116 cells.

However, whether the MAPKs functioned upstream or downstream to mediate baicalein-induced apoptosis remained to be elucidated. Therefore, on the basis of our previous results that DEPP and Gadd45a were markedly upregulated in baicalein-treated HCT116 cells by microarray, it was hypothesized DEPP and Gadd45a were important in baicalein-

induced apoptosis and the activation of caspase-3, caspase-9 and MAPKs in HCT116 cells. Consistent with this hypothesis, the absence of DEPP notably decreased the apoptotic rate and the levels of pro-apoptotic proteins cleaved-caspase-3, cleaved-caspase-9, p-JNK/ERK/p38, p21, p53 and Gadd45a, all of which were induced by baicalein. Similar results were observed in HCT116 cells transfected with Gadd45a siRNA. Furthermore, the overexpression of DEPP provoked the phosphorylation of MAPKs and the expression of p21, p53 and Gadd45a. Taken together, these results indicated that as DEPP upregulated Gadd45a at the protein and mRNA levels, both these genes contribute to the activation of JNK/ERK/p38 during baicalein-induced apoptosis. However, further *in vitro* and *in vivo* investigations are required to examine whether baicalein has a direct impact on the expression of DEPP.

DEPP is a progesterone-induced gene that is regulated by progesterone in endometrial stromal cells and by insulin in adipose tissue. Additionally, it is induced in malignant glioma cells under hypoxic conditions (9,10,17). However, there are few reports on its physiological function. Salcher *et al* found that the expression of DEPP contributes to Forkhead Box O3 (FOXO3)-induced apoptosis, as DEPP knockdown significantly reduced FOXO3-induced cell death (8). Another study demonstrated that the upregulation of DEPP activated MAPK signaling pathways to stimulate the transcription factor ELK1 (17). Therefore, the findings in the present study that

DEPP promoted baicalein-induced apoptosis by the activation of Gadd45a, JNK/ERK/p38, caspase-3 and caspase-9, can drive further investigations regarding the physiological function of DEPP. However, there remain several questions in terms of how baicalein increases the mRNA and protein levels of DEPP and how DEPP induces the phosphorylation of MAPKs, two activities requiring thorough investigation.

In contrast to DEPP, the induction of Gadd45a and apoptosis via the MAPK signaling pathway has been examined extensively (13,14). However, detailed conclusions regarding the feedback loop between Gadd45a and MAPKs remain to be fully elucidated as reports have conflicting data. Certain reports have shown that Gadd45a is upstream of MAPK signaling and activates the JNK pathway (18), whereas others have suggested that Gadd45a is downstream of MAPK pathways, and is positively regulated by JNK and negatively regulated by ERK/p38 (14). Accordingly, the analyses in the present study of the mediation between the expression of Gadd45a and baicalein-induced activation of JNK/ERK/p38 in human colon cancer cells is useful as a supplement to previously published data. The *in vitro* experiments suggested the existence of a positive feedback loop between Gadd45a and JNK/p38. However, further investigations on the detailed role of Gadd45a and JNK/p38 on this loop, and the role of ERK outside this loop, in baicalein-induced apoptosis are required.

In conclusion, the present study provided evidence of a novel mechanism of baicalein-induced apoptosis in human colon cancer cells and found for the first time, to the best of our knowledge, that baicalein upregulated DEPP and Gadd45a, leading to apoptosis via the MAPK signaling pathway and the activation of caspase-3 and caspase-9 in HCT116 cells. In this context, the existence of a positive feedback loop between Gadd45a and JNK/p38 was also confirmed. In general, the findings of the present study may encourage the development of novel options to target DEPP and the Gadd45a-JNK/p38 feedback loop in the treatment of colon cancer.

## Acknowledgements

Not applicable.

## Funding

This study was supported by the National Natural Science Foundation of China (grant no. 81472233), the Jiangsu National Natural Science Foundation of China (grant no. BK20150700) and the '111 Projec' from the Ministry of Education of China and the State Administration of Foreign Expert Affairs of China (grant no. 111-2-07).

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

MS, YZ and JD designed and performed the experiments; XR, HY, XZ and XK contributed to the data analysis; BS and CZ

contributed to the conception of the study and helped with data interpretation; GP drafted the manuscript and was responsible for methodology; JD and CZ revised the manuscript critically; all authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Liu X, Liu S, Chen J, He L, Meng X and Liu S: Baicalein suppresses the proliferation of acute T-lymphoblastic leukemia Jurkat cells by inhibiting the Wnt/ $\beta$ -catenin signaling. *Ann Hematol* 95: 1787-1793, 2016.
2. Han Z, Zhu S, Han X, Wang Z, Wu S and Zheng R: Baicalein inhibits hepatocellular carcinoma cells through suppressing the expression of CD24. *Int Immunopharmacol* 29: 416-422, 2015.
3. Chung H, Choi HS, Seo EK, Kang DH and Oh ES: Baicalin and baicalein inhibit transforming growth factor- $\beta$ 1-mediated epithelial-mesenchymal transition in human breast epithelial cells. *Biochem Biophys Res Commun* 458: 707-713, 2015.
4. Wang XF, Zhou QM, Du J, Zhang H, Lu YY and Su SB: Baicalin suppresses migration, invasion and metastasis of breast cancer via p38MAPK signaling pathway. *Anticancer Agents Med Chem* 13: 923-931, 2013.
5. Ma X, Yan W, Dai Z, Gao X, Ma Y, Xu Q, Jiang J and Zhang S: Baicalein suppresses metastasis of breast cancer cells by inhibiting EMT via downregulation of SATB1 and Wnt/ $\beta$ -catenin pathway. *Drug Des Devel Ther* 10: 1419-1441, 2016.
6. Zhou QM, Wang S, Zhang H, Lu YY, Wang XF, Motoo Y and Su SB: The combination of baicalin and baicalein enhances apoptosis via the ERK/p38 MAPK pathway in human breast cancer cells. *Acta Pharmacol Sin* 30: 1648-1658, 2009.
7. Kim HJ, Park C, Han MH, Hong SH, Kim GY, Hong SH, Kim ND and Choi YH: Baicalein induces caspase-dependent apoptosis associated with the generation of ROS and the activation of AMPK in human lung carcinoma A549 Cells. *Drug Dev Res* 77: 73-86, 2016.
8. Salcher S, Hagenbuchner J, Geiger K, Seiter MA, Rainer J, Kofler R, Hermann M, Kiechl-Kohlendorfer U, Ausserlechner MJ and Obexer P: C10ORF10/DEPP, a transcriptional target of FOXO3, regulates ROS-sensitivity in human neuroblastoma. *Mol Cancer* 13: 224, 2014.
9. Kuroda Y, Kuriyama H, Kihara S, Kishida K, Maeda N, Hibuse T, Nishizawa H, Matsuda M, Funahashi T and Shimomura I: Insulin-mediated regulation of decidual protein induced by progesterone (DEPP) in adipose tissue and liver. *Horm Metab Res* 42: 173-177, 2010.
10. Chen S, Gai J, Wang Y and Li H: FoxO regulates expression of decidual protein induced by progesterone (DEPP) in human endothelial cells. *FEBS Lett* 585: 1796-1800, 2011.
11. Sytnikova YA, Kubarenko AV, Schäfer A, Weber AN and Niehrs C: Gadd45a is an RNA binding protein and is localized in nuclear speckles. *PLoS One* 6: e14500, 2011.
12. Tong T, Ji J, Jin S, Li X, Fan W, Song Y, Wang M, Liu Z, Wu M and Zhan Q: Gadd45a expression induces Bim dissociation from the cytoskeleton and translocation to mitochondria. *Mol Cell Biol* 25: 4488-4500, 2005.
13. Tront JS, Hoffman B and Liebermann DA: Gadd45a suppresses Ras-driven mammary tumorigenesis by activation of c-Jun NH2-terminal kinase and p38 stress signaling resulting in apoptosis and senescence. *Cancer Res* 66: 8448-8454, 2006.

14. Yin F, Bruemmer D, Blaschke F, Hsueh WA, Law RE and Herle AJ: Signaling pathways involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells. *Oncogene* 23: 4614-4623, 2004.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the  $2(-\Delta \Delta C(T))$  Method. *Methods* 25: 402-408, 2001.
16. Ye F, Wang H, Zhang L, Zou Y, Han H and Huang J: Baicalein induces human osteosarcoma cell line MG-63 apoptosis via ROS-induced BNIP3 expression. *Tumour Biol* 36: 4731-4740, 2015.
17. Watanabe H, Nonoguchi K, Sakurai T, Masuda T, Itoh K and Fujita J: A novel protein Depp, which is induced by progesterone in human endometrial stromal cells activates Elk-1 transcription factor. *Mol Hum Reprod* 11: 471-476, 2005.
18. Song L, Li J, Zhang D, Liu ZG, Ye J, Zhan Q, Shen HM, Whiteman M and Huang C: IKK $\beta$  programs to turn on the GADD45 $\alpha$ -MKK4-JNK apoptotic cascade specifically via p50 NF- $\kappa$ B in arsenite response. *J Cell Biol* 175: 607-617, 2006.