

Baicalin inhibits breast cancer development via inhibiting I κ B kinase activation *in vitro* and *in vivo*

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Abstract. The aim of the present study was to investigate the effect and therapeutic potential of baicalin in breast cancer. Baicalin is used to treat inflammatory diseases. The effects of baicalin were assessed in breast cancer MCF-7 and MDA-MB-231 cells, and human breast cancer xenograft mice. Cells were treated with 0, 20 or 30 μ M baicalin for 48 h, while xenograft mice were treated with intraperitoneal injection of 0, 100 or 200 mg/kg baicalin for 30 days. The results demonstrated that treatment with baicalin dose-dependently suppressed breast cancer cell invasion, migration and proliferation, and also induced G1/S-phase cell cycle arrest *in vitro* and *in vivo*. Baicalin alleviated inflammation injury and inhibited the secretion of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , thus suppressing nuclear factor (NF)- κ B-p65 activation via inhibition of I κ B kinase. Investigation of the mechanism underlying baicalin activity indicated that it inhibited protein expression of NF- κ B-p65, leading to NF- κ B-induced increased expression of CCND1, BCL2, BIRC2 and BIRC3, thus inhibiting cell proliferation, invasion and migration and suppressing anti-apoptotic factors *in vitro* and *in vivo*. In addition, baicalin did not affect non-tumorigenic normal breast epithelial cells. These results indicate that baicalin may exert therapeutic effects in breast cancer.

Introduction

Breast cancer is the most common type of malignancy worldwide and the second leading cause of cancer-related mortality

in women (1,2). Recent advances in breast cancer detection and treatment have decreased the mortality rate attributable to breast cancer. However, the side effects of radiotherapy and chemotherapy, such as immunosuppression, recurrence and metastasis, remain challenging and adversely affect the quality of life of breast cancer patients (3,4). A number of traditional Chinese herbal medicines have been suggested as potential therapeutic options, due to their relative lack of side effects.

Constitutive activation of the transcription factor nuclear factor (NF)- κ B stimulates proliferation and metastasis and inhibits apoptosis of breast cancer cells. NF- κ B activity-stimulating signals have been shown to cause dissociation of I κ B, allowing NF- κ B dimers to translocate to the nucleus and alter gene expression, in order to activate anti-apoptotic genes and promote the transcription of genes associated with cancer growth, invasion and metastasis. This makes NF- κ B a key factor in enabling precancerous and malignant cells to escape apoptosis (5-7). Inhibition of NF- κ B activity results in the partial release of cells from the G2/M arrest following curcumin treatment of human breast cancer MCF-7 cells (8,9). Therefore, NF- κ B may represent an effective target for controlling tumor invasion and metastasis.

Baicalin is one of the predominant flavonoids isolated from the dry root of *Scutellaria baicalensis* Georgi (Huang-Qin, a medicinal plant), an important traditional Chinese medicinal herb used to treat diseases of the central nervous system (CNS), hepatic disorders and inflammatory conditions, among others (10,11). Furthermore, baicalin has multiple biological functions, including anti-inflammatory, antioxidant, anti-apoptotic and immune regulation properties (12,13). Baicalin reduces the acute hepatic injury induced by CCl₄ and promotes early recovery of liver function (14). Baicalin inhibits HepG2 hepatoblastoma cells through oxidative/nitrative stress (15). Evidence suggests that the potential antioxidant and anti-inflammatory properties of this compound are largely due to its ability to enhance an antioxidant status and to suppress the expression of several inflammatory cytokines by attenuating the activity of NF- κ B (12,16). Dinda *et al* reported that baicalin inhibited reactive oxygen species (ROS) production in arteriosclerotic vascular disease by suppressing the activation of the NF- κ B signaling pathway (17). Wang *et al* reported that

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baicalin exerts an inhibitory effect on inflammation-related collagen-induced arthritis via inhibition of the activation of the NF- κ B signaling pathway (18). Given its ability to inhibit the activation of NF- κ B signaling, it was hypothesized that baicalin may suppress breast cancer growth. To the best of our knowledge, no previous studies have investigated this possible effect of baicalin.

In the present study, the breast cancer cell lines MCF-7 and MDA-MB-231 and a xenograft mouse model were treated with baicalin, with the aim of determining the therapeutic potential of this compound in breast cancer and elucidating the role of NF- κ B in the effects of baicalin.

Materials and methods

Cell treatment. The breast cancer cell lines MCF-7, MDA-MB-231 and MCF-10 were purchased from Cell Applications, Inc. (San Diego, CA, USA). The cells were cultured in cell growth medium supplemented with 100 IU/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% carbon dioxide (CO₂) and 95% air.

The cell growth medium was replaced with serum-free medium for an additional 24-h culture prior to further treatments. Cells were suspended at a density of 2x10⁶ cells/ml for experiments. In the mechanism experiments, cells treated with serum-free medium alone served as the control group. The cells were treated with 20 or 30 μ M baicalin in Dulbecco's modified Eagle's medium for 48 h. The concentrations of baicalin were selected as previously described (10-13,18).

In vivo tumorigenicity and baicalin treatment. The present study was approved by the Ethics Committee for Animal Experimentation of the School of Life Science and Technology, Harbin Institute of Technology. All animal experiments were performed according to the Guidelines for the care and use of experimental animals approved by the Heilongjiang Province People's Congress (<http://www.nicpbp.org.cn/sydw/CL0249/2730.html>). Female BALB/c nude mice, 5 weeks old and weighing ~15 g, were purchased from the Harbin Veterinary Research Institute (Harbin, China). The animals were housed in a clean and ventilated environment under standard laboratory conditions (temperature 22±1°C, relative humidity 50-70%, and a 12-h light/dark cycle). Standard food and water were provided *ad libitum* throughout the experiments. The animals were allowed to acclimatize to their surroundings over 7 days to eliminate the effect of stress prior to the initiation of the experiments, and then randomly divided into three groups (control, 100 and 200 mg/kg groups; n=6 per group) for the following tumorigenicity and baicalin treatment experiments.

MDA-MB-231 cells (1x10⁵/injection) were suspended in 100 μ l phosphate-buffered saline (PBS) containing 50% Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and injected into the mammary fat pad of 4-5-week-old female nude mice. One week later, the control mice received PBS (1 ml/kg, once daily i.p.), while 100 and 200 mg/kg group mice received daily i.p. injections of baicalin (>95% purity; Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA) at doses of 100 and 200 mg/kg, respectively. The concentrations of baicalin were selected as previously described (10-13,18).

Tumor size was measured every 5 days for 4 weeks, starting 5 days after tumor cell implantation. The tumor volume was calculated according to the following formula: $V = L \times W^2/2$, where V, volume (mm³); L, length (mm); and W, width (mm).

Cell viability. Cells were cultured using the abovementioned method, diluted to 2x10⁶ cells/ml, seeded into 96-well plates (100 μ l/well) and incubated for 24 h, followed by the addition of 20 or 30 μ M baicalin and further incubation for 72 h. According to the manufacturer's recommendations, 20 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide solution (5 mg/ml in PBS; Sigma-Aldrich; Merck KGaA) was added to each well and incubated with cells under standard conditions for 4 h. Subsequently, the formazan crystals in each well were dissolved in dimethyl sulfoxide, after removal of the medium. Finally, the optical density at 490 nm was measured using an enzyme-linked immunosorbent microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results are expressed as the mean of triplicate experiments.

Cell cycle analysis. Untreated cells and those treated with baicalin were dissociated and washed with PBS for cell cycle analysis. The cells were incubated in PBS containing 500 U/ml RNase (Sigma-Aldrich; Merck KGaA) at 37°C for 15 min, incubated with 50 μ g/ml propidium iodide (PI) at 4°C in the dark for 2 h, and analyzed to determine the percentage of 10,000 cells in each of three cell cycle compartments (G0/G1, S and G2/M) using a fluorescence-activated cell analyzer (Becton-Dickinson, Mountain View, CA, USA). Modifit LT 2.0 software was used to perform data analysis, and CELLQuest software (both from Becton-Dickinson) was used to quantify the percentage of cells in each compartment.

Transwell assay. To measure cell invasion, 8- μ m pore 24-well Matrigel invasion chambers (Corning Inc., Corning, NY, USA) were used according to the manufacturer's instructions. A total of 2x10⁴ cells were seeded into each well. DMEM with 0.1% FBS was added to the upper chamber, while DMEM supplemented with 10% FBS was added to the lower chamber well to promote cell invasion. After 24 h of incubation at 37°C with 5% CO₂, non-invading cells were removed from the top well, while migrated cells were quantified by capturing photographs of 6 independent visual fields under an Olympus BX51 microscope (Olympus Co., Tokyo, Japan), and was stained with 0.1% crystal violet solution. Independent experiments were repeated at least three times.

Scratch wound healing assay. A scratch wound healing assay was performed to evaluate the migration of untreated cells and cells treated with baicalin. In brief, MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and cultured with DMEM supplemented with 10% FBS. After reaching confluence, each well was scratched with a 200- μ l pipette tip. After 12 and 24 h of incubation, 6 wound healing areas were imaged and the distance between the two edges was analyzed by ImageJ software, version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Dual-luciferase reporter assay. The pRL-TK and recombinant NF- κ B luciferase reporter plasmids (Thermo Fisher Scientific,

Inc., Waltham, MA, USA) were co-transfected into MCF-7 and MDA-MB-231 cells for measurement of NF- κ B reporter activity. Transfected cells were harvested 48 h after transfection, and luciferase activity was determined by Fluoroskan Ascent FL (Thermo Fisher Scientific, Inc.) configured for dual assays. The relative luciferase activity was normalized to *Renilla* luciferase activity. All experiments were performed in triplicate wells.

Enzyme-linked immunosorbent assay (ELISA) measurement of TNF- α and IL-1 β levels in mouse serum and cell culture supernatants. At day 30 of baicalin treatment, the mice were anesthetized with intraperitoneal injection of 10% chloral hydrate (400 mg/kg body weight). Blood samples were collected from the abdominal aorta using syringes, and centrifuged for 30 min (3,000 \times g at 4°C). Serum samples were stored at -80°C until use. The mice were then euthanized by cervical dislocation. To determine cytokine levels *in vitro*, MCF-7 and MDA-MB-231 cells were treated with or without baicalin using the method described above. The supernatants of culture plates were harvested from each well. TNF- α and IL-1 β levels in the serum and culture supernatants were measured using mouse TNF- α and IL-1 β ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Western blotting. MCF-7 and MDA-MB-231 cells and 0.3-g tumor samples from mice were treated with RIPA lysis buffer supplemented with protease inhibitor cocktail and protein phosphatase inhibitor. The protein concentration was quantified using a DC protein kit (Bio-Rad Laboratories). Samples were then separated, transferred to PVDF membranes, and incubated with 1:1,000 dilution primary antibodies overnight at 4°C. The primary antibodies used were anti-phospho-NF- κ B-p65 (Ser536) (rabbit mAb, catalog no. 3033), anti-phospho-IKK α / β (Ser176/180) (rabbit mAb, catalog no. 2697) and anti-IkB α (rabbit mAb, catalog no. 4812). The anti-total NF- κ B-p65 (rabbit mAb, catalog no. 8242), anti-total-IKK β (rabbit mAb, catalog no. 2370) and anti- β -actin antibodies (rabbit mAb, catalog no. 4970) were used as loading controls. On the following day, the membranes were incubated with secondary antibody (anti-rabbit IgG, catalog no. 4414) for 2 h at room temperature. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The bands were visualized using an ECL Plus western blotting detection system (GE Healthcare, Milwaukee, WI, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (RT-qPCR). Total RNA was extracted from MCF-7 and MDA-MB-231 cells and xenograft tumor tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Grand Island, NY, USA) according to the manufacturer's instructions. Equal amounts (1 μ g) of total RNA were used to perform reverse transcription using PrimeScript™ RT reagent kit (Takara, Tokyo, Japan). For the determination of the expression of various genes in MCF-7 and MDA-MB-231 cells and xenograft tumor tissues, RT-qPCR was performed using the following primers: *CCND1*, forward, 5'-AGAAGCTGTGCATC TACACCGACA-3' and reverse, 5'-TGATCTGTTTGTCTCCT CCGCCT-3'; *BCL2*, forward, 5'-TGAGCAGAGTCTTCAGAG ACAGCC-3' and reverse, 5'-ATGTGTGTGGAGAGCGTCA

ACC-3'; *BIRC2*, forward, 5'-CCCAAAGACTTTTCCCAGGT CCC-3' and reverse, 5'-ACTGAGCTTCCCACCACAGGCA-3'; *BIRC3*, forward, 5'-GAATACTCCCTGTGATTAATGCTG CCGTGG-3' and reverse, 5'-TCTCTTGCTTGTAAGACGTC TGTGTCTTC-3'. The *ACTB* (β -actin) control primers were 5'-CGTTGCTATCCAGGCTGTGCTA-3' and 5'-CCAGGT CCAGACGCAGGATGGC-3'. Quantification of gene expression was determined by comparative quantity, using *ACTB* gene expression as internal control. The PCR conditions were denaturation at 94°C for 45 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min, for 40 cycles.

Statistical analysis. Data are expressed as mean \pm standard error of the mean and were analyzed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). All calculated significant differences were based on one-way analysis of variance and the post hoc Tukey's test. For direct comparisons between two groups, the Student's t-test was used. $P < 0.05$ was considered to indicate statistically significant differences.

Results

Baicalin suppresses proliferation and induces G1/S arrest in breast cancer cells. To evaluate the effect of baicalin on breast cancer, cell proliferation was assessed. The cells were manually counted in 6 wells per group. The results demonstrated that baicalin reduced cell proliferation from 48 to 72 h in MCF-7 and MDA-MB-231 cells, indicating that this compound can inhibit breast cancer cell proliferation (Fig. 1A). In addition, the effect of baicalin on non-tumorigenic normal breast epithelial cells was assessed by comparing the proliferation of MCF-7 and MCF-10 cells. Baicalin did not affect the proliferation of MCF-10 cells, indicating that this compound mediated growth arrest of breast cancer cell lines without affecting the non-tumorigenic normal breast epithelial cells (Fig. 1A). To further elucidate the mechanism of action of baicalin in breast cancer development, after 48 h of treatment, cell cycle analysis was performed by flow cytometry. In MCF-7 cells, the cell phase distributions before and after treatment with 20 and 30 μ M baicalin were as follows: G0/1 phase, 49.53 \pm 0.62 vs. 55.34 \pm 1.74 and 63.52 \pm 3.64%; S phase, 31.75 \pm 1.24 vs. 25.73 \pm 2.79 and 25.51 \pm 3.61%; and G2/M phase, 18.71 \pm 0.45 vs. 17.93 \pm 1.87 and 17.66 \pm 2.2, respectively. In MDA-MB-231 cells, the cell phase distributions before and after treatment with 20 and 30 μ M baicalin were as follows: G0/1 phase, 42.96 \pm 2.01 vs. 53.1 \pm 1.74 and 64.96 \pm 3.33%; S phase, 29.53 \pm 1.12 vs. 24.17 \pm 2.71 and 24 \pm 2.29%; and G2/M phase, 17.51 \pm 1.79 vs. 18.17 \pm 1.74 and 13.41 \pm 2.48%, respectively (Fig. 1B and C). These results indicate that the proportion of cells in the S phase was significantly reduced following treatment with baicalin, whereas the proportion of cells in the G0/1 phase was significantly increased, suggesting that baicalin induces G1/S arrest. Compared with MCF-7 cells, baicalin did not affect the cell cycle distribution of MCF-10 cells (Fig. 1B and C).

Baicalin suppresses the invasion and migration of breast cancer cells. The Transwell assay was employed to evaluate the effect of baicalin on the invasion of MCF-7 and MDA-MB-231 cells. The cells were manually counted in 6 wells per group. The mean number of invasive cells, as determined by microscopy,

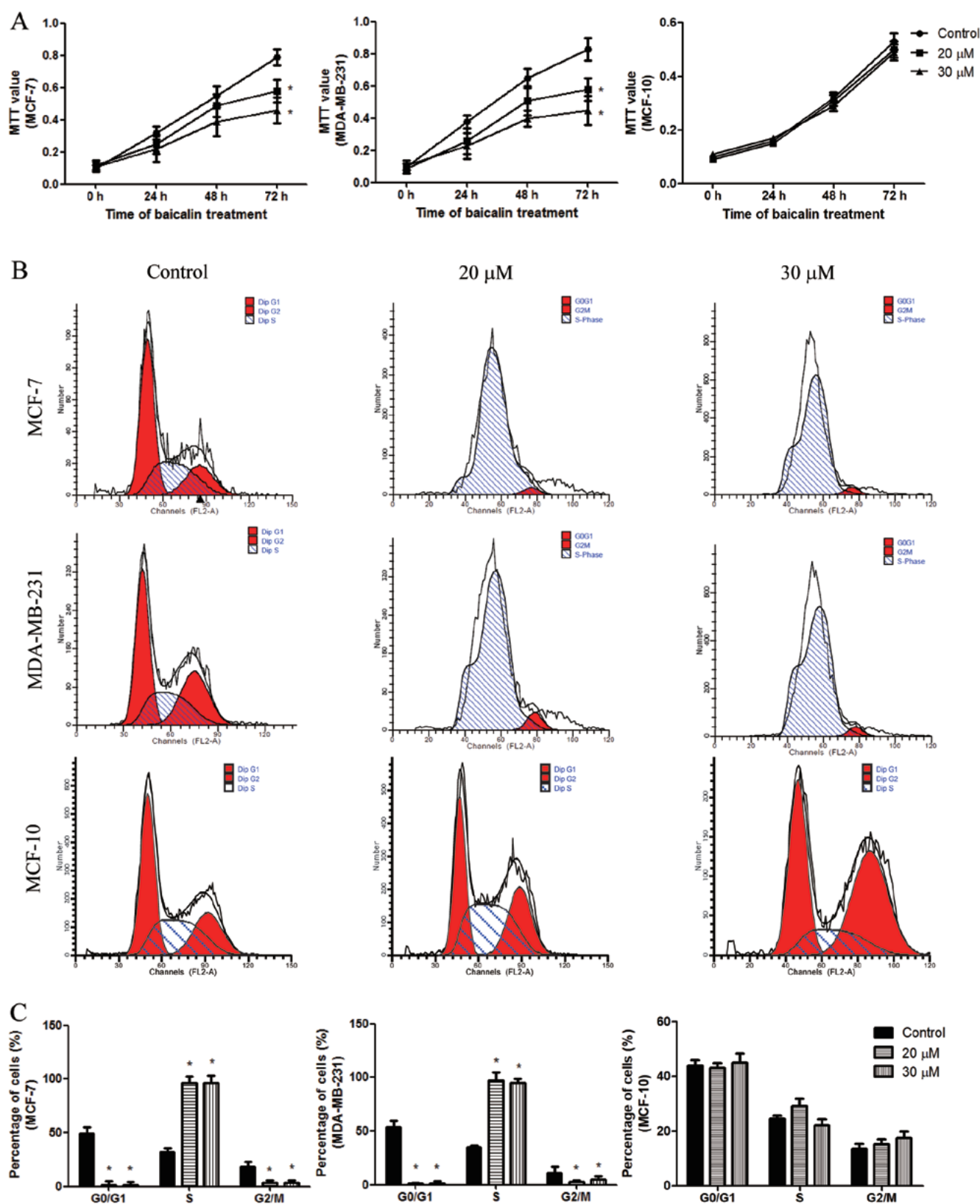


Figure 1. Baicalin suppresses breast cancer cell proliferation. (A) Growth curves represent the effect of baicalin on cell proliferation in MCF-7 and MDA-MB-231 breast cancer cells and MCF-10 normal breast epithelial cells. (B) Flow cytometry results demonstrate the effect of baicalin on cell phase distribution in MCF-7 and MDA-MB-231 breast cancer cells and MCF-10 normal breast epithelial cells. (C) Quantitative results of flow cytometry. * $P < 0.05$ vs. the control group. The results are presented as mean \pm standard error ($n = 6$).

was 100 ± 19.5 , 43 ± 13.7 and 41 ± 13.3 in untreated MCF-7 cells, and those treated with 20 and 30 μ M baicalin, respectively. The mean number of invading cells, as determined by

microscopy, was 100 ± 13.6 , 54 ± 15.3 and 44 ± 16.3 in untreated MBA-MB-231 cells, and those treated with 20 and 30 μ M baicalin, respectively. Therefore, the number of invading cells

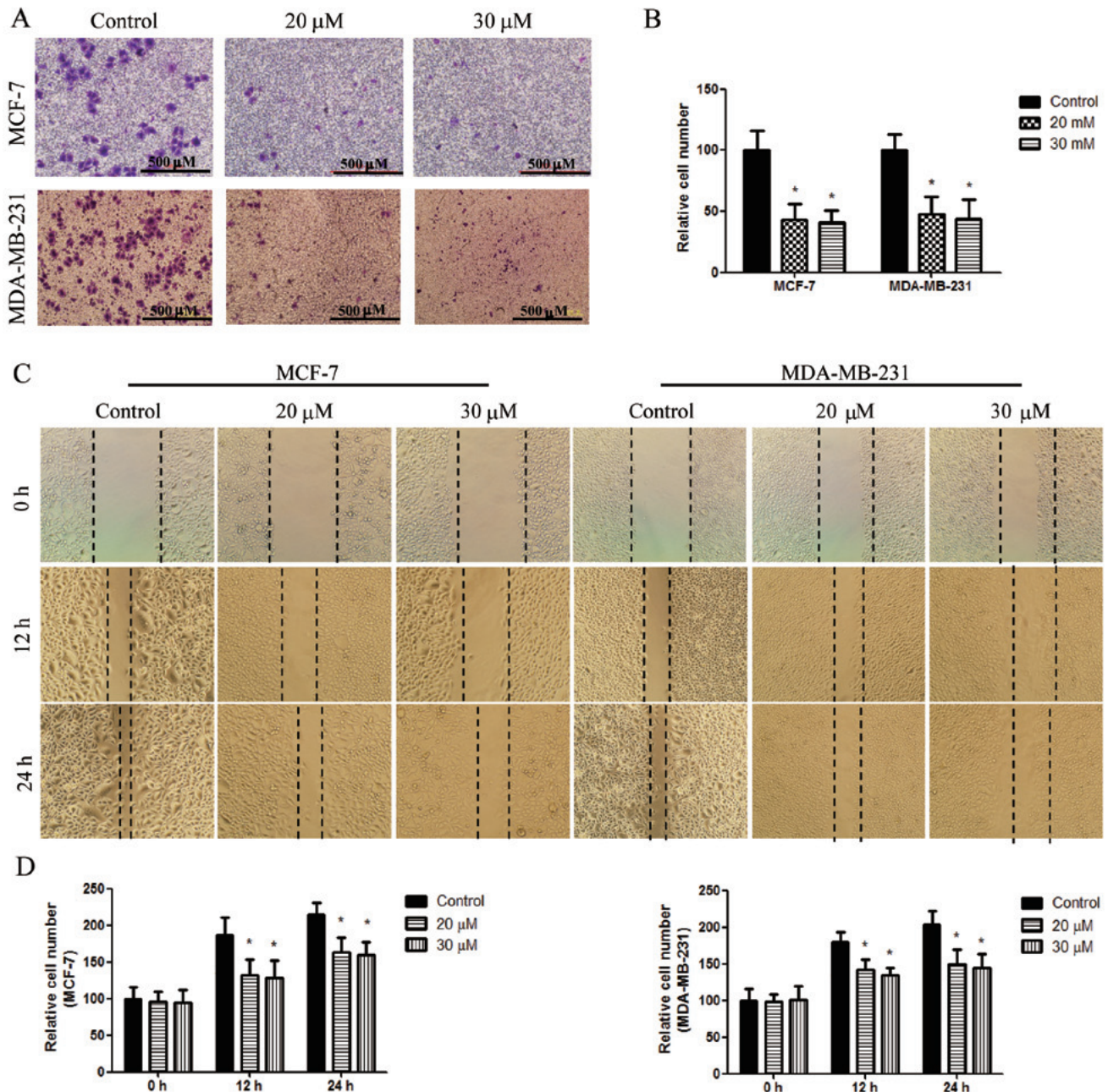


Figure 2. Baicalin suppresses breast cancer cell invasion and migration. (A) Transwell migration assays in baicalin-treated MCF-7 and MDA-MB-231 breast cancer cells and control cells. (B) Quantitative results of the invasion assays. (C) Scratch wound in baicalin-treated MCF-7 and MDA-MB-231 breast cancer cells and control cells. (D) Quantitative results of migration assays. * $P < 0.05$ vs. the control group. The results are presented as mean \pm standard error ($n = 6$).

in the treatment groups was significantly reduced compared with that in the untreated control group (Fig. 2A and B).

Next, a wound healing assay was performed to explore the effects of baicalin on the migration of MCF-7 and MDA-MB-231 cells. The cells were manually counted in 6 wells per group. After 24 h, the number of migrating MCF-7 cells was 115 ± 16.3 , 67 ± 7.7 and 64 ± 10.1 in untreated cells, and those treated with 20 and 30 μ M baicalin, respectively. The number of migrating MDA-MB cells was 103 ± 12.6 , 52 ± 10.6 and 44 ± 7.3 in untreated cells, and those treated with 20 and 30 μ M baicalin, respectively. Therefore, the number of migrating cells in the treatment groups was significantly reduced compared with that in the untreated control group (Fig. 2C and D).

Baicalin suppresses breast cancer tumor growth in xenograft mice. To further explore the effects of baicalin on breast cancer growth, an MBA-MB-231 xenograft mouse model was employed, using 6 mice per group. After 25 days, the tumor volumes were smaller in the MBA-MB-231 cell xenograft mice treated with 100 and 200 mg/kg baicalin compared with the untreated group (124.77 ± 38.33 and 116.22 ± 35.64 vs. 363.63 ± 47.63 mm³, respectively; Fig. 3A-C). In the control group, the longest diameter of the largest subcutaneous tumor was 15.62 mm. The tumor weights were also reduced in the 100 and 200 mg/kg baicalin treatment groups compared with the untreated group (0.12 ± 0.03 and 0.10 ± 0.05 vs. 0.25 ± 0.02 g, respectively; Fig. 3B and D). No multiple subcutaneous tumors or peritonitis were observed in mice after being injected with MDA-MB-231 cells or baicalin.

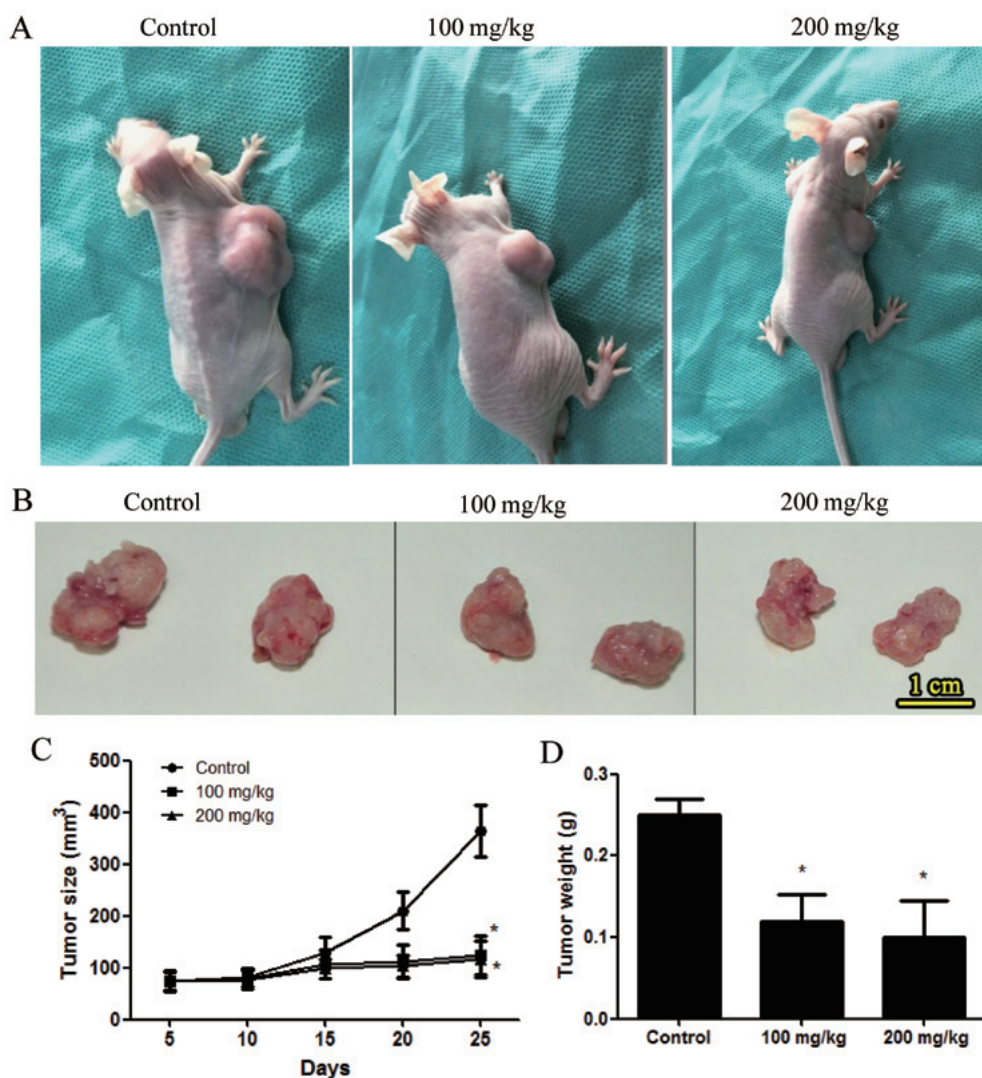


Figure 3. Baicalin suppresses tumor growth in xenograft mice. (A) Representative results of the effects of baicalin in xenograft tumors in nude mice. (B) Representative results of baicalin treatment in xenograft tumors. (C) Subcutaneous tumor growth curves for baicalin-treated and untreated xenograft mice in at different time points. (D) Tumor weight in nude mice was measured at 30 days. * $P < 0.05$ vs. the control group. The results are presented as mean \pm standard error ($n = 6$).

Baicalin inhibits the secretion of TNF- α and IL-1 β into cell media and mouse serum. To investigate whether baicalin modulates the inflammatory process by regulating the secretion of cytokines, the serum levels of TNF- α and IL-1 β were measured *in vitro* and *in vivo* by ELISA. The cells were manually counted in 6 wells per group; the number of mice per group was $n = 6$. As shown in Fig. 4A and B, high levels of the pro-inflammatory cytokines TNF- α and IL-1 β were detected in the medium of MCF-7 and MBA-MB-231 cells in the control group. By contrast, the levels of these cytokines were significantly lower in the baicalin-treated groups (20 and 30 μ M) compared with the untreated group. However, there was no significant difference in the cytokine levels between mice treated with 20 and those treated with 30 μ M baicalin. Similar results were observed in serum samples obtained from mice on day 30 following treatment with 100 or 200 mg/kg baicalin (Fig. 4C).

Baicalin suppresses the activation of NF- κ B. Based on the key role of NF- κ B in the regulation of tumor-associated inflammation and cancer progression, we next investigated

the association between baicalin and NF- κ B. The cells were manually counted in 6 wells per group, and the number of mice per group was $n = 6$. In a dual luciferase reporter assay, a 3-fold lower luminescence intensity was observed in cells treated with 20 and 30 μ M baicalin compared with the control group *in vitro*, indicating that the activation of NF- κ B was inhibited by baicalin (Fig. 5A). Western blot analysis further confirmed that NF- κ B was inhibited by baicalin in breast cancer cells *in vivo* and *in vitro*. The phosphorylation levels of I κ B kinase β (IKK β) and NF- κ B-p65 were reduced, whereas the expression of I κ B α , an essential NF- κ B inhibitor, was markedly upregulated in response to baicalin treatment (Fig. 5B and C).

NF- κ B also regulates oncological behavior. The expression of cyclin D1 (*CCND1*), *BCL2*, *BIRC2* (*cIAP1*) and *BIRC3* (*cIAP2*) were examined by RT-qPCR. The results demonstrated that baicalin reduced the expression of these genes, indicating that baicalin may suppress cell proliferation by inhibiting NF- κ B-induced expression of *CCND1*, and suppress cell invasion and migration by inhibiting NF- κ B-induced expression of *BCL2*, *BIRC2* and *BIRC3* (Fig. 5D).

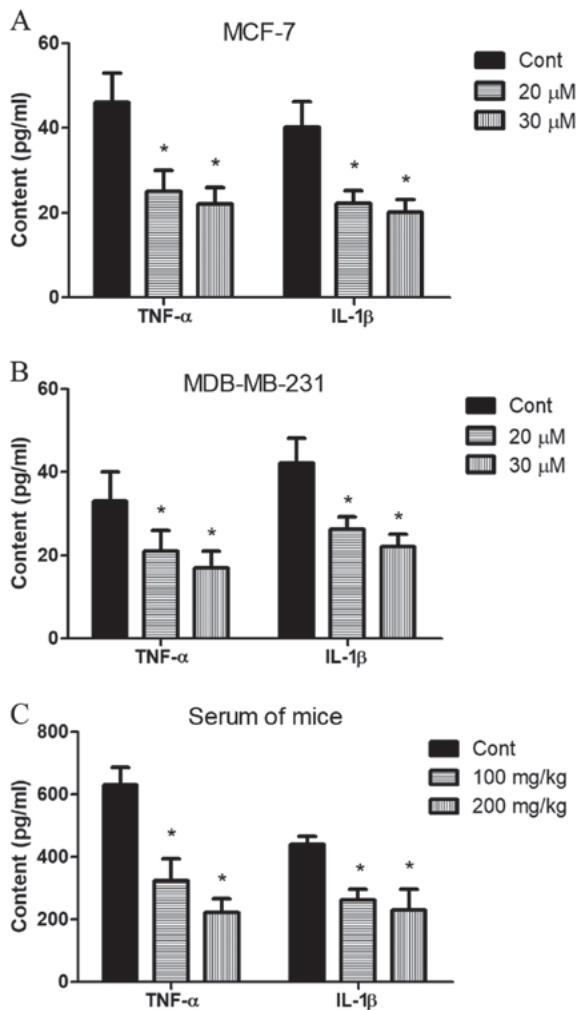


Figure 4. Baicalin suppresses the production of TNF- α and IL-1 β *in vivo* and *in vitro*. (A) The levels of TNF- α and IL-1 β were measured in the medium of baicalin-treated and untreated MCF-7 cells. (B) The levels of TNF- α and IL-1 β were measured in the medium of baicalin-treated and untreated MDA-MB-231 cells. (C) The levels of TNF- α and IL-1 β were measured in the serum of baicalin-treated and untreated xenograft mice at day 30. *P<0.05 vs. the control group. The results are presented as mean \pm standard error (n=6). TNF, tumor necrosis factor; IL, interleukin.

Discussion

Breast cancer is one of the most commonly diagnosed types of malignancy among women worldwide (1,2). The current standard therapies for breast cancer are associated with numerous side effects. Traditional Chinese herbal medicines have been suggested as potential new therapeutic drugs with a more tolerable toxicity profile (3,4). NF- κ B has been demonstrated to be a key factor enabling precancerous and malignant cells to escape apoptosis (5-7). Inhibition of NF- κ B activity results in the partial release of cells from the G2/M arrest after curcumin treatment of MCF-7 cells (8,9). Baicalin is a chemical commonly used to treat diseases of the CNS, hepatic disorders and inflammatory conditions (10,11), and it inhibits lipopolysaccharide-induced inflammation caused by endotoxin shock (12,16). Baicalin also inhibits ROS production in arteriosclerotic vascular disease by reducing the activation of the NF- κ B signaling pathway (17). Given its ability to inhibit the activation of the NF- κ B signaling pathway, it

was hypothesized that baicalin, a traditional Chinese herbal medicine with few known side effects (18,19), may suppress breast cancer growth. In the present study, the therapeutic efficacy of baicalin in regulating the inflammatory reaction in breast cancer was determined.

It was herein demonstrated that 20 and 30 μ M baicalin suppressed the invasion and migration of MCF-7 and MDA-MB-231 breast cancer cells (Fig. 2A-D). Furthermore, the results indicated that the mechanism through which baicalin suppresses breast cancer cell proliferation involves induction of G1/S arrest (Fig. 1A-C). The results of *in vivo* experiments demonstrated that 100 and 200 mg/kg baicalin suppressed breast tumor growth in xenograft mice (Fig. 3A-D). Furthermore, baicalin mediated growth arrest of breast cancer cell lines without affecting non-tumorigenic normal breast epithelial cells (Fig. 1A-C). Treatment doses <20 μ M did not induce significant effects *in vitro*, whereas treatment doses <100 mg/kg also did not exert significant effects *in vivo* (data not shown). Taken together, these results indicate that baicalin is therapeutically effective in breast cancer.

The development of breast cancer has been strongly associated with inflammation (20). The conventional inflammatory reaction is mediated by numerous cytokines, including TNF- α , IL-1 β , IL-6, TGF- β , IL-8 and IL-10, among others (20,21). In the present study, it was demonstrated that baicalin significantly decreased the secretion of TNF- α and IL-1 β into breast cancer cell medium *in vitro* (Fig. 4A and B). Furthermore, baicalin significantly reduced the levels of TNF- α and IL-1 β in the plasma of xenograft mice (Fig. 4C). Collectively, these data suggest that baicalin suppresses the inflammatory response by reducing the secretion of key inflammatory cytokines by MCF-7 and MDA-MB-231 breast cancer cells *in vitro*, with similar effects in xenograft mice.

TNF- α and IL-1 β are key pro-inflammatory cytokines that exert their effects by regulating the NF- κ B pathway through interaction with specific receptors on cell membranes (22,23). The NF- κ B pathway is a key mediator of inflammatory response in cancer (24). Several studies on acute and chronic inflammation have demonstrated that the anti-inflammatory activity of baicalin is associated with the regulation of NF- κ B. For example, in *Staphylococcus aureus*-induced mastitis and cigarette smoke-induced inflammation, baicalin effectively attenuated inflammation through inhibition of NF- κ B activation (25,26). In addition, NF- κ B activation plays a pathogenic role in cell proliferation and has an anti-apoptotic effect in breast cancer (5-7). A recent study also demonstrated that treatment with the NF- κ B inhibitor, BAY117082, suppressed breast cancer tumor growth in xenograft mice (24). Consistent with these reports, we herein observed that baicalin suppressed the activation of NF- κ B *in vitro* (Fig. 5A). Furthermore, the phosphorylation levels of NF- κ B-p65 were reduced by baicalin treatment in a dose-dependent manner, not only in MCF-7 and MDA-MB-231 breast cancer cells, but also in xenograft mice (Fig. 5B and C). Taken together, these data suggest that the NF- κ B pathway mediates the effect of baicalin inhibition of breast cancer progression.

To the best of our knowledge, no previous studies have investigated the regulatory mechanism baicalin on NF- κ B to date. In the present study, the effects of baicalin on NF- κ B were found to involve I κ B. In its inactive form, NF- κ B is

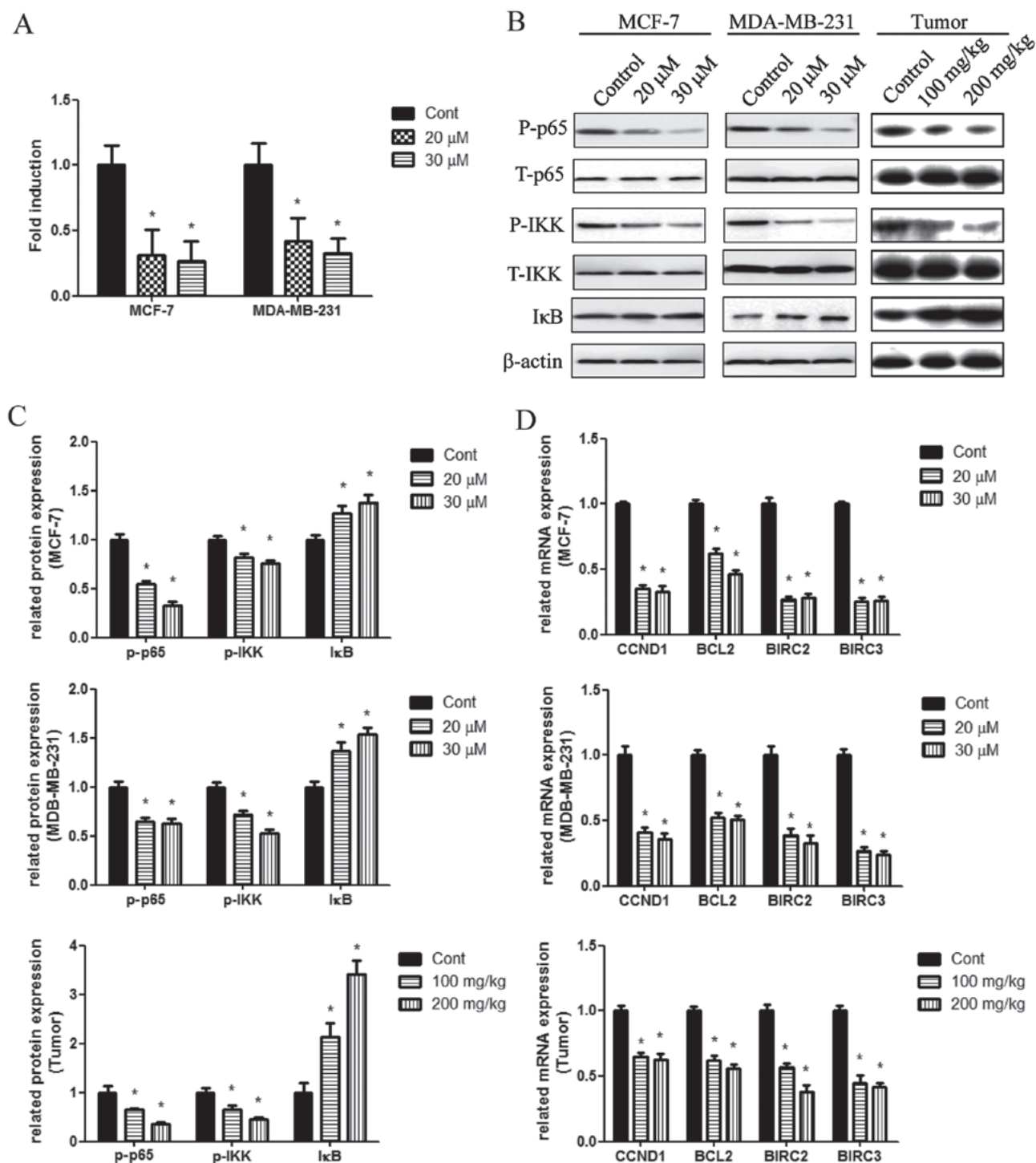


Figure 5. Baicalin suppresses the activation of NF- κ B *in vivo* and *in vitro*. (A) NF- κ B activation of baicalin-treated and untreated MCF-7 and MDA-MB-231 cells measured by dual luciferase reporter assay. (B) Phosphorylation of NF- κ B-p65 and IKK α / β , the expression of I κ B α , NF- κ B-p65, IKK β and β -actin in baicalin-treated and untreated MCF-7 and MDA-MB-231 cells and tumors of baicalin-treated and untreated xenograft mice. (C) Phosphorylation of NF- κ B-p65/total NF- κ B-p65, phosphorylation of IKK α / β /total IKK β , and I κ B α / β -actin ratios of baicalin-treated and untreated MCF-7 and MDA-MB-231 breast cancer cells and tumors in xenograft mice at day 30. (D) Gene expression of *CCND1*, *BCL2*, *BIRC2* and *BIRC3* of baicalin-treated and untreated MCF-7 and MDA-MB-231 breast cancer cells and tumors in xenograft mice at day 30. * $P < 0.05$ vs. the control group. The results are presented as mean \pm standard error (n=6). NF- κ B, nuclear factor- κ B; *CCND1*, cyclin D1.

sequestered in the cytoplasm and bound by members of the I κ B family of inhibitor proteins. In tumors, the various stimuli that activate NF- κ B cause phosphorylation of I κ B, which is followed by its ubiquitination and subsequent degradation (24,26). I κ B proteins are phosphorylated by the I κ B kinase complex (IKK). TNF- α and IL-1 β can activate IKK

and, thus, regulate the NF- κ B pathway (24,27). IKK-knockout mice have a dysfunctional NF- κ B pathway, leading to embryonic fatality (28). The present study demonstrated that the levels of phosphorylated IKK β were lower in the baicalin group, suggesting that baicalin suppresses the activity of IKK β in breast cancer cells and xenograft mice (Fig. 5A-C).

In addition, activated IKK phosphorylates I κ B α (Ser32 and 36), and I κ B α is ubiquitinated by Lys21 and Lys22, the ubiquitinated I κ B α is degraded and, thus, activated NF- κ B is released (29). In the present study, the protein expression level of I κ B α was higher in the baicalin treatment groups, suggesting that, through suppressing the activity of IKK β , baicalin inhibited the degradation of I κ B α , thus inhibiting the activation of NF- κ B in breast cancer cells and xenograft mice. However, further investigation is required to elucidate the underlying molecular mechanisms.

CCND1 is involved in regulation of the cell cycle, and NF- κ B promotes the expression of *CCND1* and, thus, cell proliferation (30,31). Therefore, baicalin treatment leads to a reduction of *CCND1* expression, which indicates that baicalin likely inhibits cell proliferation via downregulation of NF- κ B-induced expression of *CCND1*. Inflammation-activated NF- κ B also induces expression of *BCL2*, *BIRC2* and *BIRC3*, the proteins encoded by which inhibit the activation of various apoptosis-related enzymes, thereby reducing the apoptosis of tumor cells (31,32). The BCL2 family of proteins has anti-apoptotic members that are expressed in several types of cancer, including lymphoma, lung, prostate and breast cancer (33). *BIRC2* and *BIRC3* are members of the inhibitor of apoptosis protein family, which are expressed in several types of cancer, and are associated with chemoresistance, disease progression and poor prognosis (34). The present study demonstrated that baicalin significantly inhibited the expression of these apoptosis-inhibiting genes (Fig. 5D). NF- κ B is crucial for cell invasion and migration in breast cancer (35). Therefore, it may be inferred that the ability of baicalin to inhibit invasion and migration of MCF-7 and MDA-MB-231 breast cancer cells is likely to be associated with inhibition of the activation of NF- κ B. However, the detailed mechanism of NF- κ B-induced cell invasion and migration remains unclear. Therefore, the detailed mechanism underlying baicalin-induced inhibition of cell invasion and migration requires further research.

In summary, the results of the present study clearly demonstrated that baicalin, an important component isolated from the dry root of *S. baicalensis* Georgi, has therapeutic properties against breast cancer via inhibition of the inflammatory response, through the suppression of inflammatory cytokine-activated NF- κ B-p65, and the expression of *CCND1*, *BCL2*, *BIRC2* and *BIRC3*. This process inhibits breast cancer cell proliferation, invasion and migration, and downregulates the expression of anti-apoptotic factors *in vitro* and *in vivo*. Furthermore, baicalin did not affect the non-tumorigenic normal breast epithelial cells.

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Ethics approval

The present study was approved by the Ethics Committee for Animal Experimentation of the School of Life Science and Technology, Harbin Institute of Technology. All animal experiments were performed according to the Guidelines for the Care and Use of Experimental Animals and approved by the Heilongjiang Province People's Congress (<http://www.nicbpb.org.cn/sydw/CL0249/2730.html>).

Patient consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed in the present study are all included in this published article.

Authors' contributions

QW conceived the idea. YG, HL and HZW designed the experiments. YG, HLH and HJH performed the *in vitro* experiments. HZW, HJS and CJJ performed the *in vivo* experiments. XH, DL and SC contributed to gene expression analysis. NG and QG contributed to protein expression analysis. HL performed statistical analysis. HJH and NG made figures. YG and HLH wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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