Bufalin attenuates triple-negative breast cancer cell stemness by inhibiting the expression of SOX2/OCT4

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Abstract. Triple-negative breast cancer (TNBC) has the poorest prognosis among all types of breast cancer and there is yet no effective therapy. Chemotherapy is the traditional standard of care for patients with TNBC; however, treatment of TNBC with chemotherapy may lead to the enrichment of cancer stem cells (CSCs), which exhibit an enhanced capacity for self-renewal, tumor initiation and metastasis. The present study demonstrated that bufalin, a small molecular compound used in traditional Chinese medicine, exerted anticancer effects on a wide range of cancer cell lines, inhibited cell proliferation through inducing G2/M cell cycle arrest, and triggered apoptosis in the TNBC cell lines MDA-MB-231 and HCC-1937. Consistently, bufalin markedly suppressed TNBC growth in a cell line-derived xenograft model. More importantly, unlike common chemotherapeutic drugs, bufalin reduced the stemness of TNBC stem cells. A mechanistic study suggested that bufalin may suppress the proliferation of TNBC stem cells by inhibiting the expression of octamer-binding transcription factor 4 (OCT4) and sex determining region Y-box 2 (SOX2) in MDA-MB-231 and HCC-1937 cells. These results indicated that bufalin may hold promise as a therapeutic agent in TNBC, and its effects may be mediated through the SOX2/OCT4 axis.

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Introduction

Triple-negative breast cancer (TNBC) accounts for 12-17% of all breast cancers and it is a highly aggressive malignancy, displaying a high propensity for metastasis and the worst short-term prognosis among all types of breast cancer (1,2). At present, there are no available targeted therapy options due to the inherent heterogeneity of TNBC (3,4), and the standard of care includes surgery, radiotherapy and/or chemotherapy for both early- and advanced-stage disease. However, the majority of patients with TNBC will ultimately develop drug resistance, tumor relapse and/or metastasis (5-7).

In recent years, several trials investigating therapeutic combinations have led to promising advances in TNBC therapy (3,8). Zhang *et al* demonstrated that agents that inhibit cancer cell stemness may complement the antitumor activity of chemotherapy by eliminating chemo resistant cancer stem cells (CSCs) (9). CSCs are a small proportion of cancer cells that possess normal stem cell markers. Compared with non-CSCs, they exhibit lower sensitivity and higher degrees of resistance to drugs and irradiation (5,10). Furthermore, numerous chemotherapeutic agents have been found to increase the proportion of CSCs and increase the tumor-initiating potential of breast cancer cells *in vitro* and *in vivo* (11). Thus, CSCs are considered to be responsible for breast cancer recurrence and metastasis (12-14), and there is an urgent need to develop innovative and more effective therapeutic approaches that achieve a more durable response to TNBC treatment. TNBC treatment may be improved by identifying a single agent with bioactivities targeted at eliminating or inhibiting both CSCs and non-CSCs.

Toad skin has long been used for cancer therapy in traditional Chinese medical practice, and toad skin extract is currently widely used as a traditional Chinese medicine (15). Bufalin, a cardiotonic steroid isolated from toad venom, is an active compound that may be used for its functions in pain relief, myocardial contraction stimulation, blood pressure stimulation and anti-inflammation, among others (16). Since 2010, bufalin has been known for its anticancer effects on a wide range of cancer cell lines, including breast cancer cell lines (17-21). It has been reported that bufalin possesses the ability to inhibit cell proliferation, induce cell apoptosis, and inhibit metastasis and invasion of human cancer cells (10,17,21-24). Furthermore,

bufalin may be used safely over an extended period of time without marked side effects (21). All these findings indicate that bufalin may be a promising candidate for anticancer treatment. However, it remains unknown whether bufalin can inhibit CSCs.

The transcription factor sex determining region Y-box 2 (SOX2) has been reported to be highly expressed in TNBC cell lines and patient tissues (25). High SOX2 levels are correlated with poor differentiation of TNBC and short survival time of the patients (25). The expression of octamer-binding transcription factor 4 (OCT4) was also found to be associated with worse prognosis of TNBC patients after surgery (26). Moreover, SOX2 may promote TNBC cell proliferation and metastasis *in vitro* as well as *in vivo*, suggesting that SOX2 acts as a tumor promoter in TNBC (27). However, the role of SOX2 and OCT4 in the regulation of CSCs in TNBC remains unclear.

The purpose of the present study was to investigate whether bufalin can inhibit the stemness of TNBC cells and elucidate the underlying mechanism.

Materials and methods

Cell culture and transient transfection. The TNBC cell lines MDA-MB-231 and HCC-1937 were obtained from the Cell Bank of the Representative Culture Preservation Committee of the Chinese Academy of Sciences. All the cells were cultured in DMEM supplemented with 10% FBS (HyClone; Cytiva) at 37°C in 5% CO_2 and subcultured every 2-3 days. siRNA or negative control RNA were obtained from Shanghai GenePharma Co. Ltd. The cells were seeded in six-well plates at a density of $3x10^5$ cells/well. The transfections were performed using Transfection Reagent (Polyplus Transfection SA) according to the manufacturer's instructions. The transfection efficiency was confirmed via quantitative PCR. For the spheroid formation assay, the cells were transfected with siRNA targeting SOX2 or OCT4 for 24 h prior to treatment with bufalin (0.5 μM).

Cell Counting Kit-8 (CCK-8) assay for cell proliferation. The MDA-MB-231 and HCC-1937 cells were plated in 96-well plates at a density of $3x10^3$ cells/well and incubated at 37°C for 24, 48 or 72 h. At the end of the incubation period, CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well and incubated at 37°C for 1 h according to the manufacturer's instructions. After staining, the absorbance was measured at 570 nm (Multiskan Spectrum; Thermo Fisher Scientific, Inc.).

Cell apoptosis and cell cycle distribution analysis. Following incubation with 0.5 μM bufalin for 48 h, the MDA-MB-231 and HCC-1937 cells were harvested for PI staining or Annexin V-PI staining according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Data acquisition and analysis were performed using a FACSCanto II Flow Cytometer (BD Biosciences). A total of $1x10^4$ cells were scanned in each analysis. Each experiment was repeated at least three times.

Colony formation assay. Following treatment with 0.5 μM bufalin or vehicle, MDA-MB-231 and HCC-1937 cells were reseeded in 12-well plates at a density of 10,000 cells/well and cultured at 37°C to form natural colonies. After 7 days, the cells were washed with PBS 3 times and fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed colonies were stained with 20% crystal violet solution at room temperature for 10 min and captured.

Western blot analysis. The cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). Equal amounts of proteins (25 μg) were separated using 10% SDS-PAGE, transferred onto nitrocellulose membranes (Beyotime Institute of Biotechnology) and blocked with 5% non-fat milk for 1 h at room temperature. After incubation with an antibody specific for poly (ADP-ribose) polymerase (anti-PARP; 1:1,000; cat. no. 9532, Cell Signaling Technology, Inc.) for 2 h at room temperature, the blots were incubated with anti-rabbit secondary antibody (1:10,000; cat. no. 3900, Cell Signaling Technology, Inc.) for 2 h at room temperature and then detected by enhanced chemiluminescence (Beyotime Institute of Biotechnology). β-actin was used as a loading control.

In vivo tumorigenicity assays. MDA-MB-231 cells ($2x10^6$) were suspended in 100 μl PBS and injected into the lower flank of 14 4-6-weeks old nude mice housed in a room at 23-28°C and approximately 70% humidity, with an alternating 12 h light (from 7 a.m.)/dark cycle (from 7 p.m.) and free access to sterilized food and water. All experiment operations complied with laboratory animal ethics requirements approved by IACUC. Tumor diameter was measured with calipers. When the tumor diameter reached ~5 mm, bufalin (T1719; Shanghai Topscience Co., Ltd.) was injected into the tumor at a dose of 1 mg/kg three times per week (n=7), whereas control group animals were injected with an equal volume of DMSO (n=7). After 2 weeks, the tumors were weighed with an electronic balance. The animal experimental protocols were approved by the Ethics Committee of the University of Traditional Chinese Medicine.

Immunohistochemistry (IHC) staining. The xenografted tumors were fixed in 10% formalin at room temperature for 48 h and embedded in paraffin. Then, the paraffin blocks were cut into 4-mm sections and deparaffinized. Routine IHC staining for Ki-67 (1:100; Cell Signaling Technology, Inc.) was performed on the slides and staining for SOX2 (1:100; Proteintech, Inc.) or OCT4 (1:100; Proteintech, Inc.) was performed on the TNBC tissue microarray (Shanghai Outdo Biotech, Inc). Detection was performed using Ventana's UltraView diaminobenzidine (DAB) detection kit (P0202; Beyotime Institute of Biotechnology). Apoptotic cells were identified by TUNEL colorimetric staining according to the manufacturer's instructions (Roche Applied Science, Inc.). The tissue microarray (HBreD050Bc01), including 40 TNBC patient tissue samples was purchased from Shanghai Outdo Biotech Co., Ltd., and subjected to IHC staining

Spheroid formation assay. Approximately 500 viable single cells were plated on ultra-low attachment 96-well plates (Thermo Fisher Scientific, Inc.) and cultured in cell growth medium (Thermo Fisher Scientific, Inc.) with or without bufalin at a dose of 0.5 μM for 7 days. The number of spheroids was counted under an inverted microscope (IX51; Olympus Corporation) at a magnification of x100.
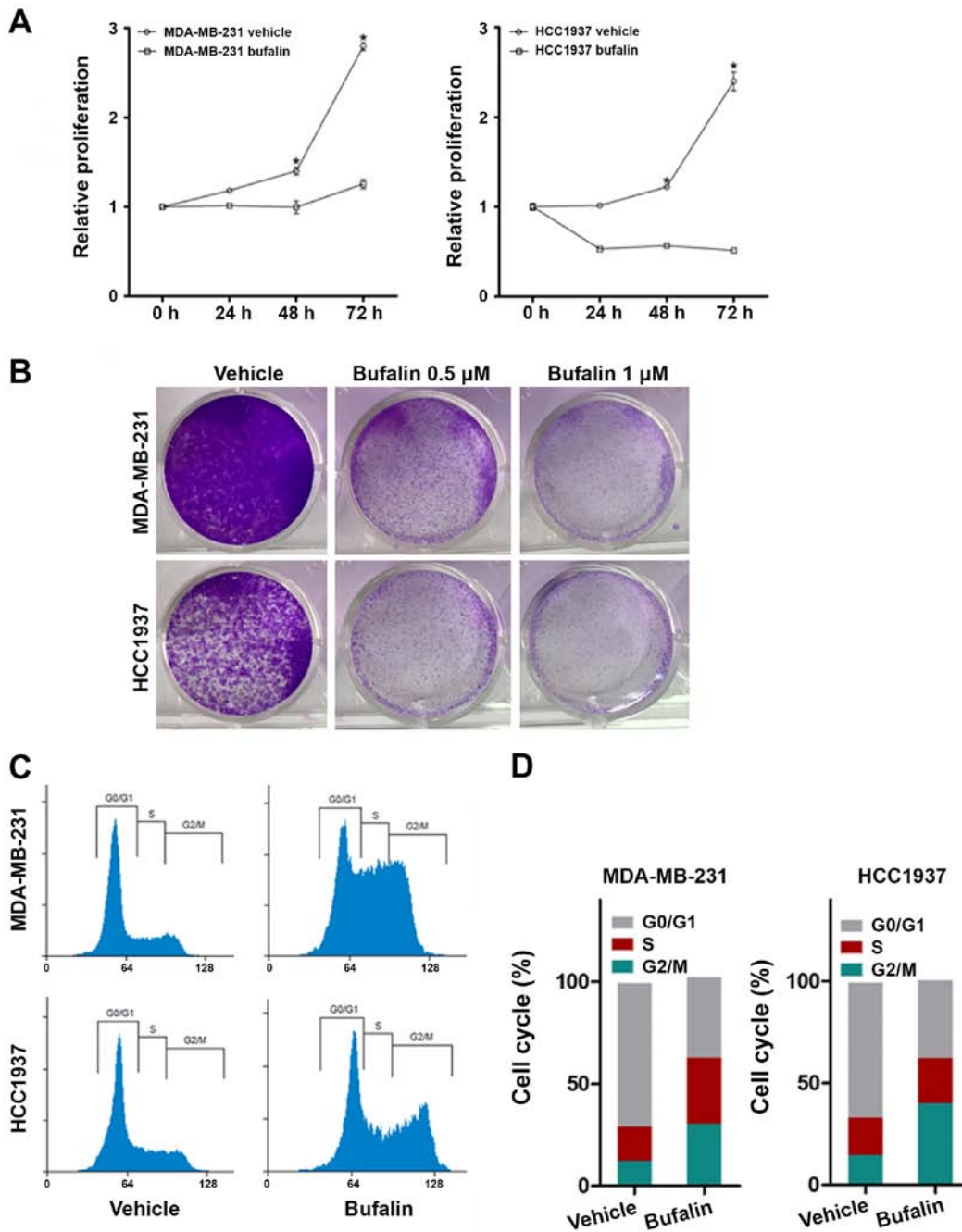
Figure 1. Bufalin inhibits TNBC cell proliferation *in vitro*. (A) The proliferation of MDA-MB-231 and HCC-1937 cells was measured by the Cell Counting Kit-8 assay following treatment with 0.5 μM bufalin or vehicle for the indicated times. The data are shown as mean $\pm$ standard deviation; Student's t-test *P<0.05. (B) Colony formation assays were performed on MDA-MB-231 and HCC1937 cells following treatment with vehicle, 0.5 or 1 μM bufalin for 7 days. (C and D) Quantitative cell cycle analysis. MDA-MB-231 and HCC-1937 cells were treated with vehicle or 0.5 μM bufalin for 48 h and cell cycle distribution was analyzed. (C) Representative cell cycle distribution following treatment of MDA-MB-231 and HCC1937 cells with 0.5 μM bufalin. (D) Quantitative analysis of the proportion of the cells in each phase of the cell cycle was performed from at least 10,000 cells per sample.

Gene expression analysis. Total RNA was extracted from MB-231 and HCC-1937 cells using TRIzol®(Invitrogen; Thermo Fisher Scientific, Inc.). For reverse transcription-quantitative PCR (RT-qPCR) analysis, 1 μg total RNA was reverse-transcribed (37°C for 1 h and 70°C for 10 min) by using Superscript III RT (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using the ABI PRISM 7300HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 1 cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec (denaturation), 60°C for 30 sec (annealing), 72°C for 30 sec (extension) and 72°C for 5 min (final extension). The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Cq}$ method ($\Delta\Delta Cq=\Delta Cq_{bufalin}-\Delta Cq$) (28). The primers used for SOX2, OCT4, c-Myc, β-catenin, Nanog and β-actin are listed in Table SI.

Statistical analysis. For statistical analysis, Student's t-test was used for parametric variables. One-way ANOVA followed by Dunnett's test was used for comparisons among multiple
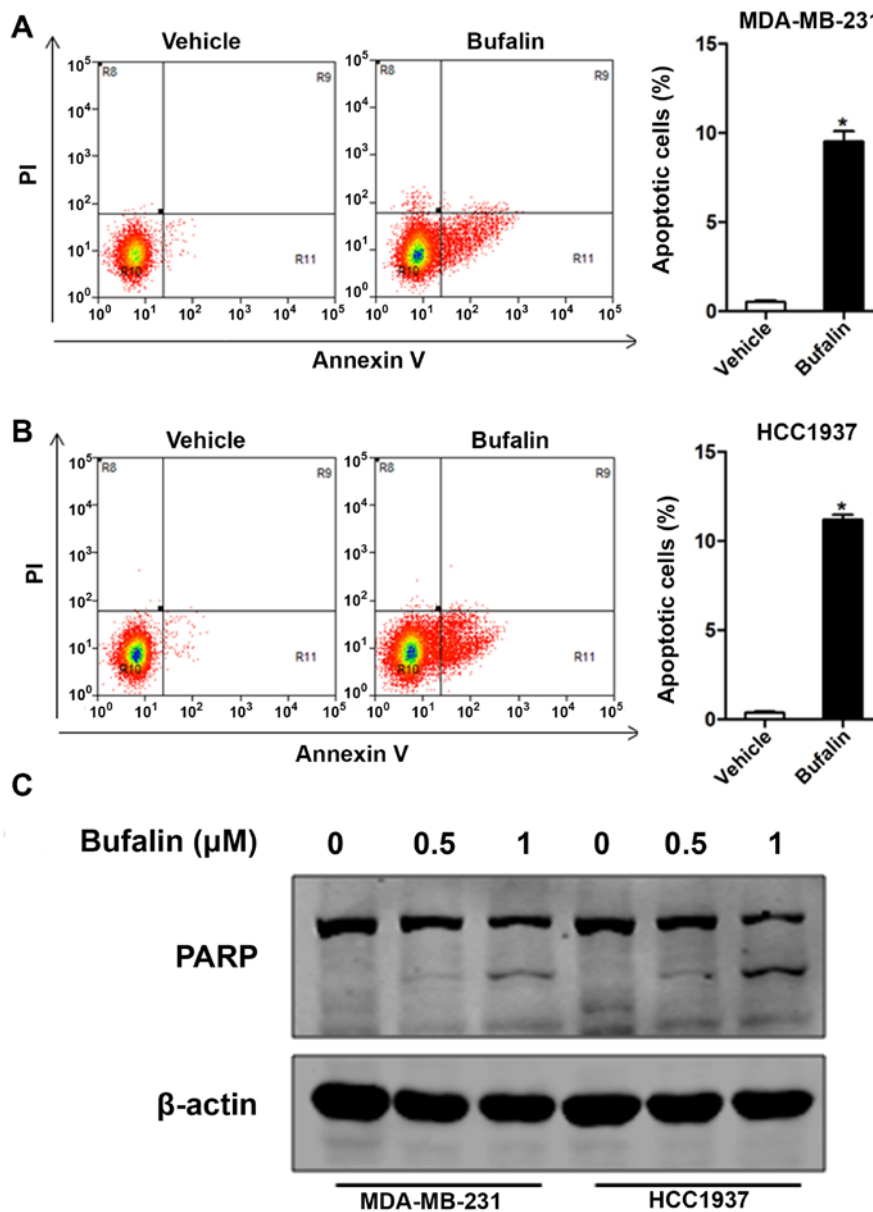
Figure 2. Bufalin induces apoptosis in triple-negative breast cancer cells. (A and B) Flow cytometry was used to determine the apoptotic rates of MDA-MB-231 and HCC-1937 cells following incubation with 0.5 μM bufalin or vehicle for 48 h. The data are shown as mean ± standard deviation; Student's t-test *P<0.05. (C) The protein expression levels of PARP were determined by western blot analysis followingtreatment of MDA-MB-231 and HCC-1937 cells with 0, 0.5 or 1 μM bufalin. PARP, poly(ADP-ribose) polymerase.

groups. All tests were performed three times, and P<0.05 was considered to indicate statistically significant differences.

Results

Bufalin inhibits TNBC cell proliferation and induces apoptosis. To investigate the biological effect of bufalin in TNBC, *in vitro* assays were performed in MDA-MB-231 and HCC1937 cells using the dosages mentioned in previous studies (29,30). First, the CCK-8 assay was used to examine the effect of bufalin on cell proliferation. As shown in Fig. 1A, bufalin significantly inhibited the proliferation of both MDA-MB-231 and HCC1937 cells (P<0.05). Second, the antiproliferative effects of bufalin were further determined by colony formation assays, and the data revealed that the number and size of the colonies were markedly reduced

by bufalin treatment (Fig. 1B). The effect of bufalin on the regulation of the tumor cell cycle was then assessed using FACS analysis. As shown in Fig. 1C and D, bufalin led to increased accumulation of cells in the G2/M phase of the cell cycle. Next, the apoptotic rates induced by bufalin were evaluated by flow cytometry, and our data demonstrated that bufalin triggered apoptosis of MDA-MB-231 and HCC1937 cells (Fig. 2A and B). Consistently, the induction of PARP, an apoptosis regulator, also confirmed this effect (Fig. 2C).

Bufalin suppresses TNBC growth in vivo. After confirming that bufalin inhibits cell proliferation and enhances apoptosis *in vitro*, it was examined whether bufalin can inhibit breast cancer growth in a xenograft TNBC model. As shown in Fig. 3A and B, bufalin significantly decreased the tumor volume and weight compared with vehicle control. Ki-67 and
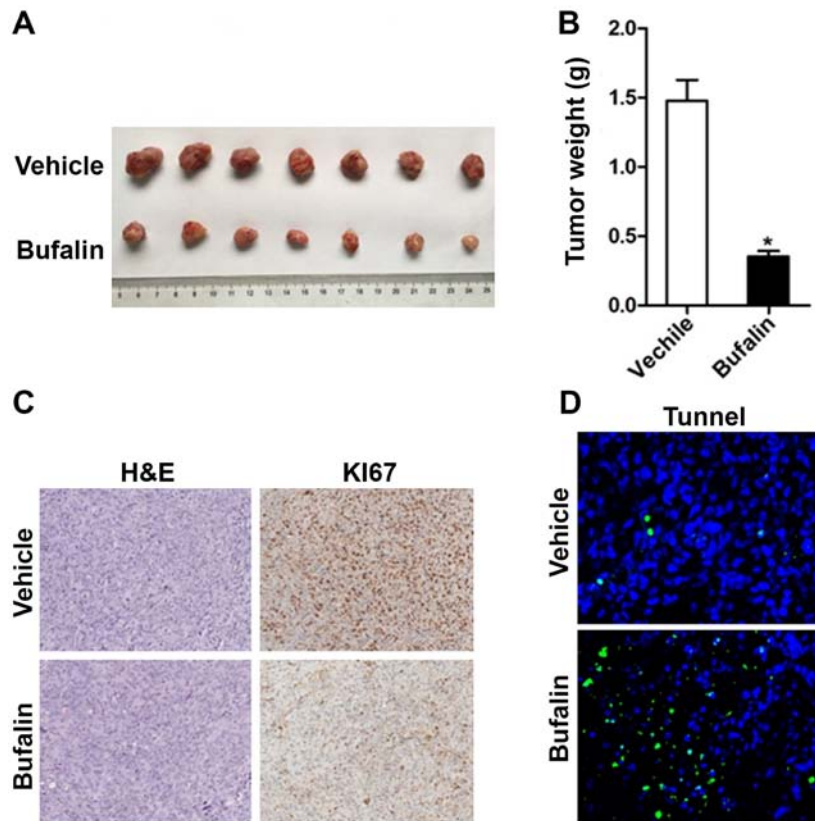
Figure 3. Bufalin suppresses triple-negative breast cancer growth *in vivo*. (A) In order to establish a xenograft nude mouse model, MDA-MB-231 cells were inoculated into the lower flank of nude mice. When the tumor diameter reached ~5 mm, bufalin was injected into the tumor at a dose of 1 mg/kg three times per week, whereas the control group was injected with an equal volume of DMSO. (B) The tumor weight was analyzed by the two-samples t-test. The data are shown as mean ± standard deviation; Student's t-test *P<0.05. (C) Representative images of H&E (left) and immunohistochemistryKi-67 (right) staining of tissue samples from each group. Images were captured at a magnification of x200. (D) Representative TUNEL staining images. The apoptotic cells appear as TUNEL-positive. Images were captured at a magnification of x200. H&E, hematoxylin and eosin.
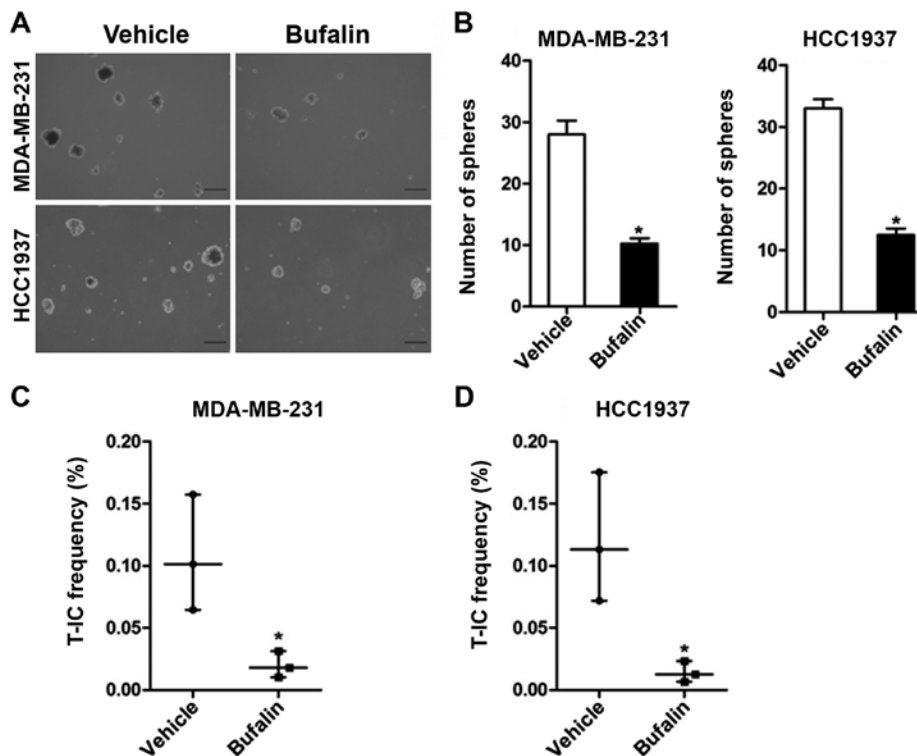

Figure 4. Bufalin inhibits the ability of triple-negative breast cancercells to form spheroids. (A) Representative images of MDA-MB-231 and HCC1937 spheroids grown in Matrigel incubated with 0.5 μM bufalin or vehicle. (B) Numbers of spheroids incubated with vehicle or bufalin; the data are shown as mean ± standard deviation; Student's t-test *P<0.05. (C and D) Tumor-initiating cell (T-IC) frequency from an *in vitro* limiting dilution assay. *P<0.05.
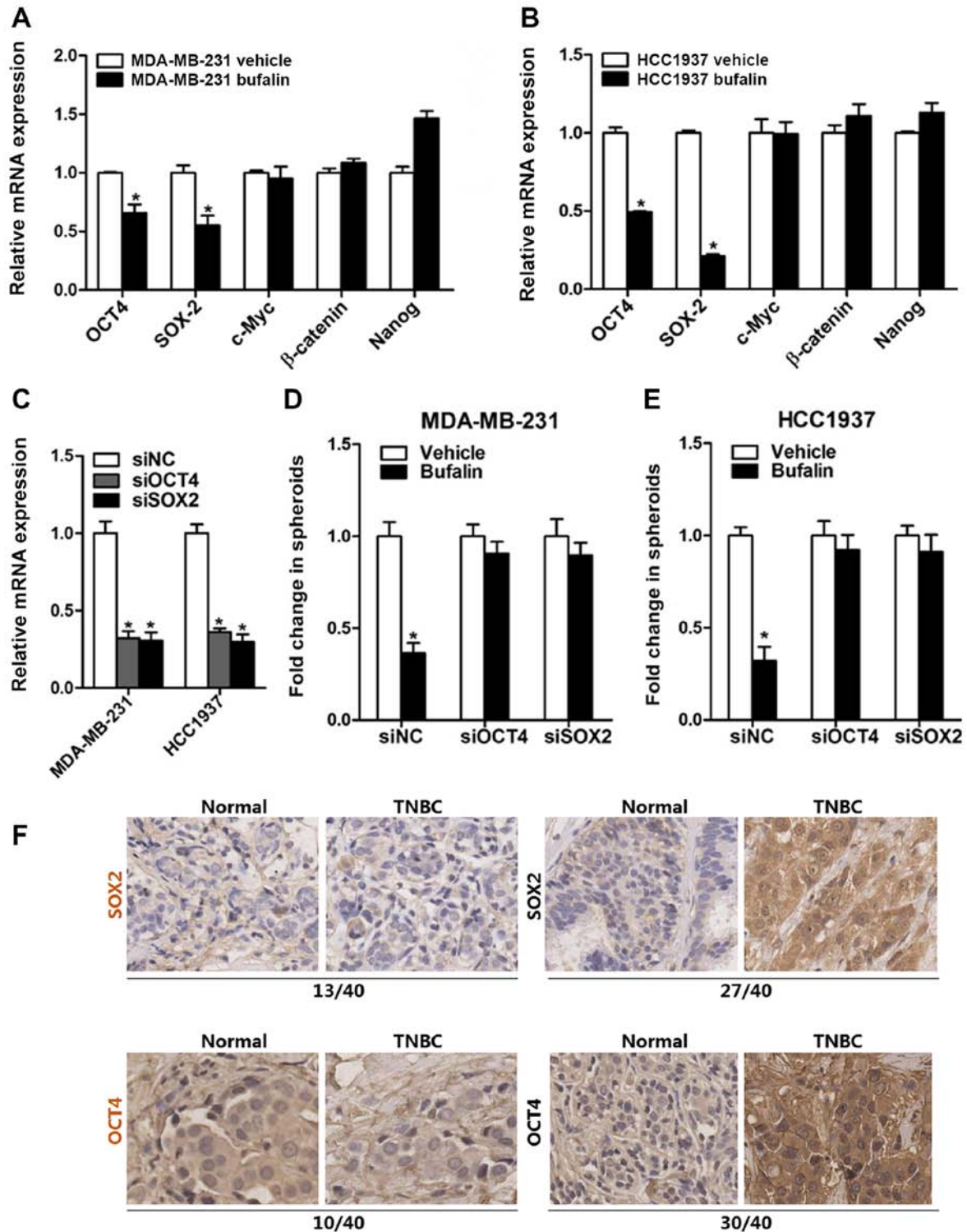
Figure 5. Bufalin attenuates the stem cell characteristics of TNBC cells via suppressing SOX2/OCT4. Genes expression levels were determined by reverse transcription-quantitative PCR analysis and normalized to the GAPDH level. (A and B) mRNA levels of OCT4, SOX2, c-Myc, β-catenin and Nanog in the indicated groups and (C) mRNA levels of OCT4 and SOX2 following transfection with siRNAs targeting OCT4 or SOX2. (D and E) Number of spheroids resulting from incubation with vehicle or bufalin following transfection with siRNAs targeting OCT4 or SOX2; the data are shown as mean ± standard deviation; Student's t-test *P<0.05. (F) The expression of SOX2 and OCT4 in 40 TNBC patient tissues was determined by routine immunohistochemical examination. The pictures were taken via microscopy at a magnification of 400X. Brown font indicates downregulation and black font indicates upregulation. The number of tumor tissues with low (13) or high (27) SOX2 and low (10) or high (30) Oct4 expression among 40 TNBC tissues were shown under each set of panels. TNBC, triple-negative breast cancer; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2.

TUNEL staining were also performed on paraffin sections of tumor samples collected from xenografts. A reduction in Ki-67 expression and an increase in apoptosis were observed in tumors treated with bufalin compared with those treated with vehicle (Fig. 3C and D). These results further confirmed the therapeutic effect of bufalin in TNBC.

Bufalin inhibits the self-renewal of TNBC stem cells. To explore whether bufalin attenuates the stemness of TNBC cells, a spheroid formation assay was performed. MDA-MB-231 and HCC1937 cells exhibited a reduced capacity to form spheroids when treated with 0.5 μM bufalin compared with control cells (Fig. 4A and B). Furthermore, the proportion of sphere-forming cells was determined by performing a limiting dilution analysis of cells incubated with or without bufalin, and the results demonstrated that the proportion of MDA-MB-231 and HCC1937 cells forming spheroids was significantly decreased by bufalin (P<0.05; Fig. 4C and D). These data indicated that bufalin effectively suppressed the self-renewal of TNBC stem cells.

Bufalin attenuates TNBC cell stemness via suppressing SOX2/OCT4. To elucidate the mechanisms through which bufalin attenuates the stem cell characteristics of TNBC cells, RT-qPCR analysis was performed to analyze the expression level of stemness-related genes following bufalin treatment, as shown in Fig. 5A and B. The expression levels OCT4 and SOX2 were found to be significantly downregulated following treatment with bufalin (P<0.05). To further confirm whether bufalin attenuates the stemness characteristics of TNBC cells by inhibiting SOX2/OCT4 expression, MDA-MB-231 or HCC1937 cells were transfected with siRNAs targeting OCT4 and SOX2, followed by a spheroid formation assay. The results demonstrated that the ability of TNBC cells to form spheroids was not affected by bufalin after SOX2 or OCT4 expression interference (P<0.05), suggesting that bufalin may attenuate the stemness of TNBC cells via downregulating the stemness-associated factors SOX2/OCT4 (Fig. 5C-E). In addition, SOX2/OCT4 protein expression was examined in 40 tissue samples from patients with TNBC. As expected, most TNBC tissues exhibited upregulated SOX2 (27/40) and OCT4 (30/40) expression (Fig. 5F).

Discussion

Bufalin is a potential polygenic and multi target anticancer agent (22). Numerous studies have reported that bufalin can induce cell cycle arrest and apoptosis and even trigger autophagic cell death in various human cancer cell lines (17,31,32). Furthermore, bufalin can suppress cancer growth and metastasis by inhibiting distinct cancer-associated signaling pathways, including transforming growth factor-β, phosphoinositide 3-kinase/AKT, Wnt/β-catenin and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways in different types of cancer (22,23,33,34). Consistent with these studies, the results of the present study demonstrated that bufalin can inhibit TNBC growth by inducing G2/M cell cycle arrest and promoting apoptosis of MDA-MB-231 or HCC1937 cells. The decreased proportion of cells in the G1/G0 phase of the cell cycle may be explained by increased S entry or decreased G1/G0 entry. The data in Fig. 1A and B demonstrated that bufalin suppressed the proliferation of TNBC cellsby inducing G2/S arrest and causing decreased G1/G0 entry, which explains the decreased proportion of G1/G0 phase cells.

At present, cytotoxic chemotherapy remains the mainstay of treatment for patients with TNBC, as these patients respond poorly to other types of therapies (35,36). However, following chemotherapy, tumor recurrence or metastasis may develop from chemo resistant preexisting CSCs or from resilient cancer cells

that eventually acquire CSC properties (11,37,38). Conventional chemotherapeutic agents target proliferating cells to induce their apoptosis, while they exert little effect on CSCs (39). It was recently demonstrated that cancer cells may acquire stemness characteristics following chemotherapy (40). Moreover, a recent study reported that agents inhibiting cancer cell stemness may complement the antitumor activity of chemotherapy in eradicating breast cancer patient-derived xenografts (9). Therefore, it is crucial to identify novel agents that can effectively target both CSCs and non-CSCs. In the present study, it was examined whether bufalin could inhibit TNBC cell stemness. The spheroid formation assay results demonstrated that bufalin reduced the ability of MDA-MB-231 and HCC-1937 cells to form spheroids. Other previous studies suggested that enhancement of CSCs may be detected following activation of stemness-associated factors, such as SOX2, OCT4, β-catenin, c-Myc and Nanog, in cancer cells (41-44). To investigate the molecular mechanism underlying the bufalin-mediated stemness reduction, the mRNA expression levels of SOX2, OCT4, c-Myc, β-catenin and Nanog were examined. The results demonstrated that SOX2 and OCT4 were significantly downregulated following bufalin treatment. Furthermore, the ability of MDA-MB-231 and HCC-1937 cells to form spheroids was not affected by bufalin in cells transfected with siRNAs targeting SOX2 or OCT4. All these data indicate that bufalin successfully inhibited stemness in MDA-MB-231 and HCC-1937 cells.

In conclusion, bufalin was shown to not only inhibit the proliferation and induce apoptosis in TNBC cells, but was also able to significantly attenuate the stemness of TNBC cells. However, further studies are warranted to confirm whether bufalin may be of value for preventing cancer recurrence or chemo resistance.

Availability of materials and data

All data that support the findings of this study are available from the corresponding author to the researchers upon reasonable request.

Authors' contributions

FC, LZ and ZL conceived and designed the study. FC, LZ, ZL, SJ, HL, JZ, JW, FW performed the experiments. FC, LZ, JH and ZL analyzed the data. FC, LZ, SJ and ZL interpreted

results of experiments. FC and LZ prepared figures. FC, LZ, SJ and ZL drafted the manuscript. FC, LZ, JH and ZL edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experimental protocols were approved by the Ethics Committee of the University of Traditional Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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