Ellagic acid and Sennoside B inhibit osteosarcoma cell migration, invasion and growth by repressing the expression of c-Jun

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Abstract. Osteosarcoma is a mesenchymally derived, high-grade bone sarcoma that is the most frequently diagnosed primary malignant bone tumor. Today, chemoprevention is regarded as a promising and realistic approach in the prevention of human cancer. Previous studies have suggested ellagic acid (EA) and Sennoside B have potential in this regard. The aim of the present study was to elucidate the anti-osteosarcoma effects of EA and Sennoside B by using Saos-2 and MG63 osteosarcoma cells. It was identified that EA or Sennoside B treatment could inhibit the growth, migration and invasion of the cells, and induce G_1 cell cycle arrest by repressing the transcription of c-Jun. These results may provide a cellular basis for the application of EA or Sennoside B in the treatment of patients with osteosarcoma.

Introduction

Osteosarcoma (OS) is one of the most malignant types of bone tumor, which is most frequent in children and adolescents and characterized by the formation of neoplastic bone tissue (1). There is a high tendency to for OS to undergo metastasis (2). The treatment of OS typically includes surgery, radiation, chemotherapy, or the combination of chemotherapy and radiotherapy; however, these treatments are not successful in the long-term (3). Numerous studies have indicated the abilities of natural products to inhibit the development of different types of cancer in multiple animal models, as previously reviewed (4). These compounds may be used as anti-proliferation or anti-metastasis agents.

Sennoside, an extract from senna, is widely used as a stimulant laxative and its safety and efficiency have been established (5); however, little is known regarding its usefulness as an anti-tumor agent. Ellagic acid (EA) is a polyphenol compound with strong antioxidant properties that is found as ellagitannins in the fruits and nuts of several plants. The oral administration of EA can protect the system from alcohol toxicity by decreasing the expression of liver marker enzymes and increasing the activity of the antioxidant cascade (6,7); it was previously demonstrated to induce apoptosis and cell cycle arrest in various types of cancer cell (8-11). However, its potential anti-tumor role in osteosarcoma and the mechanisms for its effects remain elusive.

The c-Jun N-terminal kinase (JNK) signal transduction pathway is associated with malignant cellular transformation (12). Studies with cultured cells have demonstrated that the JNK signal transduction pathway participates in the proliferation, differentiation, apoptosis and metastasis of osteoblasts (13). Phosphorylated JNKs activate the Jun proto-oncogene, AP-1 transcription factor subunit (c-Jun), which homodimerizes and/or heterodimerizes with Fos proto-oncogene, AP-1 transcription factor subunit to generate the activator protein-1 transcription complex (AP-1) (14), which binds to specific DNA sequences at target promoters, and regulates the expression of cognate genes that participate in the differentiation and function of osteoblasts, as well as in the pathogenesis of osteosarcoma (15-17).

It the present study, it is reported that EA and Sennoside B can inhibit Saos-2 and MG63, two malignant osteosarcoma cell lines, growth, migration and invasion, and induce cell cycle arrest by the inhibition of c-Jun expression.

Materials and methods

Antibodies and reagents. Rabbit anti-poly(ADP-ribose) polymerase (PARP; cat no. 88817), p-p38 (cat no. 4511), p-extracellular signal-regulated kinase (ERK) 1/2 (cat no. 4370) and c-Jun (cat no. 9165) antibodies were obtained

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from Cell Signaling Technology, Inc. (Danvers, MA, USA); mouse anti-GAPDH (cat no. sc-32233) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The EA, Sennoside B and propidium iodide were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. Saos-2 and MG63 human osteosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 atmosphere.

Wound closure assay. Saos-2 and MG63 cells were plated in a 12-well cell culture plate. When the cell density reached ≥90%, the monolayer was scratched with a 200 μl pipette. Wound closure was monitored with phase contrast microscopy and quantified as the relative wound closure rate (wound distance at a specific time point/the original wound distance).

Transwell assay. A total of $1x10^4$ of Saos-2 or MG63 cells were seeded in the upper chamber of Transwell plates (BD Bioscience) with Matrigel, in serum-free conditions. DMEM supplemented with 10% fetal calf serum (Hyclone; GE Healthcare Life Sciences) and 50 ug/ml fibronectin (BD Biosciences, Franklin Lakes, NJ, USA) was used as a chemoattractant in the lower chamber. At 48 h, cells remaining on the upper chamber were removed with a cotton swab, while cells adhering to the lower membrane were stained with 0.1% crystal violet for 30 min at room temperature and photographed with an inverted microscope (Zeiss AG, Oberkochen, Germany). The area of positive staining was measured using image analysis software (Image-Pro Plus 6.0; Media Cybernetics, Inc., Rockville, MD, USA); the rate of invasion was calculated as the positive area percentage. At least three independent experiments were performed for each condition.

MTT assays. Cell viability was assessed with an MTT assay in replicates. Cells were seeded in 96-well plates at $2.5x10^3$ cells per well (Saos-2 cells) or $1.0x10^3$ cells per well (MG63 cells) and incubated for 0, 24, 48 or 72 h. Then the medium was replaced with 200 ml fresh DMEM containing 0.5 mg/ml MTT, the cells were incubated for a further 4 h, then MTT formazan crystals were dissolved in DMSO and absorbance at 490 nm was measured and analyzed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To assess mRNA levels, RNA was isolated from cells using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) and cDNA was synthesized using the MLV reserve transcriptase from Promega Corporation (Madison, WI, USA), according to the manufacturers' protocols. The cDNA was subjected to qPCR (95.0˚C for 10 min for 1 cycle, then 95.0˚C for 15 sec followed by 60.0˚C for 1 min for 40 cycles) using SYBR-Green master mix (Toyobo Life Science, Osaka, Japan) in the Mx3005P Real-Time PCR system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). The comparative cycle quantification method ($2^{\Delta\Delta Cq}$) was employed to analysis the gene expression with housekeeping gene 18S as an internal normalization control (18). Each experiment was performed in duplicate and repeated three times. The primers for qPCR were as follows: c-Jun forwards, TCCAAG TGCCGAAAAAGGAAG; reverse, CGAGTTCTGAGCTTT CAAGGT; 18S forwards, GGACACGGACAGGATTGACA; reverse, GACATCTAAGGGCATCACAG. I8S was used as an internal control.

Cell cycle analysis. Saos-2 cells or MG63 cells were treated with EA or Sennoside B for 24 h. Cells were trypsinized, collected, fixed and stained with propidium iodide solution following the manufacturer's protocol of the Propidium Iodide Flow Cytometry kit (cat no. ab139418, Abcam, Cambridge, UK). The cell cycle distribution was then analyzed by the BD FACSCalibur (BD Biosciences) using CellQuest Pro, version 5.1 (BD Biosciences).

Western blotting. Saos-2 or MG63 cells were treated with EA or Sennoside B for 24 h, then scraped into ice-cold PBS and lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol and protease inhibitors. The protein concentration was determined using the Pierce BCA protein assay kit (cat no. 23225, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Same quantities of protein (25 μg/lane) were resolved using 12% PAGE-SDS gel, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA; cat no. 1620112), then followed by 5% non-fat milk blocking at room temperature for 1 h. The proteins were analyzed with antibodies against PARP (1:1,000 diluted in 5% BSA), p-ERK (1:2,000 diluted in 5% BSA), c-Jun (1:1,000 diluted in 5% BSA), p-p38 (1:1,000 diluted in 5% BSA), and GAPDH (1:1,000 diluted in 5% BSA). The primary antibodies were incubated with membrane at 4˚C overnight, followed by a 1 h secondary antibody incubation at room temperature. HRP-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; cat no. 115-035-146; 1:5,000 in 5% non-fat milk) and goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.; cat no. 115-035-045; 1:5,000 in 5% non-fat milk) secondary antibodies were used to visualize the protein of interest using Amersham ECL Prime Western Blotting Detection Reagent (cat no. RPN2236; GE Healthcare, Chicago, IL, USA). All data were the results from 3 replicates.

Statistics. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Quantitative data are expressed as the means ± standard deviation. Statistical analysis was performed with a one-way analysis of variance followed by Dunnett's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EA and Sennoside B inhibit the migration and invasion of osteosarcoma cells. Previous reports have indicated the anti-tumor potential of EA and Sennoside B (8). In order to

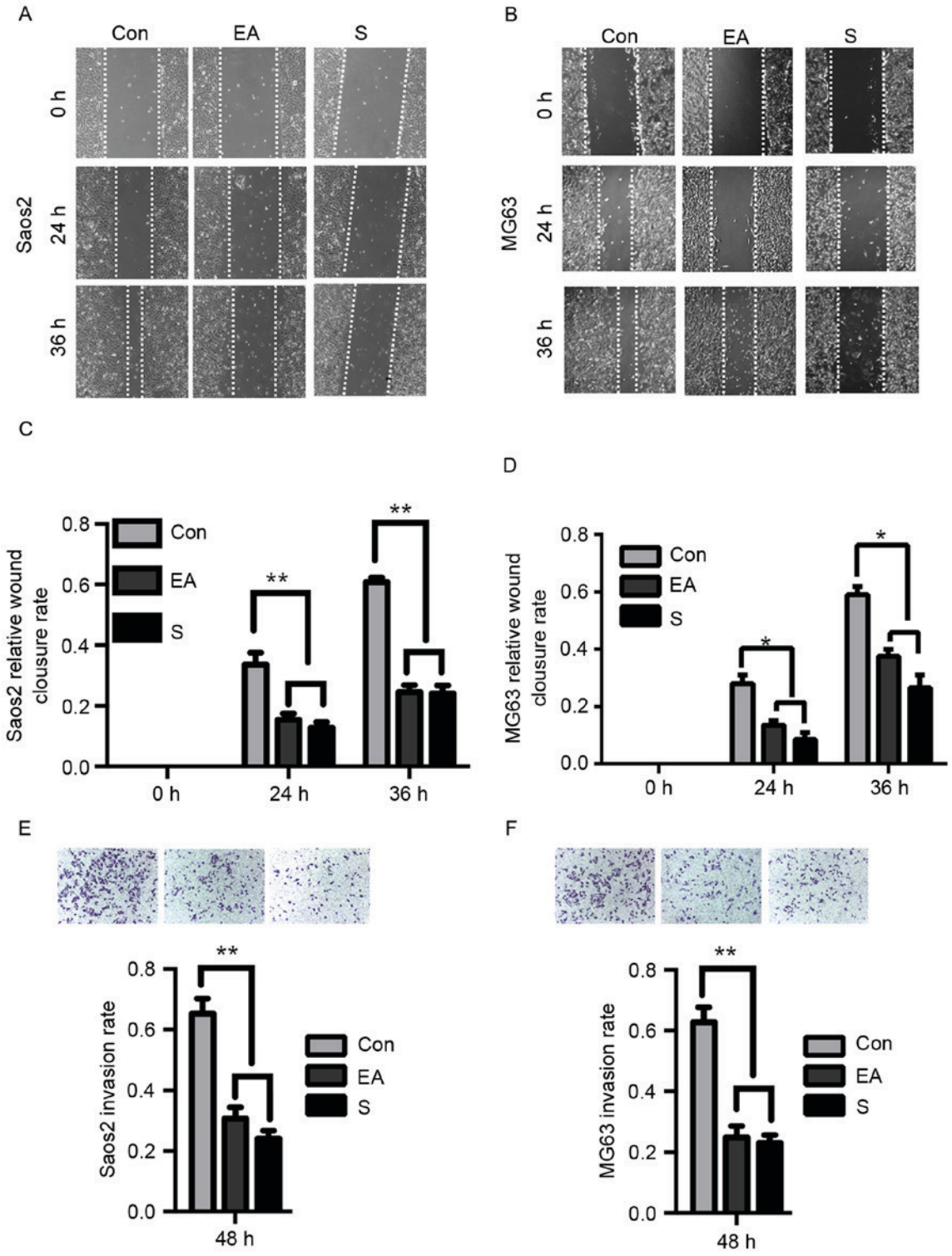

Figure 1. EA and Sennoside B inhibit the migration of osteosarcoma cells. (A) Saos-2 or (B) MG63 cells were treated with 20 μM EA or Sennoside B, and wound closure was monitored. Magnification, x10. The closure rate was quantified for (C) Saos-2 and (D) MG63 cells in order to compare the rate of migration. (E) Saos-2 or (F) MG63 cells were seeded on transwell membranes with or without EA or Sennoside B treatment. Magnification, x20. The migrated cells were fixed and stained with crystal violet (upper panel). The proportion of migrated cells was then quantified (lower panel). Data represent the mean ± standard deviation of 3 experimental repeats. *P<0.05, **P<0.01. EA, ellagic acid; Con, control; S, Sennoside B.

investigate their effect on osteosarcoma, Saos-2 and MG63 cells were analyzed. A series of concentrations of each agent were used in a wound-healing assay to verify their influence on the migration of OS cells. It was indicated that the 20 μM EA or Sennoside B was the minimal requirement for the inhibition of the migration ability of Saos-2 cells (data not shown and Fig. 1A), which was consistent with the result for MG63 cells (data not shown and Fig. 1B). The analysis of the wound-healing assay demonstrated that when the cells were exposed to 20 μM EA or Sennoside B for 24 or 36 h, the inhibition rate was 40-70% (Fig. 1C and D; Saos-2, P<0.01; MG63, P<0.05). Then, the invasion abilities of these cells in

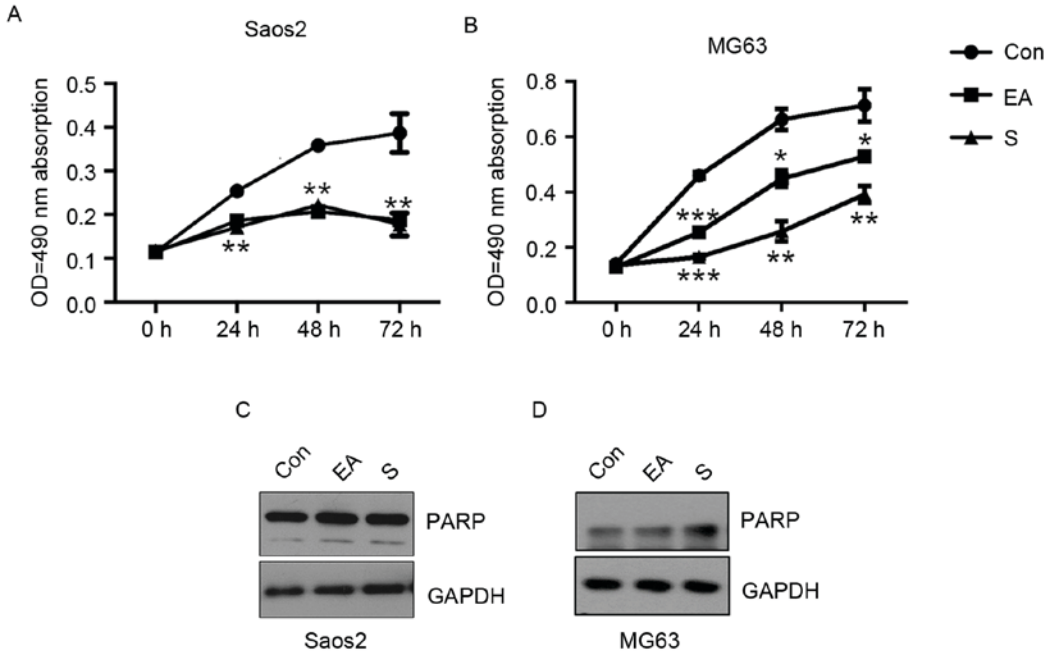

Figure 2. EA and Sennoside B inhibit osteosarcoma cell growth. (A) Saos-2 or (B) MG63 cells were cultured with 20 μM EA or Sennoside B for the indicated times. The rate of cell growth was determined by MTT assays. Data represent the mean ± standard deviation of 3 experimental repeats performed in triplicate. **P<0.01, ***P<0.0001. The (C) Saos-2 or (D) MG63 cells were treated with 20 μM EA or Sennoside B for 24 h. The indicated proteins were detected by western blotting; representative images are included. EA, ellagic acid; Con, control; S, Sennoside B.

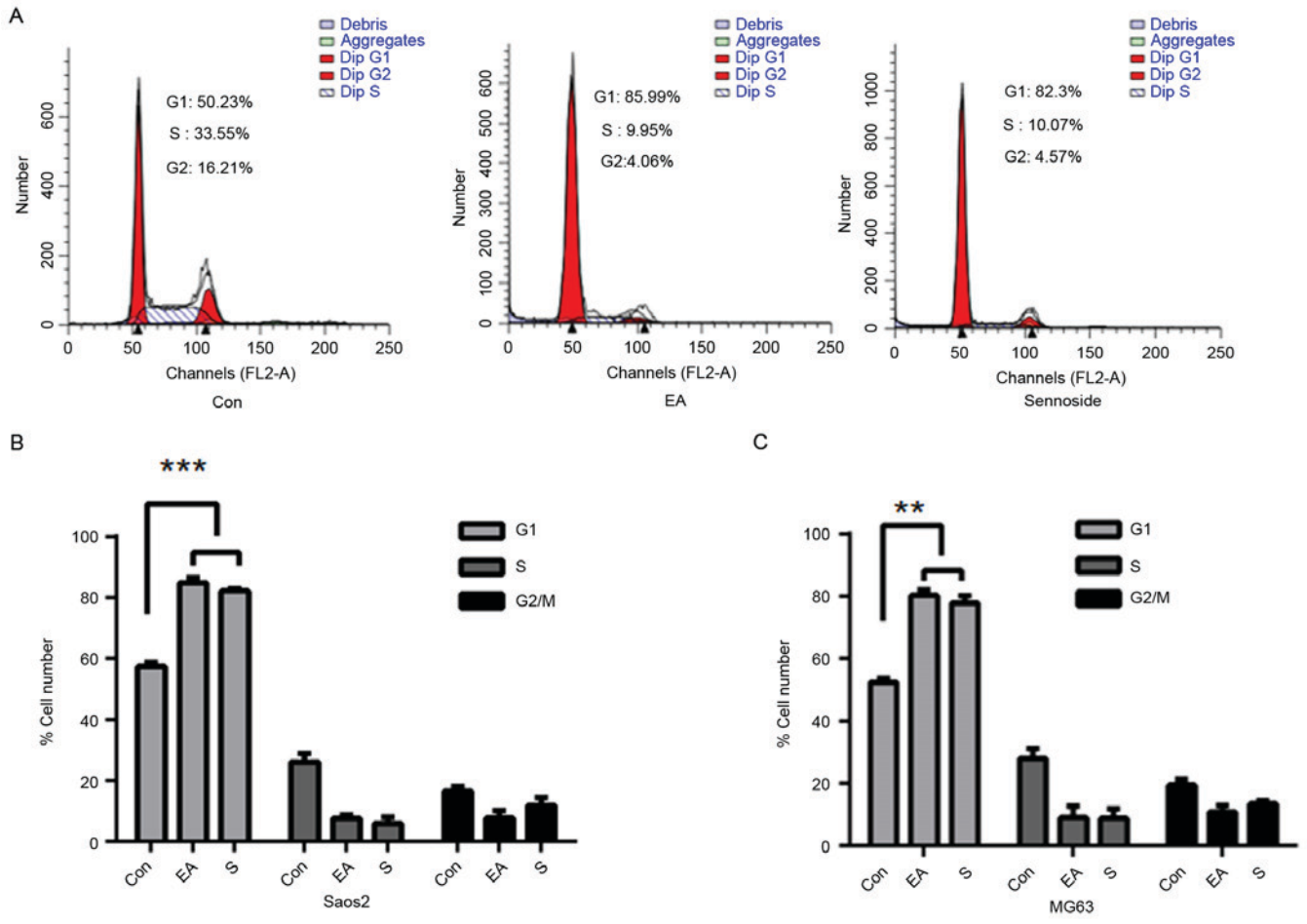

Figure 3. EA and Sennoside B induce cell cycle arrest in osteosarcoma cells. (A) Cells were treated with EA or Sennoside B for 24 h, stained with propidium iodide and analyzed by fluorescence-activated cell sorting. A representative image with Saos-2 cells is depicted. The compounds induced the accumulation of (B) Saos-2 and (C) MG63 cells in the G_1 phase. Data represent the mean ± standard deviation of 3 experimental repeats. **P<0.01, ***P<0.0001. EA, ellagic acid; Con, control; S, Sennoside B.

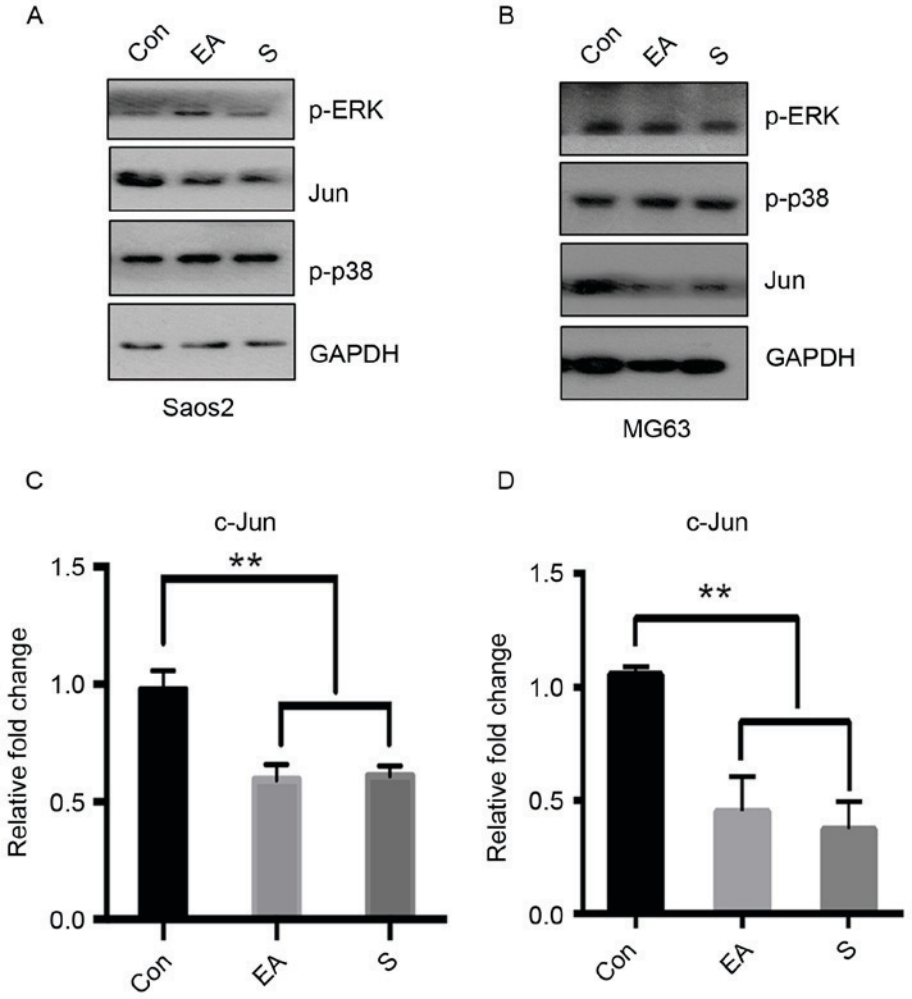

Figure 4. EA and Sennoside B inhibit c-Jun expression in osteosarcoma cells. (A and B) Following 24 h treatment with EA or Sennoside B, the indicated proteins were detected by western blotting in (A) Saos-2 and (B) MG63 cells. The (C) Saos-2 or (D) MG63 cells were treated with EA or Sennoside B for 12 h before the total RNA was extracted and the c-Jun mRNA expression level was examined by reverse transcription-quantitative polymerase chain reaction with specific primers. Data represent the mean ± standard deviation of 3 experimental repeats performed in duplicate. **P<0.01. EA, ellagic acid; c-Jun, Jun proto-oncogene, AP-1 transcription factor subunit; Con, control; S, Sennoside B; p-, phosphorylated-; ERK, extracellular signal-regulated kinase.

the presence or absence of EA or Sennoside B were examined by transwell assays. The EA- or Sennoside B-treated cells invaded significantly less frequently than the control cells (Fig. 1E and F; P<0.01). Thus, it was determined that EA or Sennoside B treatment can suppress the migration and invasion of osteosarcoma cells.

EA and Sennoside B inhibit the growth of osteosarcoma cells without inducing apoptosis. Saos-2 and MG63 cells were treated with 20 μM EA or Sennoside B, and viability was examined by an MTT assay. The results suggested both EA and Sennoside B significantly inhibited cell growth from 24 h at 20 μM, compared with the control groups (Fig. 2A and B; Saos-2, P<0.01; MG63, P<0.0001). To assess whether the induction of apoptosis served a role in the repression of the proliferation of OS cells, the cells were subjected to EA and Sennoside B treatment for 24 h and were collected for western blotting. No significant difference in cleaved PARP expression was observed between the control and treated groups (Fig. 2C and D), even though a previous study demonstrated that EA inhibited proliferation by the induction of apoptosis in colon and prostate cancer cells (19).

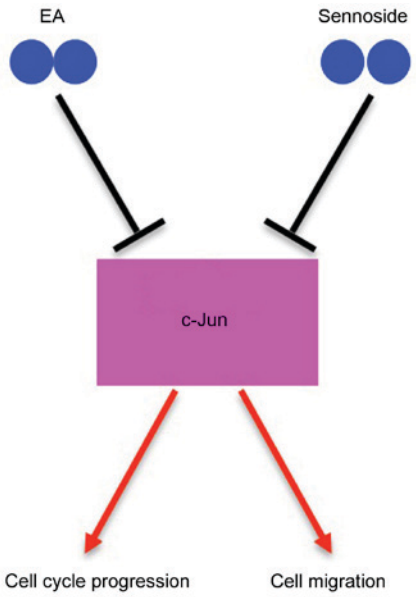

Figure 5. A schematic of the potential mechanism for the inhibition of the migration and growth of osteosarcoma cells by EA or Sennoside B. EA, ellagic acid; c-Jun, Jun proto-oncogene, AP-1 transcription factor subunit.

EA and Sennoside B induce cell cycle arrest. As EA and Sennoside B inhibited cell growth without inducing apoptosis, the cell cycle distribution of cells treated with 20 μM EA or Sennoside B for 24 h was analyzed. It was identified that >80% of Saos-2 cells in the treated groups were in the G_1 stage, compared with <60% in the control groups (Fig. 3A and B; P<0.0001). Similar results were detected for MG63 cells (Fig. 3C; P<0.01).

EA and Sennoside B repress c-Jun expression. The accumulation of cells in the G_1 phase indicated the inhibition of the signals required for cell cycle progression. The expression of factors from key signaling pathways associated with G_1 cell cycle progression was thus examined following exposure to EA or Sennoside B. After 24 h of treatment, the c-Jun protein level was observed to decrease to a greater extent than the other assessed proteins (Fig. 4A and B). In order to further confirm the effect of EA or Sennoside B on c-Jun, mRNA was extracted for RT-qPCR following the same treatment. These results suggested that EA or Sennoside B treatment repressed the transcription of c-Jun mRNA (Fig. 4C and D). A proposed schematic was drawn to represent the potential mechanism for the EA or Sennoside B inhibition of the growth, migration and invasion of osteosarcoma cells (Fig. 5).

Discussion

In the present study, it was demonstrated that both EA and Sennoside B treatment can inhibit cell migration, invasion and proliferation. The inhibitory effect of EA or Sennoside B on proliferation was attributed to the induction of cell cycle arrest. The data regarding the effects of EA on osteosarcoma are consistent with previous studies in other cancer types (7-11). However, the inhibitory effects of Sennoside B were demonstrated for the first time, to the best of our knowledge.

The high incidence of mortality in osteosarcoma is associated with tumor metastasis (1). Metastasis is a complex cascade, including various physiological alterations to induce extracellular matrix (ECM) degradation (20). Matrix metallopeptidases (MMPs), particularly MMP-2 and MMP-9, have been reported as the most important factors in the degradation of type IV collagen, a major component of the basement membrane, which induces tumor cell growth and metastasis (21). It has been reported that EA inhibits either MMP2 activity or the secretion of collagenases and gelatinases (22). AP-1 may serve a dominant role in the transcriptional activation of the MMP promoters, and increasing evidence has indicated the key role of c-Jun in the regulation of MMPs (23). In the present study, both EA and Sennoside B treatment repressed the transcription of c-Jun, ultimately leading to a decrease in its protein level. This mechanism partially explains why two different compounds inhibited Saos-2 and MG63 cells in a similar manner.

Previous studies indicated that EA could inhibit cancer cell growth by inducing apoptosis and cell cycle arrest (8-11,19), but Sennoside B's effect on cell growth was previously unreported. The results of the present study suggested that both EA and Sennoside B could inhibit cell proliferation without inducting apoptosis. The oncogenic protein c-Jun regulates a range of cell cycle progression-associated genes, including cyclin D1 (24). Cell cycle arrest in the present study was likely to be caused by the repression of c-Jun expression. However, other factors can induce the cell cycle arrest of osteosarcoma cells. For example, the knockdown of ROR2 in osteosarcoma cells inhibited cell proliferation and colony formation by inducing cell cycle arrest (25). In addition, it was previously reported that the exogenous expression of miR-497 in human osteosarcoma MG63 cells suppressed cell proliferation, colony formation, migration and invasion, and induced apoptosis and arrest at the G_0/G_1 phase of the cell cycle (26).

In conclusion, it was identified that EA and Sennoside B may share a common mechanism to inhibit the growth, migration and invasion of Saos-2 and MG63 cells, specifically, the repression of c-Jun expression at the mRNA level. Further studies are required to understand the detailed molecular mechanisms of action of EA and Sennoside B on the regulation of the expression of c-Jun, which may provide useful information for their possible application in osteosarcoma prevention and therapy.

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