The nutritional herb *Epimedium grandiflorum* inhibits the growth in a model for the Luminal A molecular subtype of breast cancer

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Abstract. The Luminal A subtype of breast cancer expresses the estrogen receptor (ER)-α and progesterone receptor (PR), but not the human epidermal growth factor receptor (HER)-2 oncogene. This subtype of breast cancer responds to endocrine therapy involving the use of selective estrogen receptor modulators and/or inhibitors of estrogen biosynthesis. However, these therapeutic agents are frequently associated with long-term systemic toxicity and acquired tumor resistance, emphasizing the need to identify non-toxic alternative treatments for chemo-endocrine therapy responsive breast cancer. The present study utilized the human mammary carcinoma-derived, $ER^+/PR^+/HER\text{-}2^-$ MCF-7 cell line as a model of the Luminal A subtype of breast cancer to examine the growth inhibitory effect of the Chinese nutritional herb *Epimedium grandiflorum* (EG) and determine the mechanisms underlying this effect. MCF-7 cells maintained in a serum-depleted culture medium retained their ability to grow in response to 17β-estradiol (E_2). Treatment of the MCF-7 cells with EG resulted in dose-dependent inhibition of E_2-promoted growth. Mechanistically, EG inhibited E_2-promoted cell cycle progression through G_1 stage arrest and modulated the cellular metabolism of E_2, increasing the formation of the anti-proliferative metabolites 2-hydroxyestrone and estriol. Long-term treatment of MCF-7 cells with EG inhibited E_2-promoted anchorage independent growth, a surrogate *in vitro* biomarker of tumorigenesis. In conclusion, the results of the present study demonstrate the growth inhibitory effects of EG on MCF-7 cells and identified clinically relevant mechanistic leads for its anti-tumorigenic efficacy.

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Introduction

Metastatic breast cancer is one of the leading causes of breast-cancer associated mortality in the United States, and the American Cancer Society (Atlanta, GA, USA) projections for breast cancer incidence and mortality rates estimate 246,660 newly diagnosed cases of invasive breast cancer and 40,450 metastatic breast cancer-associated mortalities in women in 2017 (1). These figures emphasize the requirement for a more precise molecular classification of breast cancer for subtype-specific targeted therapy, in addition to the necessity to identify novel, non-toxic and efficacious modalities as alternatives for cancer prevention and therapy.

The global gene expression profiling of breast cancer has led to the identification of molecularly distinct subtypes. Patients with the molecular subtype Luminal A express estrogen receptor-α (ER)-α and progesterone receptor (PR), and they lack the expression of human epidermal growth factor receptor-2 (HER-2). Patients with this subtype respond to endocrine-based targeted therapy, such as selective ER modulators and specific small molecule inhibitors of estrogen biosynthesis (2-4). However, long-term endocrine-based therapy has been associated with adverse systemic toxicity, acquired tumor resistance and the emergence of drug resistant cancer stem cells that compromise therapeutic efficacy and promote disease progression (4-6).

Complementary and alternative medicine using herbal products has been applied to endocrine therapy-responsive patients with breast cancer, as a potential method of reducing therapy-associated toxicity. Several nutritional herbs, including *Lycium barbarum* (LB), *Cornus offcinalis* (CO) and *Epimedium grandiflorum* (EG), are essential components of herbal formulations that are used in traditional Chinese medicine as palliative and/or adjuvant treatment options for women with breast cancer (7,8). The majority of nutritional herbs display potent immunomodulatory and antiangiogenic properties *in vivo* (7). However, the underlying molecular signaling pathways and targets that are responsible for the growth inhibitory effects of these herbs on breast cancer cells remain to be systematically elucidated.

The human mammary carcinoma-derived cell line MCF-7 expresses ER-α and PR, and lacks HER-2 expression, thus representing an accepted pre-clinical cell model for the Luminal A molecular subtype of breast cancer (2-4). Previous studies using MCF-7 cells as a model for the Luminal A breast cancer subtype have demonstrated the direct growth inhibitory efficacy of extracts from Chinese nutritional herbs, such as LB and CO, that function through distinct mechanistic pathways (9,10). The present study utilized MCF-7 cells to investigate the direct growth inhibitory effects of the Chinese nutritional herb EG and identify the potential molecular mechanisms underlying its efficacy.

Materials and methods

Cell culture. The ER-α^+/PR^+/$HER\text{-}2^-$ human mammary carcinoma-derived cell line MCF-7 was obtained from the Michigan Cancer Foundation (Detroit, MI, USA). The cells were cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with heat inactivated 7% fetal calf serum, 2 mM L-glutamine, 1% penicillin-streptomycin mixture (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 μg/ml insulin (Ely Lilly and Company, Indianapolis, IN, USA) and 1 μM dexamethasone (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), following published protocols (9-11).

In the present study, MCF-7 cells were adapted for long-term growth in serum-depleted medium through maintaining the cells in DMEM/F-12 supplemented with 0.7% fetal calf serum [17β-estradiol (E_2) <1 nM] for ≥5 passages. Stock cultures were routinely maintained in DMEM/F-12 supplemented with 0.7% fetal calf serum in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were sub-cultured in a 1:4 ratio once they reached 80% confluency.

Growth parameters. Cell population doubling time was determined during the exponential growth phase. Viable cell counts were performed 24, 48, 72 and 96 h post-seeding of $1x10^5$ cells using a trypan blue exclusion cell viability assay kit (Sigma-Aldrich; Merck Millipore), following the protocol supplied by the vendor. The data are expressed as the mean of the four time points. Similarly, saturation density was determined by the viable cell count on day 7 post-seeding, using the trypan blue cell viability assay kit. The experiment for anchorage independent (AI) growth was performed using 12-wells/treatment group. AI growth was evaluated by determining the number of AI colonies that had developed in 0.33% Bacto-agar (Sigma-Aldrich; Merck Millipore) on day 21 post-seeding of $1x10^3$ cells, following published optimized protocols (9-11).

Aqueous extract of EG. To prepare an aqueous extract, 20 g of leaves and stems from EG were boiled in 200 ml of deionized water until the volume had reduced to 100 ml, and the supernatant was subsequently collected (extract 1). The resultant residue was further boiled in 100 ml of water until the volume had reduced to 50 ml (extract 2). The two extracts (100 and 50 ml) were combined and further concentrated through boiling until the volume had reduced to 25 ml. These combined extracts were centrifuged at 500 x *g* at room temperature for 10 min. The resulting supernatant (20 ml) was collected and stored in 5 ml aliquots at -20˚C. The stock solution (100%) was serially diluted in DMEM/F-12 to obtain working solutions of 2.0, 1.0 and 0.5% for subsequent experiments.

Dose-response analysis. To determine the growth inhibitory effects, and the minimum effective and maximum cytostatic concentrations of EG, the MCF-7 cells were treated with increasing concentrations of EG (0.5, 1.0 and 2.0%). The experiments for the dose-response of EG were performed using 6 flasks/treatment group. Viable cell counts were determined on day 7 post-seeding of $1.0x10^5$ cells using the trypan blue exclusion cell viability assay as described above. Dose-response analysis identified the half maximal inhibitory concentration (IC_{50}), the maximum cytostatic concentration (IC_{90}) and the toxic concentration of EG for growth inhibition. The IC_{90} was defined as the highest dose of EG that resulted in a viable cell count ≥ the initial seeding density. The toxic concentration was defined as the lowest dose of EG that resulted in a viable cell count < the initial seeding density.

Cell cycle progression. To examine the effect of EG on cell cycle progression, MCF-7 cell cultures treated for 48 h with 20 nM E_2 (Sigma-Aldrich; Merck Millipore) alone (control) or with 20 nM E_2+1.0% EG were analyzed using fluorescence-activated cell sorting, following an optimized protocol (12,13). Briefly, trypsinized cell cultures from the control and treatment groups were stained with propidium iodide (Calbiochem; Merck Millipore), and the percentage of the cell population in G_1, S and G_2/M phases of the cell cycle was monitored using the EPICS® 752 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), equipped with a 488 nM excitation filter and a 630 nM longpass filter. Cell cycle phase distribution was analyzed using MultiCycle MPLUS 2.0 software (Phoenix Flow Systems, San Diego, CA, USA). Results are expressed as the percentage of cells in G_1, S and G_2/M phases of the cell cycle. In addition, the G_1: S+G_2/M ratio of the number of cells in each phase is presented. This assay was performed using 3 flasks/treatment group.

Cellular metabolism of E_2. To examine the effect of EG on the cellular metabolism of E_2, on day 1 post-seeding, $1x10^6$ MCF-7 cells were treated with 20 nM E_2 alone (control) or E_2+1.0% EG for 48 h. The media was subsequently collected and analyzed for the presence of select E_2 metabolites, such as estrone (E_1), 2-hydroxyestrone (2-OHE_1), 16α-hydroxyestrone (16α-OHE_1) and estriol (E_3), using gas chromatography-mass spectrometry. An Agilent 6908 N gas chromatograph equipped with an Agilent 5973 mass selection detector, Agilent 7683 injector and HPGI 701 CA MSD Chemstation (all Agilent Technologies, Inc., Santa Clara, CA, USA) was used following a previously optimized protocol (9,10). Primary data are expressed as metabolite concentration (ng)/$1x10^6$ cells. Additionally, the primary data was analyzed and expressed as 2-OHE_1:16α-OHE_1 and E_3:16α-OHE_1 ratios for E_2 metabolites. This assay was performed using 3 flasks/treatment group.

Statistical analysis. Statistical analyses were performed using the Student's t-test and one-way analysis of variance,

followed by a Dunnett's Multiple Range Test (α=0.05) where appropriate. All tests were performed using GraphPad Prism® software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. The threshold of α=0.05 was selected for comparison of multiple treatment groups using the Dunnett's Multiple Range Test.

Results

Growth-promoting effects of E_2. Results of the investigation into the effects of E_2 on MCF-7 cells adapted for growth in 0.7% serum (E_2, <1 nM) vs. those in 20 nM E_2 are presented in Table I. MCF-7 cells treated with 20 nM E_2 demonstrated a 17.6% decrease in the population doubling time, a 91.9% increase in the saturation density and a 1.2-fold increase in the number of AI colonies compared with the control group that had been grown in 0.7% serum (E_2, <1 nM). The statistical significance of the data was analyzed by one-way analysis of variance and Dunnett's Multiple Range Test (α=0.05).

Dose-response to EG. Results of the investigation into the growth inhibitory effects of EG on MCF-7 cells are presented in Fig. 1. A 7-day treatment with 20 nM E_2 resulted in a viable cell count of 26.1±4.7x10^5, a 26-fold increase compared with the initial seeding density (α=0.05). Furthermore, treatment with 20 nM E_2+0.5% EG and 20 nM E_2+1.0% EG resulted in a significant decrease in the viable cell counts compared with the 20 nM E_2 alone-treated control group (20 nM E_2+0.5% EG, 12.8±2.3x10^5; 52.6% decrease; α=0.05) and (20 nM E_2+1.0% EG, 2.4±0.4x10^5; 87.0% decrease; α=0.05). This dose-response analysis identified that EG had an IC_{50} of 0.49±0.18% and an IC_{90} of 1.03±0.17% (data not shown). An EG concentration of 2.0% was toxic to MCF-7 cells.

Effect of EG on cell cycle progression. Data from the investigation into the effect of E_2 and E_2+EG on the cell cycle progression of MCF-7 cells are presented in Table II. The data demonstrates that treatment with 20 nM E_2 induced an increase in the percentage of cells in the S and G_2/M phases, with a simultaneous decrease in the percentage of cells in the G_1 phase, compared with the serum-treated control group. Furthermore, treatment with 20 nM E_2 and the IC_{90} concentration of EG (1.0%) induced pronounced G_1 cell cycle arrest with a simultaneous decrease in the percentage of cells in the S and G_2/M phases, compared with the E_2 alone-treated control. Compared with the serum-treated control group, treatment with E_2 resulted in a 54.3% decrease (α=0.05) in the G_1: S+G_2/M ratio of cells. In addition, treatment with EG+E_2 resulted in a 76.2% increase in the G_1: S+G_2/M ratio of cells compared with the E_2 alone-treated control group (α=0.05). These results indicate that E_2 accelerates cell cycle progression and that EG can attenuate the effect of E_2.

Effect of EG on the cellular metabolism of E_2. Data from the investigation into the effect of EG on E_2 metabolism are presented in Table III. The results demonstrate that the E_2+EG group had a significantly increased formation rate of E_1 (P=0.01), 2-OHE_1 (P=0.006) and E_3 (P=0.001) compared with the E_2 alone-treated control group. EG induced a 3.3-fold increase in E_1 formation, a 5.0-fold increase in 2-OHE_1 formation and a 9.0-fold increase in E_3 formation compared with the E_2 alone-treated control group. Additionally, the primary data were further analyzed and expressed as the 2-OHE_1:16α-OHE_1 and E_3:16α-OHE_1

Table I. Growth promoting effects of E_2 on MCF-7 cells.

	Culture conditions	
Growth parameter	0.7% serum (<1 nM E_2)	0.7% serum + 20 nM E_2
Population doubling (h)[a]	33.0±1.9	27.2±1.6
Saturation density (x10^5)[b]	13.6±4.5	26.1±4.7
AI colonies (no.)[c]	16.5±1.5	37.2±2.1

[a]Determined from the exponential growth phase (n=3/treatment group). [b]Viable cell number determined at day 7 post-seeding of 1x10^5 cells (n=3/treatment group). [c]Determined at day 21 post-seeding of 1x10^3 cells (n=12/treatment group). Results are presented as the mean ± standard deviation. α=0.05 <1 nM E_2 vs. 20 nM E_2. Data was analyzed by one-way analysis of variance and Dunnett's test (α=0.05). E_2, 17β-estradiol; AI, anchorage independent.

Table II. Inhibition of MCF-7 cell cycle progression by EG.

	Cell cycle distribution[a]		
Treatment	% G_1	% S+G_2/M	G_1: S+G_2/M ratio
Serum 0.7% (<1 nM E_2)	82.3±8.2	17.8±1.8	4.6±0.5[b]
20 nM E_2	67.3±6.7	32.5±3.2	2.1±0. 2[c]
20 nM E_2 + 1.0% EG	78.6±7.8	21.4±3.8	3.7±0.4[d]

[a]Determined following 48 h treatment. Results are presented as the mean ± standard deviation (n=3/treatment group). α=0.05 [b] vs. [c]; α=0.05 [b] vs. [d]; α=0.05 [c] vs. [d]. Data was analyzed by one-way analysis of variance and Dunnett's test (α=0.05). E_2, 17β-estradiol; EG, *Epimedium grandiflorum*.

Table III. EG modulates the metabolism of E_2 in MCF-7 cells.

	E_2 metabolite concentration[a] (ng/1.0x10^6 cells)			
Treatment	E_1	2-OHE_1	16α-OHE_1	E_3
20 nM E_2	2.7±0.1	0.7±0.3	1.8±0.8	0.3±0.1
20 nM E_2 + 1.0% EG	11.7±0.6[b]	4.2±0.3[c]	2.2±0.5	3.0±0.9[d]

[a]Determined following 48 h. treatment. Results are presented as the mean ± standard deviation (n=3/treatment group). [b]P=0.01, [c]P=0.006, [d]P=0.001 compared with the 20 nM E_2 control group. Data was analyzed using the Student's t-test. E_2, 17β-estradiol; E_1, estrone; OHE_1, hydroxyestrone; E_3, estriol; EG, *Epimedium grnadiflorum*.

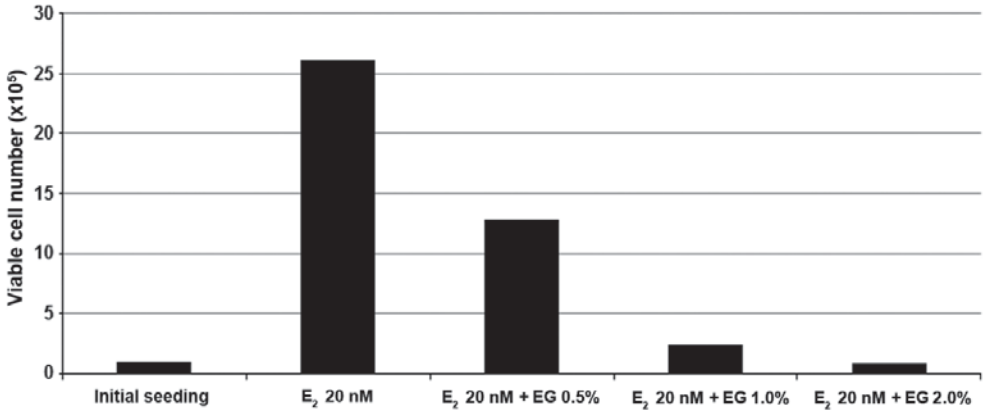

Figure 1. Dose-response analysis of EG in MCF-7 cells. The growth promoting effect of E_2, represented by viable cell number, decreased in response to treatment with EG in a dose-dependent manner. EG IC_{50}, 0.49±0.18%; IC_{90}, 1.03±0.17%. Results are presented as the mean ± standard deviation (n=6/treatment group). E_2 20 nM vs. E_2 20 nM + EG 0.5% (E_2>E_2+EG, α=0.05), E_2 20 nM vs. E_2 20 nM + EG 1.0% (E_2>E_2+EG, α=0.05), and E_2 20 nM vs. E_2 20 nM + EG 2.0% (E_2>E_2+EG, α=0.05). Data was analyzed using one-way analysis of variance and Dunnett's test (α=0.05). E_2, 17β-estradiol; EG, *Epimedium grandiflorum*.

ratios. This analysis revealed that the 2-OHE_1:16α-OHE_1 ratio was 0.39±0.16 for the E_2 alone-treated control group and 1.9±0.4 for the E_2+EG-treated group, demonstrating a significant 3.9-fold increase (P=0.01) compared with the E_2 alone-treated control group. Similarly, the E_3:16α-OHE_1 ratio was 0.16±0.06 for the E_2 alone-treated control and 0.58±0.14 for the E_2+EG-treated group, demonstrating a significant 2.6-fold increase (P=0.03) compared with the E_2 alone-treated control group.

Effect of EG on AI cell growth. Results from the investigation into the effect of EG on AI growth are presented in Fig. 2. The AI colony count in the serum-treated control group was 16.8±1.8 compared with 36.9±2.1 in the E_2-treated group, illustrating a 119.6% increase compared with the control group (α=0.05). The AI colony count in the 0.5% EG and 1.0% EG-treated groups was 18.1±1.0 and 4.7±0.4, respectively, compared with the E_2 alone-treated control (α=0.05). Thus, treatment with E_2+0.5% EG and E_2+1.0% EG resulted in a substantial 50.9 and 87.3% decrease in the AI colony count, respectively, compared with the E_2-treated control.

Discussion

The Luminal A molecular subtype of clinical breast cancer, defined by the expression of ER-α and PR, is typically treated with selective ER modulators and/or aromatase inhibitors, with or without cytotoxic drugs (3-6). These treatments are frequently associated with systemic toxicity and acquired tumor resistance, compromising therapeutic efficacy. Relatively non-toxic nutritional herbal products are used as therapeutic alternatives in traditional Chinese medicine. However, the long-term clinical safety, efficacy and potential interaction of nutritional herbs with traditional therapies remain unclear. The present study utilized the human mammary carcinoma-derived MCF-7 cell line ($ER^+/PR^+/HER\text{-}2^-$) as a preclinical cell model of the Luminal A molecular subtype of breast cancer, in order to investigate the growth inhibitory effects of the nutritional herb EG and to identify possible molecular mechanisms for its efficacy.

MCF-7 cells that were maintained in a medium supplemented with 0.7% serum (E_2, <1 nM) retained their responsiveness to physiologically relevant concentrations of E_2. This response was demonstrated by an increase in the number of viable cells following treatment with 20 nM of E_2 compared with the serum-treated control group. In addition, at this physiological concentration E_2 decreased cell population doubling time, increased saturation density and enhanced AI growth compared with the corresponding control groups. These data are consistent with a recent study, where E_2 concentrations within the physiological range induced progressive increases in viable cell number (14). These results indicate that the method used in the current study produced an optimized E_2-responsive cell culture model for the Luminal A molecular subtype of breast cancer.

Dose-response analysis demonstrated that E_2-promoted cell growth was inhibited by EG in a dose-dependent manner, with an IC_{50} of 0.49% and IC_{90} of 1.03%. EG at a concentration of 2.0% demonstrated a cytotoxic response, indicated by a significantly reduced viable cell count, relative to the initial seeding density. These data indicate that EG antagonizes the growth-promoting effect of E_2.

The growth-promoting effect of E_2 on MCF-7 cells was demonstrated when investigating the effect of E_2 on cell cycle progression. These was a reduction in the G_1: S+G_2/M ratio of cells in the E_2-treated group compared with the serum-treated control group. Treatment of MCF-7 cells with E_2 resulted in a decreased percentage of cells in the G_1 phase, with a simultaneous increase in the percentage of cells in the S phase of the cell cycle. At its IC_{90} concentration, EG inhibited the E_2-induced cell cycle progression of MCF-7 cells, as demonstrated by a pronounced G_1 stage arrest and simultaneous decrease in the number of cells in the S+G_2/M phases of the cell cycle. This alteration in cell cycle progression was associated with an increase in the G_1: S+G_2/M ratio and suggests that the cytostatic effect of EG is predominantly due to the inhibition of E_2-promoted cell cycle progression.

Data from the present study on the cytostatic effect of EG at a relatively low dose are consistent with previous studies investigating the effects of extracts from other nutritional

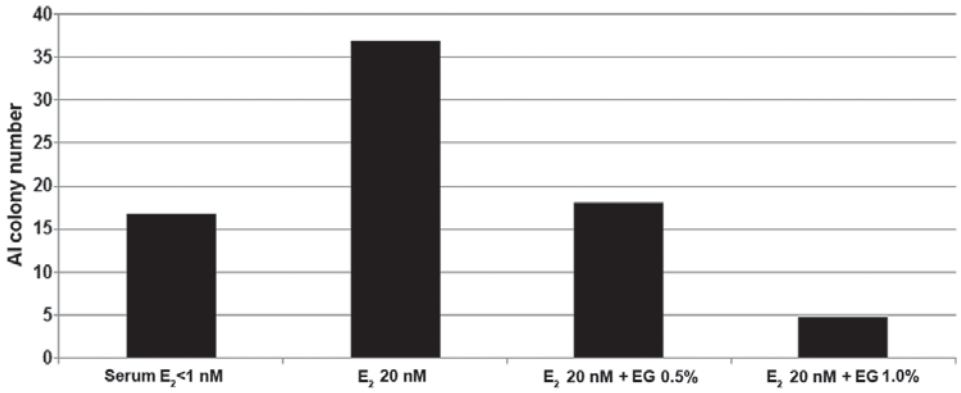

Figure 2. Effect of EG on the AI growth of MCF-7 cells. Results are presented as the mean ± standard deviation (n=12/treatment group). E_2 promoted AI growth of the cells, represented by the number of AI colonies, decreased in response to treatment with EG in a dose-dependent manner. Serum vs. E_2 20 nM (serum<E_2 α=0.05), E_2 20 nM vs. E_2 20 nM + EG 0.5% (E_2>E_2+EG α=0.05), E_2 20 nM vs. E_2 20 nM + EG 1.0% (E_2>E_2+EG α=0.05). Data was analyzed using one-way analysis of variance and Dunnett's test (α=0.05). E_2, 17β-estradiol, EG, *Epimedium grandiflorum*; AI, anchorage independent.

herbs, such as *Tabebuia avellandae (TA)*, *CO* and *LB,* on MCF-7 cells (9,10,13,14). The signaling pathways susceptible to extracts from TA, CO and LB include cell proliferation, cellular apoptosis, ER, xenobiotic metabolism and cell cycle progression through the modulated expression of specific genes (9,10,13,14). Additionally, these extracts affect the cellular metabolism of E_2, as demonstrated through the increased formation of specific anti-proliferative metabolites of E_2 (9-11,13,14). These results suggest that low-dose treatment with extracts from nutritional herbs is associated with an acceptable toxicity profile for future animal experiments and clinical trials.

The organ-selective growth-promoting effects of E_2 have been well established in cell culture and *in vivo* in animal models. Previously, E_2 has been demonstrated to promote the growth of ER-positive MCF-7 cells *in vitro,* and promote tumor development *in vivo* (15). In addition to the effects of E_2 on ER-dependent cellular signal transduction pathways (5,6), specific cellular metabolites of E_2 have been demonstrated to modulate the growth of mammary carcinoma-derived cells (16,17). The intermediate metabolite E_1 functions as a common precursor for the formation of the pro-mitogenic 16α-OHE_1 through the C16α-hydroxylation pathway, or for the formation of anti-proliferative 2-OHE_1 through the C2-hydroxylation pathway (16-21). The results of the present study demonstrated that treatment with EG significantly increased the formation of E_1, 2-OHE_1 and E_3, while the formation of 16α-OHE_1 remained essentially unaltered, when compared with the E_2-treated control group. Since the C2- and C16α-hydroxylation pathways represent two divergent metabolic pathways generating distinct E_2 metabolites, and since E_3 represents a proximate metabolite of 16α-OHE_1, the relative ratios of these metabolites serve as important endocrine biomarkers for carcinogenic risk (9,10). In the present study, E_2 metabolite ratios demonstrated that in response to the treatment with EG, the 2-OHE_1:16α-OHE_1 and the E_3:16α-OHE_1 ratios were substantially increased compared with the E_2-treated control group. This indicates that EG selectively induces increased formation of 2-OHE_1 and accelerates conversion of 16α-OHE_1 into E_3. Notably, similar modulation of the cellular metabolism of E_2 by extracts from CO and LB has been revealed (9,10,14).

AI growth represents a specific and sensitive *in vitro* surrogate end-point biomarker for tumorigenic phenotypes (9-11,14,20-22). In the current study, E_2 promoted the formation of AI colonies by MCF-7 cells. In response to treatment with EG, the number of AI colonies decreased in a dose-dependent manner, supporting the potential of EG as an anti-tumorigenic agent.

The results of the present study provide mechanistic evidence for the growth inhibitory effect of EG in a cellular model of ER-α^+/PR^+/HER-2^- Luminal A subtype breast cancer. A recent study compared the efficacy of several Chinese nutritional herbs on ER non-functional (ER-NF) and ER functional (ER-F) phenotypes of breast cancer (14), where EG demonstrated comparable growth inhibition in ER-NF and ER-F phenotypes, suggesting potential efficacy in ER-α^- and ER-α^+ breast cancer. In addition, an ongoing study into the growth inhibitory effects of EG in a ER-α^-/PR^-/HER-2^- triple-negative MDA-MB-231 breast cancer cell model has elucidated the potential molecular mechanisms underlying the cell cycle arrest and induction of cellular apoptosis (Telang *et al*, unpublished results). These results suggest that EG is an anti-tumorigenic agent in ER-α^- and triple-negative breast cancer, in addition to the Luminal A subtype investigated in the present study.

It is well known that traditional herbal medicine combines the use of several herbal preparations and that these herbs contain several bioactive agents. Traditionally, herbs are boiled in water and consumed by patients as herbal tea (7,8,23-25). Therefore, to simulate patient consumption, non-fractionated aqueous extracts from EG were used as the test agent in the present study. Despite the growth inhibitory effects of the non-fractionated aqueous extract of EG demonstrated in the current study, the identity of the water-soluble bioactive agent(s) in this extract remain unclear. Methanolic extracts from EG containing icariin and icaritin have been demonstrated to effect multidrug resistance in HepG2/ADR cells (26). In contrast, the same methanol soluble bioactive agents induce growth promoting effects through the upregulation of epidermal growth factor receptor/mitogen-activated protein kinase

signaling pathways in a model of HER-2$^+$ breast cancer (27). Furthermore, organic solvent fractionated extracts from *Epimedium brevicornum* containing the prenylflavone breviflavone B have been demonstrated to exhibit inhibitory effects on ER-α expression, in addition to on the growth of ER-α$^+$ breast cancer cells *in vitro* and *in vivo* (28-30). The growth inhibitory effect of non-fractionated aqueous extract from EG in the present study and the growth modulatory effects of organic solvent fractionated extracts from different *Epimedium* species in previous studies, suggest that EG has a pleiotropic effect, which is likely due to the presence of distinct constitutive bioactive components. In addition to the MCF-7 cell line representing a model of the Luminal A subtype of breast cancer, MCF-7 cells have provided a model for endocrine therapy-resistant cancer stem cells (31). Investigations into the response of cancer stem cell models to EG may identify potential therapeutic targets for stem cell therapy. A study using a similar approach has identified the antitumorigenic efficacy of the phytochemicals quercetin and sulforaphane in pancreatic cancer stem cells (32).

In conclusion, the present study has identified distinct phenomenological and mechanistic evidence for the antitumorigenic efficacy of EG in the Luminal A molecular subtype of breast cancer. The results of the present study validate a mechanism-driven cell culture approach to prioritize efficacious herbal medicinal products for the treatment of patients with the ER$^+$ Luminal A molecular subtype of breast cancer.

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