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Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells

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Abstract

Phytoestrogens have attracted attention as being safer alternatives to hormone replacement therapy (HRT) and as chemopreventive reagents for breast cancer because dietary soy isoflavone intake has been correlated with reduction in risk. To identify safe and effective phytoestrogen candidates for HRT and breast cancer prevention, we investigated the effects of daidzein, genistein, coumestrol, resveratrol and glycitein on cell growth, cell cycle, cyclin D1 expression, apoptosis, Bcl-2/Bax expression ratio and p53-dependent or NF-κB-dependent transcriptional activity in MCF-7 breast cancer cells. Phytoestrogens, except for glycitein, significantly enhanced estrogen-response-element-dependent transcriptional activity up to a level similar to that of 17β-estradiol (E_2). E_2 increased cell growth significantly, coumestrol increased cell growth moderately, and resveratrol and glycitein reduced cell growth. Phytoestrogens, except for glycitein, stimulated the promotion of cells to G_1/S transition in cell cycle analysis, similar to E_2. This stimulation was accompanied by transient up-regulation of cyclin D1. While genistein, resveratrol and glycitein all increased apoptosis and reduced the Bcl-2/Bax ratio, resveratrol reduced this ratio more than either genistein or glycitein. Moreover, resveratrol significantly enhanced p53-dependent transcriptional activity, but slightly reduced NF-κB-dependent transcriptional activity. On knockdown analysis, genistein, resveratrol and glycitein all reduced the Bcl-2/Bax ratio in the presence of apoptosis-inducing stimuli, and estrogen receptor (ER) α silencing had no effect on these reductions. In contrast, in the absence of apoptosis-inducing stimuli, only resveratrol reduced the ratio, and ERα silencing abolished this reduction. Thus, resveratrol might be the most promising candidate for HRT and chemoprevention of breast cancer due to its estrogenic activity and high antitumor activity.

Keywords: Phytoestrogens; Resveratrol; Estrogen receptor; Breast cancer; Apoptosis; Cell cycle

1. Introduction

Estrogens and estrogen receptor (ER) play an important role in the etiology of breast cancer. In ER-positive MCF-7 breast cancer cells, 17β-estradiol (E_2) induces rapid and transient up-regulation of cyclin D1 [1–4], followed by promotion to G_1/S transition of the cell cycle [2–4]. Although the mechanism of cyclin D1 up-regulation is still controversial, it may be indirectly regulated *via* CRE [5], AP-1 [5,6] and/or Sp1 site [7] by ligand-bound ER through interaction with other DNA-binding transcriptional factors. Alternatively, it could be up-regulated *via* nongenomic signaling pathways of the ER [8]. E_2 also induces antiapoptotic effects *via* an increased Bcl-2/Bax ratio [9,10], the reduction of which is considered a marker for mitochondrial contribution to apoptosis [9,11,12]. Taken together, E_2 is likely to contribute to the development and/or progression of breast cancer through cell cycle progression and antiapoptotic effects, as well as through other known or yet unknown mechanisms for tumor growth regulation.

Estrogens with or without progestin have been used for hormone replacement therapy (HRT) in postmenopausal women, but they increase the risk of breast cancer. In recent years, phytoestrogen supplements such as extracts from soy and red clover have become attractive as safer alternatives, and their efficacy has been investigated in clinical trials [13–15]. Phytoestrogens are natural plant substances found in food that exert weak estrogen-like activity toward mammals. For example, daidzein, genistein and glycitein exist in soybeans and soy products; coumestrol exists in mung bean sprouts and alfalfa sprouts; and resveratrol exists in grape skins and red wine. Previous epidemiological studies have reported a correlation between dietary intake of soy isoflavones such as genistein and a reduction in risk for breast cancer in China [16], the Netherlands [17] and Japan [18]. A meta-analysis of 18 epidemiological studies has revealed that soy intake is associated with a small reduction in breast cancer risk [19]; however, the effects of phytoestrogens on cancer prevention are still controversial [20–22]. Furthermore, genistein and daidzein, commonly studied soy isoflavones, have been reported to stimulate the growth of cancer cells *in vivo* and *in vitro* [21,23–27], suggesting an elevated breast cancer risk associated with some phytoestrogens. Therefore, it is important to assess the estrogen-like effects and antitumor effects of individual phytoestrogens.

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In this study, we investigated phytoestrogens to identify safer and more effective candidates for HRT and breast cancer prevention. We examined the effects of several kinds of dietary phytoestrogens, including daidzein, genistein, coumestrol, resveratrol and glycitein, on cell growth, cell cycle progression, expression of cyclin D1, apoptosis and the Bcl-2/Bax expression ratio, as well as p53-dependent or NF-κB-dependent transcriptional activity, on ER-positive breast cancer cells. We also performed experiments with and without E_2 to simulate premenopausal or postmenopausal status and to acquire useful information for HRT in postmenopausal women and breast cancer prevention in premenopausal and postmenopausal women.

2. Materials and methods

2.1. Reagents

E_2, daidzein, genistein, coumestrol, resveratrol, glycitein and activated charcoal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextran was obtained from Fluka Biochemica (Buchs, Switzerland). The ER antagonist ICI 182,780 was obtained from Tocris Bioscience (Ellisville, MO, USA).

2.2. Cells and culture

The human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Equitech-bio, Inc., Kerrville, TX, USA), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Plasmids and reporter gene assay

The estrogen-responsive reporter plasmid ptk-ERE-Luc [28] was kindly provided by Dr. S. Hayashi. The pRL-SV40 control vector (Toyo Ink Mfg. Co., Tokyo, Japan) was used as internal control for transfection efficiency. Estrogen response element (ERE)-dependent transcriptional activity was measured using a luciferase assay, as described previously [29], with slight modification. Briefly, after 4 days of cultivation in phenol-red-free DMEM (SAFC Biosciences, Lenexa, KS, USA) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), cells were seeded in phenol-red-free DMEM supplemented with 10% DCC-FBS on 24-well plastic culture plates. Cells were transiently transfected with 200 ng of ptk-ERE-Luc reporter plasmid and 4 ng of pRL-SV40 control vector using Lipofectamine Reagent (Invitrogen), in accordance with the manufacturer's instructions. The cells were then cultured in the presence or in the absence of 10^{-5} M phytoestrogen with or without 10^{-8} M E_2 for 24 h, and the luciferase activity of lysates was measured using the PicaGene Dual SeaPansy Luminescence Kit (Toyo Ink Mfg. Co.) and a MicroLumatPlus microplate luminometer LB96V (EG&G Berthold, Bad Wildbad, Germany). Transfection efficiency was normalized against *Renilla* luciferase activity using the pRL-SV40 control vector. To assess p53-dependent or NF-κB-dependent transcriptional activities, we transfected the p53-Luc plasmid (Stratagene, La Jolla, CA, USA) or pNF-κB-Luc plasmid (Stratagene) into cells using the same method as for the ptk-ERE-Luc plasmid. For analysis of p53-dependent transcriptional activities, cells were cultured with or without reagents for 48 h.

2.4. Colony formation assay

Colony formation assays were performed as previously described [29], with slight modification. Briefly, after 4 days of cultivation in phenol-red-free DMEM supplemented with 10% DCC-FBS, 1×10^4 cells were seeded in phenol-red-free DMEM supplemented with 10% DCC-FBS and agar (0.3% agar for the top layer and 0.5% agar for the base layer) in the presence or in the absence of 10^{-5} M phytoestrogen with or without 10^{-8} M E_2 or 10^{-6} M ICI 182,780, and then colonies were counted in triplicate, under a microscope, after 14 days.

2.5. Flow cytometry

Cell cycle profile was analyzed by staining DNA with propidium iodide in preparation for flow cytometry with the BD LSR/CellQuest system (Becton Dickinson, San Jose, CA, USA). Briefly, after 4 days of cultivation in phenol-red-free DMEM supplemented with 5% DCC-FBS, cells were cultured in the presence or in the absence of 10^{-5} M phytoestrogens with or without 10^{-8} M E_2 for 24 h, harvested and suspended in 1.12% sodium citrate containing 0.1% Nonidet P40 (Nacalai Tesque, Kyoto, Japan) and 250 μg/ml RNase A (Sigma-Aldrich), and incubated for 30 min at room temperature. Thereafter, cells were stained with 50 μg/ml propidium iodide (Calbiochem, San Diego, CA, USA) for 30 min at room temperature. A DNA histogram was obtained by analyzing 25,000 cells with the ModFitLT program (Verity Software House, Inc., Topsham, ME, USA). For analysis of apoptosis, after cells had been cultured in the presence or in the absence of 10^{-5} M phytoestrogens with or without 10^{-8} M E_2 or 10^{-6} M ICI 182,780 for 5 days in media containing 0.5% DCC-FBS, attached cells, together with floating cells, were collected and fixed with 80% ethanol and subjected to flow cytometry. Apoptosis was quantified as the fraction of the cell population in sub-G_1.

2.6. Real-time reverse transcription–polymerase chain reaction analysis

To determine the expression levels of cyclin D1, Bcl-2 and Bax, we performed real-time reverse transcription–polymerase chain reaction (RT-PCR) using a Thermal Cycler Dice Real Time System (Takara Bio, Inc., Shiga, Japan), in accordance with the manufacturer's instructions. After 4 days of cultivation in phenol-red-free DMEM supplemented with 5% DCC-FBS, cells were synchronized at G_0/G_1 by two additional days of cultivation in phenol-red-free DMEM supplemented with 0.5% DCC-FBS. Thereafter, we stimulated cells in the presence or in the absence of 10^{-5} M phytoestrogens with or without 10^{-8} M E_2 for 2 or 24 h, and total RNA was extracted from cells using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). Total RNA was subjected to cDNA synthesis using the PrimeScript RT reagent kit (Takara Bio, Inc.), and real-time RT-PCR was performed using the SYBR Premix Ex Taq (Takara Bio, Inc.). The Bcl-2/Bax ratio was calculated based on the expression levels of Bcl-2 and Bax.

2.7. Knockdown analysis for ERα

To investigate the involvement of ERα in the regulation of the Bcl-2/Bax mRNA ratio by E_2 and phytoestrogens, we seeded cells in phenol-red-free DMEM supplemented with 10% DCC-FBS on a 24-well plastic culture plate and then transfected them with 100 nmol of nontargeting negative control or ERα siRNA (On-TARGETplus siRNA SMARTpool Reagents; Dharmacon, Lafayette, CO, USA). After 48 h of cultivation under low (0.5%) or normal (5%) DCC-FBS conditions, cells were stimulated in the presence or in the absence of 10^{-8} M E_2 or 10^{-5} M phytoestrogen for 24 h, and real-time RT-PCR for Bcl-2 and Bax was performed. The Bcl-2/Bax ratio was calculated as described above. Silencing of ERα was confirmed by Western blot analysis, as described previously [29], using anti-ERα rabbit polyclonal antibody (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin mouse monoclonal antibody (AC-15; Sigma, St. Louis, MO, USA) as internal controls 48 h after siRNA transfection.

2.8. Statistical analysis

Data are expressed as the mean±standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using Tukey's multiple comparison test, and $P<.05$ was considered significant. In knockdown experiments, results in control cells were compared with those in ERα siRNA-transfected cells using Student's *t* test. Correlations between colony formation activity and G_1/S transition of the cell cycle, expression of cyclin D1, apoptosis and Bcl-2/Bax expression ratio were analyzed using the Pearson Product–Moment Correlation Coefficient.

3. Results

3.1. Effects of estrogen and phytoestrogens on ERE-dependent transcriptional activity

To confirm the estrogen-like effects of individual phytoestrogens, we performed luciferase assays using ERE reporter plasmids. The binding affinities of the phytoestrogens daidzein, genistein and coumestrol for ERα are reported to be about 20,000-fold, 3000-fold and 2000-fold weaker than that of E_2, respectively [30,31]. We performed experiments using a phytoestrogen concentration of 10^{-5} M, as this concentration is reportedly required for significant activation of ERE-dependent transcriptional activity by daidzein, genistein and resveratrol [32]. An E_2 concentration of 10^{-8} M was used in this study because the maximal plasma level of endogenous E_2 in premenopausal women is between 10^{-9} and 10^{-8} M. As expected, all the tested phytoestrogens, except for glycitein, significantly enhanced transcriptional activity up to the level to which E_2 alone increases it ($P<.01$), around a 40-fold to 70-fold increase. In comparison, glycitein induced only about a 5-fold activation compared with control (Fig. 1A). In the presence of E_2, all phytoestrogens stimulated ERE-dependent transcriptional activity cooperatively with E_2 (Fig. 1B). Since the transactivating properties of the phytoestrogens at 10^{-5} M concentration were similar to those of E_2 at 10^{-8} M concentration, the following experiments were performed using these concentrations.

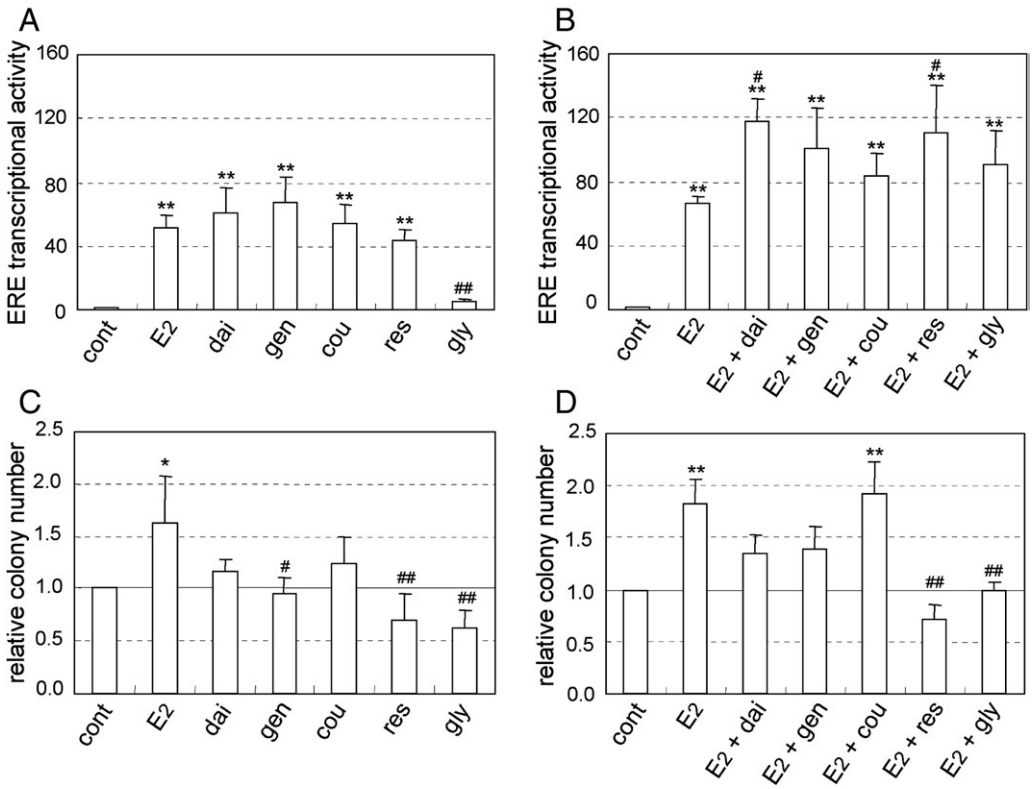

Fig. 1. Effects of estrogen and phytoestrogens on ERE-dependent transcriptional activity and cell growth. To assess transcriptional activity, we transiently transfected MCF-7 cells with the ptk-ERE-Luc reporter plasmid, together with the pRL-SV40 control vector, and then cultured them in the presence or in the absence of 10^{-5} M phytoestrogen alone (A) or with 10^{-8} M E_2 (B). The lysates were assayed for luciferase activity, and activities relative to those of control were determined. To determine the effects on cell growth, we performed colony formation assays in the presence or in the absence of 10^{-5} M phytoestrogen alone (C) or with 10^{-8} M E_2 (D). Results are representative of at least three independent experiments. Columns, means; bars, S.D. *P<.05, compared with control; **P<.01, compared with control; $^{\#}P$<.05, compared with E_2; $^{\#\#}P$<.01, compared with E_2; Tukey's multiple comparison test. cont, control; dai, daidzein; gen, genistein; cou, coumestrol; res, resveratrol; gly, glycitein.

3.2. Effects of estrogen and phytoestrogens on colony formation activity in soft agar

To evaluate the effects of E_2 and phytoestrogens on cell growth, we performed colony formation assays in soft agar. E_2 significantly increased the number of colonies (P<.05), while coumestrol moderately increased the number of colonies. Resveratrol and glycitein reduced the number of colonies by 30% and 38% of the original colony numbers, respectively. The number of colonies that resulted when cells were treated with genistein, resveratrol or glycitein was significantly less than the number of colonies that resulted when cells were treated with E_2 (P<.05 for genistein and P<.01 for resveratrol and glycitein; Fig. 1C). In the presence of E_2, only coumestrol significantly increased the number of colonies compared with control (P<.01). On the other hand, resveratrol and glycitein dramatically inhibited the stimulatory effect of E_2 on cell growth, resulting in 28% and 1% reduction of the original colony numbers, respectively. Daidzein and genistein treatment moderately increased cell growth in the presence of E_2 (Fig. 1D). These data suggest that resveratrol might have its greatest antitumor effect independent of the presence of E_2, despite showing inconsistency with estrogenic effects measured by reporter gene assay (Fig. 1A and B).

3.3. Effects of estrogen and phytoestrogens on cell cycle distribution and expression of cyclin D1

Tumor growth is likely to be regulated both by cell cycle control and by apoptosis. To understand the mechanism of the effects of phytoestrogens on tumor growth, we first analyzed cell cycle distributions using flow cytometry. E_2 and all the phytoestrogens significantly decreased the size of the fractions in G_1 and increased S and G_2/M fractions compared with control (P<.01 for all); however, the effect of glycitein was less than those of E_2 and other phytoestrogens (Fig. 2A). The combination of the phytoestrogens with E_2 showed no effect on E_2-mediated cell cycle distributions (Fig. 2B).

We also examined the expression of cyclin D1 in G_0/G_1-phase-synchronized cells using real-time RT-PCR. Cyclin D1 is a gene product that has been reported to be involved in the G_1/S transition of the cell cycle [2–4]. E_2 and all the phytoestrogens, except for glycitein, transiently up-regulated cyclin D1 expression in 2 h (Fig. 2C), comparable to the effect of E_2 previously reported [1–4]. The combination of the phytoestrogens with E_2 showed no cooperative or inhibitory effect on cyclin D1 expression (Fig. 2D). These results are consistent with those of the G_1/S transition in flow cytometry (Fig. 2A and B). However, these effects on cell cycle regulation and cyclin D1 up-regulation were not consistent with cell growth results (Fig. 1A and B), suggesting that regulation of the cell cycle may not be central to the involvement of phytoestrogens in controlling cell growth.

3.4. Effects of estrogen and phytoestrogens on apoptosis, Bcl-2/Bax ratio and p53-dependent or NF-κB-dependent transcriptional activity

In addition to regulation of cell cycle progression, tumor growth is also regulated by apoptosis. To investigate the proapoptotic or antiapoptotic effects of E_2 and phytoestrogens, we next used flow cytometry to determine the sub-G_1 fraction, which is defined as the fraction of cells containing less DNA than cells in G_1 after culturing

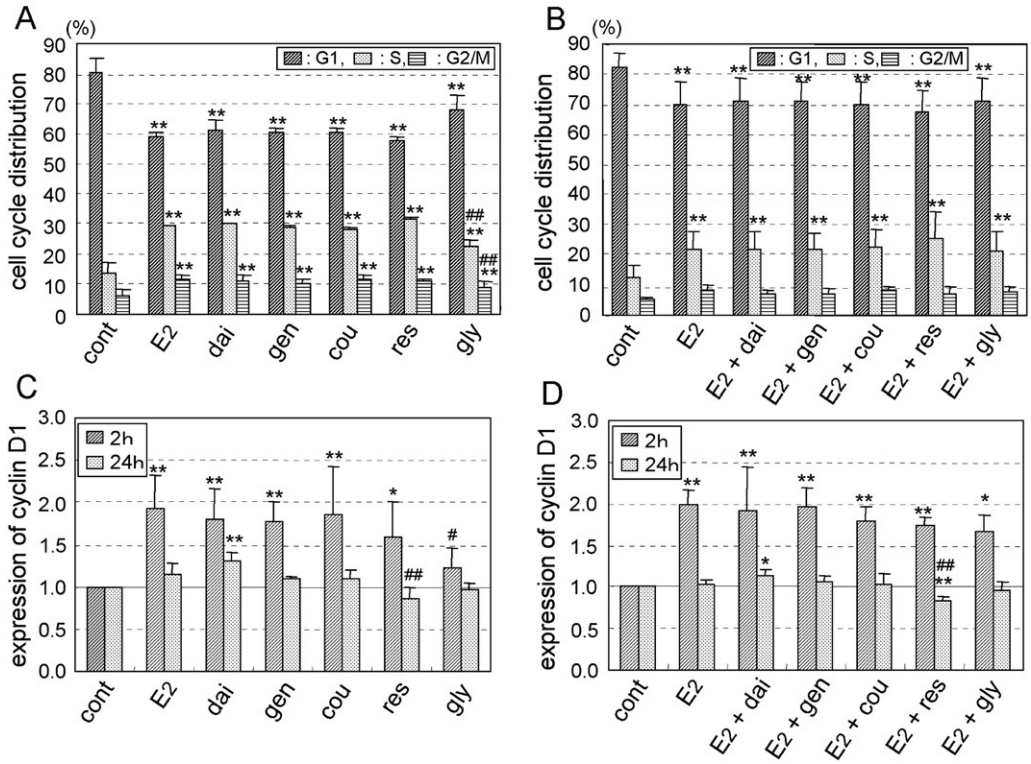

Fig. 2. Effects of estrogen and phytoestrogens on cell cycle distribution and cyclin D1 mRNA expression. For cell cycle analysis, MCF-7 cells were cultured in the presence or in the absence of 10^{-5} M phytoestrogen alone (A) or with 10^{-8} M E_2 (B) for 24 h, and cell cycle profile was analyzed by flow cytometry. To detect the level of cyclin D1 mRNA expression, we cultured G_0/G_1-phase-synchronized cells in the presence or in the absence of 10^{-5} M phytoestrogen alone (C) or with 10^{-8} E_2 (D) for 2 or 24 h. Total RNA extracted from the cells was subjected to cDNA synthesis and real-time RT-PCR. Results are representative of at least three independent experiments. Columns, means; bars, S.D. *P<.05, compared with control; **P<.01, compared with control; #P<.05, compared with E_2; ##P<.01, compared with E_2; Tukey's multiple comparison test. cont, control; dai, daidzein; gen, genistein; cou, coumestrol; res, resveratrol; gly, glycitein.

cells in low serum conditions to induce apoptosis. E_2 decreased the fraction of apoptotic cells, whereas genistein, resveratrol and glycitein increased it. Daidzein and coumestrol induced no obvious changes (Fig. 3A). The combination of phytoestrogens with E_2 induced effects similar to those induced by phytoestrogens alone (Fig. 3B).

To examine the molecular mechanisms of the effects of phytoestrogens on apoptosis, we investigated the expression of the Bcl-2 antiapoptotic and Bax proapoptotic genes in G_0/G_1-phase-synchronized cells using real-time RT-PCR and determined the Bcl-2/Bax ratio. A reduction in this ratio is a marker for mitochondrial involvement in apoptosis. The ratio was reduced by genistein, resveratrol and glycitein in 24 h independent of the presence of E_2, with a significant reduction by resveratrol (Fig. 3C and D). These results are consistent with apoptosis status determined by flow cytometry (Fig. 3A and B).

The Bcl-2 promoter is regulated negatively by p53 [33] and positively by NF-κB [34]. Conversely, the Bax promoter is regulated positively by p53 [35] and negatively by NF-κB [36]. Taken together, activation of the p53 signaling pathway and/or inhibition of the NF-κB signaling pathway might decrease the Bcl-2/Bax ratio. To investigate the mechanisms of the effects of phytoestrogens on the Bcl-2/Bax ratio, we determined p53-dependent and NF-κB-dependent transcriptional activities. Daidzein, genistein, resveratrol and glycitein significantly enhanced p53-dependent transcriptional activity (P<.01 for daidzein, genistein and resveratrol; P<.05 for glycitein), while E_2 and coumestrol had no effect on the p53-dependent transcriptional activity (Fig. 3E). On the other hand, daidzein, genistein, coumestrol and glycitein tended to increase NF-κB-dependent transcriptional activity, whereas resveratrol slightly reduced it by 20% (Fig. 3F). As only resveratrol enhanced p53-dependent transcriptional activity, but not NF-κB-dependent transcriptional activity, this alteration might be a possible mechanism of reducing the Bcl-2/Bax ratio by resveratrol.

3.5. Correlations between colony formation activity and G_1/S transition, cyclin D1 expression, apoptosis and Bcl-2/Bax ratio

To understand whether cell cycle regulation or apoptosis is more important in regulating tumor growth mediated by phytoestrogens, we investigated whether colony formation activity correlates with G_1/S transition, cyclin D1 expression, apoptosis or the Bcl-2/Bax ratio in the presence or in the absence of estrogen and phytoestrogens. In cells treated with or without estrogen or phytoestrogens, no correlation was observed between cell growth activity and G_1/S transition (r=−.254, P=.583; Fig. 4A), and little correlation was seen between cell growth and expression of cyclin D1 after 2 h (r=.598, P=.156; Fig. 4B). On the other hand, a significant negative correlation was observed between cell growth activity and apoptosis (r=−.944, P=.001; Fig. 4C), and a weaker correlation was observed between cell growth activity and the Bcl-2/Bax ratio (r=.703, P=.078; Fig. 4D), suggesting considerable involvement of apoptosis and reduction in the Bcl-2/Bax ratio in the regulation of cell growth mediated by phytoestrogens.

3.6. Analysis of ER involvement in cell growth, apoptosis and regulation of the Bcl-2/Bax ratio

As ERs have an important role in the etiology of breast cancers, it is important to evaluate the involvement of ER in the effects of

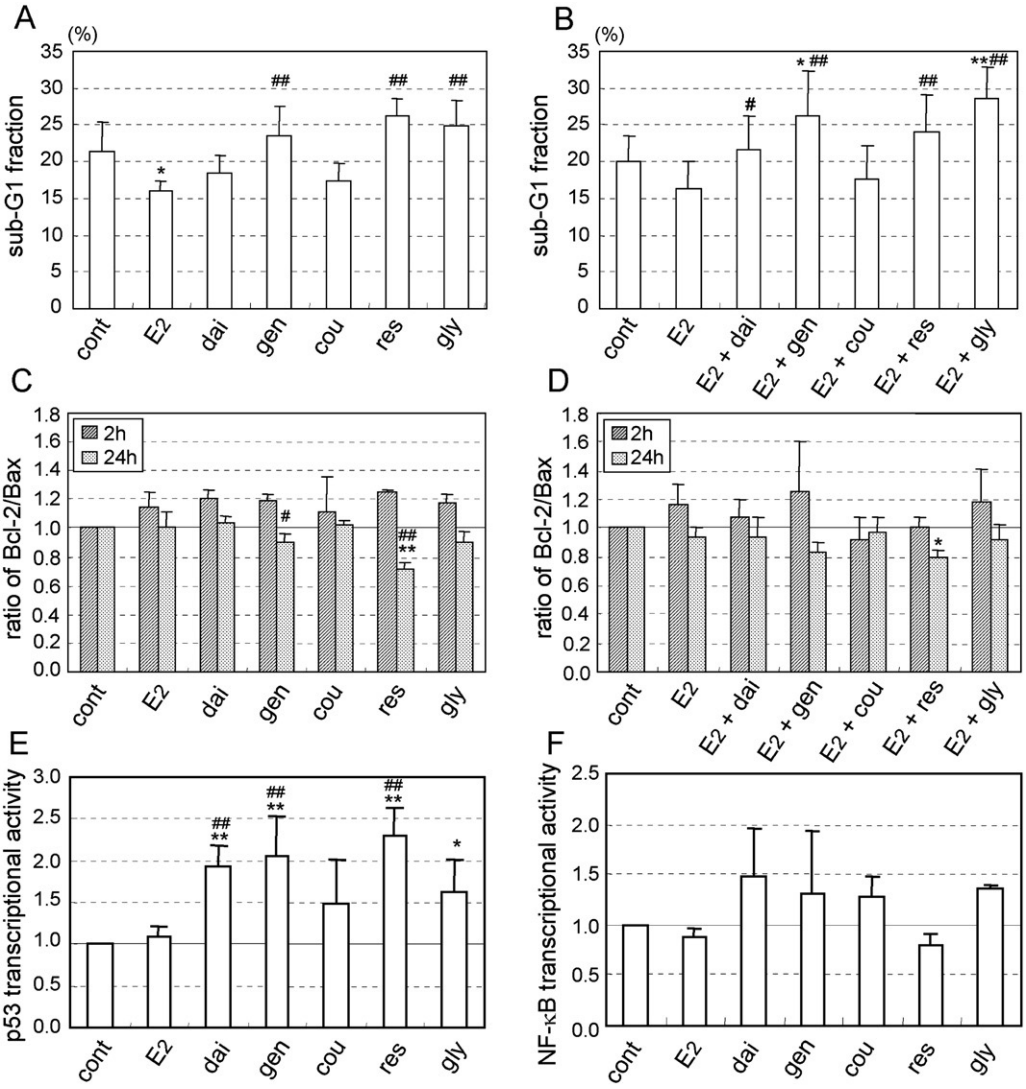

Fig. 3. Effects of estrogen and phytoestrogens on apoptosis, Bcl-2/Bax mRNA ratio and p53-dependent or NF-κB-dependent transcriptional activity. To examine apoptosis, we cultured MCF-7 cells in the presence or in the absence of 10^{-5} M phytoestrogens alone (A) or with 10^{-8} M E_2 (B) for 5 days under 0.5% serum conditions, and the ratio of the sub-G_1 fraction was analyzed with flow cytometry. The expression of Bcl-2 and Bax mRNA was determined using real-time RT-PCR in the presence or in the absence of 10^{-5} M phytoestrogen alone (C) or with 10^{-8} M E_2 (D) for 2 or 24 h, and then the Bcl-2/Bax mRNA ratios were calculated. To assess p53-dependent (E) or NF-κB-dependent (F) transcriptional activity, we transiently transfected MCF-7 cells with the p53-Luc or pNF-κB-Luc plasmid, together with the pRL-SV40 control vector, and then cultured them in the presence or in the absence of 10^{-8} M E_2 or 10^{-5} M phytoestrogen. Each lysate was assayed for luciferase activity, and activities relative to those of control were determined. Results are representative of at least three independent experiments. Columns, means; bars, S.D. *P<.05, compared with control; **P<.01, compared with control; $^{\#}P$<.05, compared with E_2; $^{\#\#}P$<.01, compared with E_2; Tukey's multiple comparison test. cont, control; dai, daidzein; gen, genistein; cou, coumestrol; res, resveratrol; gly, glycitein.

phytoestrogens. Therefore, we investigated the effects of phytoestrogens on colony formation and apoptosis in the presence of ICI 182,780. Furthermore, to determine whether ERα is involved in the reduction of the Bcl-2/Bax ratio by genistein, resveratrol and glycitein, we performed ERα knockdown analysis under normal (5% serum) or apoptosis-inducing (0.5% serum) conditions.

On colony formation assay, ICI 182,780 and the combination of all phytoestrogens with ICI 182,780 significantly reduced the number of colonies by 81% and 80–88% of the original colony numbers, respectively (P<.01). Resveratrol and glycitein especially enhanced the inhibitory effect of ICI 182,780 with 88% and 85% reduction of the original colony numbers, respectively, but they were not significant compared to that of ICI 182,780 alone (Fig. 5A). On apoptosis analysis, ICI 182,780 and the combination of all phytoestrogens with ICI 182,780 significantly increased the number of apoptotic cells (P<.01 for all); combined treatment of genistein, resveratrol or glycitein with ICI 182,780 significantly increased the number of apoptotic cells compared to treatment with ICI 182,780 alone (P<.01; Fig. 5B). These data suggested that the apoptosis-inducing effect of genistein, resveratrol and glycitein is exerted *via* an ER-independent pathway.

Transfection of siRNA for ERα drastically decreased the level of ERα protein expression (Fig. 5C). Under low serum conditions, genistein, resveratrol and glycitein reduced the Bcl-2/Bax ratio in control siRNA-transfected cells (Fig. 5D), resembling the results for G_0/G_1-phase-synchronized cells (Fig. 3C). ERα silencing showed no effect on the reduction of the Bcl-2/Bax ratio by genistein, resveratrol and glycitein (Fig. 5D). These data suggest that these three phytoestrogens enhance induction of apoptosis in the presence of apoptosis-inducing stimuli *via* an ERα-independent pathway. In the presence of E_2, the Bcl-2/Bax ratio in ERα siRNA-transfected cells was significantly lower than that in control siRNA-transfected cells (Fig. 5D), indicating functional repression of ERα as a positive control of siRNA transfection. Under normal serum conditions, only resveratrol significantly reduced the Bcl-2/Bax ratio in control siRNA-transfected

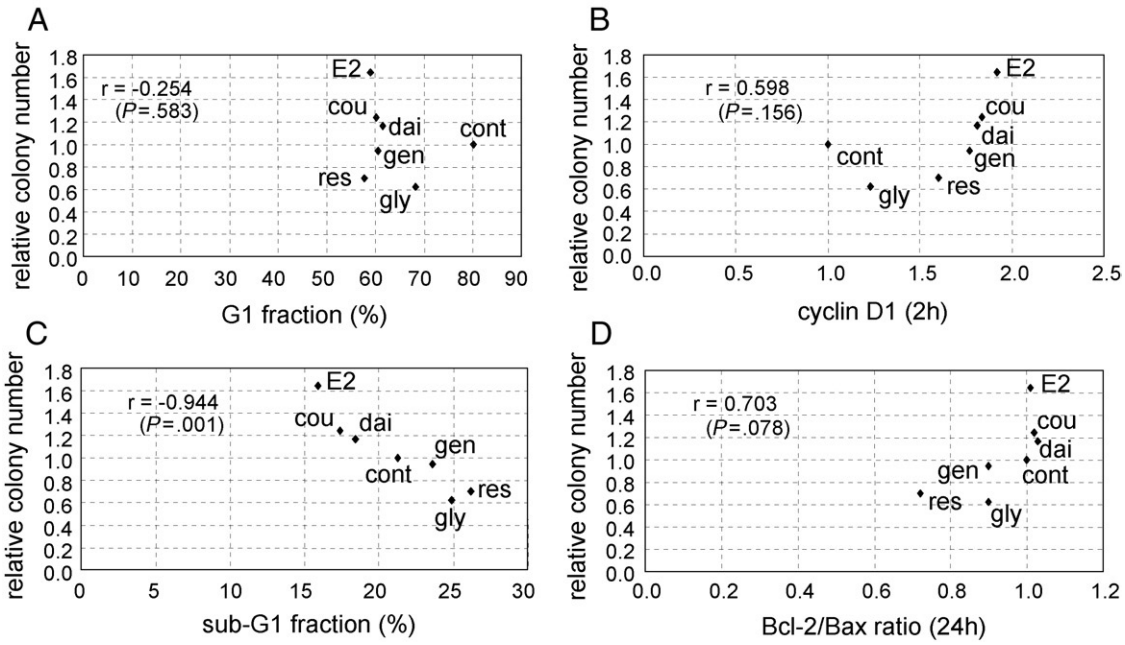

Fig. 4. Correlations between cell growth and G_1/S transition, cyclin D1 expression, apoptosis and Bcl-2/Bax ratio in MCF-7 cells cultured in the presence or in the absence of 10^{-8} M estrogen or 10^{-5} M phytoestrogen. Cell growth (from Fig. 1C) is compared with the G_1 fraction of the cell cycle (from Fig. 2A) (A), as well as with cyclin D1 expression (from Fig. 2C) (B), sub-G_1 fraction (from Fig. 3A) (C) and the Bcl-2/Bax ratio (from Fig. 3C) (D). Data were analyzed using the Pearson Product–Moment Correlation Coefficient. cont, control; dai, daidzein; gen, genistein; cou, coumestrol; res, resveratrol; gly, glycitein.

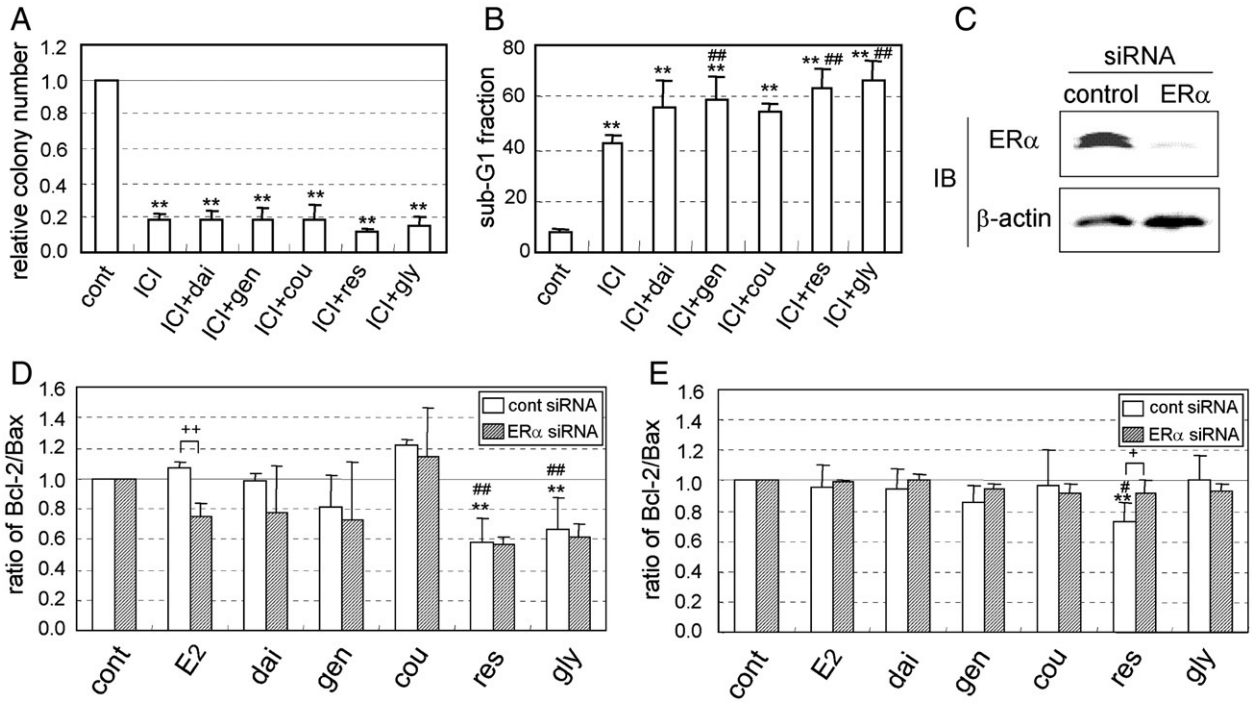

Fig. 5. Analysis of ER involvement in cell growth, apoptosis and regulation of the Bcl-2/Bax ratio. To determine the involvement of ER in the effects of phytoestrogens on cell growth, we performed colony formation assays in the presence or in the absence of 10^{-5} M phytoestrogen with 10^{-6} M ICI 182,780 (A). To examine the involvement of ER in the effects of phytoestrogens on apoptosis, we cultured MCF-7 cells in the presence or in the absence of 10^{-5} M phytoestrogen with 10^{-6} M ICI 182,780 for 5 days under 0.5% serum conditions, and the ratio of the sub-G_1 fraction was analyzed with flow cytometry (B). The expression of ERα protein in MCF-7 cells transfected with a nontargeting negative control or ERα siRNA was confirmed by Western blot analysis 48 h after transfection with siRNA (C). MCF-7 cells were then cultured for 24 h in the presence or in the absence of 10^{-8} M E_2 or 10^{-5} M phytoestrogen, and the Bcl-2/Bax ratio was determined under 0.5% (D) or 5% (E) serum conditions. Results are representative of at least three independent experiments (A, B, D and E). Columns, means; bars, S.D. $^{**}P<.01$, compared with control; $^{\#}P<.05$, compared with E_2; $^{\#\#}P<.01$, compared with E_2; Tukey's multiple comparison test; $^{\dagger}P<.05$, $^{\dagger\dagger}P<.01$, Student's t test. cont, control; dai, daidzein; gen, genistein; cou, coumestrol; res, resveratrol; gly, glycitein; ICI, ICI 182,780.

cells, and this effect was abolished by ERα siRNA transfection (Fig. 5E). Thus, the proapoptotic effect by resveratrol may be mediated, at least in part, *via* ERα, in contrast with the effect observed in the presence of apoptosis-inducing stimuli.

4. Discussion

Recently, some phytoestrogens have been considered as alternatives to HRT in postmenopausal women and as chemopreventive reagents, especially against hormone-dependent tumors. In this study, we investigated potential candidates for HRT and/or chemoprevention of breast cancer by analyzing the effects of several kinds of dietary phytoestrogens on tumor cell growth, cell cycle and apoptosis. Our results indicate that coumestrol stimulates growth, but not apoptosis, independent of the presence of E_2. Although coumestrol is found in several foods such as mung bean sprouts [37], alfalfa sprouts [37] and supplements made from kudzu [38], the amount is considerably low [37,38]. Therefore, it is unlikely that coumestrol from foods or supplements would increase the risk of breast cancer, even though the relationship between coumestrol and risk of cancer has not been well elucidated.

We also investigated the effects of soy isoflavones such as daidzein, genistein and glycitein, which are contained in soybeans, soy products [22,37] and supplements made from soy extract, red clover extract and semipurified isoflavones [15,38,39]. Their concentrations in foods and supplements are relatively high compared with the concentrations of other phytoestrogens [15,38,39]. In this study, specific characteristics were found for each phytoestrogen. Daidzein showed no antitumor activity in the absence of E_2 and only a partial suppressive effect on E_2-stimulated tumor growth. Genistein showed a similar effect on cell growth as daidzein, and only genistein induced apoptosis, as well as a slight reduction in the Bcl-2/Bax ratio. Glycitein repressed cell growth, induced apoptosis and slightly reduced the Bcl-2/Bax ratio. The effect of glycitein on endogenous ER transactivation was much weaker compared with the effect of the other phytoestrogens. A higher dose of glycitein may induce a stronger transactivation of endogenous ER; however, this was not examined in this study.

Among the isoflavones obtained from foods such as soy and soy products, genistein is the most abundant phytoestrogen. On the other hand, some supplements predominantly contain daidzein [13,38]. Therefore, the risk of breast cancer may be a concern when administering these supplements, as our results indicate that daidzein causes a slight cell-stimulating effect in the absence of E_2, which may lead to an increased risk of breast cancer in postmenopausal women taking supplements containing these phytoestrogens. In fact, elevated serum concentrations of phytoestrogens (which are higher than those in our experiments) in postmenopausal women administered these supplements, especially daidzein, have been reported [13]. However, the effect of such supplements on the risk of breast cancer has not been well documented.

Resveratrol, which is contained in grape skin, and its products such as red wine repressed cell growth, induced apoptosis and significantly reduced the Bcl-2/Bax ratio independent of the presence of E_2. Because it stimulated the transcription of endogenous ER and proapoptotic effects, this phytoestrogen is the most promising candidate as an HRT alternative and chemopreventive reagent for breast cancer. However, the mechanism by which resveratrol represses cell growth needs to be further investigated. In synchronized cells, genistein appears to up-regulate both Bcl-2 and Bax in 24 h, resveratrol appears to up-regulate only Bax and glycitein appears to induce small changes in the expression of these two genes (data not shown). These effects result in a greater reduction in the Bcl-2/Bax ratio by resveratrol compared to reduction by genistein or glycitein, suggesting different mechanisms involved in the induction of apoptosis mediated by these three phytoestrogens. Moreover, resveratrol appears to enhance p53-dependent transcriptional activity and to slightly reduce NF-κB-dependent transcriptional activity, indicating a possible mechanism of reducing the Bcl-2/Bax ratio through regulation of the Bcl-2 and Bax promoters by p53 and NF-κB [33–36].

In breast cancer, ER status is one of the most important prognostic factors and determinants of treatment strategy. Therefore, we determined whether ER is involved in the induction of apoptosis by genistein, resveratrol and glycitein. Under low serum conditions, which are apoptosis-inducing stimuli, all three phytoestrogens induced a reduction in the Bcl-2/Bax ratio, with significant decreases by resveratrol and glycitein. As ERα silencing was ineffective at reducing the Bcl-2/Bax ratio (Fig. 5D), these phytoestrogens appear to enhance the proapoptotic effect by low serum stimuli *via* an ERα-independent pathway. In addition, genistein, resveratrol and glycitein also induced ER-independent apoptosis (Fig. 5B). Resveratrol and glycitein enhanced the inhibitory effect of ICI 182,780 (Fig. 5A), suggesting the ER-independent cell growth inhibition by these two phytoestrogens. Although we could not detect ER-independent cell growth inhibition by genistein, it might be possible that the inhibitory effect of ICI 182,780 on cell growth may have been too strong to detect additional minor effects by genistein. As resveratrol has been reported to induce apoptosis *via* other signaling pathways such as p53 activation [40] or rapid dissociation of mitochondrial membrane potential [41], these mechanisms may be involved in the ERα-independent induction of apoptosis.

Under normal serum conditions, which are poor apoptosis-inducing stimuli, resveratrol, but not genistein or glycitein, significantly reduced the Bcl-2/Bax ratio, which is abolished by ERα silencing. Thus, resveratrol may induce proapoptotic effects even in the absence of apoptosis-inducing stimuli, at least in part *via* ERα, contrary to the mechanism in the presence of apoptosis-inducing stimuli. Among the phytoestrogens we examined, resveratrol seems to exert the strongest proapoptotic effect, most likely through ER-independent mechanisms and, at least in part, ER-dependent mechanisms.

E_2 acts through the ER signaling pathway, including the classical pathway *via* ERE; transcriptional regulation by ER through interaction with other DNA-binding transcriptional factors such as c-Fos/c-JunB (AP-1) [42,43] and Sp1 [44]; or a nongenomic pathway such as rapid activation of Src/MAP kinase [45], PI3 kinase/Akt [46], IP3-PKC-α [47] and cAMP [48] signaling pathways. Although the intact helix 12 of ERα is required for E_2-induced ERE-dependent transactivation, it is not necessarily required for transactivation by resveratrol [49]. Therefore, E_2 and phytoestrogens are considered to show different effects on apoptosis, *via* both ER-dependent and ER-independent signaling pathway. In this study, genistein, resveratrol and glycitein seemed to induce apoptosis *via* an ER-independent pathway and did not activate MAP kinase and Akt (preliminary data; data not shown), which are reported to be regulated by E_2 through ER nongenomic pathways [45,46]. From these findings, we speculated that MAP kinase and Akt activation *via* ER might not be involved in apoptosis induction by phytoestrogens at least under our experimental conditions. Although Bcl-2 is up-regulated by estrogen [9,50,51] and we found significantly enhanced p53-dependent transcriptional activity and slightly reduced NF-κB-dependent transcriptional activity by resveratrol, further analysis is needed to understand the detailed mechanism of apoptosis regulation by individual phytoestrogens.

In addition to the type of phytoestrogen, concentration may play a major factor. Genistein administered at a lower physiological level elicits an expression pattern suggestive of increased mitogenic activity, and genistein administered at a higher pharmacological level induces a pattern that likely contributes to increased apoptosis [52]. Furthermore, *in vitro* and *in vivo* studies have shown that low levels of phytoestrogens stimulate cell growth [21,23–27], while higher levels of phytoestrogens repress cell growth [21,24]. This study

used the higher pharmacological level of phytoestrogens (10^{-5} M), which is comparable to the serum level when dietary supplements are administered [13]. Under these conditions, most of the phytoestrogens maintained their estrogenic activities and cell-cycle-stimulating effects similar or equivalent to those observed at the physiological level of endogenous estrogen. Thus, the pharmacological concentration of phytoestrogens may be potent enough to stimulate cell growth.

In conclusion, we found a significant negative correlation between tumor growth activity and apoptosis, suggesting that apoptotic action might be the major factor leading to repression of cell growth. Moreover, resveratrol might be the most promising candidate for HRT and breast cancer chemoprevention due to its estrogen-like activity and high antitumor activity. Although further animal experiments and studies of molecular mechanisms in other cancer cell lines are necessary to clarify the risks and benefits associated with phytoestrogens, our findings can be applied to the development of novel, safer strategies for HRT in postmenopausal women and chemoprevention of breast cancer.

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References

[1] Frasor J, Danes JM, Komm B, Chang KCN, Lyttle CR, Katzenellenbogen BS. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003;144:4562–74.
[2] Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, et al. 17β-Estradiol induces cyclin D_1 gene transcription, $p36^{D1}$–$p34^{cdk4}$ complex activation and $p105^{Rb}$ phosphorylation during mitogenic stimulation of G_1-arrested human breast cancer cells. Oncogene 1996;12:2315–24.
[3] Planas-Silva MD, Weinberg RA. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol Cell Biol 1997;17:4059–69.
[4] Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW, Sutherland RL. Estrogen-induced activation of Cdk4 and Cdk2 during G_1–S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem 1997;272:10882–94.
[5] Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, et al. Opposing action of estrogen receptors α and β on cyclin D1 gene expression. J Biol Chem 2002;277:24353–60.
[6] Shiozawa T, Miyamoto T, Kashima H, Nakayama K, Nikaido T, Konishi I. Estrogen-induced proliferation of normal endometrial glandular cells is initiated by transcriptional activation of cyclin D1 *via* binding of c-Jun to an AP-1 sequence. Oncogene 2004;23:8603–10.
[7] Castro-Rivera E, Samudio I, Safe S. Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. J Biol Chem 2001;276:30853–61.
[8] Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A. Distinct nongenomic signal transduction pathways controlled by 17β-estradiol regulate DNA synthesis and cyclin D_1 gene transcription in HepG2 cells. Mol Biol Cell 2002;13:3720–9.
[9] Huang Y, Ray S, Reed JC, Ibrado AM, Tang C, Nawabi A, et al. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits Taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res Treat 1997;42:73–81.
[10] Lewandowski SA, Thiery J, Jalil A, Leclercq G, Szczylik C, Chouaib S. Opposite effects of estrogen receptors alpha and beta on MCF-7 sensitivity to the cytotoxic action of TNF and p53 activity. Oncogene 2005;24:4789–98.
[11] Rafi MM, Rosen RT, Vassil A, Ho CT, Zhang H, Ghai G, et al. Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid. Anticancer Res 2000;20:2653–8.
[12] Leung LK, Wang TTY. Differential effects of chemotherapeutic agents on the Bcl-2/Bax apoptosis pathway in human breast cancer cell line MCF-7. Breast Cancer Res Treat 1999;55:73–83.
[13] Khaodhiar L, Ricciotti HA, Li L, Pan W, Schickel M, Zhou J, et al. Daidzein-rich isoflavone aglycones are potentially effective in reducing hot flashes in menopausal women. Menopause 2008;15:125–32.
[14] Tice JA, Ettinger B, Ensrud K, Wallace R, Blackwell T, Cummings SR. Phytoestrogen supplements for the treatment of hot flashes: the Isoflavone Clover Extract (ICE) Study: a randomized controlled trial. JAMA 2003;290:207–14.
[15] Booth NL, Piersen CE, Banuvar S, Geller SE, Shulman LP, Farnsworth NR. Clinical studies of red clover (*Trifolium pratense*) dietary supplements in menopause: a literature review. Menopause 2006;13:251–64.
[16] Dai Q, Franke AA, Jin F, Shu XO, Hebert JR, Custer LJ, et al. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. Cancer Epidemiol Biomarkers Prev 2002;11:815–21.
[17] Verheus M, van Gils CH, Keinan-Boker L, Grace PB, Bingham SA, Peeters PHM. Plasma phytoestrogens and subsequent breast cancer risk. J Clin Oncol 2007;25:648–55.
[18] Iwasaki M, Inoue M, Otani T, Sasazuki S, Kurahashi N, Miura T, et al. Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case–control study from the Japan Public Health Center-based prospective study group. J Clin Oncol 2008;26:1677–83.
[19] Trock BJ, Hilakivi-Clarke L, Clarke R. Meta-analysis of soy intake and breast cancer risk. J Natl Cancer Inst 2006;98:459–71.
[20] Peeters PHM, Keinan-Boker L, van der Schouw YT, Grobbee DE. Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res Treat 2003;77:171–83.
[21] Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr 2004;91:513–31.
[22] Dixon RA. Phytoestrogens. Annu Rev Plant Biol 2004;55:225–61.
[23] Rice S, Whitehead SA. Phytoestrogens and breast cancer — promoters or protectors? Endocr Relat Cancer 2006;13:995–1015.
[24] Hsieh CY, Santell R, Haslam SZ, Helferich WG. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. Cancer Res 1998;58:3833–8.
[25] Ju YH, Allred KF, Allred CD, Helferich WG. Genistein stimulates growth of human breast cancer cells in a novel, postmenopausal animal model, with low plasma estradiol concentrations. Carcinogenesis 2006;27:1292–9.
[26] Seo HS, DeNardo DG, Jacquot Y, Laïos I, Vidal DS, Zambrana CR, et al. Stimulatory effect of genistein and apigenin on the growth of breast cancer cells correlates with their ability to activate ER alpha. Breast Cancer Res Treat 2006;99:121–34.
[27] Ju YH, Fultz J, Allred KF, Doerge DR, Helferich WG. Effects of dietary daidzein and its metabolite, equol, at physiological concentrations on the growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in ovariectomized athymic mice. Carcinogenesis 2006;27:856–63.
[28] Saji S, Okumura N, Eguchi H, Nakashima S, Suzuki A, Toi M, et al. MDM2 enhances the function of estrogen receptor α in human breast cancer cells. Biochem Biophys Res Commun 2001;281:259–65.
[29] Sakamoto T, Eguchi H, Omoto Y, Ayabe T, Mori H, Hayash S. Estrogen receptor-mediated effects of tamoxifen on human endometrial cancer cells. Mol Cell Endocrinol 2002;192:93–104.
[30] Lehmann L, Esch HL, Wagner J, Rohnstock L, Metzler M. Estrogenic and genotoxic potential of equol and two hydroxylated metabolites of Daidzein in cultured human Ishikawa cells. Toxicol Lett 2005;158:72–86.
[31] Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, et al. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem Pharmacol 2006;71:1459–69.
[32] Harris DM, Besselink E, Henning SM, Go VLW, Hebe D. Phytoestrogens induce differential estrogen receptor alpha- or beta-mediated responses in transfected breast cancer cells. Exp Biol Med 2005;230:558–68.
[33] Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the *bcl-2* gene. Cancer Res 1994;54:3131–5.
[34] Catz SD, Johnson JL. Transcriptional regulation of *bcl-2* by nuclear factor κB and its significance in prostate cancer. Oncogene 2001;20:7342–51.
[35] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell 1995;80:293–9.
[36] Bentires-Alj M, Dejardin E, Viatour P, Van Lint C, Froesch B, Reed JC, et al. Inhibition of the NF-κB transcription factor increases Bax expression in cancer cell lines. Oncogene 2001;20:2805–13.
[37] Thompson LU, Boucher BA, Liu Z, Cotterchio M, Kreiger N. Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. Nutr Cancer 2006;54:184–201.
[38] Clarke DB, Bailey V, Lloyd AS. Determination of phytoestrogens in dietary supplements by LC-MS/MS. Food Addit Contam 2008;25:534–47.
[39] Thompson LU, Boucher BA, Cotterchio M, Kreiger N, Liu Z. Dietary phytoestrogens, including isoflavones, lignans, and coumestrol, in nonvitamin, nonmineral supplements commonly consumed by women in Canada. Nutr Cancer 2007;59:176–84.
[40] She QB, Bode AM, Ma WY, Chen NY, Dong Z. Resveratrol-induced activation of p53 and apoptosis is mediated by extracellular-signal-regulated protein kinases and p38 kinase. Cancer Res 2001;61:1604–10.
[41] Sareen D, Darjatmoko SR, Albert DM, Polans AS. Mitochondria, calcium, and calpain are key mediators of resveratrol-induced apoptosis in breast cancer. Mol Pharmacol 2007;72:1466–75.
[42] Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson JÅ, Kushner PJ, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 1997;277:1508–10.

[43] Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McInerney E, et al. The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 1999;13:1672–85.
[44] Safe S. Transcriptional activation of genes by 17β-estradiol through estrogen receptor–Sp1 interactions. Vitam Horm 2001;62:231–52.
[45] Migliaccio A, Di Domenico M, Castoria G, de Falco A, Bontempo P, Nola E, et al. Tyrosine kinase/p21^{ras}/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996;15:1292–300.
[46] Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, et al. PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J 2001;20:6050–9.
[47] Marino M, Pallottini V, Trentalance A. Estrogens cause rapid activation of IP_3–PKC-α signal transduction pathway in HEPG2 cells. Biochem Biophys Res Commun 1998;245:254–8.
[48] Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action *via* the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci USA 1994;91:8517–21.
[49] Wu F, Safe S. Differential activation of wild-type estrogen receptor α and C-terminal deletion mutants by estrogens, antiestrogens and xenoestrogens in breast cancer cells. J Steroid Biochem Mol Biol 2007;103:1–9.
[50] Perillo B, Sasso A, Abbondanza C, Palumbo G. 17β-Estradiol inhibits apoptosis in MCF-7 cells, inducing *bcl-2* expression *via* two estrogen-responsive elements present in the coding sequence. Mol Cell Biol 2000;20:2890–901.
[51] Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, et al. Mechanisms of transcriptional activation of *bcl-2* gene expression by 17β-estradiol in breast cancer cells. J Biol Chem 1999;274:32099–107.
[52] Lavigne JA, Takahashi Y, Chandramouli GVR, Liu H, Perkins SN, Hursting SD, et al. Concentration-dependent effects of genistein on global gene expression in MCF-7 breast cancer cells: an oligo microarray study. Breast Cancer Res Treat 2008;110:85–98.