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Genistein Modulates Proliferation and Mitochondrial Functionality in Breast Cancer Cells Depending on ERalpha/ERbeta Ratio

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ABSTRACT

Breast cancer is the most common malignancy in women of developed countries. The aim of this study was to investigate whether genistein, a soy phytoestrogen, and 17β-estradiol (E2) could have effects on the cell cycle and mitochondrial function and dynamics. Three human breast cancer cell lines with different estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) ratio were used: MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio) and MDA-MB-231 (ER-negative). Cell proliferation, cell cycle, mitochondrial functionality, and mitochondrial dynamics parameters were analyzed. E2 and genistein treatment induced cell proliferation and apoptosis inhibition in MCF-7, but not in T47D and MDA-MB-231. Moreover, genistein treatment produced an up-regulation of ERβ and a rise in cytochrome *c* oxidase activity in T47D cells, decreasing the ATP synthase/cytochrome *c* oxidase ratio. Finally, genistein treatment produced a drop in mitochondrial dynamics only in MCF-7 cells. In summary, the beneficial effects of genistein consumption depend on the ERα/ERβ ratio in breast cells. Therefore, genistein treatment produces cell cycle arrest and an improvement of mitochondrial functionality in T47D cells with a low ERα/ERβ ratio, but not in MCF-7 (high ERα/ERβ ratio) and MDA-MB-231 (ER-negative) ones. J. Cell. Biochem. 115: 949–958, 2014.

KEY WORDS: GENISTEIN; MITOCHONDRIA; 17β-ESTRADIOL; ESTROGEN RECEPTORS; CELL CYCLE; APOPTOSIS; CELL PROLIFERATION

Breast cancer is the most commonly diagnosed malignancy in women of developed countries [Siegel et al., 2011]. Epidemiological studies have showed a disparity incidence of breast cancer between Eastern and Western countries, where on average 1 in every 8 women will suffer breast cancer compared to 1 in every 30 women in Japan [Bouker and Hilakivi-Clarke, 2000].

Estrogens, especially 17β-estradiol (E2), are risk factors for the development of breast cancer, and produce tumorigenesis in epithelial breast cells [Yager and Liehr, 1996]. Through diet, humans are exposed to many different phytoestrogens [Patisaul and Jefferson, 2010], and especially those originating from soy products or legumes were initially identified as cancer potential causative factors [Messina et al., 1994]. However, phytoestrogens consumption has also been linked to cancer prevention, most notably prostate and breast cancer [Adlercreutz, 2002; Orlando et al., 2011]. Moreover, high phytoestrogen consumption leads to a lower risk of cardiovascular disease [Klaunig and Kamendulis, 2004] and osteoporosis [Mantovani et al., 2008]; as well as it has been shown to relieve climacteric symptoms [Manju and Nalini, 2007].

The phytoestrogen genistein is the major isoflavonoid found in soybeans, and some beneficial effects have been attributed to its consumption, including anti-carcinogenic effects [Zhou et al., 1998]. Previous studies showed that people in the Eastern countries have higher blood levels of genistein, 10 times than people from Western

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countries [Morton et al., 2002]. Genistein has a very similar chemical structure to E2 and it binds and activates both estrogen receptors (ER), ERα and ERβ. Different functions have been associated with the two ER; while ERα mediates proliferative effects of estrogens, ERβ is more related to cytostatic effects [Chang et al., 2006; Sotoca et al., 2008; Rajah et al., 2009].

Previous studies in our laboratory demonstrate that E2 increases cell proliferation in MCF-7 breast cancer cells with a high ERα/ERβ ratio, but not in the T47D cell line, with a lower ERα/ERβ ratio than MCF-7, nor in the MDA-MB-231 breast cancer cell line, ER-negative [Sastre-Serra et al., 2010; Nadal-Serrano et al., 2012]. Moreover, similar results were obtained in prostate cancer cell lines [Miro et al., 2011]. Genistein has been postulated to be an inhibitor of cell proliferation and inductor of apoptosis in breast cancer cell lines [Davis et al., 2008] and as a cytostatic agent in colon cells [Schleipen et al., 2011].

Recently, Adams et al. [2012] observed that genistein treatment could restore mitochondrial functionality, while some studies have shown that E2 treatment produced mitochondrial dysfunction and an increase in radical oxygen species (ROS) production [Sastre-Serra et al., 2012b], and a greater production of ROS has been related with a higher cell proliferation [Sastre-Serra et al., 2010]. Mitochondria are organelles which are involved in constant cycles of division and fusion, processes related to mitochondrial dynamics [Rambold et al., 2011]. This process of mitochondrial dynamics has been related to mitochondrial function and cell apoptosis [Grandemange et al., 2009; Youle and van der Bliek, 2012].

The aim of this study was to investigate the effects of physiological concentrations of 17β-estradiol and phytoestrogen genistein on cell proliferation, cell cycle and apoptosis, and mitochondrial functionality in breast cancer cell lines with different ERα/ERβ ratios. To tackle this aim we performed a cell proliferation and cell cycle analysis and we studied the proliferation- and apoptosis-related proteins, the ATP synthase and cytochrome *c* oxidase activities and mitochondrial dynamics-related genes mRNA expression in MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio), and MDA-MB-231 (ER-negative) breast cancer cell lines.

MATERIALS AND METHODS

MATERIALS

17β-Estradiol (E2), genistein, dimethyl sulfoxide (DMSO), and Propidium Iodide were purchased from Sigma–Aldrich (St. Louis, MO). Mitotracker Green (MTG) and Lysotracker Red (LTR) were purchased from Life Technologies Ltd (Paisley, UK). Primers were purchased from TIB MOLBIOL (Berlin, Germany) and from Metabion (Martinsried, Germany). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA).

CELL CULTURE AND TREATMENTS

Breast cancer cell lines MCF-7, T47D, and MDA-MB-231 were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37°C. To evaluate the effects of E2 and genistein, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 h prior to treatment. Experiments were performed when cell cultures reached confluence by providing fresh media supplemented with 1 nmol/L E2, 1 μmol/L genistein, or 0.001% DMSO as vehicle for 48 h. For the cell proliferation assay with different genistein concentrations, cells were cultured in the same way as described above and treated with genistein concentrations from 10 nM to 10 μM. For cell cycle analysis in subconfluence, experiments were performed when cell cultures reached 75% of confluence.

CELL LINES ESTROGEN RECEPTORS CHARACTERIZATION

Figure 1 shows estrogen receptor (α and β) level analysis which was carried out by Western blot in normal conditions of each breast cancer cell line. MCF-7 was considered as high ERα/ERβ ratio cell line, T47D as a low ERα/ERβ ratio cell line and MDA-MB-231 as ERα-negative, in spite of the lowest levels of ERβ, so that we considered this cell line to be an ER-negative breast cancer cell line.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

After harvesting the cells with trypsin–EDTA, cells were fixed with methanol 100% and DNA staining was carried out with an RNAase and propidium iodide mix. After 30 min of room temperature incubation, samples were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL). The red fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. Ten thousand events were acquired and analyzed with Expo32 ADC analysis software (Beckman Coulter).

CELL PROLIFERATION ASSAY

Cells were plated at 10,000 cells per well in 96-well plates in DMEM, supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37°C, and shifted to phenol red-free DMEM with 10% charcoal-FBS and 1% antibiotics (penicillin and streptomycin) 24 h before treatment with the vehicle, either E2 or

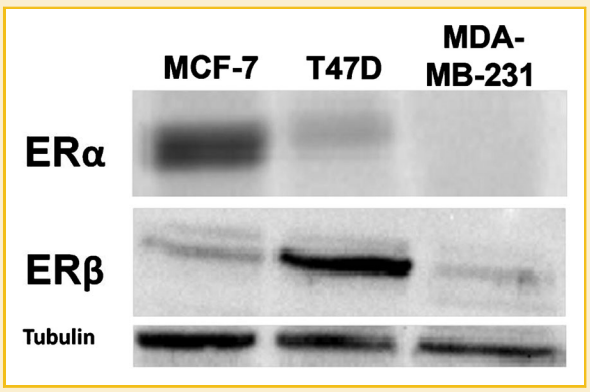


Fig. 1. Cell lines estrogen receptors characterization by Western blot analysis. Measurements were made with cells in basal conditions. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta and Tubulin as a housekeeping protein.

genistein, or both treatments. The number of cells was determined by the crystal violet method [Nagamine et al., 2009] with modifications. Briefly, after 48 h of treatment, 20 μl of a Violet Crystal solution (0.5% of Violet Crystal in 30% acetic acid) was added and plates were incubated for 10 min at room temperature. The medium was removed and washed twice with distilled water. Finally, water was removed and 100 μl of methanol were added to wells and plate was shaken for 1 min. Absorbance was measured at 570 nm using a microplate reader (Power Wave XS, Bio-Tek).

WESTERN BLOT ANALYSIS

For Western blot analysis, 40 μg of cell lysate protein was fractioned by SDS–PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. ERα (1:200) and ERβ (1:200) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Stat3 (1:1,000), Phospho-Stat3 (1:1,000), and PARP/Cleaved PARP (1:1,000) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Protein bands were visualized by the Immun-Star Western C kit reagent (Bio-Rad Laboratories) Western blotting detection system. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One software (Bio-Rad Laboratories).

ATP SYNTHASE AND CYTOCHROME *C* OXIDASE ACTIVITIES

After 48 h of vehicle, E2, genistein, or both E2 and genistein treatments, cells were harvested by scraping the culture into PBS buffer and then centrifuged at 5,000 rpm for 5 min at 4°C to remove cell debris. Cell pellet were resuspended in RNAase free water. Lysates were kept on ice and protein content was determined with the bicinchoninic acid protein assay kit (Pierce, Bonn, Germany), with the enzyme assays run immediately after.

Cytochrome *c* oxidase (Complex IV, EC 1.9.3.1) activity was measured using a spectrophotometric method [Chrzanowska-Lightowlers et al., 1993]. Briefly, cell lysate was incubated in 0.1 M $NaPO_4H_2$, pH 7.0, in the presence of 2 μg/ml catalase and 5 mM substrate DAB (3,3′-diaminebenzidine-tetrachloride) and then 100 μM of reduced cytochrome *c* was added to start the reaction, with the absorbance variation followed for 20 min at 450 nm.

ATP synthase (ATP phosphohydrolase, Complex V, EC 3.6.1.3) activity was measured by following the oxidation of NADH at 340 nm and 37°C [Ragan et al., 1987]. The extinction coefficient used was 6.22 $mM^{-1}\,cm^{-1}$.

REAL-TIME PCR

After 4 h of genistein or vehicle (0.001% DMSO), total RNA was isolated from cultured cells using TriPure® Isolation Reagent (Roche) and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U of MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNAase inhibitor, and 500 μM each dNTP. Each resulting cDNA was diluted 1/10.

PCR was done for six target genes: mitofusin-1 (mfn1), mitofusin-2 (mfn2), optic atrophy 1 (opa1), dynamin-related protein (drp1), fission 1 (fis1), and 18S, using SYBR green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μl, containing 1 μl Lightcycler-FastStart DNA Master SYBR Green I, 0.5 μM sense and antisense specific primers, 2 mM $MgCl_2$, and 3 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation (10 min 95°C) followed by 40 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depends on each pair of specific primers), and an extension step (12 s, 72°C for all). A negative control without cDNA template was run in each assay.

The specific primers used were forward 5′-ttcgatcaagttccggattc-3′ and reverse 5′-ttggagcggagacttagcat-3′ for mfn1 with 51°C as annealing step, forward 5′-gcagaactttgtcccagagc-3′ and reverse 5′-agaggcatcagtgaggtgct-3′ for mfn2 with 56°C as annealing step, forward 5′-acaatgtcaggcacaatcca-3′ and reverse 5′-ggccagcaagattagctacg-3′ for opa1 with 51°C as annealing step, forward 5′-gttcacggcatgacctttt-3′ and reverse 5′-aagaaccaaccacaggcaac-3′ for drp1 with 51°C as annealing step, forward 5′-gctgaaggacgaatctcagg-3′ and reverse 5′-cttgctgtgtccaagtccaa-3′ for fis1 with 53°C as annealing step, and forward 5′-ggacacggacaggattgaca-3′ and reverse 5′-acccacggaatcgagaaaga-3′ for 18S with 61°C as annealing step.

The C_t values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (MultiDAnalises, Sweden).

STATISTICAL ANALYSIS

All statistical analyses were performed with the Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL). Data are presented as means ± standard error of the mean (SEM). Statistical differences between control, E2-treated, genistein-treated, and E2 + genistein-treated cells were analyzed by univariate ANOVA and the Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

EFFECTS OF E2 AND GENISTEIN TREATMENT ON CELL PROLIFERATION

In Table I can be observed that after 48 h of treatment with E2, genistein, and their combination the cell proliferation was significantly increased in the order of 20% in MCF-7 breast cancer cell line. However, in T47D breast cancer cell line there were no differences between control and treated cells. In MDA-MB-231 there is a small increase (5% maximum) in cell proliferation in genistein-treated cells.

Figure 2 shows the effect of different concentrations of genistein (10 nM–10 μM) on the proliferation of cell lines studied in the absence (solid line) or presence (dashed line) of E2 at 1 nM concentration. Genistein-treated MCF-7 cells were always above the control line for the vehicle-treated ones (the solid line representing 100% of control) and around the E2-treated cells (the dotted line representing E2-

TABLE I. Effects of 17β-Estradiol and Genistein on Cell Proliferation in MCF-7, T47D, and MDA-MB-231 Cells Measured by Violet Crystal Assay

	C	E2	GEN	E2 + GEN	ANOVA
MCF-7	100 ± 1.79	118 ± 0.50*	118 ± 2.01°	121 ± 2.29°	E, G, E × G
T47D	100 ± 2.69	96.7 ± 1.35	101 ± 1.18	104 ± 2.11	NS
MDA-MB-231	100 ± 0.51	102 ± 1.07	105 ± 0.62	105 ± 1.67	G

C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells.
All measurements were made after 48 h of 1 nM 17β-estradiol, 1 μM genistein, combination of 1 nM 17β-estradiol, and 1 μM genistein or vehicle (0.1% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. ANOVA analysis was carried out where E means E2 effect, G means genistein effect and E × G means combinatory effect of E2 and genistein and NS means no significant differences. As a result, in MCF-7 a Student's *t*-test ($P < 0.05$, n = 8) was carried out:
*Differences between vehicle- and E2-treated cells.
°Differences between vehicle- and GEN-treated cells.

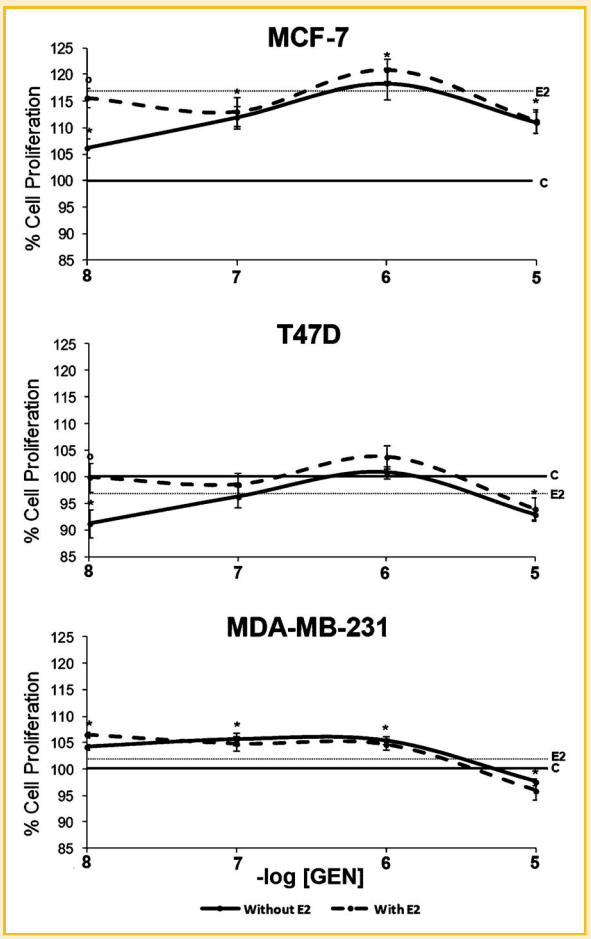


Fig. 2. Effects of different concentrations of genistein in combination or not with 17β-estradiol on cell proliferation in MCF-7, T47D, and MDA-MB-231 cells measured by Crystal Violet assay. All measurements were made after 48 h of 0.01, 0.1, 1, and 10 μM genistein or vehicle (0.001% DMSO) treatment in the absence or presence of 17β-estradiol at 1 nM. The value of vehicle-treated cells was set at 100% as an abscissa perpendicular solid line and the value of 17β-estradiol-treated cells is represented as an abscissa perpendicular dotted line. Values in ordinate represent −log of genistein concentration (mol/L). C, Vehicle-treated; E2, 17β-estradiol; GEN, Genistein. *Significant difference between vehicle- and genistein-treated cells and ° significant difference between genistein- and genistein + E2-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

treated cells) indicating a cell proliferation induction by genistein treatment. In T47D cells genistein treatment produced no differences or a small drop in cell proliferation, while MDA-MB-231 cells presented a biphasic effect: low concentrations of genistein (10 nM–1 μM) produced a small increase in cell proliferation although higher concentrations (10 μM) produced a slight drop in cell proliferation. There are no differences between E2 and genistein treatments but in MCF-7 and T47D cells the lowest genistein concentration (10 nM) without E2 treatment shows lower proliferation than the E2-treated one, probably due to the presence of ERα in these breast cancer cell lines. GEN have higher affinity for ERβ than for ERα [Kuiper et al., 1997], but at low concentrations of this phytoestrogen the presence of 1 nM of E2 could act through ERα enhancing cell proliferation in those cells that possess ERα. The physiological concentrations of genistein, 1 μM [Morton et al., 2002], mask the effects of E2 because genistein is binding to both estrogen receptors.

EFFECTS OF E2 AND GENISTEIN TREATMENT ON CELL CYCLE

The effect of E2 and genistein treatments on cell cycle of breast cancer cell lines in confluence and subconfluence with different ERα/ERβ ratio is represented in Figure 3. Cell cycle analysis determined that both treatments, E2 and genistein, produced an increase in proliferating cells (S + G2/M) and a decrease in apoptotic and cytostatic cells (G0/G1) in the MCF-7 breast cancer cell line. Furthermore, the genistein effect is in the manner but more attenuated than E2. These effects were more pronounced with subconfluence.

However, in T47D breast cancer cell line, E2 and genistein treatments produced a decrease in proliferating cells, and, moreover, genistein treatment produced an increase in cytostatic cells. In the subconfluence there was no significant difference between treated and non-treated cells.

Finally, in MDA-MB-231 breast cancer cells there was no significant difference between control and treated cells in the confluence situation. However, in the subconfluence cells treated with E2 showed a decrease in apoptosis and cytostatic cells, and a significant rise in proliferating cells.

EFFECTS OF GENISTEIN TREATMENT ON ESTROGEN RECEPTORS LEVELS

Genistein treatment produced a strongly increase (more than 11 times) of ERβ protein levels in T47D breast cancer cell line.

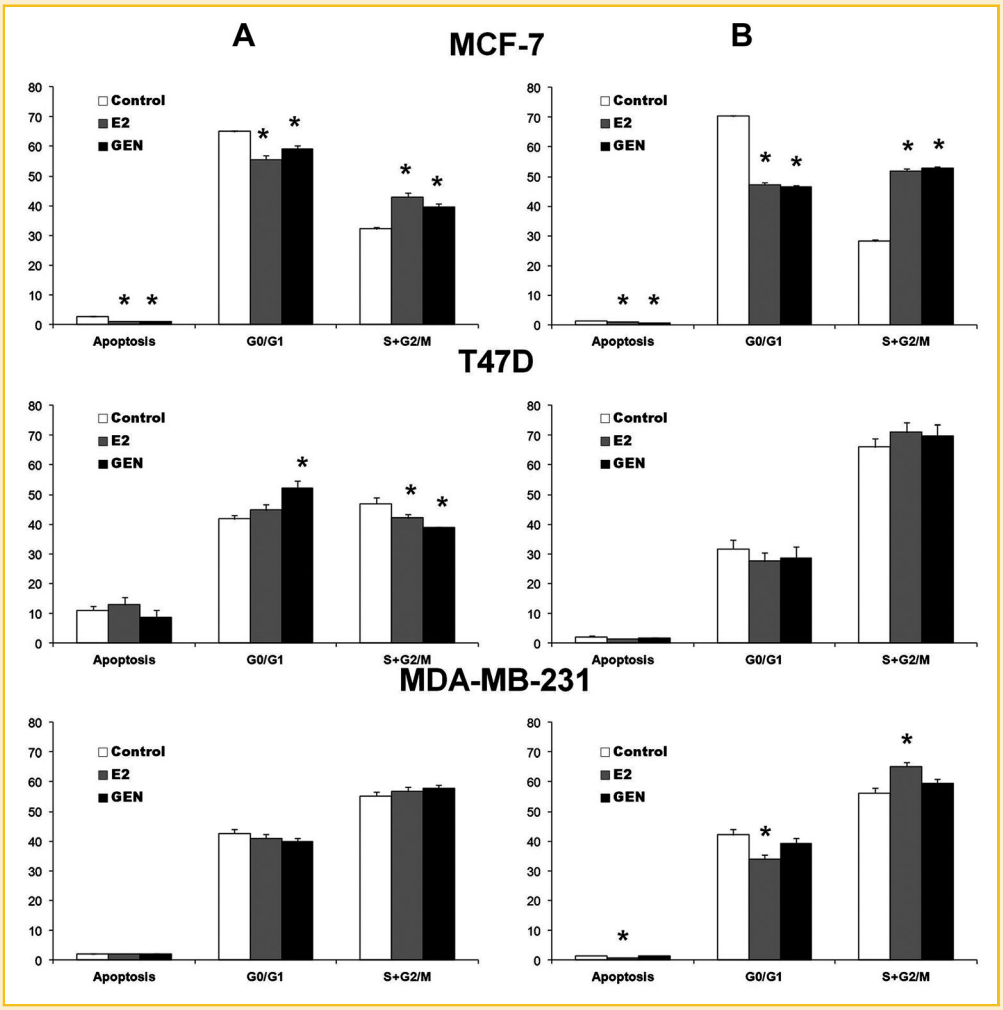


Fig. 3. Effects of E2 and genistein on cell cycle in MCF-7, T47D, and MDA-MB-231 cells measured by propidium iodide staining in flow cytometer. All measurements were made after 48 h of 1 μM genistein (dark bar), 1 nM E2 (gray bar), or vehicle (white bar) (0.001% DMSO) treatment. A-column represents the cell cycle analysis in confluence conditions and B-column represents the cell cycle analysis in subconfluence conditions. Bars represent means ± SEM of percentage of a total of 10,000 cells present in different cell cycle phases. *Significant difference between genistein- and E2-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

Nevertheless, genistein treatment produced no changes in ERβ protein levels in both MCF-7 and MDA-MB-231 cells. ERα protein levels showed no variation with genistein treatment in MCF-7 and T47D breast cancer cell lines, considering that MDA-MB-231 does not have this receptor. These results can be observed in Table II.

STAT3 PHOSPHORYLATION AND PARP CLEAVAGE ANALYSIS

The results of P-STAT3/STAT3 and Cleaved PARP/PARP protein levels are represented in Table III.

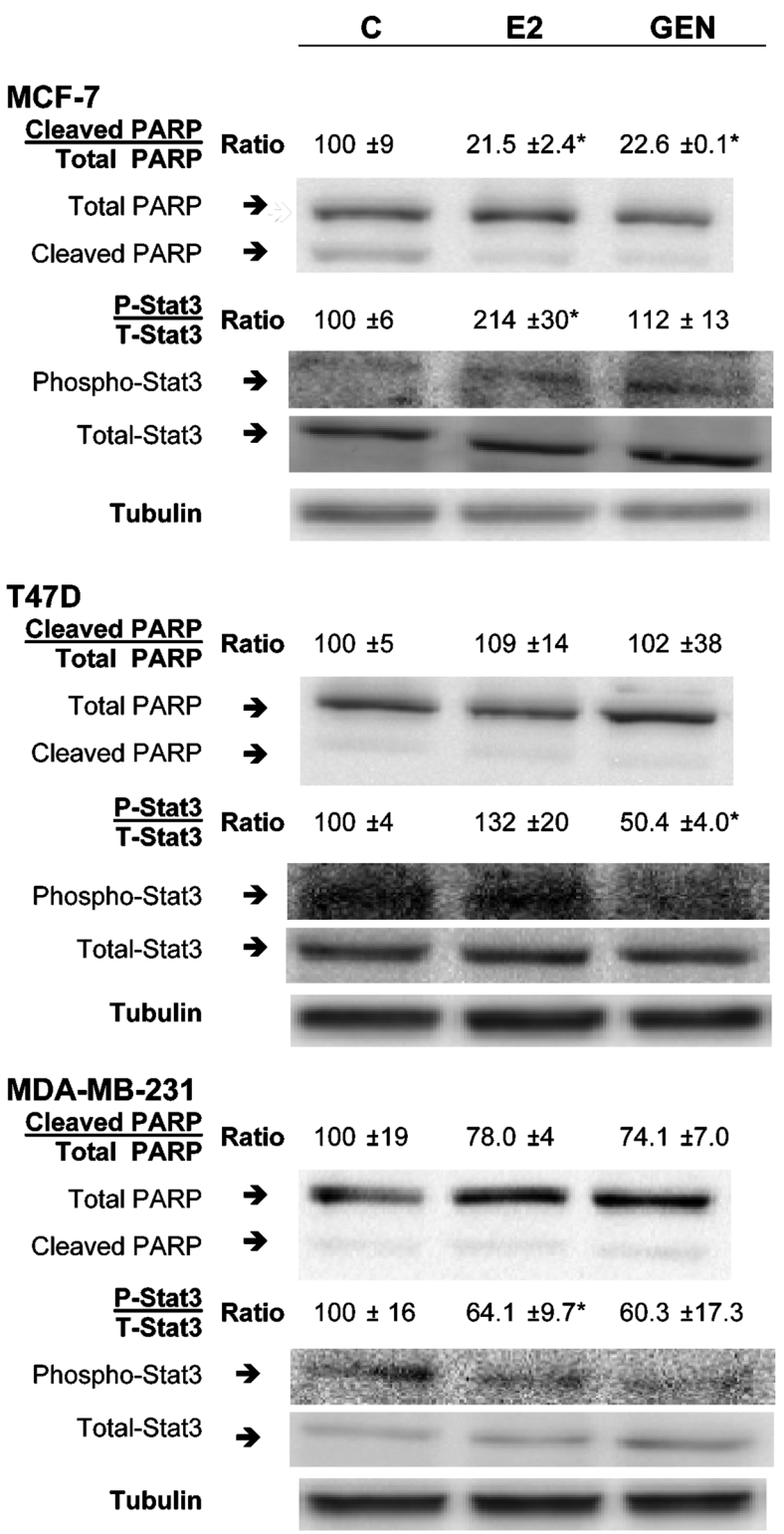
The MCF-7 breast cancer cell line showed no significant differences with E2 and genistein treatment in P-STAT3/STAT3 ratio. In the

TABLE II. Estrogen Receptors Protein Levels Analysis in MCF-7, T47D, and MDA-MB-231 Cells by Western Blot

	T47D		MCF-7		MDA-MB-231	
	C	GEN	C	GEN	C	GEN
ERα	100 ± 17.5	86.3 ± 14.5	100 ± 11.4	91.1 ± 6.84	UND	UND
ERβ	100 ± 9.18	93.7 ± 14.7	100 ± 30.9	1,100 ± 353*	100 ± 15.0	120 ± 23.0

C, vehicle-treated cells; GEN, genistein-treated cells; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; UND, undetected.
All measurements were made after 48 h of 1 μM genistein or vehicle (0.001% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%.
*Significant difference between genistein-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

TABLE III. Cleaved PARP/PARP and P-STAT3/STAT3 Ratios Analysis Measured by Western Blot in MCF-7, T47D, and MDA-MB-231 Cells



C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells; PARP, poly-ADP ribose polymerase; P-STAT3, phosphorylated signal transducer and activator of transcription 3, T-STAT3, total signal transducer and activator of transcription 3.

All measurements were made after 48 h of 1 μM genistein, 1 nM 17β-estradiol, or vehicle (0.001% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. Tubulin was used as a housekeeping protein.

*Significant difference between GEN- and E2-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

T47D breast cancer cell line the genistein treatment caused a significant decrease in P-STAT3/STAT3 ratio, while the E2 treatment had no effects. In the MDA-MB-231 breast cancer cell line, both treatments produced significant lower levels of P-STAT3/STAT3 ratio, although the genistein treatment produced a greater decrease than the E2 one.

Likewise, both treatments caused a significant decrease in Cleavage PARP/PARP ratio in MCF-7 breast cancer cell line. However, in T47D and MDA-MB-231 breast cancer cell lines, the E2 and genistein treatment did not make a significant difference in Cleaved PARP/PARP ratio compared to vehicle treated cells.

MITOCHONDRIAL FUNCTIONALITY

Mitochondrial functionality was measured by ATP synthase and cytochrome *c* oxidase enzymatic activities and the resulting ratio of these activities, represented in Table IV, is considered to be the mitochondrial functionality parameter.

In the MCF-7 breast cancer cell line, treatment with E2 triggered a decrease of the cytochrome *c* oxidase activity with no change in ATP synthase activity, resulting in an increase of the ATP synthase/cytochrome *c* oxidase ratio. However, genistein treatment, as well as E2 + genistein treatment, produced no changes in enzyme activities and the ATP synthase/cytochrome *c* oxidase ratio had no alterations caused by this phytoestrogen.

In the T47D breast cancer cell line, treatment with E2 caused no differences between treated and control cells for either enzyme activity. Nevertheless, genistein treatment and, to a lesser extent, combination of E2 and genistein treatment resulted in an increase in cytochrome *c* oxidase activity and a subsequent decrease in ATP synthase/cytochrome *c* oxidase ratio in both treatments.

In MDA-MB-231 breast cancer cell line, E2, genistein, and combination of E2 and genistein treatments produced no changes in the ATP synthase/cytochrome *c* oxidase activity ratio.

MITOCHONDRIAL DYNAMICS IS AFFECTED BY GENISTEIN TREATMENT IN MCF-7 CELLS

As shown in Table V, mitochondrial dynamics is affected just in MCF-7 cells after genistein treatment, resulting in a drop in Opa1 (mitochondrial fusion) and Fis1 (mitochondrial fission) mRNA expression. However, in T47D and MDA-MB-231 cells genistein treatment did not produce any alteration in mitochondrial fusion and fission.

DISCUSSION

In this study we demonstrated the importance of ERα/ERβ ratio on the effects of 17β-estradiol and genistein at physiological concentrations in breast cancer cell lines. While in MCF-7 cells (high ERα/ERβ ratio) E2 and genistein treatment produced proliferation effects and decreased mitochondrial functionality; in T47D cells (low ERα/ERβ ratio) E2 and especially genistein, exerted antiproliferative and proapoptotic effects, and a better mitochondrial functionality.

The present study shows a relationship between ERα/ERβ ratio and genistein effects, suggesting that a high ERα/ERβ ratio is related to cell proliferation induction and apoptosis inhibition, while a low ERα/ERβ ratio is linked to cell cycle arrest and apoptosis induction. According to our results, previous studies in our laboratory have shown that E2 has proliferative effects in MCF-7 cells and no effects in T47D and MDA-MB-231 ones [Sastre-Serra et al., 2010, 2012b; Nadal-Serrano et al., 2012]. Sotoca et al. [2008] observed that a breast cancer cell line with only ERα, showed an induction of cell proliferation after E2 (1 nM) and genistein (1 μM) treatments, and over-expression of ERβ reversed this situation producing no changes in cell proliferation. In fact, the presence of ERβ in the MCF-7 breast cancer cell line could induce cell proliferation inhibition [Paruthiyil et al., 2004] and studies in vivo have observed that βERKO mice prostate cells have lower apoptosis and differentiation and higher cell

TABLE IV. ATP Synthase and Cytochrome Oxidase Enzymatic Activities in MCF-7, T47D, and MDA-MB-231 Cells, Measured by Spectrophotometric Method

	C	E_2	GEN	E2 + GEN	ANOVA
MCF-7					
ATPase	100 ± 5.17	95.6 ± 8.82	97.2 ± 3.25	92.3 ± 3.14	NS
COX	100 ± 8.75	51.3 ± 8.50*	106 ± 8.06	100 ± 6.60	E, G, E × G
ATPase/COX ratio	1.00	1.86	0.92	0.92	
T47D					
ATPase	100 ± 1.99	111 ± 8.98	100 ± 4.41	102 ± 3.52	NS
COX	100 ± 5.85	107 ± 11.8	130 ± 9.70	119 ± 8.95	G
ATPase/COX ratio	1.00	1.04	0.77	0.86	
MDA-MB-231					
ATPase	100 ± 1.95	108 ± 3.54	96.9 ± 2.13	102 ± 11.1	NS
COX	100 ± 4.03	87.0 ± 10.4	102 ± 16.8	106 ± 5.91	NS
ATPase/COX ratio	1.00	1.24	0.95	0.96	

C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells; ATPase, ATP synthase and COX, cytochrome *c* oxidase.
All measurements were made after 48 h of 1 nM 17β-estradiol, 1 μM genistein, combination of 1 nM 17β-estradiol and 1 μM genistein or vehicle (0.01% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. ANOVA analysis was carried out where E means E2 effect, G means GEN effect and E × G means combinatory effect of E2 and GEN and NS means no significant differences. As a result, in MCF-7 a Student's *t*-test ($P < 0.05$ n = 6) was carried out:
*Differences between vehicle- and E2-treated cells.

TABLE V. Fusion- and Fission-Related Genes mRNA Expression in Breast Cancer Cell Lines After 4 h of 1 μM Genistein or Vehicle (0.001% DMSO) Treatment Analyzed by Real-Time PCR

	MCF-7		T47D		MDA-MB-231	
	C	GEN	C	GEN	C	GEN
mfn1	1.00 ± 0.05	0.98 ± 0.12	1.00 ± 0.09	1.07 ± 0.06	1.00 ± 0.08	1.00 ± 0.13
mfn2	1.00 ± 0.10	0.84 ± 0.07	1.00 ± 0.07	0.99 ± 0.06	1.00 ± 0.05	0.83 ± 0.10
opa1	1.00 ± 0.10	0.71 ± 0.07*	1.00 ± 0.11	1.06 ± 0.05	1.00 ± 0.05	1.01 ± 0.16
drp1	1.00 ± 0.07	0.85 ± 0.07	1.00 ± 0.09	0.96 ± 0.03	1.00 ± 0.08	1.19 ± 0.11
fis1	1.00 ± 0.05	0.77 ± 0.02*	1.00 ± 0.06	0.98 ± 0.04	1.00 ± 0.07	1.11 ± 0.06

C, vehicle-treated cells; GEN, genistein-treated cells; mfn1, mitofusin 1; mfn2, mitofusin 2; opa1, optic atrophy 1; drp1, dynamin-related protein q; fis1, mitochondrial fission 1 protein.

Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%.

*Significant difference between genistein-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

proliferation [Imamov et al., 2004]. Some authors have described that genistein treatment has proapoptotic effects in the colon and could prevent colorectal cancer, as colon cells are close to absorption sites for genistein-bearing foods [Schleipen et al., 2011]. Our results indicate that the presence of ERβ and, therefore, a low ERα/ERβ ratio in the T47D breast cancer cell line, either produced no effects or inhibited cell proliferation after E2, genistein or their combination treatment. Cell cycle analysis revealed a cell cycle arrest in G0/G1 phase in the T47D cell line after genistein treatment only in confluence conditions. Similar results were described by Yu et al. [2008], who observed an inhibition of cell proliferation after genistein treatment through a G0/G1 cell cycle phase arrest in rat aortic smooth muscle cell. In breast cancer, another phytoestrogen, formononetin, inhibits cell growth through G0/G1 cell cycle arrest [Chen et al., 2011]. The increase in P-STAT3/STAT3 ratio, cell proliferation indicator [Catlett-Falcone et al., 1999], after E2 treatment and the reduction in cleaved PARP/PARP ratio, apoptosis indicator [Hoffman et al., 2012], after both treatments in MCF-7 cells agree with our cell cycle analysis results and with previous similar studies involving regulation of PARP cleavage and STAT3 phosphorylation in an ER-dependent manner [Catlett-Falcone et al., 1999; Bjornstrom and Sjoberg, 2005; Hoffman et al., 2012]. Here we observed a biphasic effect of genistein treatment on MDA-MB-231 cell proliferation depending on genistein concentrations, with slight changes. Regarding to cell cycle analysis, MDA-MB-231 showed an induction of cell cycle progression after E2 treatment in subconfluence. This effect (among others) could be due to an ERx membrane element able to induce ERs independent gene transcription leading to the modulation of significant cellular functions such as apoptosis [Kampa et al., 2012]. A down-regulation of ERα and ERβ after E2 treatment has been reported [Nadal-Serrano et al., 2012]. In contrast, genistein treatment induced an over-expression of ERβ in T47D cells, effect described previously with a soy extract treatment by other authors [Gallo et al., 2005]. Therefore, all effects produced by genistein treatment in T47D could be magnified due to the over-expression of ERβ when cells are treated with this phytoestrogen, taking into account the fact that genistein possesses higher affinity for ERβ than for ERα [Kuiper et al., 1997; Muthyala et al., 2004] is considered.

In this study, we observed a loss of mitochondrial function (drop in cytochrome *c* oxidase activity) in high ERα/ERβ ratio cells after E2 treatment, and no effects in low ERα/ERβ ratio cells. In contrast, genistein treatment produced no changes in mitochondrial functionality in MCF-7 breast cancer cell line, as well as the combination of both treatments; while in T47D breast cancer cell line genistein treatment produced a better mitochondrial functionality due to a significant increase in cytochrome *c* oxidase activity. Some studies have shown that E2 treatment produces a mitochondrial dysfunction [Sastre-Serra et al., 2012b], and a greater production of free oxygen radicals related to a higher cell proliferation [Sastre-Serra et al., 2010]. These different effects of genistein compared to the E2 treatment could be due to the higher affinity of genistein for ERβ than for ERα [Kuiper et al., 1997; Muthyala et al., 2004]. Results obtained have also been observed in neurons which can restore the mitochondrial functionality after genistein treatment [Adams et al., 2012]. These results related to the improvement of mitochondrial functionality after genistein treatment in T47D cells may be linked to the inhibition of cell proliferation due to the reduction of free oxygen radicals [Sastre-Serra et al., 2010]. In the same way, previous studies in our lab showed an increased mitochondrial fission and a decreased mitochondrial fusion gene expression in MCF-7 cells after E2 treatment associated with a lower expression of mitochondrial respiratory chain proteins [Sastre-Serra et al., 2012a, 2013]. In the present study we have observed a drop in mitochondrial fission gene expression in MCF-7 cells after genistein treatment which has been associated with lower apoptosis [Lee et al., 2004; Parra et al., 2008]. Lee et al. [2004] found that a drop in Opa1 seemed to be correlated with an increase in apoptosis, but they postulated that Opa1 could act counteracting the proapoptotic effect of Fis1 [Lee et al., 2004], therefore if Fis1 is down-regulated the action of Opa1 could not be necessary for avoiding this proapoptotic effect of Fis1.

In summary, this study demonstrates that genistein treatment could inhibit cancer cell proliferation and induce apoptosis and cell cycle arrest depending on the ERα/ERβ ratio. While high ERα/ERβ ratio breast cancer cells genistein-treatment, as well as E2- and E2 + genistein-treatment, increase cell proliferation; genistein treatment in low ERα/ERβ ratio breast cancer cells show cytostatic effects and a better mitochondrial functionality. However, further studies are necessary to investigate genistein as a possible anticancer therapy that would be dependent on patient ERα/ERβ ratios in malignant

cells, so it would be appropriate the study of the endowment of these two estrogen receptors subtypes in breast cancer patients' cells.

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