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High levels of arachidonic acid and peroxisome proliferator-activated receptor-alpha in breast cancer tissues are associated with promoting cancer cell proliferation☆

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Abstract

Fatty acids are endogenous ligands of peroxisome proliferator-activated receptor-alpha (PPARα), which is linked to the regulation of fatty acid uptake, lipid metabolism and breast cancer cell growth. This study was designed to screen candidate fatty acids from breast cancer tissue and to investigate the effects of these candidate fatty acids on PPARα expression, cell growth and cell cycle progression in breast cancer cell lines. One breast cancer tissue and one reference tissue were each taken from 30 individual breasts to examine for fatty acid composition and PPARα expression. The cancer cell lines MDA-MB-231 (ER–), MCF-7 (ER++++) and BT-474 (ER++) were used to explore the mechanisms regulating cell proliferation. We found that arachidonic acid (AA) and PPARα were highly expressed in the breast cancer tissues. AA stimulated the growth of all three breast cancer cells in a time- and dose-dependent manner. The growth stimulatory effect of AA was associated with PPARα activation, and the most potent effect was found in MCF-7 cells. The stimulation of cell proliferation by AA was accompanied by the increased expression of cyclin E, a reduced population of G1 phase cells, and a faster G1/S phase transition. In contrast, AA had no effects on the levels of CDK2, CDK4, cyclin D1, p27, Bcl-2 and Bax. Our results demonstrate that high levels of AA and PPARα expression in human breast cancer tissues are associated with ER-overexpressed breast cancer cell proliferation, which is involved in activating PPARα, stimulating cyclin E expression, and promoting faster G1/S transition.

Keywords: Arachidonic acid; PPARα; Cyclin E; Estrogen receptor; Breast cancer

1. Introduction

The fatty acid composition of dietary lipids and breast cancer tissues were reported to be associated with breast cancer risk, survival and recurrence [1–5]. The Women's Intervention Nutrition Study provided evidence that a reduction in dietary fat intake to 22% of total energy intake led to a 24% reduction in the breast cancer recurrence rate [4]. Many studies have shown that dietary fatty acids are potential regulators of breast cancer cell growth [4,6,7]. An increased ratio of long-chain ω-3/total ω-6 fatty acids in breast adipose tissue was also reported to be associated with a decreased risk of breast cancer [2,8]. Fatty acids are the major components of cell membranes and are essential for various biological functions, including cell growth and the division of normal and malignant tissues. Peroxidation of polyunsaturated fatty acids affects essential components of the cell membrane and may be involved in tumorigenic processes in breast cancer [9–12]. In particular, fatty acids are an important energy source for the body; when bound to nuclear peroxisome proliferator-activated receptors (PPARs), they mediate the transcription of genes involved in glucose and lipid metabolism [13].

The PPAR family is composed of three nuclear hormone receptors: PPARα, PPARβ and PPARγ [14]. PPARα is primarily expressed in organs such as the liver, kidney, heart and brown adipose tissue [15–17]. A diverse range of chemicals that include hypolipidemic fibrate drugs, plasticizers and herbicides are known exogenous PPARα ligands [18]. Endogenous ligands for PPARα include linoleic acid, arachidonic acid and leukotriene B4 [18]. PPARα was shown to regulate lipid metabolism by controlling the gene expression of β-oxidation enzymes, apolipoproteins and fatty acid transport proteins [16,19]. An increase in PPARα mRNA expression was found in rat mammary gland carcinoma [20]. However, activation of PPARα by arachidonic acid (AA) was reported to either stimulate [18] or inhibit [21] breast cancer cell proliferation. The suggested regulatory mechanism was that PPARα activation may be involved in cell cycle control or apoptosis pathways in breast cancer cell lines [18,21].

Abbreviations: AA, arachidonic acid; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; PPARα, peroxisome proliferator-activated receptor-alpha; PR, progesterone receptor.

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The major effective endocrine therapy for breast cancer targets anti-estrogen effects. The mechanism of action of endocrine therapy is to either repress the binding of estrogen to estrogen receptors (ER) or to inhibit aromatase activity [7,22]. However, one of the mechanisms of resistance to endocrine therapy in breast cancer involves crosstalk between the ER and HER2 (also known as c-erbB-2, human epidermal growth factor receptor-2) signaling pathways [23–25]. Signaling through HER2 can result in down-regulation of ER expression in cultured cells [26]. Therefore, two important tumor markers currently used in breast cancer patients are ER and HER2 expression levels [22].

When activated by fatty acids, PPARα plays an important role in regulating fatty acid uptake and lipid metabolism, suggesting that PPARα signaling may influence pathways that regulate breast cancer cell growth [16,19]. Furthermore, treatment options for women with early and advanced breast cancer are strongly related to the presence of ER-positive and/or HER2-positive tumors, indicating that estrogen stimulates tumor growth and that hormonal therapy may be effective in preventing breast cancer progression [7,22]. Therefore, to investigate the role of PPARα signaling in ER- and/or HER2-related tumor cell growth, we evaluated the changes in fatty acid composition and PPARα expression in breast cancer tissue and non-malignant reference tissue each taken from the same breast. We then explored the effects of arachidonic acid, a candidate fatty acid suggested by previous data, on PPARα expression, cell growth and cell cycle progression in the breast cancer cell lines MDA-MB-231 (ER–, PR– and HER2–), MCF-7 (ER++++, PR++ and HER2+/++) and BT-474 (ER++, PR+/++ and HER2++++).

2. Materials and methods

2.1. Materials

Lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), margaric acid (17:0), stearic acid (18:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), 8,11,14-eicosatrienoic acid (20:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosahexaenoic acid (22:6), insulin, estrogen, propidium iodide, Triton X-100 and MK886 sodium hydrate were purchased from the Sigma-Aldrich Company (MO, USA). MDA-MB-231, MCF-7 and BT-474 breast cancer cell lines were obtained from the American Type Culture Collection. Iscove's Modified Dulbecco's Media (IMDM), containing fetal bovine serum (FBS), penicillin and streptomycin, was purchased from Invitrogen (CA, USA). Antibodies recognizing cyclin-dependent kinase 2 (CDK2), Bax, Bcl-2, p27, cyclin D1, cyclin E, histone 1, ERK1/2, JNK and β-actin and anti-IgG horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CDK4 and p38 antibodies were purchased from BD Transduction Laboratories (CA, USA). PPARα was purchased from Cayman Chemical Company (MI, USA). Phospho-ERK1/2, phospho-p38 and phospho-JNK were purchased from Cell Signaling technology (MA, USA). The enhanced chemiluminescence (ECL) detection system was purchased from Thermo Scientific Pierce (IL, USA). RNase was purchased from Worthington Biochemical Corporation (NJ, USA). All other chemicals were of the highest commercially available grade and were supplied either by Merck or the Sigma-Aldrich Company.

2.2. Breast tissue preparation

Thirty breast cancer patients were recruited in this study. Breast tissue samples obtained after primary surgery from the Changhua Christian Hospital in Taiwan were frozen immediately and stored at −80°C until analyzed. This study was approved by the institutional review board of the Changhua Christian Hospital. All breast tissue samples from female patients with unexplained uterine bleeding, pre-existing endometrial cancer, thyroid function abnormalities, diabetes mellitus or other endocrinopathies were excluded from this study. Breast tissue samples included 30 cancer samples and 30 reference tissue samples (normal and/or benign tissues) that were obtained from the margins of the excised tumor of the same breast where no carcinoma was detected. Histopathological analysis was performed by pathologists. The average age of patients was 51.9±11.9 years (mean±S.D.). The premenopausal/postmenopausal ratio was 14/16. The average body mass index of patients was 23.8±3.3 (mean±S.D.). Histopathological reports indicated that 90% (27/30) of the cancer samples were infiltrating ductal carcinoma; the remaining cancer samples (3/30) were ductal carcinoma in situ.

2.3. Analysis of total cholesterol, triglyceride, phospholipid and fatty acid composition

The breast tissue samples were homogenized, and then total lipids were extracted in chloroform-methanol (2:1, v/v), as previously described [27]. The total cholesterol level of each breast sample was determined by the method of Abell [28]. Triglyceride and phospholipid levels were determined according to the method of Soloni [29] and Stewart [30], respectively. The pattern of fatty acids was analyzed by reversed-phase high-performance liquid chromatography (HPLC) after derivation with 2-nitrophenylhydrazine hydrochloride in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; margaric acid (C17:0) was used as an internal standard [31].

2.4. Cell lines and culture conditions

The MDA-MB-231 (ER–, PR– and HER2–), MCF-7 (ER++++, PR++ and HER2+/++) and BT-474 (ER++, PR+/++ and HER2++++) breast cancer cells [32] were grown in IMDM containing 5% fetal bovine serum (FBS, v/v), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The base medium was supplemented with 10 μg/ml insulin and 10^{-10} M estrogen for MCF-7 and BT-474 cell growth. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experimental purposes, the FBS content of each culture medium was changed from 5% to 1%. Because the cultured cells did not grow well when starved of essential fatty acids (linoleic and linolenic acids) for 96 h, the cells were grown in IMDM supplemented with 1% FBS, which was replaced every 2 days for growth rate determination, cell cycle examination and Western blotting analysis.

2.5. Cell growth rate and viability determination

The MDA-MB-231, MCF-7 and BT-474 cells were seeded in 24-well plates at a final density of 3×10^4 cells/well and were then cultured for 24 h. After the cells attached to the plate, the medium was removed and replaced with fresh 1% FBS-IMDM containing arachidonic acid (AA) at a range of concentrations with or without 5 μM MK886 [33,34], a PPARα inhibitor, for 24, 48, 72 and 96 h. Viability and cell number counts were calculated with a Beckman Coulter Z1 Dual cell and particle counter.

2.6. Analysis of cell cycle

The MDA-MB-231, MCF-7 and BT-474 cells were cultured in 1% FBS, treated with or without 10 μM AA for varying lengths of time and then harvested, fixed in 95% ice-cold ethanol and stored at −20°C overnight. Prior to cell cycle analysis, the cells were centrifuged at 300×*g* for 5 min and then resuspended in 1×PBS. Reagent A (0.5% Triton X-100, 0.5 mg/ml RNase and 0.05 mg/ml PI; 0.5 ml) was added to the cell pellets, which were then incubated at 37°C for 30 minutes. Reagent B (0.05 mg/ml propidium iodide; 0.5 ml) was then added to the suspension. Samples, protected from light, were next incubated at 4°C for 1 h. Cells were stained for DNA cell cycle analysis using a previously described method [35]. DNA content was measured by flow cytometric analysis (FACS CaliburTM, Becton Dickinson, NJ, USA). The percentage of cells present in each cell cycle phase was analyzed with ModFit software (Becton Dickinson, NJ, USA). Samples were measured in three replicates, and each experiment was repeated at least three times.

2.7. Protein extraction and Western blotting

For breast tissue analysis, each sample (0.25 g) was homogenized and the nuclear proteins were extracted as previously described [36]. Nuclear protein content was determined using the bicinchoninic acid assay (Thermo Scientific Pierce, IL, USA) with bovine serum albumin as a standard. For the analysis of cell lines, cell lysates were prepared and subjected to western blot analysis as previously described [37,38]. Total protein content was determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA, USA) with bovine serum albumin as a standard. Equal amounts (50 μg) of extracted proteins from each condition were fractionated by SDS-PAGE. Proteins were transferred to PVDF (polyvinylidene fluoride) membranes in a Bio-Rad electroblotting device. PVDF membranes were blocked overnight at 4°C in 20 mM Tris buffer (pH 7.6) containing 5% non-fat milk, 137 mM NaCl, 0.25% Tween-20. The blots were washed in PBST and then incubated with primary antibodies for 2 h at room temperature. PPARα, cyclin E, CDK2 and CDK4 antibodies were used at a 1:500 dilution. Cyclin D1, ERK1/2, phospho-ERK1/2, Bax, Bcl-2, p27, p38, phospho-p38, JNK, phospho-JNK, histone 1 and β-actin antibodies were used at a 1:1000 dilution. Following primary antibody incubation, the blots were washed and incubated with anti-IgG HRP at a dilution of 1:5000 for 1 h at room temperature. The blots were then finally washed and developed with the ECL kit as directed by the manufacturer. Western blots were quantitated by densitometric analysis using KODAK image analysis software (Kodak EDAS290, Eastman Kodak, Rochester, NY, USA).

2.8. Statistical analysis

All data were analyzed using SPSS 15.0 for Windows (Microsoft). Paired *t* tests were used to test for significant differences in lipid parameters between reference and cancer tissues. The association between lipid levels and breast tissue samples (reference versus cancer) was evaluated with a multivariate logistic regression model. Significant differences between the AA-treated group and the control (untreated) group in cell growth rate, cell viability, cell cycle distribution and protein expression were analyzed using a one-way analysis of variance (ANOVA) followed by a multiple

comparison test. All values are expressed as means with standard deviation (means±S.D.), and differences were considered significant at $P<.05$.

3. Results

3.1. Lipid levels and fatty acid compositions in breast reference and cancer tissues

The total lipid content and fatty acid composition of the breast tissues are shown in Table 1. We found that the total cholesterol ($P<.005$) and phospholipid ($P<.005$) levels were significantly higher in the breast cancer tissue than in the reference tissue, while the triglyceride level was significantly lower ($P<.005$) in the breast cancer tissue. The major fatty acids in the breast tissue of patients were oleic acid (18:1, ω9), linoleic acid (18:2, ω6) and palmitic acid (16:0). Based on HPLC analysis, these fatty acids comprised approximately 80% of the total fatty acids. Using paired t-test analyses, we found that the stearic acid (18:0; $P<.001$), eicosatrienoic acid (20:3, ω6; $P<.05$), arachidonic acid (AA, 20:4, ω6; $P<.001$) and docosahexaenoic acid (22:6, ω3; $P<.005$) levels were significantly higher in the breast cancer tissues than in the reference tissues. In contrast, the palmitoleic acid (16:1, ω7; $P<.005$) and linoleic acid (18:2, ω6; $P<.001$) levels were significantly lower in the breast cancer tissues. In addition, through a multivariate logistic regression analysis, we found that both stearic acid ($P<.001$, OR=2.968, 95% CI: 1.619–5.44) and AA ($P=.009$, OR=8.370, 95% CI: 1.713–40.897) were associated with mammary carcinogenesis. AA and stearic acid were significantly higher in the breast cancer tissues than in the reference tissues. Stearic acid is a non-essential fatty acid and is difficult to trace in the human body. We therefore used AA, a semi-essential fatty acid synthesized from linoleic acid (ω-6) in humans, for the following studies in the breast cancer cell lines.

3.2. PPARα expression in breast tissues

We examined the PPARα protein levels in three breast cancer patients with infiltrating ductal carcinoma and in one patient with ductal carcinoma in situ. Each paired breast tissue sample was obtained from the same patient. The results showed that the PPARα protein was highly expressed in all four breast cancer tissues in comparison to the reference tissues (Fig. 1).

Table 1
Lipid parameters in breast reference and cancer tissues

	Reference tissue[a] (n=30)	Cancer tissue (n=30)	Paired t-test[b]	Logistic regression[c] (Reference vs. Cancer)
Total lipids	mg/g tissue		*P* value	*P* value
Cholesterol	1.81±0.78[d]	2.82±1.34	.001	.028
Triglyceride	459.45±242.46	200.59±137.35	<.001	.021
Phospholipid	1.37±0.59	5.46±3.10	<.001	.006
Saturated fatty acids	% of total lipids			
12:0	1.40±2.45	1.07±2.06	.586	.430
14:0	2.57±1.19	2.21±0.73	.093	.321
16:0	22.26±2.18	21.93±2.22	.820	.926
18:0	4.28±1.16	6.44±1.91	<.001	<.001
Unsaturated fatty acids	% of total lipids			
16:1	3.87±1.35	2.89±1.11	.002	.094
18:1	33.48±3.62	32.85±4.09	.517	.389
18:2	27.29±3.96	23.94±3.97	<.001	.481
18:3	1.89±0.98	1.44±0.71	.064	.557
20:3	0.87±1.23	2.07±2.21	.016	.512
20:4	1.02±0.89	3.46±2.08	<.001	.009
20:5	0.47±0.80	0.35±0.42	.458	.257
22:6	0.61±0.44	1.35±1.24	.001	.524

[a] Reference tissues (normal and/or benign) were obtained from the margins of the excised tumor of the same breast where no carcinoma was detected.
[b] Paired t-test was used to test for significant differences in parameters between reference and cancer tissues.
[c] The association between lipids levels and breast tissue samples (reference versus cancer) was evaluated with a multivariate logistic regression model.
[d] Values are means±S.D.

3.3. Effect of arachidonic acid on breast cancer cell growth rate and PPARα expression

To evaluate the effect of AA on cell growth rate, the breast cancer cell lines MDA-MB-231 (ER–, PR– and HER2–), MCF-7 (ER++++, PR++ and HER2+/++) and BT-474 (ER++, PR+/++ and HER2 ++++) were treated with various doses of AA for 12, 24, 72 and 96 h. As shown in Fig. 2A, the treatment of MDA-MB-231 cells with 10 μM AA induced a maximum, time-dependent increase in the cell growth rate. Similar results were also found in MCF-7 (Fig. 2B) and BT-474 cells (Fig. 2C). The growth-promoting effects of 10 μM AA became significant in both MDA-MB-231 and MCF-7 cells after 48 h ($P<.05$) and in BT-474 cells after 72 h ($P<.05$). After 96 h, the MCF-7 cells appeared to become more susceptible to the growth stimulatory effect of 10 μM AA in comparison to the untreated control cells at 0 h. The BT-474 cells showed the smallest effect of AA on cell growth.

In addition, the PPARα expression levels were also examined in these three cell lines induced with 10 μM AA for 24, 48 and 72 h. After 10 μM AA treatment for 48 h, we observed that the PPARα levels were significantly increased 44% in MDA-MB-231 cells and 51% in MCF-7 cells in comparison to the control cells. A 93% increase in PPARα expression level was not observed until after a 72 h induction in BT-474 cells. Taken together, AA stimulated cell proliferation in a time- and dose-dependent manner in all three cancer cell lines; the most potent concentration was 10 μM. This concentration of AA also induced high levels of expression of PPARα protein in breast cancer cells. We therefore used AA at a concentration of 10 μM in further studies.

3.4. Cell viability induced by AA with or without MK886

To determine the relevance of the cell growth promotion and the PPARα activation, we used MK886 to explore cell viability after a combination of AA and MK886 exposure for 24, 48 and 72 h. MK886 inhibited PPARα expression by a noncompetitive mechanism, which was confirmed by examining the binding of AA to the cloned PPARα ligand binding domain [33]. As can be observed in Fig. 3A, cell viability in MDA-MB-231 cells was lower after exposure to 5 μM MK886 than after treatment with combination of 10 μM AA and 5 μM MK886. The same results were found in MCF-7 (Fig. 3B) and BT-474 (Fig. 3C) cells. In addition, the stimulatory effect of AA on cell viability was reduced when cells were co-treated with AA and MK886. We observed that cell viability was significantly higher after treatment with 10 μM AA than after treatment with a combination of AA and MK886 in MDA-MB-231 cells after 24 h, in MCF-7 cells after 48 h and in BT-474 cells after 72 h. These results indicate that PPARα activation is involved in the growth stimulatory effects of AA and the MCF-7 cells are the most sensitive cell line to MK886 exposure.

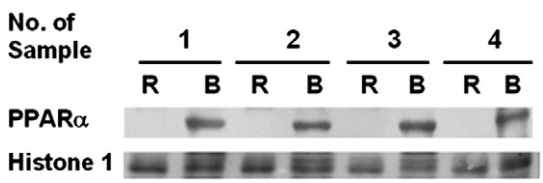

Fig. 1. PPARα expression in breast cancer tissue and reference non-malignant tissue was measured by Western blot analysis. Four paired breast tissue samples were selected randomly from the recruited patients with infiltrating ductal carcinoma (Samples 1, 2 and 3) or with ductal carcinoma in situ (sample 4). All the details are described in the Materials and methods section. B: breast cancer tissue, R: reference non-malignant tissue.

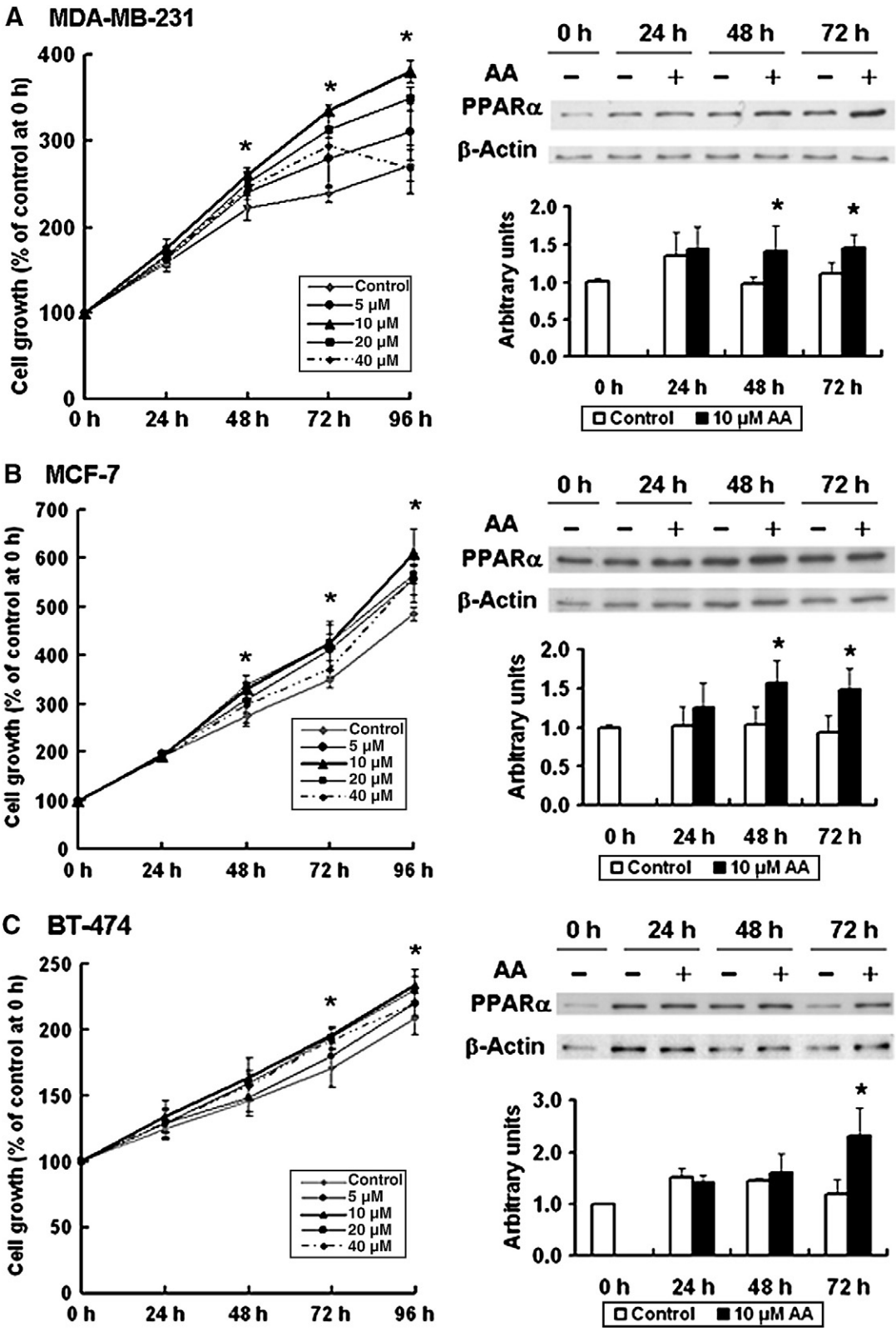

Fig. 2. Arachidonic acid stimulates cell growth and PPARα expression in (A) MDA-MB-231, (B) MCF-7 and (C) BT-474 breast cancer cells. (Left panel) All three cell lines were treated with various concentrations of arachidonic acid (AA) for 24, 48, 72 and 96 h, and then the cell numbers were calculated with a Beckman Coulter Z1 dual cell and particle counter. Cell growth rate is expressed as the percentages of control values at 0 h. All data are shown as the mean±S.D. from at least three independent experiments. Asterisks represents significant differences between 10 μM AA treatment and control (untreated) at the same time point (*P<.05, one-way ANOVA). (Right panel) All three cell lines were treated for various times in the absence (−) or presence (+) of 10 μM AA, and total cell lysates were then obtained. PPARα protein expression was analyzed by Western blotting. The densitometric values of the bands from three independent experiments are shown as the mean±S.D. Protein contents are normalized by the β-actin values and are expressed as the fold stimulation above the control values at the 0 h time point. Asterisks represent significant differences between the untreated (−) and 10 μM AA (+) stimulated samples at the same time point (*P<.05, one-way ANOVA).

3.5. Arachidonic acid modulates cell cycle progression in breast cancer cells

To explore the effects of AA on cell cycle progression, we analyzed the cell population at 4, 8, 12, 24, 32 and 40 h. In comparison to untreated control cells, MDA-MB-231 cells cultured for 8 h with 10 μM AA showed a decrease from 29.31±1.03% to 27.96±0.92% ($P<.05$) in the S phase population and an increase from 18.74±0.15% to 20.89±0.38% in the portion of G2-M cells ($P<.05$, Fig. 4A). Similar but more accelerated effects were observed after a 12 h induction (Fig. 4A), where the portion of G0/G1 phase increased from 57.57±0.22% to 59.22±0.54% ($P<.05$), the S phase population decreased from 23.81±1.01% to 20.65±0.41% ($P<.05$) and the G2/M fraction increased from 18.62±0.96% to 20.14±0.17% ($P<.05$). These results indicate that AA accelerated cell cycle progression in MDA-MB-231 cells. We also found that 10 μM AA promoted cell cycle progression after a 4 h induction in MCF-7 cells ($P<.05$, Fig. 4B) and after a 12 h induction in BT-474 cells ($P<.05$, Fig. 4C).

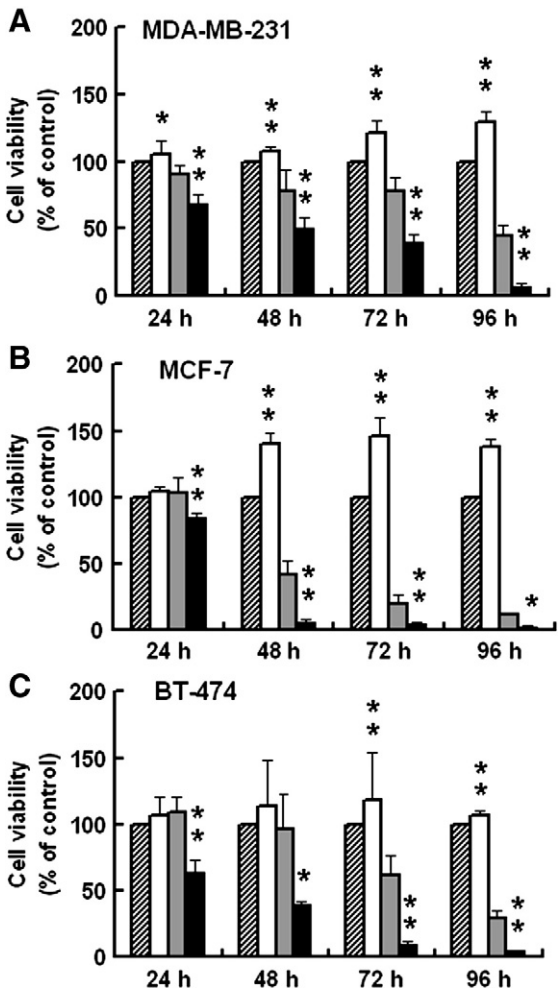

Fig. 3. Effects of arachidonic acid and MK886 on breast cancer cell proliferation. The cell lines MDA-MB-231 (A), MCF-7 (B) and BT-474 (C) were treated without AA and MK886 (control, ▨), with 10 μM AA (□), with a combination of 10 μM AA and 5 μM MK886 (■), or with 5 μM MK886 (■) for 24, 48, 72 and 96 h, and cell numbers were then calculated with a Beckman Coulter Z1 dual cell and particle counter. Cell viability is expressed as the percentages of control values at the same time point. Data from three independent experiments are presented as the mean±S.D. Values that are significantly different from the AA+MK886 group, as determined by one-way ANOVA are indicated (*$P<.05$, **$P<.005$).

3.6. Expression of proteins that play critical roles in cell cycle and cell proliferation

Because the activation of PPARα by AA is involved in cell cycle progression and MCF-7 cells show the most sensitive growth inhibition upon MK886 exposure, we therefore determined the protein levels of cyclin D1, CDK4, cyclin E, p27 and CDK2, which

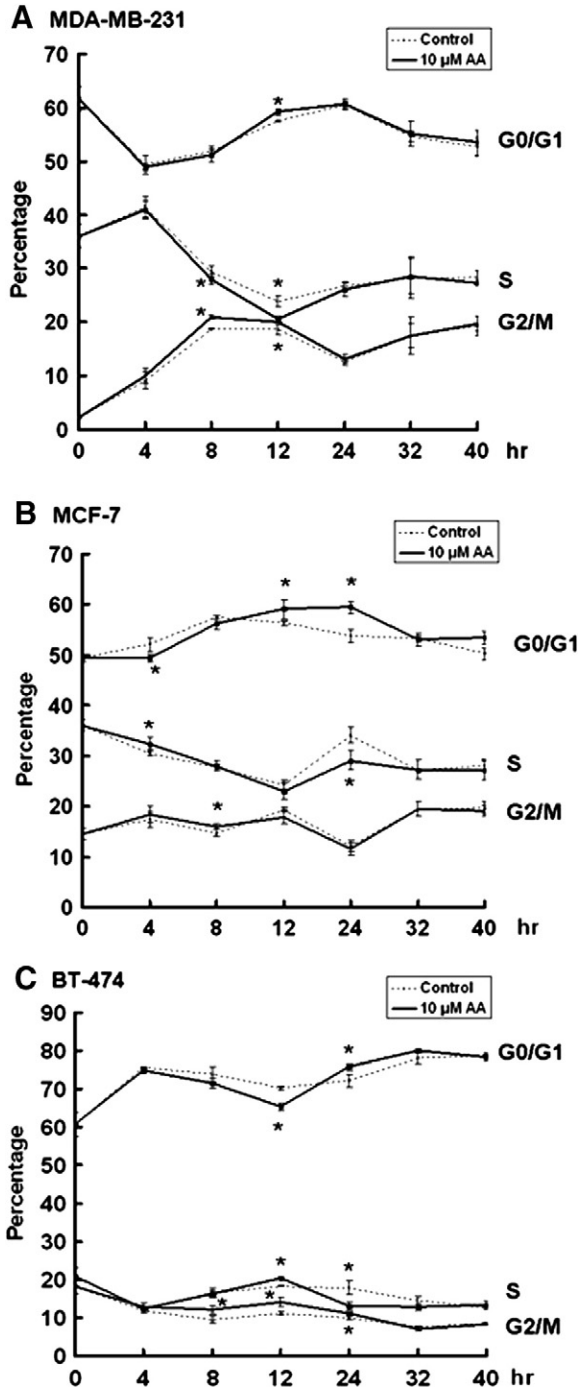

Fig. 4. Propidium iodide staining for the effects of arachidonic acid on the human breast cancer cell lines (A) MDA-MB-231, (B) MCF-7 and (C) BT-474. Cells were incubated with 10 μM AA for 0, 4, 8, 12, 24, 32 and 40 h, and the percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis, as described in the Materials and methods section. All data are shown as the mean±S.D. from three independent experiments. Asterisks represent significant differences between 10 μM AA treatment and control (untreated) at the same time point (*$P<.05$, one-way ANOVA).

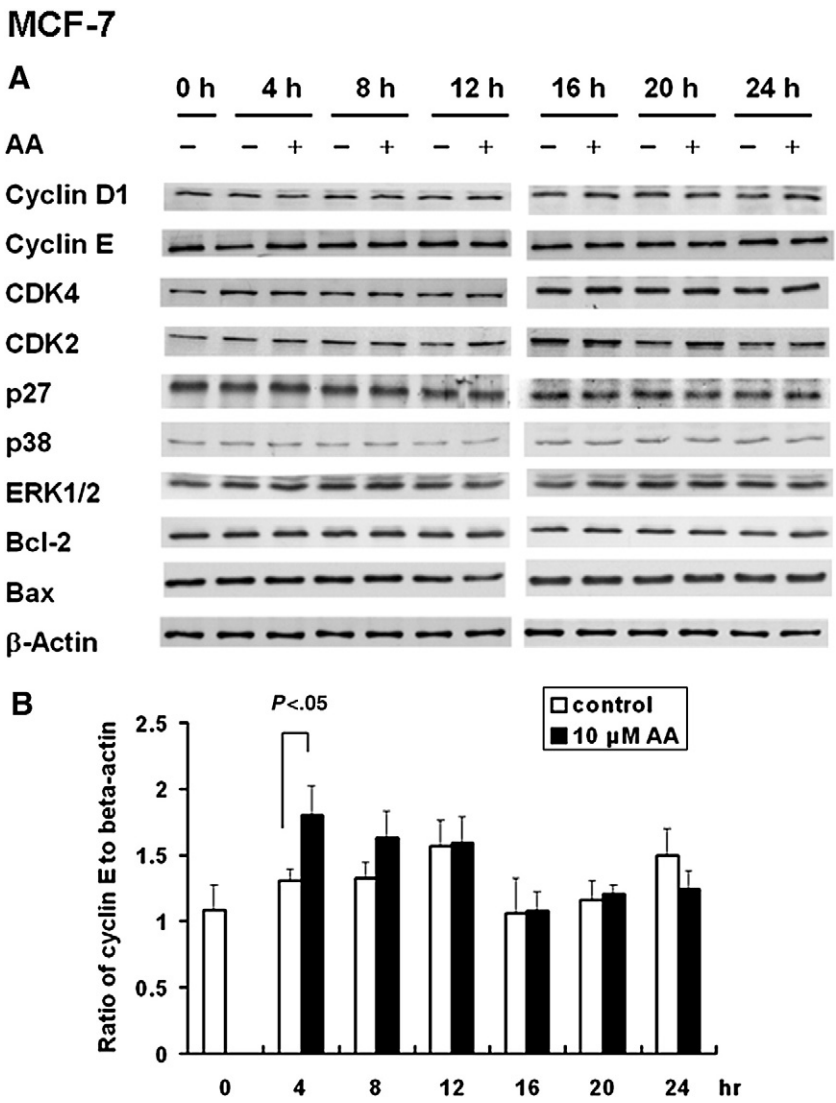

Fig. 5. Time-dependent changes in cell cycle, cell proliferation and cell survival regulatory protein levels in MCF-7 cells treated with arachidonic acid (AA). MCF-7 cells were treated with 10 μM AA for 0, 4, 8, 12, 16, 20 and 24 h. (A) Changes in the expression of cyclin D1, cyclin E, CDK2, CDK4, p38, ERK1/2 MAPK, Bcl-2 and Bax were measured by Western blot analysis. (B) The densitometric values of cyclin E expression from three independent experiments are shown as the mean±S.D. and are indicated by vertical bars. Asterisks represent significant differences between 10 μM AA treatment and control (untreated) at the same time point (*P<.05 by one-way ANOVA).

play critical roles in the G1 phase of the cell cycle and for passage from G1 into S phase in MCF-7 cells (Fig. 5). We found that stimulation of the cells with 10 μM AA promoted cyclin E expression after a 4 h treatment when compared with the control group (Fig. 5B, P<.05). However, the levels of cyclin D1, CDK4, p27 and CDK2 did not show significant differences. In addition, crosstalk between PPARα and the mitogen-activated protein kinase (MAPK) signaling pathway has previously been suggested [39]. To explore this crosstalk, we also determined the expression levels of p38, ERK1/2 MAPK, Bcl-2 and Bax, which are considered to be involved in cell proliferation and cell survival pathways. However, 10 μM AA treatment had no effect on p38, ERK1/2, Bcl-2 and Bax protein levels (Fig. 5A). Additionally, we examined the short-term treatment with AA and determined phosphorylated protein levels of p38, ERK1/2 and JNK at 1, 2 and 4 h. When MCF-7 cells were treated with 10 μM AA, the phosphorylation levels of ERK1/2 increased at 1 h treatment but declined after 2 and 4 h (Supplementary Fig. 1). The levels of phospho-p38 and phospho-JNK did not show differences from the control groups (Supplementary Fig. 1).

4. Discussion

Epidemiological studies have shown that dietary fatty acids are associated with an increased risk of breast cancer [40]. To exclude individual differences such as genotypes, dietary fats, lifestyles and environmental factors, we examined the lipid contents of cancerous and paired reference samples each taken from the same breasts. The results showed that phospholipid levels were 3-fold higher and triglyceride levels were 56.3% lower in the breast cancer tissues than in the reference tissues. Furthermore, the AA contents of the breast cancer tissues were positively correlated with mammary carcinogenesis (Table 1). Similar results have also been found in previous studies [2,27]. In contrast, no significant difference in the AA content was reported in Japanese women [1]. One explanation for the difference between our data and the Japanese study is that the average total fat intake (25% energy) in Japan in the 1990s was significantly lower than in Taiwan (~40% energy) in the 2000s.

Linoleic (ω-6) and linolenic (ω-3) acids are essential for human beings. AA (ω-6) is a semi-essential fatty acid that is produced from

the linoleic acid metabolic pathway or is obtained from dietary fats. Most studies have used serum-free medium to synchronize cells in the G0/G1 phase for cell cycle analysis. Notably, the signal transduction pathways that are related to linoleic acid or linolenic acid are also affected by serum-free conditions. In fact, human breast tumorigenesis is not an essential fatty acids-deficient condition. We therefore measured the effects of AA on breast cancer cell growth in 1% FBS-IMDM containing essential fatty acids. The three breast cancer cell lines MDA-MB-231 (ER–, PR– and HER2–), MCF-7 (ER++++, PR++ and HER2+/++) and BT-474 (ER++, PR+/++ and HER2++++) were therefore studied. We found that AA stimulated the growth rate of these three cancer cell lines in a dose- and time-dependent manner; MCF-7 cells showed the greatest response (Fig. 2).

Furthermore, we found that AA stimulation of breast cancer cell growth was linked to PPARα expression. Firstly, both AA and PPARα protein levels were higher in the breast cancer tissues than in the reference tissues (Fig. 1). Secondly, 10 μM AA treatment was found to increase PPARα protein expression in MDA-MB-231, MCF-7 and BT-474 cell lines (Fig. 2). Thirdly, up-regulation of PPARα by AA was specifically inhibited by MK886, a PPARα inhibitor (Fig. 3). A positive association between PPARα and breast tumorigenesis was also reported in isolated rat mammary gland epithelial cells [20] and breast cancer cell lines [18,41]. In contrast, it was reported that PPARα was involved in the growth inhibitory effect of AA in both MDA-MB-231 and MCF-7 cell lines [21]. This inconsistent result may be because the culture medium used in the study of Bocca et al. [21] was serum-free. According to the report of Suchanek et al., when MDA-MB-231 and MCF-7 cells were cultured in serum-free medium, cell growth did not progressively increase in a time-dependent manner over a 13-day observation period [18]. Another explanation may be the short duration time (24 h) of AA administration in the study of Bocca et al. [21]. In our cellular model, no significant difference between AA administration and PPARα expression was found after a 24-h observation; instead, the PPARα protein levels significantly increased after a 48 h AA induction (Fig. 2). Importantly, we found that when cells concomitantly treated with AA and MK886 for 48 h, AA reversed the inhibitory effects of MK886 in MCF-7 (5.8-fold), BT-474 (1.5-fold) and MDA-MB-231 (0.56-fold) cells. MK886 exhibited the highest inhibitory effects in MCF-7 cells after 48 h (Fig. 3B).

It should be noted that MCF-7 cells overexpress ER and that BT-474 cells show a moderate level of ER expression; in contrast, MDA-MB-231 cells are ER-negative. These results suggest that ER overexpression enhanced the effect of PPARα on the promotion of breast cancer cell growth. The ability of ER-modulated PPARα expression was also previously reported [42]. In addition, crosstalk between MAP kinase and ER was reported to enhance estrogen-mediated signaling and tumor growth in ER-positive breast tumors [43]. Cell cycle distribution was further determined in our study, and AA was found to accelerate the progression of cells into S phase after a 4 h induction in MCF-7 cells and a 12 h induction in BT-474 cells (Fig. 4). These results suggest that the cell proliferation stimulated by activation of PPARα was due to a shortening of the G1 phase. We therefore evaluated the expression of cyclin D1, CDK4, cyclin E, p27 and CDK2, which play critical roles in cell cycle progression in G0/G1 phase and in passage from G1 to S transition in MCF-7 cells (Fig. 5). We found that 10 μM AA promoted cyclin E expression after a 4 h treatment in MCF-7 cells, while it did not affect the expression levels of CDK2, cyclin D1 and CDK4. Cyclin E is an important cell cycle regulator that promotes the G1 to S phase transition through activation of CDK2 kinase activity [44,45]. The expression level of p27, a cyclin E/CDK2 inhibitor, was also evaluated, and no difference was found. Although no changes were found in the protein levels of ERK1/2, p38, Bcl-2 and Bax, which are considered to be involved in cell proliferation and survival after 24 h AA induction (Fig. 5A), changes in the phosphorylation level of ERK1/2 were found in the short-term AA induction (Supplementary Fig. 1). It was reported that the agonists of PPARα had the ability to phosphorylate PPARα leading to changes in transcriptional activity through MAPK family membranes (ERK, p38 and JNK) [46]. Therefore, the ability of AA to stimulate MCF-7 cell growth may be link to the effects on phospho-ERK1/2 downstream signaling [21,46]. However, the target genes involved in phospho-ERK1/2 signaling, as well as subsequent transcription events in response to PPAR-dependent or MAPK-dependent effects, require further identification.

In addition to the direct effects of AA on the activation of PPARα expression and promoting breast cancer cells proliferation, crosstalk between PPARα and ER overexpression plays a significant role in shortening the G1 phase and stimulating cyclin E expression. Cyclin E overexpression was proposed to be a marker of poor clinical outcome in breast cancer [47]. Notably, low molecular weight cyclin E, which is observed more efficiently than the full-length form, can increase the incidence of mammary tumor formation and distant metastasis [48,49]. The relationship between PPARα signaling, ER overexpression and low-molecular-weight cyclin E is not as clear. Further studies are required to clarify whether PPARα-promoted cell proliferation is related to high expression of low molecular weight cyclin E and is linked to the ER-dependent pathway.

In conclusion, in this study, we detected high levels of AA and PPARα in breast cancer tissues and provided evidence that AA could promote breast cancer cell proliferation through PPARα activation. The molecular mechanism is of this response involved the stimulation of cyclin E expression, the shortening of the G1 phase of the cell cycle and the promotion of the G1 to S phase transition in ER-over-expressing breast cancer cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.06.005.

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