Ellagic acid induces cell cycle arrest and apoptosis through TGF-β/Smad3 signaling pathway in human breast cancer MCF-7 cells

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Abstract. Breast cancer represents the second leading cause of cancer-related deaths among women worldwide and preventive therapy could reverse or delay the devastating impact of this disease. Ellagic acid (EA), a dietary flavonoid polyphenol which is present in abundance in pomegranate, muscadine grapes, walnuts and strawberries, has been shown to inhibit cancer cells proliferation and induce apoptosis. Here, we investigated the growth inhibitory effects of EA on MCF-7 breast cancer cells. In the present study, we first found that EA inhibits the proliferation of MCF-7 breast cancer cells mainly mediated by arresting cell cycle in the G0/G1 phase. Moreover, gene expression profiling of MCF-7 breast cancer cell line treated with EA for 6, 12 and 24 h was performed using cDNA microarray. A total of 4,738 genes were found with a >2.0-fold change after 24 h of EA treatment. Among these genes, 2,547 were downregulated and 2,191 were upregulated. Furthermore, the changes of 16 genes, which belong to TGF-β/Smads signaling pathway, were confirmed by real-time RT-PCR and/or western blot analysis. TGF-β/Smads signaling pathway was found as the potential molecular mechanism of EA to regulate breast cancer cell cycle arrest *in vitro*. Therefore, the regulation of TGF-β/Smads pathway in breast cancer cells could be a novel therapeutic approach for the treatment of patients with breast cancer. Further studies with *in vitro* models, as well as an analysis of additional human samples, are still needed to confirm the molecular mechanisms of EA in inhibition or prevention of breast cancer growth.

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer-related death among females in the world, accounting for 23% of the total cancer cases and 14% of the cancer deaths (1), indicating that prevention and early therapy of breast cancer is needed urgently. Currently, breast cancer is treated with surgery, chemotherapy, endocrine therapy, radiation therapy and targeted therapy or multidisciplinary synthetic therapy. Although these treatment modalities are remarkable successful, a significant number of patients either do not respond to therapy, or the tumor may recur and metastasize during therapy. The unsatisfactory prognosis strongly suggests that the evaluation of novel preventive agents is urgently needed to decrease the incidence of breast cancer.

Studies have shown that ellagic acid, a dietary flavonoid polyphenol which is abundant in pomegranate, muscadine grapes, walnuts and strawberries, can inhibit cancer cells proliferation and induce apoptosis (2-5). However, the precise molecular mechanism by which EA inhibits cancer cell growth is still unknown. Understanding the molecular biological properties of EA may lead to the clinical development of mechanism-based chemopreventive and therapeutic strategies for breast cancer. The alterations of gene expression profiles by some anticancer agents have been reported (6,7). In the present study, cDNA microarray can detect the changes of gene expression profiles and provide evidence for determining the effects of anticancer agents on cancer cells. We used the high-throughput gene chip, which contains 41,000^{+} known genes to understand the potential molecular mechanism of EA on MCF-7 breast cancer cells.

It is well known that many signal pathways play important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiologic processes, respectively (8-11). In the present study, several genes belonging to TGF-β/Smads signaling pathway were regulated remarkably by EA treatment in MCF-7 cells.

Materials and methods

EA and cell lines. Ellagic acid (EA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solution of

EA (2 mg/ml) was prepared in dimethyl sulfoxide (DMSO), and filter sterilized before use. The human breast cancer cell line MCF-7 was purchased from the Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin solution in an atmosphere of 95% air and 5% CO_2 in a 37˚C humidified incubator.

Cell proliferation assay. Cells were seeded in 96-well plates at a density designed to reach 70-80% confluency. Cells were allowed to adhere and 24 h later were treated with EA at 0, 10, 20, 30 and 40 μg/ml. After 24, 48 or 72 h of treatment, 200 μl of MTT was added to each well, and the cells were incubated for 4 h at 37˚C. The medium was discarded, and the dark blue formazan crystals were adequately dissolved with DMSO for 10 min on a rocker platform. The absorbance was measured at 570 nm using an ELISA reader (Tecan Sunrise, Männedorf, Switzerland). The cell viability was calculated according to: OD sample/OD control x 100%. The assay was performed in triplicate.

Cell cycle analysis. MCF-7 cells ($5x10^5$) were seeded in T25 culture flasks and grew for 6 h to reach 50-60% confluency. Cells were starved in serum-free medium for 24 h to achieve synchronization. After returning to regular growth medium for 6 h, cells were treated with EA at 0, 10, 20 and 30 μg/ml. DMSO (final concentration <0.1%) was used as a negative control. After treated for 24 h at 37˚C, floating and adherent cells were collected, washed with ice-cold PBS and fixed with 70% ethanol for at least 12 h at 4˚C. The cells were then treated with 80 mg/ml RNase A and 50 μg/ml PI at a density of $1x10^6$ cells/ml for 30 min, and the stained cells were analyzed using a FACScan cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Cell apoptosis analysis. The apoptotic rate was measured by FCM according to the instructions provided by the Annexin V-FITC kit (Becton-Dickinson). Briefly, following treatment with 0, 10, 20 and 30 μg/ml of EA for 24 h, cells were harvested after digestion with 0.25% trypsin. The collected cells were washed three times with ice-cold PBS containing calcium and resuspended in binding buffer (500 μl) at a density of $1x10^6$ cells/ml, in which 500 μl of cell suspension was added to a 5 ml FCM tube and Annexin V-FITC (50 μg/ml, 5 μl) and PI (50 μg/ml, 5 μl) were added. Then the cells were incubated for 30 min at room temperature in the dark. The apoptotic percentage of 10,000 cells was determined using FCM.

Morphology and ultra structure of apoptotic cells was observed by transmission electron microscopy (TEM). MCF-7 cells were seeded at a density of $1x10^6$ cells/flask and treated with 15 μg/ml EA-supplemented medium for 24 h. DMSO was used as a control. After incubation, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4˚C. Then, the cells were washed in the same buffer three times, fixed with 1% osmium tetroxide and dehydrated in graded ethanol. The 100% ethanol solution was then replaced by propylene oxide and embedded in epoxy resin, which was polymerized at 70˚C for 8 h. Sections were stained with uranyl acetate and lead citrate, and then observed with a Hitachi H-7650 TEM (Hitachi High Technologies, Tokyo, Japan).

The change of gene profiles in MCF-7 cells treated with EA. MCF-7 cells were plated at a density of $4x10^3$ cells/cm^2 in T75 culture flasks. Synchronization was achieved as previously described. The cells were harvested at 80-90% confluency. After exposure to EA (15 μg/ml) for 6, 12 and 24 h, total RNAs of the cells were harvested using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and the RNeasy kit (Qiagen, Dusseldorf, Germany) according to manufacturer's instructions. For each time-point, two preparations of RNA samples were independently subjected to array hybridization. After having passed RNA measurement using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and denaturing gel electrophoresis, the samples were amplified and labeled using the Agilent Quick Amp labeling kit and hybridized with Agilent whole genome oligo-microarray in Agilent's SureHyb hybridization chambers. After hybridization and washing, the processed slides were scanned with the Agilent DNA microarray scanner using settings recommended by Agilent Technologies (Palo Alto, CA, USA).

The resulting text files extracted from Agilent Feature extraction software (version 10.5.1.1) were imported into the Agilent GeneSpring GX software (version 11.0) for further analysis. The microarray data sets were normalized in Agilent Feature extraction software, and then the genes recorded present in all the samples were chosen for further analysis. Differentially expressed genes were identified through fold-change screening.

The gene expression profiling experiment was successfully completed on the samples. The profiling identified a subset of the total number of probes analyzed by Agilent Whole Genome Oligo microarray that are differentially expressed. GO Analysis and Pathway Analysis were performed on this subset of genes. More detailed information was found in Gene Ontology (GO) report and Pathway Analysis report.

Real-time reverse transcription-PCR analysis. Total RNA from MCF-7 cells exposed to 15 μg/ml EA for 6, 12 or 24 h were used for transcriptomics analysis by real-time reverse transcription-PCR (real-time RT-PCR) with selected target genes. Each 2 μg of RNA was reversely transcribed to cDNA using oligo(dT) primers and SuperScript II reverse transcriptase kit (Life Technologies). Primers were designed using Primer 5 software and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primers are shown in Table I. Real-time RT-PCR reactions were then performed in a total of 25 μl of reaction mixture using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the comparative Ct method and was normalized by GAPDH expression in each sample.

Western blot analysis. Western blot analysis was performed using the lysis buffer isolated protein. Briefly, 20 μg of protein was resolved in 10-15% SDS/PAGE and transferred to polyvinylidene fluoride membrane. The blots were blocked in blocking buffer overnight at 4˚C, and incubated with the primary antibody at 37˚C for 1 h. The antibody-antigen

Table I. The primer used for real-time RT-PCR analysis.

Genes	Primer sequenece
TGFβ1	TGGAAACCCACAACGAAATCTATG GCTAAGGCGAAAGCCCTCA
TGFβR II	AAAGGTCGCTTTGCTGAGGTCTA GTCGTTCTTCACGAGGATATTGGA
TGFβR I	TTCAAACGTGCTGACATCTATGC TTCCTGTTGACTGAGTTGCGATA
SMAD3	ATGGCCGGTTGCAGGTGTC GGTTCATCTGGTGGTCACTGGTTTC
p21^{Cip1}	TTAGCAGCGGAACAAGGAGT AGAAACGGGAACCAGGACA
p15^{Ink4b}	TGGTGGCTACGAATCTTCCG TCGTCGCTTGCACATCCTC
p19^{Ink4d}	CACCCTGAAGGTCCTAGTGGAG AGTGGGCAGGAGAAACAAGAAG
p57^{Kip2}	TCGGCTGGGACCGTTCA TGTATGGCAGCTACAGCTTGTG
CCND1	ATGTTCGTGGCCTCTAAGATGAAG GTGTTTGCGGATGATCTGTTTGT
CCNE	CAGTTTGCGTATGTGACAGATGGA GAGAAATGATACAAGGCCGAAGC
CCNA2	ATGATGAGCATGTCACCGTTCC TCCATTGGATAATCAAGAGGGACC
p107	AGAACCACCAAAGTTACCACGAA TCTTCAGAAGCCGTAAAGTCAGC
p130	AGAAGGGTGACTGAAGTTCGTG CAACATTGACTTGGACAGGGAAG
RB1	AAAGGACCGAGAAGGACCAACT CAGACAGAAGGCGTTCACAAAGT
E2F1	CCCCAACTCCCTCTACCCTT CTCTCCCATCTCATATCCATCCTG
E2F2	CTGGAGTGCAGTGGCCTGAT TGGCTCGTGCCTGTCATCTC
GAPDH	GGTGAAGGTCGGAGTCAACGG CCTGGAAGATGGTGATGGGATT

complexes were then detected with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies using a BCIP/NBT Alkaline Phosphatase Color Development kit. The band was recorded by a digital camera and analyzed by ImageJ software (NIH, Bethesda, MD, USA). The results were normalized with β-actin. The following monoclonal antibodies were used in the present study (source): anti-β-actin (Sigma Chemical), anti-Smad3, anti-p21^{Cip1} (Boshide Co., Wuhan, China), anti-phospho-Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Rb, anti-phospho-Rb, anti-TGFβ1, anti-TβR I and anti-TβR II (Cell Signaling Technology, Beverly, MA, USA).

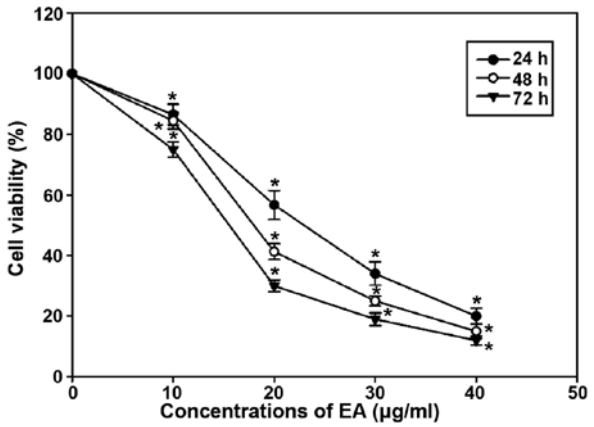

Figure 1. Effect of EA on the growth of MCF-7 cells. MCF-7 cells were exposed to increasing dosages of EA for 24, 48 or 72 h. The cell viability was expressed as the percentage of control group (DMSO). The data are presented as mean ± SD, n=6 per group. *P<0.05 compared to the vehicle control.

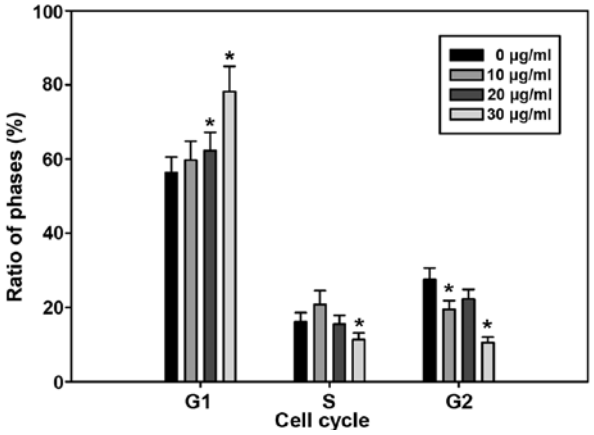

Figure 2. Effect of EA on MCF-7 cell cycle distribution. MCF-7 cells were treated with different concentrations of EA and subjected to flow cytometric analysis. The percentage of each phase is indicated in the panel. The results were representative of three independent experiments. *P<0.05 compared to the vehicle control.

Statistical analysis. The Student's t-test was used to determine the significance between treatments and untreated controls, and P<0.05 was considered significant.

Results

The effects of EA on cell proliferation in MCF-7 cells. The inhibitory effect of EA on cell proliferation in MCF-7 cells was assessed by MTT assay. As shown in Fig. 1, EA suppressed MCF-7 cell growth in a time- and dose-dependent manner. The inhibitory response was apparent as early as 24 h with a concentration range of EA from 10 to 40 μg/ml EA was found to reduce the cell number to 13.5 and 80% of the untreated control at concentrations of 10 and 40 μg/ml, respectively.

Cell cycle arrest at G0-G1 phase by EA. To determine whether decreased cell number accumulation was related to cell cycle arrest treated by EA, we assessed the effect of EA on cell cycle perturbation by flow cytometry. The data in Fig. 2 clearly showed a significant block in G0-G1 phase of the cell

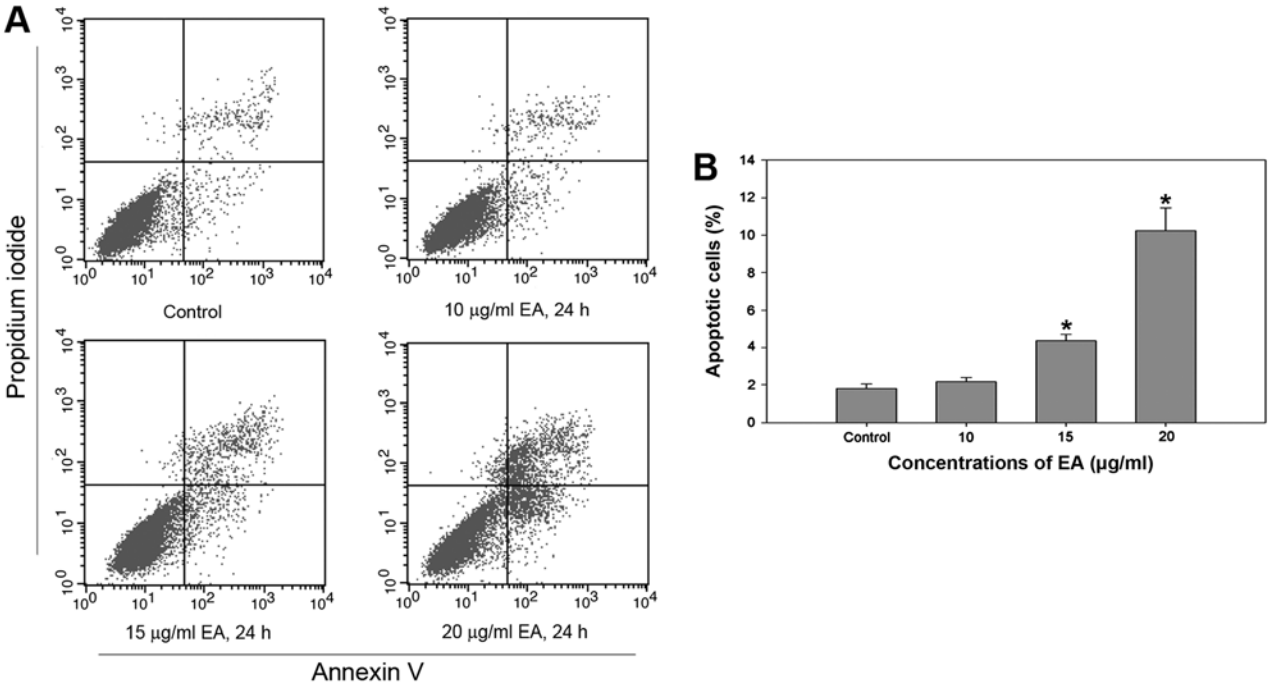

Figure 3. Effect of EA on apoptosis in MCF-7 cells. (A and B), MCF-7 cells were untreated and treated with increasing concentrations of EA for 24 h. Percentage of apoptotic cells were determined by staining with Annexin V and PI.

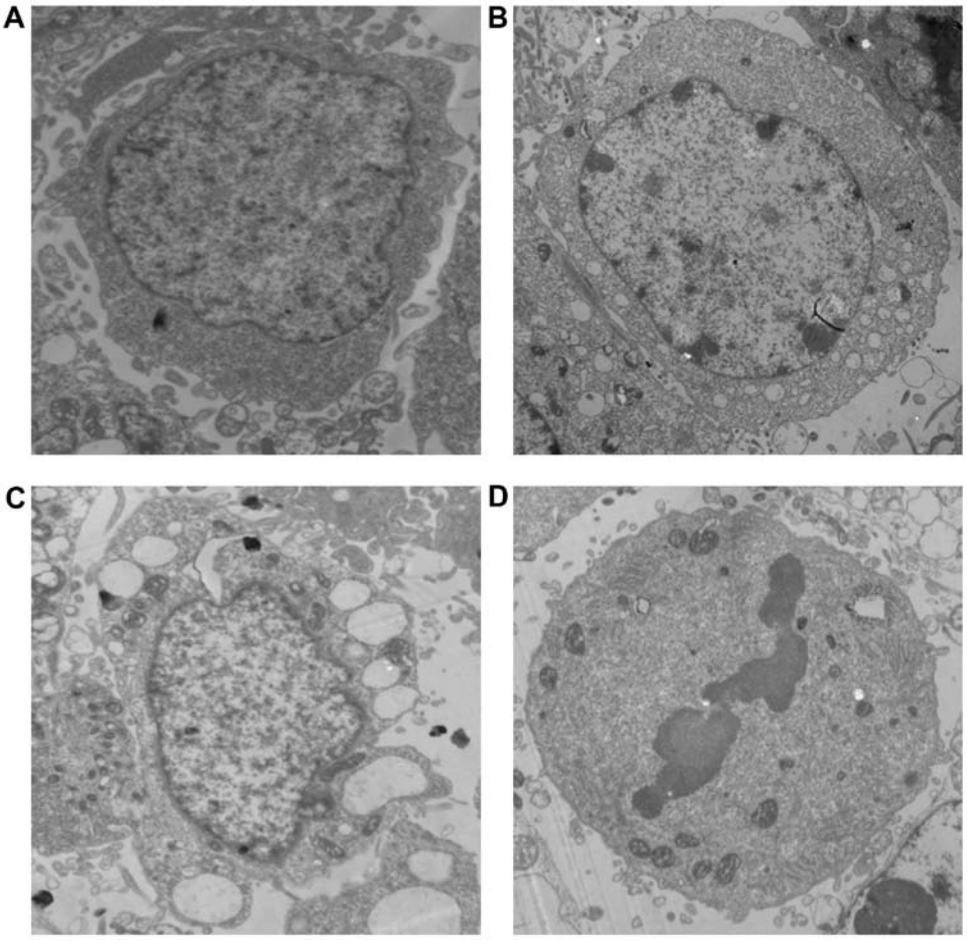

Figure 4. Morphology and ultrastructure of MCF-7 cells were analyzed by TEM. MCF-7 cells were treated with EA (15 μg/ml) for 24 h and examined by TEM at magnification x10,000. (A) In the control cells, the structure of the nucleus, abundance of cytoplasm, as well as the size and shape of the mitochondria, were all normal. (B) In the treated cells, the compaction and margination of nuclear chromatin were quite obvious. (C) Cell detachment, cell shrinkage, folded nuclear membrane, membrane blebbing, increased nuclear heterochromatin, swelling of the endoplasmic reticulum cisternae and vesicle formation (abundant vacuoles with multivesicular bodies) were observed. (D) Nuclear pyknosis and fragmentation occurred.

Table II. Fold-changes of specific genes in MCF-7 cells treated with EA.

Genes	MCF-7		
	6 h	12 h	24 h
NM_000660.4, transforming growth factor, β1 (*TGFβ1*)	NC	NC	NC
NM_001024847, transforming growth factor, β receptor II (*TGFβRII*)	NC	NC	NC
NM_004612.2 , transforming growth factor, β receptor 1 (*TGFβRI*)	NC	NC	NC
NM_005901.4, SMAD family member 2 (*SMAD2*)	NC	NC	NC
NM_005902.3, SMAD family member 3 (*SMAD3*)	NC	NC	2.7
NM_000389.1, CDK inhibitor 1A ($p21^{Cip1}$)	NC	3.4	4.8
NM_078487.3, cyclin-dependent kinase inhibitor 2B ($p15^{Ink4b}$)	NC	NC	4.8
NM_001800.3, cyclin-dependent kinase inhibitor 2D ($p19^{Ink4d}$)	NC	NC	2.8
NM_000076.2, cyclin-dependent kinase inhibitor 1C ($p57^{Kip2}$)	NC	NC	0.3
NM_053056.2, cyclin D1 (*CCND1*)	NC	0.5	2.1
NM_001238, cyclin E 1 (*CCNE1*)	2.4	NC	NC
NM_057749, cyclin E 2 (*CCNE2*)	2.3	NC	0.3
NM_001237.3, cyclin A2 (*CCNA2*)	NC	NC	0.3
NM_002895.2, retinoblastoma-like 1 (*p107*)	NC	NC	0.4
NM_005611.3, retinoblastoma-like 2 (*p130*)	NC	NC	NC
NM_000321.2, retinoblastoma 1 (*RB1*)	NC	NC	0.5
NM_005225.2, E2F transcription factor 1 (*E2F1*)	NC	0.5	0.2
NM_004091.3, E2F transcription factor 2 (*E2F2*)	NC	NC	0.4

NC, no change. <1, decrease; >1, increase.

cycle. The increase in the G0-G1 population was accompanied by a delay of passage of cells to S phase. Synchronized control cells that were not treated by EA moved into S phase.

Effect of EA on cell apoptosis. In order to determine whether EA could induce cell apoptosis, FCM and TEM were conducted in EA-treated MCF-7 cells and the control. FCM showed that the cell apoptotic rates were 4.36±0.35 and 10.24±1.23 after treatment with 15 or 20 μg/ml of EA for 24 h, respectively. MCF-7 cells treated with increasing concentrations of EA exhibited a significant increase in the apoptotic cell fraction, indicating apoptosis induction (Fig. 3).

Morphological and ultra-structural characteristics of apoptosis in MCF-7 cells exposed to EA for 24 h were examined by transmission electron microscope (TEM) (Fig. 4).

Profiling of EA-responsive genes by cDNA microarray analysis. We found a total of 4,738 genes that showed a >2.0-fold change after 24 h of EA treatment. Among these genes, 2,547 genes were downregulated and 2,191 genes were upregulated in EA-treated MCF-7 cells. The altered expressions of many genes did not occurr after 6 h of EA treatment and changes were evident with 24 h of treatment (Table II).

After clustering based analysis and pathway analysis according to their biological functions, several genes, which are related to cell cycle, apoptosis and DNA replication were found increased or decreased (Fig. 5). Especially, TGF-β/Smads pathway was found as the potential pathway, by which EA induced cell cycle arrest in G0/G1 phase and educed the inhibitory effect on MCF-7 cells (Fig. 6).

Detection of EA-responsive genes by real-time RT-PCR and western blots. The next step was to confirm the changes of a subset of 16 genes that showed pronounced regulation in TGF-β/Smads pathway by real-time RT-PCR and/or western blot analysis. We examined the genes (TGF-β1, TβR-I, TβR-II, Smad3, $p15^{Ink4b}$, $p19^{Ink4d}$, $p21C^{ip1}$, $p57^{Kip2}$, cyclin D1, cyclin E, cyclin A2, E2F1, E2F2, Rb1, *p*107 and *p*130) by real-time RT-PCR and some proteins (TGF-β1, TβR-I, TβR-II, Smad3, *p*-Smad3, Rb1, *p*-Rb1 and $p21^{Cip1}$) by western blots. We were able to verify eight proteins by western blot analysis and ten RNAs by real-time RT-PCR. This represents a success rate of 75% (12 out of 16). The results are shown in Fig. 7, respectively. The lack of complete concordance could be attributable to either false positive signals of the array data or the discrepancy between transcript and protein expression. There was a noteworthy observation. A decreased level of cyclin D1 was found at 12 h, but an upregulation of cyclin D1 expression was detected by cDNA microarray, real-time RT-PCR and western blot analyses at 24 h. Cells lacking cyclin D1 are known to stay in cell G0-G1 arrest (12,13). It is possible that at the early time-point, the repression of cyclin D1 was necessary for initiating EA-mediated cell cycle arrest, whereas at the later time-point when G0-G1 arrest was firmly established, cells produced more cyclin D1 for apoptosis to advance (14).

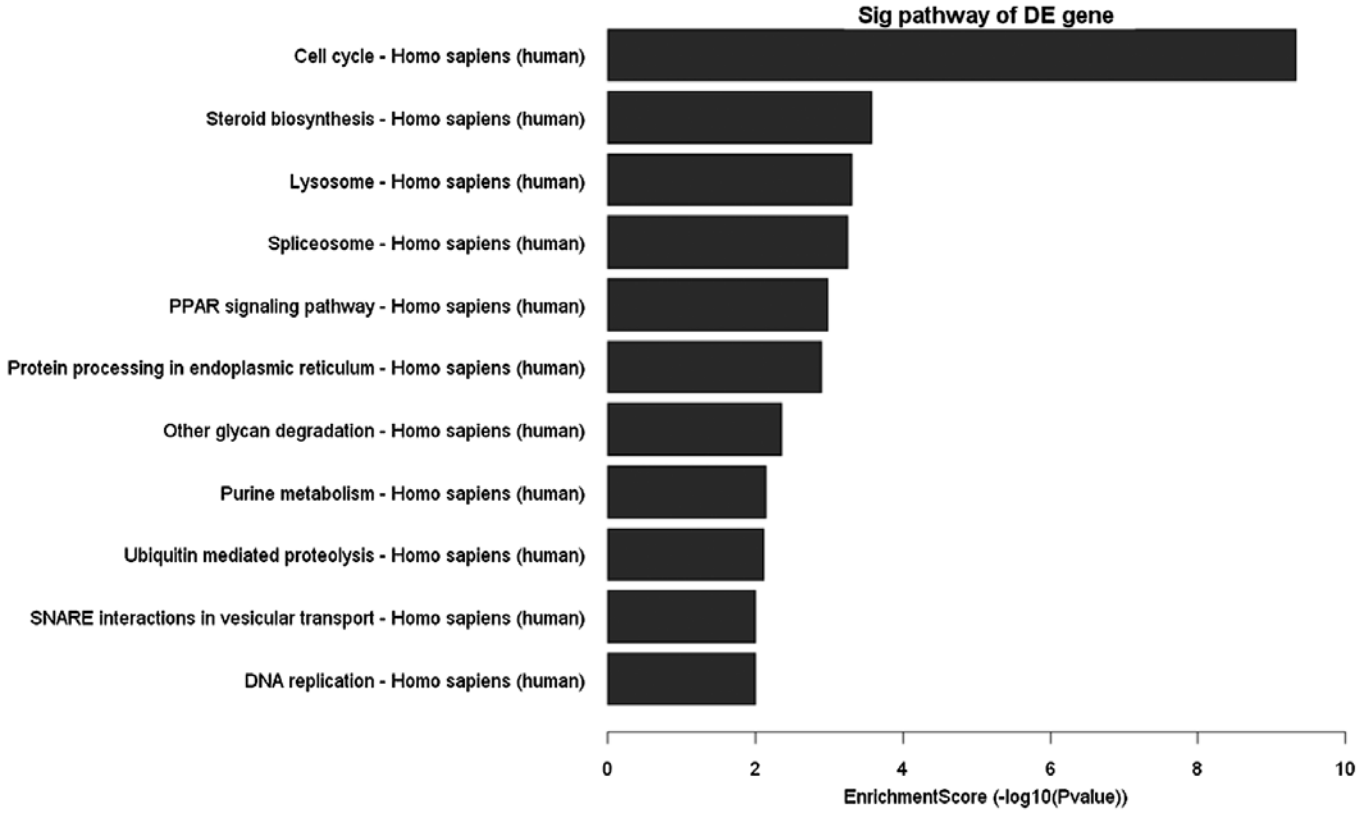

Figure 5. Pathway analysis of specific genes in MCF-7 cells treated with EA.

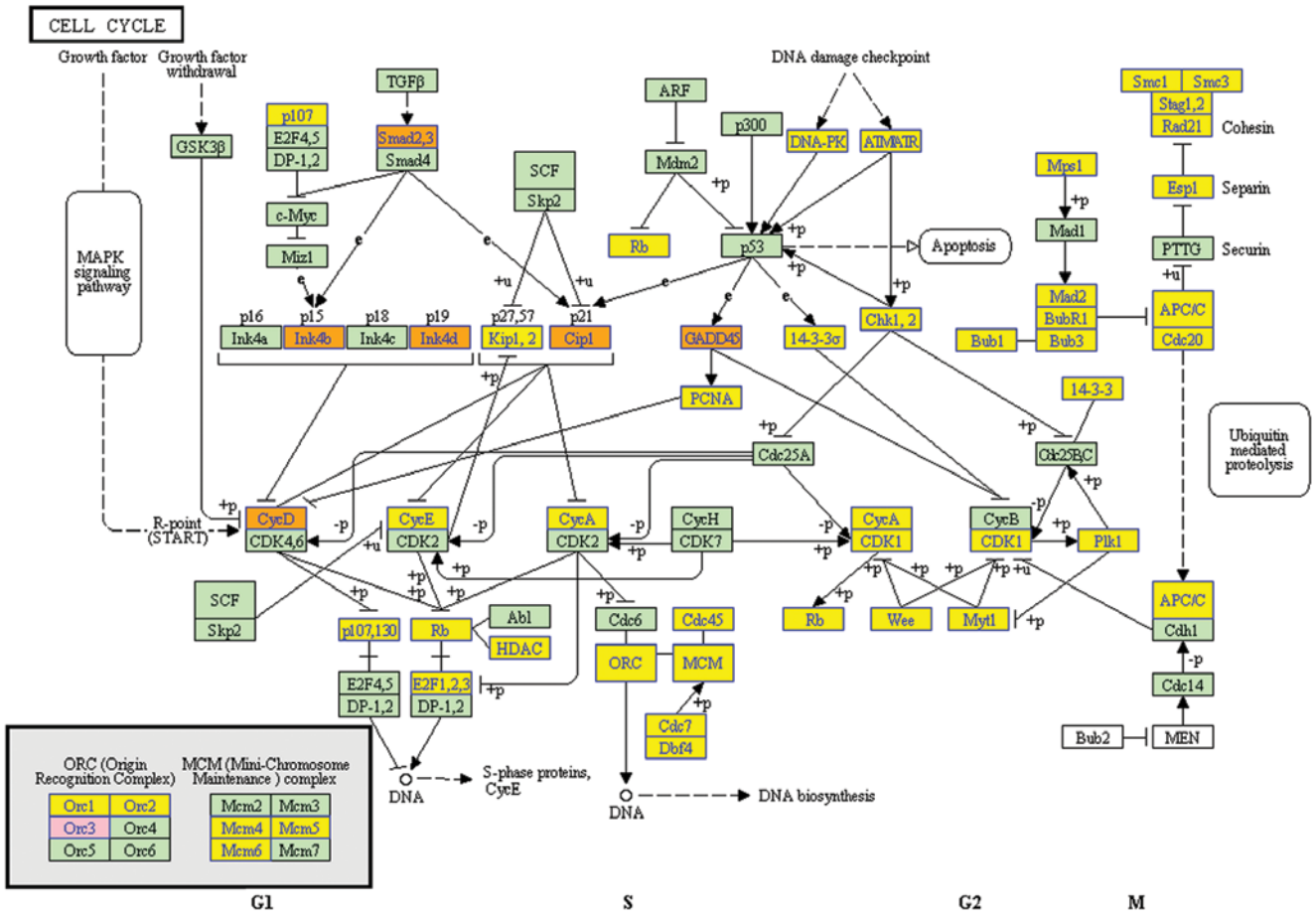

Figure 6. The change of genes in TGF-β/Smads pathway in MCF-7 cells treated with EA. Green indicates no change, yellow indicates downregulation and brown indicates upregulation.

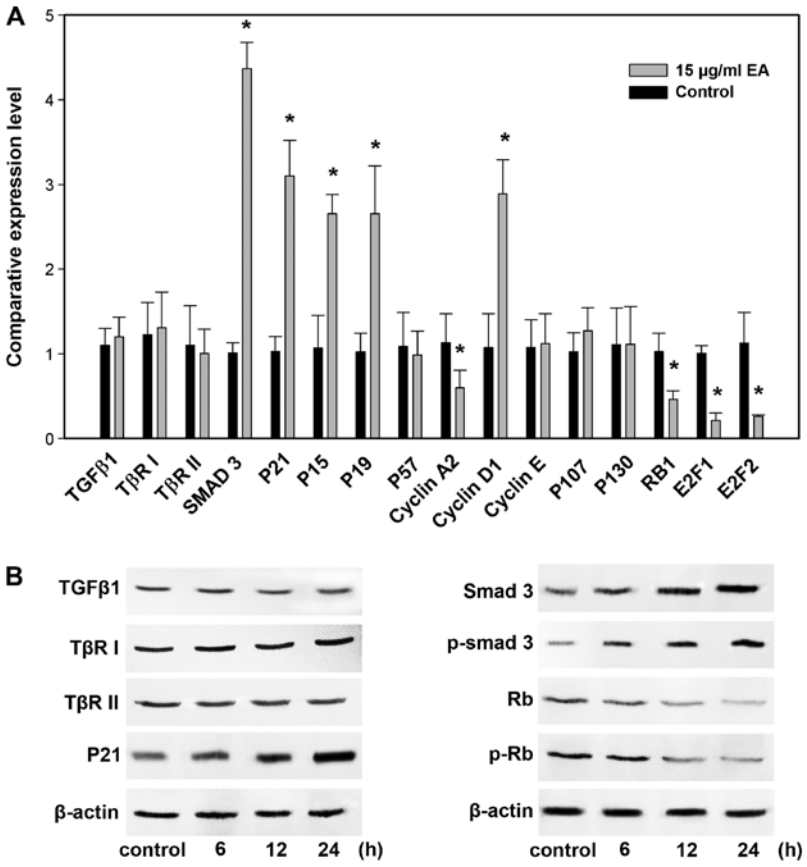

Figure 7. (A) The changes of a subset of genes in TGF-β/Smads pathway in MCF-7 cells treated with 15 μg/ml of EA for 24 h by real-time RT-PCR. (B) The changes of a subset of proteins in TGF-β/Smads pathway of MCF-7 cells treated with 15 μg/ml of EA for 6, 12 and 24 h by western blot analysis.

Discussion

To understand the molecular mechanisms leading to EA-induced growth inhibition on MCF-7 cells, in this study, cDNA microarray was used to elucidate the changes in gene expression profile. On the basis of the collection of 41,000$^+$ genes screened by the whole human genome oligo microarray of Agilent, 4,738 genes were identified responsive to EA treatment for 24 h. These genes are responsible for cell cycle arrest, apoptosis, steroid biosynthesis, lysosome, splicesome, protein processing in endoplasmic reticulum and DNA replication. TGF-β/Smads pathway was found to be the target through pathway analysis, by which EA educed the inhibitory effect on the growth of MCF-7 cells. In order to confirm the result of cDNA microarray, 16 genes belonging to TGF-β/Smads pathway were further characterized by either western blots or real-time RT-PCR. On the basis of the results obtained from the present study, we proposed that TGF-β/Smads signaling pathways could mediate the outcome of EA-induced cell cycle arrest.

Our results also showed that cyclins (cyclin A2 and cyclin E2) were downregulated in EA-treated MCF-7 cells, whereas cyclin-dependent kinase (Cdk) inhibitors ($p21^{Cip1}$, *p*15 and *p*19) were upregulated. These results suggest that EA inhibited the growth of breast cancer cell through the arrest of the cell cycle and inhibition of proliferation. Driving of the cell cycle through one phase to the other is tightly controlled by complex network events of cyclins, Cdk and transcription factors (15). During mid G1 phase, cdk4 and cdk6 interact with D type cyclins to form heterodimer kinase complex. This event follows the interaction of cyclin E with cdk2 to phosphorylate Rb in the late G1 phase (16-18). The cdk inhibitors, including $p21^{Cip1}$, *p*15 and *p*19, have been shown to inhibit activity of cyclin-cdk complex to decrease phosphorylation of Rb. Phosphorylation of Rb has been shown to be critical for the stabilization of active E2F1 to translocate into the nucleus and transcribe various genes required for the entry of the cell from G1 to S phase (19).

TGF-β/Smads signaling pathway is involved in a broad spectrum of biological responses throughout embryonic development and adult life, including cell proliferation, differentiation, epithelial-to-mesenchymal transition, apoptosis and angiogenesis (20,21). Transforming growth factor-β (TGF-β) is a member of the TGF-β superfamily. The cytokine signals carried by TGF-β were transmitted through a heterogenic complex of type I and type II serine/threonine kinase receptors (TβR-I and TβR-II). Activation of the receptor complex through ligand binding results in the phosphorylation of the TβR-I by the TβR-II. Subsequently, active TβR-I phosphorylate receptor-regulated Smads (R-Smads), which are intracellular transducers of TGF-β signals, including Smad2 and Smad3. Phosphorylated R-Smads then associate with Smad4, the common Smad (Co-Smad) and shuttle to the nucleus. The complexes interact with a large repertoire of transcription factors and result in corresponding biological function subsequently (22-25). TGF-β has been described as a potent tumor

suppressor for promoting cell growth inhibition, apoptosis and differentiation (26,27). Mutations in the components of the TGF-β signaling cascade have been identified in a number of human cancers, including hereditary non-polyposis colon cancer, hepatocellular carcinoma, and pancreatic and ovarian cancers (28).

TGF-β1 is a potent inhibitor of cell proliferation. TGF-β1-induced arrest occurs during G1 and is mediated by Smad proteins, which regulate transcriptional targets, including c-myc (29-31). Downregulation of c-myc allows induction of $p15^{Ink4b}$, which inhibits Cdk4-cyclin D (32,33). The $p27^{Kip1}$ inhibitor is also utilized by TGF-β1 to inhibit Cdk2-cyclin E (34). Cdk suppression prevents hyperphosphorylation of Rb (18), causing Rb to remain in a hypophosphorylated, growth-suppressive form (35).

TGF-β1 inhibits the growth of cells of epithelial origin by downregulating components of the cell cycle and upregulating cell cycle inhibitors (36). In most epithelial cell types TGF-β1 acts in late G1 phase and prevents further progression to the G1/S phase transition (37). Cdks and cyclin complexes phosphorylate specific target molecules, such as the retinoblastoma proteins pRb, p107 and p130 (38). The Cdk inhibitors mediate cell cycle arrest at different points of G1 (39). TGF-β1 inhibitory actions in late G1 phase are mediated in part by the inhibition of cyclin D1 and cyclin E expression which prevents Cdk kinase activity resulting in RB hypophosphorylation (40,41) and/or by the upregulation the expression of various Cdk inhibitors such as p21 and p27 (42). The transcription factor E2F regulates the expression of S and G2 phase cyclins such as cyclins E, A and B (43). Hypophosphorylated Rb binds to and represses E2F by recruiting histone deacetylases and forming a repressor complex at E2F-responsive promoters to block transcription of cyclins necessary for cell cycle progression (44,45).

In the present study, we found that EA inhibits the proliferation of MCF-7 breast cancer cells mainly mediated by arresting cell cycle in the G0/G1 phase. TGF-β/Smads signaling pathway was further found as the potential molecular mechanism of EA to regulate cell cycle arrest *in vitro*. Therefore, the regulation of TGF-β/Smads pathway in breast cancer cells could be a novel therapeutic approach for treatment of patients with breast cancer. Further studies with *in vivo* models, as well as an analysis of additional human samples are still needed to confirm the molecular mechanisms of EA in inhibition or prevention of breast cancer growth.

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