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Inhibitory actions of genistein in human breast cancer (MCF-7) cells

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Abstract

Genistein, a natural isoflavanoid phytoestrogen, is thought to be the active ingredient in soy that possesses breast cancer preventive properties. The molecular mechanisms that are involved in its cancer preventive properties have not been completely understood. The present study is designed to investigate the mechanism involved in the inhibitory action of genistein in MCF-7 cells. Genistein at 50 and 100 μM significantly arrested the growth of MCF-7 cells at G2/M phase ($P<0.05$) and decreased at the proliferative S phase ($P<0.05$). Using cDNA microarray technology, genes differentially regulated by genistein were identified. In particular, as confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR), genistein up-regulated heat shock protein 105 (HSP) mRNA and down-regulated mRNA expression of serum response factor (SRF), estrogen receptor (ER) α, disabled homolog 2 (DOC 2) and recombination activation gene 1 (RAG-1). Using real time RT-PCR, we have shown that HSP and SRF mRNA were both regulated by genistein in a time- and dose-dependent manner; however, it appears that only the effect of genistein on SRF mRNA, but not HSP mRNA expression, can be partially abolished by cotreatment with estrogen antagonist ICI 182,780. Western blotting analysis showed that the expressions of the ERα and SRF protein decreased significantly with genistein treatment ($P<0.05$). These results suggest that the inhibitory action of genistein on human breast cancer cells appears to be complex and is only partially mediated by the alteration of estrogen receptor-dependent pathways.

Keywords: Genistein; cDNA microarray technology; Gene expression; MCF-7 cell

1. Introduction

Asian women consuming a diet high in soy products have a low incidence of breast cancer [1,2]. Isoflavone phytoestrogens are the major active ingredients in soy products such as tofu, soybean milk, miso and tempeh. These phytoestrogens are found to be the normal constituents of human urine [3] and plasma [4,5] from subjects who regularly consumed large amount of soy products. The association between the lower risk of breast cancer and the consumption of soy products has led to the widespread interest in consumption of soy products, isoflavone supplements and foods to which isoflavones have been added [6].

Despite the favorable association found between soy product intakes and risk of breast cancer in epidemiological studies, conflicting data exist in experimental settings using soy isoflavones for prevention of human breast cancer [6–15]. Many factors have been shown to influence the properties of soy isoflavone on human breast cancer cells in vitro and in vivo, including the circulating/surrounding level of estrogen [7–9,12,16], presence or absence of estrogen receptor as well as dosage [12,13,16–18]. In the absence of endogenous estrogens, physiological concentrations (1 nM to 10 μM) of genistein, a major soy isoflavone, stimulate the growth of estrogen receptor positive human breast cancer (MCF-7) cells [12,17,18] but not estrogen receptor negative breast cancer (MDA-MB-435) cells in vitro [12]; whereas high concentration of genistein (>10 μM) inhibits the growth and survival of both MCF-7 cells [12,17] and MDA-MB-435 cells [19,20].

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The study of the mechanism of action of the major soy isoflavone, genistein, in human breast cancer is vital for understanding its properties in vitro and in vivo and is therefore an area that is under intense investigation. Genistein can bind to estrogen receptors, affects estrogen-regulated gene expression [21] and displays both estrogen agonist [12] and antagonist properties [16]. It can also exert its effects in many ways without direct interaction with the estrogen receptors. It was previously shown to alter estrogen metabolism via its inhibitory action on aromatase [22]. It is also a well-known protein tyrosine kinase inhibitor that competes with ATP for binding to tyrosine kinase, and thereby interferes with tyrosine kinase cascade activated by mitogens [9,23]. In addition, genistein has been demonstrated to inhibit DNA topoisomerase activity [24], suppress angiogenesis [25] and induce apoptosis [26,27] and stress [28].

The present study is designed to investigate the molecular mechanism involved in the inhibitory action of genistein in estrogen receptor positive human breast cancer (MCF-7) cells. Using cDNA microarray technology, differentially regulated genes in response to high concentrations of genistein treatment were identified. The regulations of heat shock protein (HSP) and serum response factor (SRF) mRNA, two of the newly identified genistein-regulated genes, were studied using real time reverse transcriptase-polymerase chain reaction (RT-PCR). It is hoped that this study will provide new insights for understanding the inhibitory action of genistein in human breast cancer and that the newly identified targets can serve as potential targets for cancer drug development.

2. Materials and methods

2.1. Culture of human breast cancer cell line (MCF-7)

MCF-7 cells (ATCC no. HTB-22) were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin 100 IU/ml and streptomycin 100 μg/ml. At 50% to 60% confluence, cells were transferred to phenol-red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum (sFBS), penicillin 100 unit/ml and streptomycin 100 μg/ml for 4 days before the cells were treated with genistein (50 μM, 100 μM) (Sigma, St. Louis, MO, USA) for 24 h. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For study of the regulation of SRF and HSP expression, MCF-7 cells were subjected to treatment with 100 μM genistein for different time periods (6, 24, 48 h) or different concentrations (1, 50 or 100 μM) of genistein for 24 h. For ICI 182,780 (Tocris, Bristol, UK) treatment, MCF-7 cells were exposed to 100 μM genistein in the presence or absence of estrogen antagonist ICI 182,780 (1 μM) for 24 h.

2.2. Flow cytometry

After treatment, MCF-7 cells were isolated into conical tubes, washed twice with phosphate-buffered saline (PBS) and fixed in 70% ice-cold ethanol at −20 °C. For DNA analysis, cells were centrifuged and washed two times with PBS. DNA contents of the nuclei were determined by staining nuclear DNA with propidium iodide (PI, Sigma, 50 μg/ml) solution containing 50 μg/ml of RNase A and incubated at 37 °C in the dark for 30 min. The DNA content, as reflected by the fluorescence signal of PI, was measured by using Flow Cytometer (Becton-Dickinson, Immunocytometry Systems, Mountview, CA). Distribution of cells in different phases of cell cycle was determined using a software program Modfit (Becton-Dickinson, Immunocytometry Systems) [29].

2.3. Fluorescent cDNA microarray analysis of MCF-7 cells

The human cDNA probes generated from Unigene sets were printed onto polylysine-coated slides by microarrayer (SPBIO, Hitachi Software Engineering) in a lower density format. The cDNA probes printed on the microarray slides were immobilized to the slides by 60 mJ of UV light cross-linking. The cDNA chip consists of 448 human genes, which encode for stress response, oncogene, kinase and phosphatase, G-protein, apoptosis, cell cycle, tumor suppressor, transcription factor, growth factor and receptor. The control genes including housekeeping genes are printed in duplicate. Fifty micrograms of total RNA was used as template to prepare cDNA probes labeled with Cy5-dUTP or Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, USA) by using SuperScript II RT kit (Roche Applied Science, Mannheim, Germany). Hybridization was carried out at 65 °C for 16 h and signal was obtained by a ScanArray® 4000 (Gsi Lumonic, USA). Clustering analysis was carried out by using Cluster software from Elsein' lab (Stanford University, California, USA) and a modified SOM method developed in-house. A differentially expressed gene is defined by a ratio of treated/control >2.0 or <0.5.

2.4. Verification of cDNA microarray data using RT-PCR analysis

Total RNA was isolated from cells by using Trizol reagent according to the standard protocol. Total RNA (2 μg) was used to generate cDNA in each sample using the SuperScript II reverse transcriptase with oligo(dT) 12–18 primers. Aliquots (5–10%) of total cDNA were amplified in each PCR reaction mixture that contains 0.5 μM of sense and antisense primers (Genemed Synthesis, Inc., South San Francisco, USA) of selected genes (Table 1). PCR amplification was performed on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, USA). The PCR products were analyzed on agarose gel electrophoresis. Optical densities of ethidium bromide-stained DNA bands were quantified by Luminal Imager (Roche Molecular Biochemicals) and the mRNA

Table 1
Primers used for RT-PCR verification

Primer	Sequence	Product		
		Orientation	Size (bp)	Annealing temperature (°C)
HSP	AGGAGTTCCATATCCAGAA	sense	306	46
	CAGCTCAACATTCACCAC	antisense		
ERα	AAGTTCAGGCACAATTGGATG	sense	502	50
	CCCTGCATGACACTGATTACA	antisense		
SRF	ACTGCCTTCAGTAGGAACAA	sense	595	56
	TTCAAGCACACACACTCACT	antisense		
RAG-1	GTCAGTGAGGTCAAAAGGAG	sense	597	53
	ACAGGTCCCCTGAATCAA	antisense		
DOC2	GGGATCGCCTGGTGTCACCAA	sense	300	57
	TTGTCCCTGAGACCGACCA	antisense		
GAPDH	ACCACAGTCCATGCCTACAC	sense	452	55
	TTCACCACCCTGTTGCTGTA	antisense		

expression levels were normalized to the expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5. Real-time quantitative RT-PCR

PCR amplification and analysis were achieved using a LightCycler instrument and software version 3.5, respectively (Roche Applied Science). Thermocycling were done in a final volume of 15 μl containing 1 μl cDNA sample or calibrator; 4 mM $MgCl_2$; 0.5 μM of each primers; 1.5 μl LightCycler FastStart DNA Master SYBR Green 1 mix (Roche Applied Science). After denaturing of cDNA at 95 °C for 10 min, the cycling conditions were as follows: 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at 55 °C (for SRF), 46 °C (for HSP) or 56 °C (for GAPDH) for 10 s, and extension at 72 °C for 18 s. Following the completion of the PCR amplification reaction, a melting curve analysis was performed by heating the sample to 95 °C programmed for 0 s followed by cooling down to 60 °C for 15 s and slowly heating the samples at 0.1 °C/s to 95 °C while the fluorescence was measured continuously. GAPDH was used as a reference gene for internal control. The fluorescence signal was plotted in real time against the temperature to produce melting curves of each sample. Melting curves were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature against temperature ($-dF/dT$ versus T). Thus, each specific PCR product generates a specific signal and therefore a product-specific melting peak. After completion of LightCycler analysis, PCR products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide, visualized and photographed under ultraviolet illumination. Expected band lengths were 595 bp for SRF, 306 bp for HSP and 452 bp for GAPDH. A relative standard curve of target and reference gene for quantification of RT-PCR product was

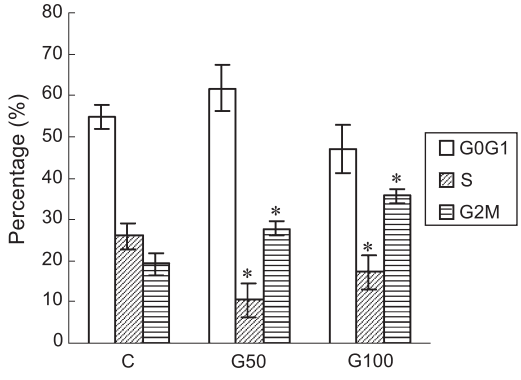

Fig. 1. Effect of genistein on cell cycle distribution in human breast cancer (MCF-7) cells. MCF-7 cells were cultured and treated with different concentrations of genistein or its vehicle for 24 h. Flow cytometric analysis of cell cycle distribution in MCF-7 cells was performed (see Materials and methods). Distribution of cells in different phases of cell cycle was determined using a software program Modfit (Becton-Dickinson, Immunocytometry Systems). Results were obtained from three independent experiments and expressed as mean ± S.E. *$P < 0.05$ vs. control, $n = 3$.

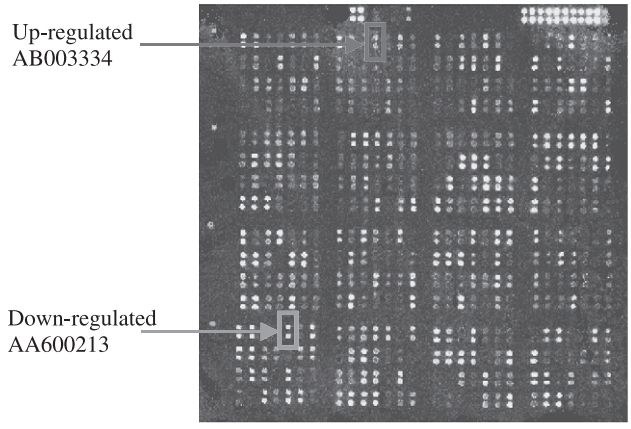

Fig. 2. Representative image of cDNA microarray showing the effect of genistein on gene expression profile in human breast cancer (MCF-7) cells. Total RNA from control samples and from genistein-treated samples were used to prepare Cy3- and Cy5-labeled cDNA, respectively. The probes were hybridized to the microarray as described in Materials and methods. In this representative image, up-regulated genes in response genistein in MCF-7 are shown as red spots while the down-regulated genes are shown as green spots. The two differentially regulated genes in MCF-7 cells treated with 100 μM of genistein, HSP and RFC4 are indicated by arrow.

Table 2
List of differentially expressed genes in MCF-7 cells treated with different concentrations (50 and 100 μM) of genistein

Accession no.	Gene name	Fold		Function
		G50	G100	
Up-regulated genes				
AB003334	heat shock protein 105 kD (HSP)	2.67	2.45	stress response
Y15801	protein kinase, Y-linked (PRKY)	2.24	1.96	transcription
K02581	thymidine kinase 1, soluble (TK1)	1.50	2.00	salvage-pathway enzyme for dTTP formation
Down-regulated genes				
X03635	ER α (ER)	0.49	0.39	transcription
J03161	serum response factor (SRF)	0.49	0.59	transcription factor
M29474	recombination activating gene 1 (RAG-1)	0.52	0.38	V(D)J recombination
X51602	fms-related tyrosine kinase 1 (VEGFR-1)	0.50	0.43	endothelial cell proliferation and angiogenesis
U39050	disabled (Drosophila) homolog 2 (DOC2)	0.49	0.57	signal transduction
AA600213	replication factor C(activator 1)4 (RFC4)	0.43	0.57	elongation DNA template
M57627	interleukin 10 (IL10)	1.07	0.43	apoptosis
X76104	thymidine kinase 2,mitochondria (TK2)	0.83	0.44	mitochondria DNA synthesis

MCF-7 cells were cultured and treated with 50 μM (G50) and 100 μM (G100) of genistein or its vehicle for 24 h as described in Materials and methods. Total RNA were extracted and labeled for cDNA microarray experiments. Results are expressed as fold change as compared to samples extracted from vehicle-treated cells and are the average of two independent microarray experiments.

generated by dilution of cDNA from the calibrator. The untreated control sample was used as a calibrator. The Relative Quantification Software was used to create a relative standard curve and stored as a coefficient (*.cof) file that can be used for each analysis. In this quantification method, the result is expressed as the target/reference ratio of each sample divided by the target/reference ratio of the calibrator.

2.6. Western blot analysis

Proteins were obtained by cell lysis in Nonidet P-40 buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40). The buffer was supplemented with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF). All of these chemicals were from Sigma. Protein concentrations were analyzed by the method of Bradford (Bio-Rad Laboratory, USA) [30]. Equal amounts of cytosolic proteins (20 μg) were separated by SDS-PAGE on 10% reducing gels, and trans-blotted to PVDF membranes (Immobilon-P, Millipore Corp., MA, USA). The membrane was treated with primary antibodies (Sigma and Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The antigen–antibody complexes were detected using an enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA) reagent [31] and visualized by the Lumi-Imager with the software Lumi Analyst version 3.10 (Roche, Mannheim, Germany).

2.7. Statistical analysis

Results are expressed as means ± standard error of mean (S.E.). The significance of differences between group means was determined by one-way ANOVA. P value <0.05 was considered statistically significant.

3. Results

3.1. Effects of genistein on cell cycle kinetics

The effects of genistein on cell cycle in MCF-7 cells were determined using flow cytometry. Representative graphs are shown in Fig. 1. When MCF-7 cells were treated with high concentration of genistein (50 μM and 100 μM), the proportion of cells in G2/M phase increased ($P<0.05$) and the proportion of cells in S phase decreased ($P<0.05$). These results confirmed the studies reported by others [9,26] that high concentration of genistein produced cyto-

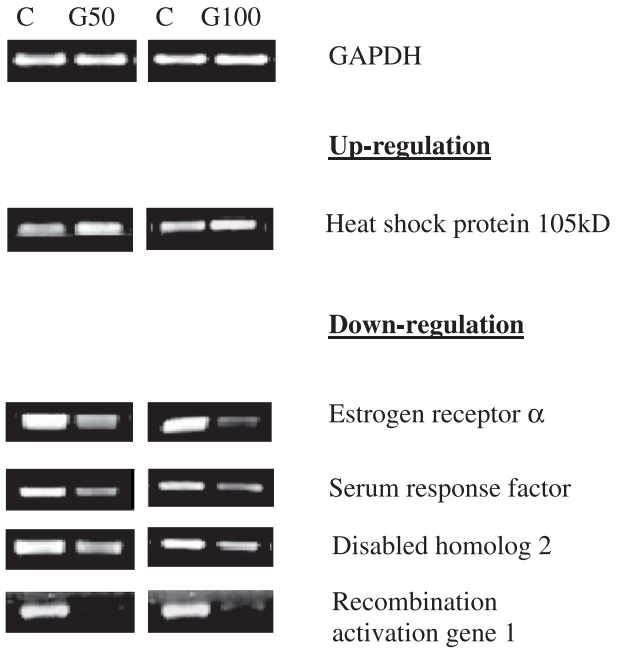

Fig. 3. RT-PCR analysis of differentially regulated genes in MCF-7 cells in response to treatment with genistein. MCF-7 cells were treated with 50 μM (G50) and 100 μM (G100) genistein or its vehicle (C) for 24 h. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. Primers for RT-PCR analysis were listed in Table 1. GAPDH was used as a control. A representative of two experiments is shown.

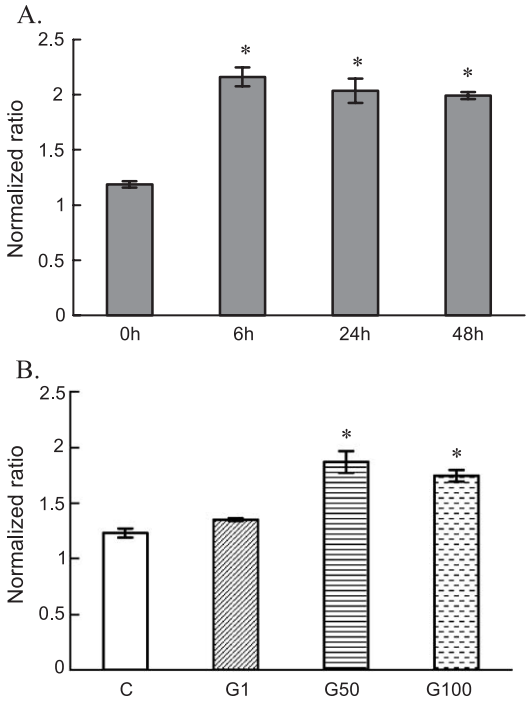

Fig. 4. Quantitative RT-PCR analysis of heat shock protein105 kD (HSP) mRNA in MCF-7 cells. (A) Time course of HSP mRNA expression in MCF-7 cells in response to 100 μM genistein. MCF-cells were cultured with 100 μM genistein for 0, 6, 24, 48 h and total RNA was isolated and subjected to quantitative RT-PCR analysis. *$P<0.05$ vs. control, $n=3$. (B) Dose response of HSP mRNA expression in MCF-7 cells treated with different concentrations of genistein. MCF-7 cells were cultured and treated with 1 μM (G1), 50 μM (G50) and 100 μM (G100) genistein or its vehicle (C) for 24 h and total RNA was isolated and subjected to quantitative RT-PCR analysis. *$P<0.05$ vs. control, $n=3$.

static effect in MCF-7 cells by decreasing DNA synthesis and arresting cells at critical checkpoint. In this study we were interested in investigating how genistein inhibits cell growth at high concentration.

3.2. Gene expression pattern of MCF-7 cells treated with genistein

The inhibitory effect of genistein could be mediated through the modulation of gene expression in human breast cancer cells. The gene expression profiles of MCF-7 cells in response to different concentrations (50 and 100 μM) of genistein were obtained by using a cDNA microarray containing 448 human genes (Fig. 2). Lists of up-regulated and down-regulated genes in MCF-7 cells treated with different doses of genistein are shown in Table 2. At 50 and 100 μM of genistein, the expressions of HSP 105 kD, protein kinase (Y-linked, PRKY) and thymidine kinase 1 (TK1) were persistently up-regulated; while the expression of SRF, ERα, disabled (*Drosophila*) homolog 2 (DOC 2), recombination activating gene 1 (RAG-1), replication factor C (RFC4) and fms-related tyrosine kinase 1 (VEGFR-1) were down-regulated. Our results also indicated that interleukin 10 (IL10) and thymidine kinase 2 (TK2) were down-regulated only by 100 μM genistein.

3.3. Verification of expression of selected genes and real-time quantitative PCR of HSP mRNA and SRF mRNA expression

Among the up-regulated genes, we selected HSP for RT-PCR verification. From the list of down-regulated genes, SRF, ERα, DOC 2 and recombination activation gene 1 (RAG-1) were selected. The RT-PCR results, as shown in Fig. 3, demonstrated identical expression pattern for all selected genes as revealed by microarray analysis. To further characterize the effect of genistein on HSP and SRF mRNA expression, time-course and dose-response experiments were performed. Using quantitative RT-PCR, genistein was shown to significantly enhance HSP mRNA expression as early as 6 h after treatment ($P<0.05$), and the up-regulation persisted for up to 48 h ($P<0.05$) (Fig. 4A). Moreover, treatment with different concentrations of genistein resulted in a dose-dependent increase in the level

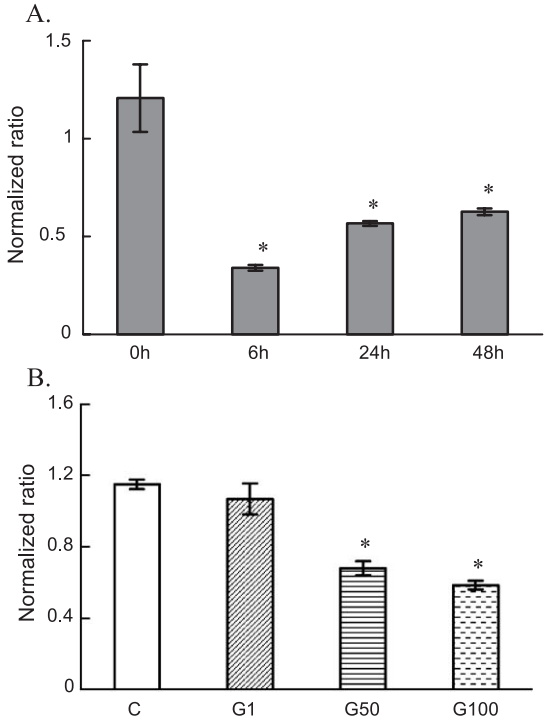

Fig. 5. Quantitative RT-PCR analysis of serum response factor (SRF) mRNA in MCF-7 cells. (A) Time course of SRF mRNA expression in MCF-7 cells in response to with 100 μM genistein. MCF-cells were cultured with 100 μM genistein for 0, 6, 24, 48 h and total RNA was isolated and subjected to quantitative RT-PCR analysis. *$P<0.01$ vs. control, $n=3$. (B) Dose response of SRF mRNA expression in MCF-7 cells treated with different concentrations of genistein. MCF-7 cells were cultured and treated with 1 μM (G1), 50 μM (G50) and 100 μM (G100) genistein or its vehicle (C) for 24 h and total RNA was isolated and subjected to quantitative RT-PCR analysis. *$P<0.05$ vs. control, $n=3$.

of HSP mRNA (50 and 100 μM, $P<0.05$) as shown in Fig. 4B. In contrast, SRF mRNA expression was down-regulated by genistein in a time-dependent manner. The down-regulation persisted throughout the duration of treatment with genistein ($P<0.01$) (Fig. 5A). In addition, genistein treatment results in a dose-dependent decrease in the mRNA expression of SRF (50 and 100 μM, $P<0.01$) (Fig. 5B).

Since previous studies showed that genistein could behave either as an estrogen agonist or estrogen antagonist [32], experiment was designed to determine if the regulations of HSP and SRF mRNA by genistein were mediated through estrogen receptor using an estrogen antagonist, ICI 182,780. The result showed that the up-regulation of HSP mRNA by genistein persisted in the presence of ICI 182,780 (Fig. 6A), suggesting the regulation of HSP by genistein is not mediated through ER-dependent pathway. On the other hand, the down-regulation of SRF was partially abolished by cotreatment with ICI 182,780 (Fig. 6B), indicating that ER pathway was at least in part involved in the inhibitory effect of genistein on SRF mRNA expression.

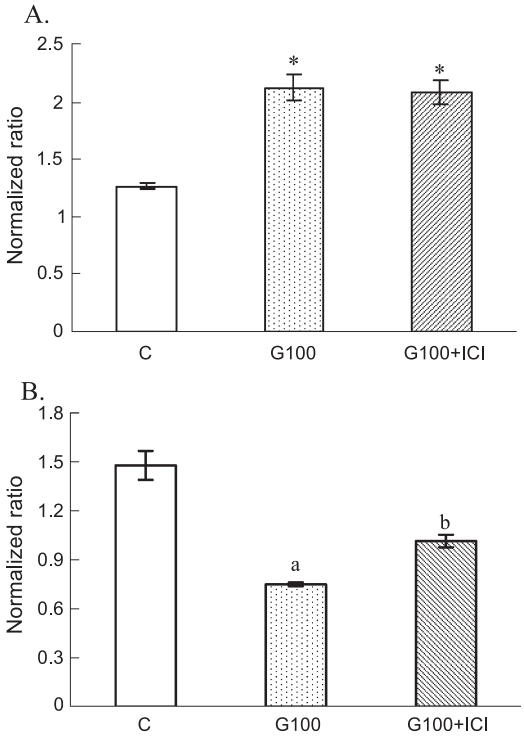

Fig. 6. Effect of estrogen antagonist ICI 182, 780 on the regulation of HSP and SRF mRNA expressions by 100 μM of genistein. (A) MCF-7 cells were cultured and treated with 100 μM genistein in the presence and absence of 1 μM ICI 182, 780 for 24 h as described in Materials and methods. Total RNA was isolated and subjected to quantitative RT-PCR analysis for HSP mRNA. *$P<0.05$ vs. control, $n=3$. (B) MCF-7 cells were cultured and treated with 100 μM genistein in the presence and absence of 1 μM ICI 182, 780 for 24 h as described in Materials and methods. Total RNA was isolated and subjected to quantitative RT-PCR analysis for SRF mRNA. a and b are significantly different from control ($P<0.05$). a and b are different ($P<0.05$).

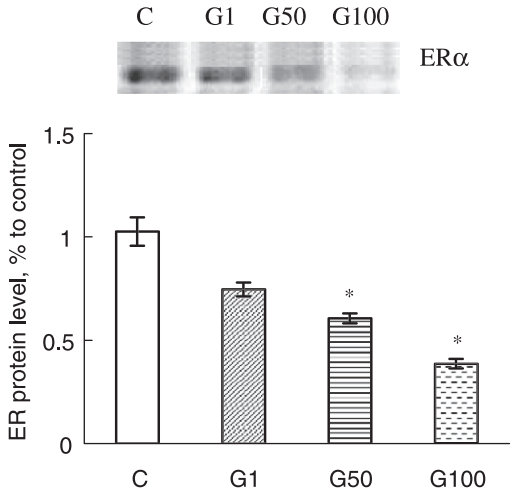

Fig. 7. Western blot analysis of the expression of ERα in MCF-7 cells treated with different concentrations of genistein. MCF-7 cells were cultured and treated with 1 μM (G1), 50 μM (G50) and 100 μM (G100) genistein or its vehicle (C) for 24 h. Cells were lysed and proteins were fractionated using 10% SDS-PAGE and immunoblotted with antibody against ERα as described in Materials and methods. The immunoblot is a representative of three independent experiments. Results were obtained from three independent experiments and expressed as mean ± S.E. *$P<0.05$ vs. control, $n=3$.

3.4. Effects of genistein on ER and SRF protein expressions

Previous studies demonstrated that genistein serves as an antiestrogen by interfering with the tumor promoting effect

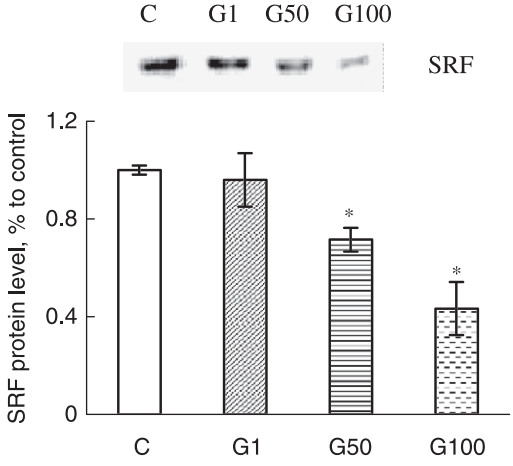

Fig. 8. Western blot analysis of the expression of SRF in MCF-7 cells treated with different concentrations of genistein. MCF-7 cells were cultured and treated with 1 μM (G1), 50 μM (G50) and 100 μM (G100) genistein or its vehicle (C) for 24 h. Cells were lysed and proteins were fractionated using 10% SDS-PAGE and immunoblotted with antibody against SRF as described in Materials and methods. The immunoblot is a representative of three independent experiments. Results were obtained from three independent experiments and expressed as mean ± S.E. *$P<0.05$ vs. control, $n=3$.

of estrogen [32]. Our microarray and RT-PCR data further demonstrated that genistein down-regulated ERα mRNA expression (Table 2 and Fig. 3). To determine if genistein also down-regulate ERα protein expression, Western blotting analysis of MCF-7 cells treated with different concentration of genistein was performed. As shown in Fig. 7, ERα protein expression in MCF-7 cells decreased significantly with increasing genistein concentrations (at 50 and 100 μM, $P < 0.05$). Thus, our results indicated that the inhibitory action of genistein on MCF-7 cells involved the down-regulation of ERα at both the mRNA and protein level.

Similar to ER that serves a pivotal role in mediating the proliferative action of estrogen in MCF-7 cells, SRF is an important transcription factor that mediates the proliferative actions of both estrogen and mitogens via its involvement in transcriptional regulation of c-*fos*, a protooncogene [33,34]. As genistein appears to down-regulate SRF mRNA in dose-dependent manner, we therefore determined if the down-regulation of SRF mRNA by genistein results in a parallel decrease in SRF protein expression in MCF-7 cells. Western blotting analysis showed that SRF protein levels were also reduced by genistein in a dose-dependent manner ($P < 0.05$) (Fig. 8), indicating that genistein at high concentration inhibited SRF expression transcriptionally and post-transcriptionally.

4. Discussion

Genistein, an active ingredient in soy products, behaves differently at different concentrations in human breast cancer MCF-7 cells. Using cDNA microarray technology, several targets or pathways that might be affected by high concentration of genistein were identified. Genes that are up-regulated by high concentration of genistein include genes that are involved in cell salvage response, while genes that are down-regulated include those that participate in different signaling pathways that lead to cell growth and differentiation.

The up-regulated genes in MCF7 cells in response to high concentration of genistein treatment are genes associated with stress response (HSP 105,), transcription (protein kinase, Y-linked, PRKY) and salvage-pathway enzyme for dTTP formation (TK1), indicating the activation of cellular salvage response in MCF-7 cells upon genistein treatment. Previous reports have shown that stress pathway is involved in the anti-proliferative effects of genistein [28]. HSPs, which function mainly as molecular chaperones, allow cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions. The events of cell stress and cell death are closely linked and HSP induction in response to stress appears to function at key regulatory points in the control of apoptosis. A recent study [35] demonstrated that overexpression of Hsp 105α can enhance stress-induced apoptosis in mouse embryonal F9 cells. Therefore, the up-regulation of HSP by genistein in MCF-7 cells provides direct evidence for the activation of stress pathways and possibly an enhancement of the process of apoptosis. In addition, our results indicate that genistein up-regulation of HSP in MCF-7 cells is independent of the estrogen receptor-mediated pathways as estrogen antagonist ICI 182,780 fails to abolish the action of genistein.

The down-regulated genes in genistein-treated MCF-7 cells are participating in different signaling pathways involved in growth and differentiation. Three of the down-regulated genes are involved in pathways that mediate mitogenic responses:

1. SRF is a transcription factor that binds to serum response element at the site of transcription initiation of many growth response-related genes such as c-*fos*. Both E_2 and IGF-I were previously shown to activate MCF-7 cell proliferation via the activation of SRF response element [33].
2. Disabled (*Drosophila*) homolog 2 (DOC 2), another down-regulated gene that is known to be the human homologous of the murine mitogen responsive phosphoprotein p96, is a cellular signal transduction component of the colony-stimulatory factor 1 (csf-1)-dependent signaling pathways. Csf-1 binds to c-fms protooncogene, triggers receptor dimerization and activates downstream signaling molecules, including DOC-2 [36].
3. Fms-related tyroinse kinase 1 (flt-1), also known as vEGFR-1, is a tyrosine-protein kinase receptor. Vascular endothelial growth factor (vEGF) and v-EGFR (flt-1) were previously showed to play important roles in stimulating vascular cell proliferation, invasion and angiogenesis in endothelial cells [37].

We have specifically studied the time-response and dose-response of SRF mRNA expression in MCF-7 cells treated with 100 μM of genistein using quantitative RT-PCR. The result indicated that genistein decreased SRF protein and mRNA expression in a dose-dependent manner. Since SRF is an important transcription factor that mediates the action of growth factors and the non-genomic actions of estrogen [33,34], our results suggest that the inhibitory effect of genistein might involve the suppression of both the ER- and IGFR-mediated pathway via the down-regulation of SRF expression in MCF-7 cells. In addition, our results also suggest that the inhibitory action on SRF mRNA is not solely mediated by estrogen receptor-dependent pathways as its action on SRF expression cannot be completely abolished by cotreatment with ICI 182,780. The down-regulation of DOC-2 and vEGFR in MCF-7 cells upon genistein treatment suggests that both csf-1- and vEGFR-dependent pathways might be partially responsible for the inhibitory action of genistein in human breast cancer cells. It is of interest to note that a recent study by Li and Sarkar [38], which employed a cDNA microarray containing 12 558 genes to characterize differential gene expression in response to genistein

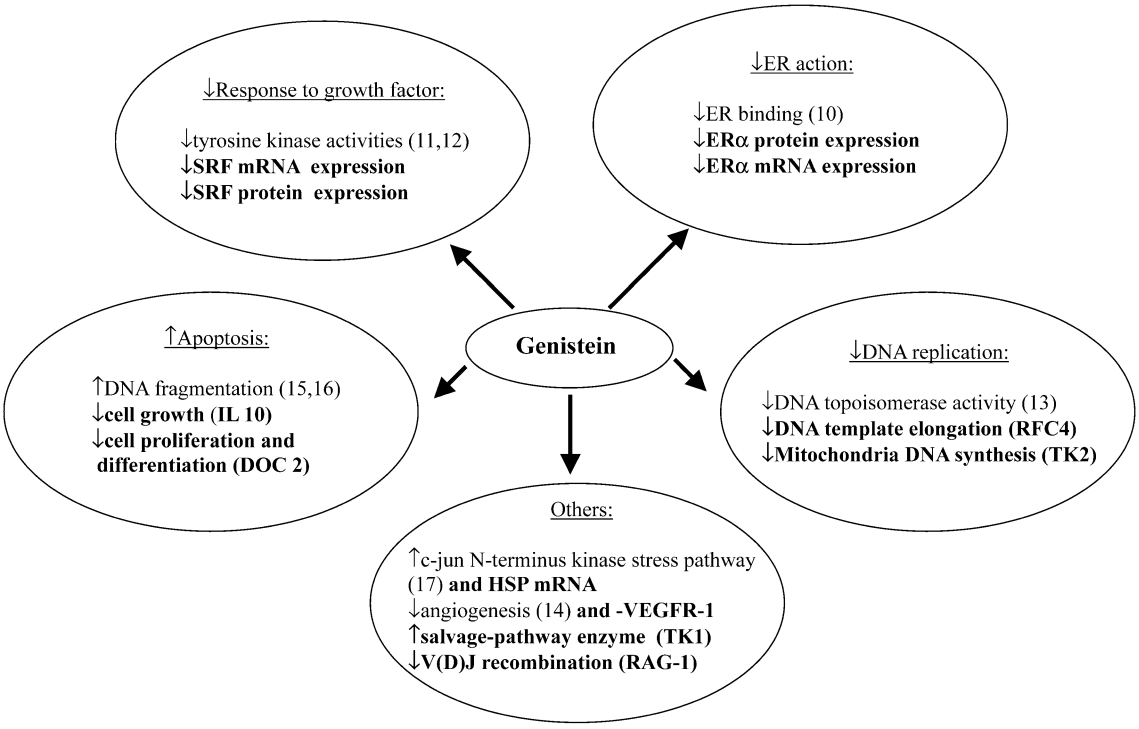

Fig. 9. Summary of current understanding of inhibitory effect of genistein in MCF-7 cells. Functions with reference number represent known mechanism and bold words without reference number represent new mechanism revealed by our cDNA microarray technology. ↓ represents inhibition, ↑ represents stimulation.

treatment in prostate cancer PC3 cells, has demonstrated that genistein could down-regulate vEGF gene expression. Together, these data support that angiogenesis-related genes, such as VEGF and vEGFR, are major targets of genistein in cancer cells.

Our results also indicated that genistein at high concentration can directly suppress ER-dependent pathways. It is well known that genistein has weak binding affinity toward ER [10] and was previously demonstrated to interfere with the binding of estrogen to estrogen receptor [32]. The present study further showed that genistein could inhibit ERα mRNA and protein expression in human breast cancer cell line in a dose-dependent manner. As estrogen is a major stimulator for the growth of many breast tumors, the inhibition of its receptor by genistein will provide a direct means of decreasing its responsiveness to circulating or surrounding estrogen, thereby limiting estrogen-dependent cell growth.

Other down-regulated genes in response to treatment with high concentration of genistein are involved in different cellular activities, including DNA replication (Replication factor C 4, RFC4), V(D)J recombination of immunoglobulin and T cell receptor genes (recombination activating gene 1, RAG-1) [39], apoptosis (IL10) [40] and mitochondria DNA synthesis (TK2) [41]. The down-regulation of RFC4, and hence DNA replication, by genistein provides a possible mechanism for the decrease in the S-phase of cell cycle in MCF-7 upon treatment with high concentration of genistein (Fig. 2). However, the exact role of each of these down-regulated genes in mediating the inhibitory action of genistein remains to be determined.

In conclusion, the present study suggests that high concentration of genistein induces stress response, interferes with ER action by down-regulation of ER both at the transcriptional and post-transcriptional level, inhibits the responses of MCF-7 cells to growth factor by decreasing tyrosine kinase activities of growth factor receptors as well as the expression of its downstream responsive transcription factor such as SRF, decreases DNA replication, and induces apoptosis. Summary of the current understanding of the inhibitory action of genistein in MCF-7 cells is shown in Fig. 9. Further investigation is needed to verify the possible roles of these differentially regulated genes in the susceptibility of human breast cancer (MCF-7) cell line to the action of genistein.

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