JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Tea Polyphenol (−)-Epigallocatechin 3-Gallate Suppresses Heregulin-β1-Induced Fatty Acid Synthase Expression in Human Breast Cancer Cells by Inhibiting Phosphatidylinositol 3-Kinase/Akt and Mitogen-Activated Protein Kinase Cascade Signaling

MIN-HSIUNG PAN,[†] CHENG-CHAN LIN,[‡] JEN-KUN LIN,*[,§,||] AND WEI-JEN CHEN*[,‡,||]

Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Road, Nan-Tzu, Kaohsiung 811, Department of Biomedical Sciences, Chung Shan Medical University, No. 110, Section 1, Chien-Kuo North Road, Taichung 402, and Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan

Tumor-associated fatty acid synthase (FAS) is implicated in tumorigenesis and connected to HER2 (human epidermal growth factor receptor 2) by systemic analyses. Suppression of FAS in cancer cells may lead to growth inhibition and cell apoptosis. Our previous study demonstrated that (−)-epigallocatechin 3-gallate (EGCG), the green tea catechin, could down-regulate FAS expression by suppressing EGFR (epidermal growth factor receptor) signaling and downstream phosphatidylinositol 3-kinase (PI3K)/Akt activation in the MCF-7 breast cancer cell line. Herein, we examined the effects of EGCG on FAS expression modulated by another member of the erbB family, that is, HER2 or HER3. We identified that heregulin-β1 (HRG-β1), a HER3 ligand, stimulated dose-dependent FAS expression in breast cancer cell lines MCF-7 and AU565, but not MDA-MB-453. The time-dependent increase in FAS expression after HRG-β1 stimulation was also observed in MCF-7 cells, and this up-regulation was de novo RNA synthesis dependent. Treatment of MCF-7 cells with EGCG markedly inhibited HRG-β1-dependent induction of mRNA and protein of FAS. EGCG also decreased the phosphorylation of Akt and extracellular signal-regulated kinase 1/2 that were demonstrated as selected downstream HRG-β1-responsive kinases required for FAS expression using dominant-negative Akt, PI3K inhibitors (LY294002 and wortmannin), or MEK inhibitor (PD98059). FAS induction by HRG-β1 was also blocked by AG825, a selective HER2 inhibitor, and by genistein, a selective tyrosine kinase inhibitor, indicating the formation of a heterodimer between HER2 and HER3, and their tyrosine kinase activities are essential for HRG-β1-mediated elevation of FAS. Additionally, growth inhibition of HRG-β1-treated cells was parallel to suppression of FAS by EGCG. Taken together, these findings extend our previous study to indicate that EGCG may be useful in the chemoprevention of breast carcinoma in which FAS overexpression results from HER2 or/and HER3 signaling.

KEYWORDS: (−)-Epigallocatechin 3-gallate (EGCG); heregulin-β1; fatty acid synthase (FAS); HER2; HER3; Akt; extracellular signal-regulated kinase1/2 (ERK1/2)

INTRODUCTION

Fatty acid synthase (FAS; E.C. 2.3.1.85) is a multifunctional enzyme that catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (*1*, *2*). It plays an important role in energy homeostasis by converting excess carbon intake into fatty acids for storage. In most normal human tissues, FAS is down-regulated due to dietary fat. Contrarily, FAS in tumor cells occurs at very high rates and is more active and highly expressed (*3*). Numerous clinical and basic studies disclose that high levels of FAS are present in many varieties of common human cancers such as human breast (*4*), prostate (*5*), colon (*6*), endometrium (*7*), and ovary (*8*) cancers. FAS is implicated in tumorigenesis through its role in cell proliferation and

* To whom correspondence should be addressed. (W.-J.C.) Phone: (886)-4-24730022 ext.11808. Fax: (886)-4-23248187. E-mail: cwj519@csmu.edu.tw. (J.-K.L.) Phone: (886)-2-2356-2213. Fax: (886)-2-2391-8944. E-mail: jklin@ha.mc.ntu.edu.tw.
[†] National Kaohsiung Marine University.
[‡] Chung Shan Medical University.
[§] National Taiwan University.
[||] These two authors contributed equally to this work.

10.1021/jf070316r CCC: $37.00
Published on Web 06/01/2007

membrane lipid incorporation of neoplastic cells. Suppression of FAS in cancer cells may lead to growth inhibition and cell apoptosis (*9*). This differential expression of FAS between normal tissues and cancers suggests that FAS may be a useful target for anticancer drug development.

The *HER2* (human epidermal growth factor receptor 2) oncogene encodes a transmembrane receptor tyrosine kinase (RTK) and belongs to the second member of the epidermal growth factor receptor (EGFR or erbB) family, which includes HER1 (EGER, erbB1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4) in humans. Amplification of the *HER2* gene or overexpression of the HER2 protein is found in 20−30% of human breast cancers, which is often associated with a poor prognosis (*10*), and occurs in a variety of other human cancers such as ovarian, gastric, and salivary cancers (*11*), implicating a critical role in the development of human cancers. Activation of erbB receptors requires ligand binding that induces dimerization of the erbB receptor to form a homodimer or heterodimer which activates the intrinsic kinase activity and leads to a wide variety of cellular signal transduction (*12*). Unlike other erbB family members, HER2 is an orphan receptor, and its natural ligand has not yet been characterized; however, HER2 can be activated by its own overexpression or transactivated by heregulins (HRGs) (*13*). HRGs are natural epidermal growth factor (EGF)-like ligands for the erbB family which are often expressed in breast cancer cells (*14*) and exert their function by binding to HER3 or HER4, thereby inducing heterodimerization with HER2 and leading to receptor tyrosine phosphorylation and activation of downstream signal transduction including the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways (*15*). Due to activation of HER2 or HER3, HRG-mediated signaling is linked to the development and the pathogenesis of breast cancer (*16*).

(−)-Epigallocatechin 3-gallate (EGCG) is the most abundant and biologically active catechin extracted from green tea and exerts its antimutagenic activity through blockage of mitotic signal transduction (*17*). Our previous study has demonstrated that EGCG could suppress FAS expression by down-regulating the EGF receptor/PI3K/Akt/Sp-1 signal transduction pathway in the MCF-7 breast cancer cell line (*18*). We postulate that EGCG would inhibit FAS expression through another erbB family pathway such as that of HER2 or/and HER3. In fact, it has been reported that EGCG down-regulates HER2 signaling in human breast, head, neck, and colon cancer cells (*19*−*21*) and inhibits HER3 activation in human colon cancer cells (*22*), implying that EGCG may inhibit the downstream phenotype controlled by HER2 or/and HER3. Furthermore, systemic analyses have revealed a molecular connection between HER2 and FAS (*23*, *24*). This raises the possibility that EGCG might inhibit HER2- or/and HER3-modulated FAS expression.

In this study, we used heregulin-β1 (HRG-β1), a specific HER3 ligand, to investigate whether HER3 mediates FAS expression and whether EGCG would down-regulate HRG-β1-stimulated FAS induction in human breast cancer cells. We also examined the effects of EGCG on several major HER2- or/and HER3-mediated downstream signal transduction pathways responsible for FAS induction and determined the coordination of HER2 and HER3 under HRG-β1 stimulation. Then we tried to define the cancer-preventive action of EGCG in HER2- or HER3-positive breast cancer cells.

MATERIALS AND METHODS

Materials. Recombinant human heregulin-β1 was purchased from R&D Systems (Minneapolis, MN). Antibody against FAS was obtained from BD Biosciences (Los Angeles, CA). The HER2 inhibitor AG825 was purchased from Calbiochem (La Jolla, CA). The antibodies to β-actin, HER3, and HER2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK1/2 (Thr42/Tyr44), and anti-ERK1/2 were from Cell Signaling Technology (Beverly, MA). RT-PCR reagents were from Promega (Madison, WI). EGCG, cycloheximide, actinomycin D, the selective tyrosine kinase inhibitor genistein, EGFR inhibitor PD153035, and the kinase inhibitors LY294002, wortmannin, SB203580, and PD98059 were obtained from Sigma (St. Louis, MO). The anti-phosphotyrosine antibody (4G10) was available from Upstate Biotechnology (Charlottesville, VA).

Cell Culture. Monolayer cultures of MCF-7 and AU565 cells were grown in Dulbecco's minimal essential medium (DMEM), and MDA-MB-453 cells were maintained in DMEM/F12 (Invitrogen). All of the cells were supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin and kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Immunoblotting (Western Blotting). Total protein extracts were prepared in a lysis buffer (50 mM Tris−HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethanesulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Equal amounts of total cellular proteins (50 μg) were resolved by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (6% for FAS, 10% for β-actin), transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA), and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham, U.K.).

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen) as recommended by the manufacturer's instructions. Total RNA (5 μg) was reverse-transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) 18 primer by incubating the reaction mixture (25 μL) at 40 °C for 90 min. Amplification of cDNA was performed by polymerase chain reaction (PCR) in a final volume of 50 μL containing 2 μL of RT product, dNTPs (each at 200 μM), 1× reaction buffer, a 1 μM concentration of each primer (FAS, forward 5′-CTGCAACACCTTCTGCAGTTCTG-3′, reverse 5′-TCGAATTTGCCAATTTCCAGGAAGC-3′; GAPDH, forward 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, reverse 5′-CATGTAGGCCATGAGGTCCACCAC-3′), and 50 units/mL Pro Taq DNA polymerase. After an initial denaturation for 5 min at 95 °C, 30 cycles of amplification (95 °C for 30 s, 58 °C for 2 min, and 72 °C for 2 min) were performed, followed by 72 °C for 10 min. A 5 μL sample of each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Expression Plasmid and Transient Transfection. Vectors (pcDNA3) expressing the kinase-dead forms of Akt (K179A, dnAkt) were kindly provided by Dr. M. L. Kuo (*18*). p38MAPK dominant-negative mutant (dnp38) was generously provided by Dr. C. C. Chen (Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan). MCF-7 cells were transiently transfected with dnAkt, dnp38, or pcDNA3 using Lipofectin reagent (GIBCO, Invitrogen, Grand Island, NY) according to the manufacturer's instructions. After transient transfection, the cells were serum-starved for 18 h, then stimulated with 50 ng/mL HRG-β1 for 9 h, and then lysed for analysis.

Immunoprecipitation. A 500 μg sample of MCF-7 total cellular proteins was first precleared by being incubated with protein A agarose (10 μL, 50% slurry, Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min. The clarified supernatants were collected by microfugation and then incubated with primary antibody for 2 h at 4 °C. The reaction mixtures were added with 20 μL of protein A agarose to absorb the immunocomplexes at 4 °C overnight. Immunoprecipitated proteins were subjected to 8% SDS−PAGE and then transferred onto a PVDF membrane (Millipore). The native- or phospho-form proteins were visualized by immunoblotting.

MTT Assay. MCF-7 cells were seeded at a density of 5×10^3 cells/mL into 96-well plates and grown overnight. Then the cells were transferred into serum-free medium and incubated for an additional 16 h. After serum starvation, the cells were treated with the indicated

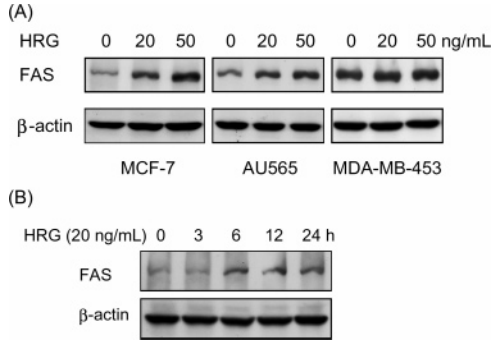

Figure 1. HRG-β1 up-regulates FAS expression in various breast cancer cell lines. (**A**) Dose effects of heregulin-β1 on FAS expression in breast cancer cell lines. Serum-starved MCF-7, AU565, or MDA-MD-453 cells were treated with 0, 20, or 50 ng/mL HRG-β1 for 24 h. (**B**) Time-dependent induction of FAS expression by heregulin-β1 in MCF-7 cells. Serum-starved MCF-7 cells were treated with 20 ng/mL HRG-β1 for the indicated times. After incubation, cell lysates were harvested and the FAS and β-actin protein levels were determined by immunoblotting as described in the Materials and Methods. Simultaneous immunoblotting of β-actin was used as an internal control for equivalent protein loading.

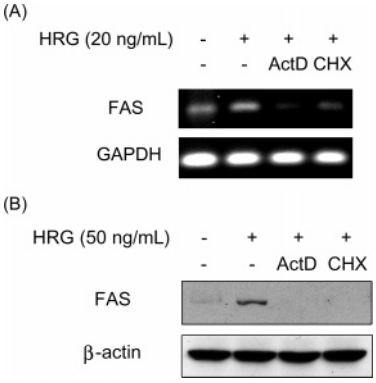

Figure 2. Transcriptional regulation of FAS expression by HRG-β1 in MCF-7 cells. (**A**) RT-PCR analysis of cDNA from MCF-7 cells. The cells were pretreated with 4 μg/mL actinomycin D (ActD) or 30 μg/mL cycloheximide (CHX) for 1 h before HRG-β1 (20 ng/mL). After 6 h of incubation, total RNA was isolated and analyzed by RT-PCR. (**B**) Immunoblotting analysis of FAS protein in MCF-7 cells. The cells were pretreated under the same circumstance and then incubated with HRG-β1 (50 ng/mL) for 9 h. Total cell lysates were analyzed by immunoblotting using FAS or β-actin antibody.

concentrations of EGCG for 30 min prior to stimulation with 20 ng/mL HRG-β1. After incubation, the proliferating cell numbers were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 20 μL of MTT solution (5 mg/mL, Sigma, St. Louis, MO) was added to each well and incubated for 4 h at 37 °C. Then the supernatant was aspirated, and the MTT−formazan crystals formed by metabolically viable cells were dissolved in 200 μL of Me_2SO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm.

Statistical Analysis. Mean values between the groups were compared using the Student's unpaired two-tailed *t* test. All statistical tests were two-sided, and differences were considered significant when $p < 0.05$.

RESULTS

HRG-β1 Up-Regulates FAS in Human Breast Cancer Cells. Initially, we used immunoblotting to analyze FAS expression in human breast cancer MCF-7, AU565, and MDA-MB-453 cells treated with various concentrations of HRG-β1. As shown in **Figure 1A**, HRG-β1 increased FAS expression dramatically in MCF-7 and AU565 cells, and this induction was dosage-dependent. However, serum-starved MDA-MB-453 cells expressed a high level of FAS protein; there was even no HRG-β1 stimulus. MDA-MB-453 cells are HER2-overexpressing cells and more malignant than MCF-7 and AU565 cells. We suggest that HER2 in MDA-MD-453 cells are highly active through HER2 homodimer formation in spite of serum deprivation. Therefore, the expression of fully level FAS due to HER2 overexpression is predictable in serum-starved MDA-MD-453 cells. **Figure 1B** shows that the time-dependent induction of FAS appeared after HRG-β1 stimulation and occurred as early as after 6 h in MCF-7 cells. The FAS signal was detectable in both MCF-7 and AU565 cells without HRG-β1 treatment, suggesting that both cell lines expressed a basal level of FAS (**Figure 1A**).

HRG-β1 Regulates FAS Expression at the Transcriptional Level. We further determine whether nascent RNA or protein synthesis is required for HRG-β1-mediated *FAS* mRNA up-regulation. To this end, we pretreated MCF-7 cells with 4 μg/mL actinomycin D (ActD), an RNA synthesis inhibitor, or 30 μg/mL cycloheximide (CHX), a protein synthesis inhibitor, for 1 h before HRG-β1 was added to the culture medium. **Figure 2A** shows that ActD completely abolished HRG-β1-mediated *FAS* mRNA up-regulation, whereas CHX had less effect on *FAS* mRNA induction by HRG-β1. However, CHX treatment completely suppressed FAS protein expression (**Figure 2B**) even though *FAS* mRNA induction by HRG-β1 occurred (**Figure 2A**). It was interesting to note that CHX treatment led to a slight inhibition on *FAS* mRNA induction by HRG-β1, suggesting that CHX might prevent a newly synthesized protein(s) or transcriptional factor(s) which is/are involved in *FAS* mRNA induction by HRG-β1 in MCF-7 cells. Totally, these findings suggest that HRG-β1-induced FAS up-regulation requires both de novo RNA synthesis and new protein synthesis.

Regulation of FAS Expression by HRG-β1 Is Mediated by PI3K/Akt and MAPK Cascade Pathways. Since HRG-β1 facilitates nascent FAS mRNA expression, we predicted that HRG-β1 downstream kinase signaling may participate in the up-regulation of FAS mRNA expression. Many of the kinase signaling pathways have been shown to be activated and involved in HRG-β1-induced diversified cellular functions (*25*). To elucidate the underlying regulatory mechanism of kinases in HRG-β1 signaling, we tested the effect of several protein kinase inhibitors on FAS expression in HRG-β1-treated MCF-7 cells. As PI3K is an upstream regulator of Akt, the PI3K inhibitors LY294002 and wortmannin were added to observe the effect of PI3K on FAS induction in the cells. The addition of LY294002 and wortmannin suppressed the HRG-β1-induced FAS expression (**Figure 3A**). On the other hand, mitogen-activated protein/extracellular signal-related kinase (MEK) inhibitor PD98059 also blocked FAS up-regulation by HRG-β1 (**Figure 3A**). However, p38MAPK inhibitor SB203580 did not induce a remarkable change in FAS expression in HRG-β1-treated cells (**Figure 3A**). To further confirm the importance of Akt in the FAS induction by HRG-β1, we established an MCF-7 cell line transient expressing dominant-negative Akt or p38MAPK and examined the expression level of FAS protein. The induced FAS protein was significantly suppressed by transient transfection dominant-negative Akt but not vector (pcDNA3) or dominant-negative p38MAPK (**Figure 3B**). Collectively, the data obtained here strongly suggest that Akt and ERK1/2 may act as upstream kinases to activate FAS up-regulation in response to HRG-β1.

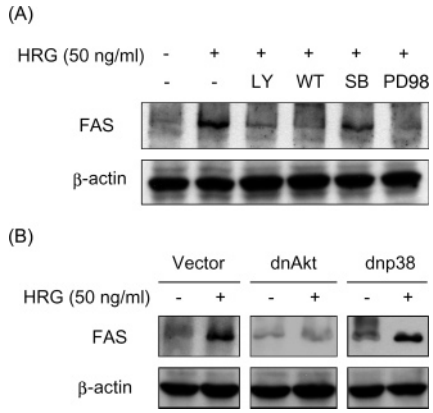

Figure 3. FAS up-regulation by HRG-β1 requires Akt and ERK1/2. (**A**) PI3K and MEK inhibitors suppress FAS expression induced by HRG-β1. Serum-starved MCF-7 cells were pretreated with 10 μM LY294002 (LY), 1 μM wortmannin (WT), 20 μM SB203580 (SB), or 50 μM PD98059 (PD98) for 1 h and then stimulated with 50 ng/mL HRG-β1. The FAS and β-actin protein levels were determined by immunoblotting. (**B**) Dominant-negative Akt inhibits HRG-β1-regulated FAS expression. MCF-7 cells were transiently transfected with dominant-negative Akt (dnAkt), dominant-negative p38MAPK (dnp38), or control vector (pcDNA3) and subsequently treated with HRG-β1 (50 ng/mL) for 9 h. The cell lysates were analyzed by immunoblotting using anti-FAS antibody. β-Actin was used as a loading control.

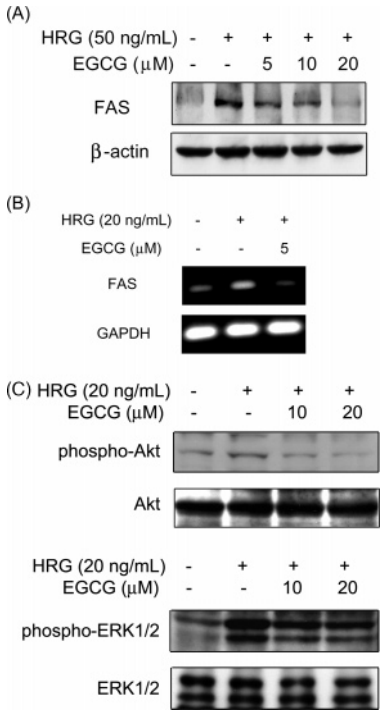

Figure 4. EGCG inhibits HRG-β1-induced FAS expression accompanied by down-regulation of Akt and ERK1/2 in MCF-7 cells. (**A**) EGCG reduces the protein level of FAS induction by HRG-β1 in a dose-dependent manner. Serum-starved MCF-7 cells were preincubated with various dosages of EGCG for 30 min and then stimulated with 50 ng/mL HRG-β1 for 9 h. Immunoblotting was performed using specific antibodies to FAS or β-actin. (**B**) EGCG down-regulates mRNA expression of FAS. Serum-starved MCF-7 cells were pretreated with 5 μM EGCG for 30 min before HRG-β1 (20 ng/mL) treatment. After incubation for 6 h, total RNA was isolated and RNA expression was analyzed by RT-PCR as described in the Materials and Methods. GAPDH cDNA was used as an internal control. (**C**) EGCG down-regulates the phosphorylation of Akt and ERK1/2. Serum-starved MCF-7 cells were pretreated with 0, 10, or 20 μM EGCG for 30 min and then stimulated by HRG-β1 (20 ng/mL) for 10 min. The phosphorylated Akt or ERK1/2 was detected by immunoblotting using specific anti-phospho-form antibody as described in the Materials and Methods. The native protein was used as a loading control.

EGCG Inhibits HRG-β1-Induced FAS Expression in MCF-7 Cells. In our previous study, we demonstrated that EGCG suppresses FAS expression by down-regulating the EGFR signaling pathway (*18*). Herein, the effect of EGCG on FAS expression stimulated by HRG-β1 was investigated. As shown in **Figure 4A**, the protein level of FAS induced by HRG-β1 was gradually decreased following the appearance of EGCG, and the decrease in the protein level of FAS was dosage-dependent in MCF-7 breast cancer cells. Next, to elucidate whether the decreased FAS induction by EGCG treatment was due to the down-regulation of mRNA, RT-PCR was performed on HRG-β1-treated MCF-7 cells with or without EGCG exposure. The data in **Figure 4B** exhibit that EGCG lowered HRG-β1-mediated *FAS* mRNA expression in MCF-7 cells. The present data suggest that EGCG inhibits the HRG-β1-induced FAS expression through transcriptional regulation.

EGCG Inhibits Akt and ERK1/2 Activation in HRG-β1-Stimulated MCF-7 Cells. To elucidate whether EGCG down-regulated FAS expression by virtue of the inhibition of activation of Akt and ERK1/2, we performed immunoblotting to detect the effects of EGCG on HRG-β1-induced Akt and ERK1/2 phosphorylation which has a direct positive correlation with their activation. **Figure 4C** shows that EGCG dramatically reduced ERK1/2 phosphorylation in HRG-β1-treated MCF-7 cells. Although the phosphorylation of Akt was moderately induced by HRG-β1, EGCG also exhibited inhibitory ability on Akt phosphorylation in **Figure 4C**. Taken together, these results demonstrate that EGCG suppresses FAS expression through the inhibition of Akt and ERK1/2 activation in HRG-β1-stimulated MCF-7 cells.

HER2 is Essential for FAS Induction by HRG-β1. To ascertain whether HER2 is involved in HRG-β1-mediated FAS induction, the effect of HRG-β1 on FAS expression was tested in the presence of selective ErbB inhibitors and genistein, a protein kinase inhibitor, with or without EGCG. As shown in **Figure 5A**, AG825, a selective HER2 inhibitor, and genistein blocked HRG-β1-induced FAS expression. However, the effect of HRG-β1 was not inhibited by the selective EGFR inhibitor, PD153035, suggesting that HER2 but not EGFR is needed for the induction of FAS by HRG-β1, and tyrosine kinase activity is necessary for HRG-β1 signal transduction. **Figure 5A** also shows that EGCG redoubled the inhibitory effect of AG825 and genistein on FAS induction by HRG-β1, providing additional evidence that EGCG inhibits FAS induction by down-regulating HRG-β1 signaling.

EGCG Interrupts HRG-β-Induced HER2−HER3 Coordination in MCF-7 Cells. To further determine the effects of EGCG on HER2 and HER3 functions, we analyzed the status of tyrosine phosphorylation of HER2 and HER3 in response to HRG-β1. HER2 or HER3 protein from HRG-β1-stimulated cell lysates was immunoprecipitated, and then phosphorylated proteins were visualized by immunoblotting with anti-phosphotyrosine antibody (4G10 monoclonal antibody). **Figure 5B** displays that either HER3 or HER2 tyrosine phosphorylation was markedly increased in HRG-β1-treated cells, and EGCG treatment resulted in a concentrated reduction in these phosphorylations. Co-immunoprecipitation assay also shows down-regulation of HER2−HER3 heterodimer formation after EGCG

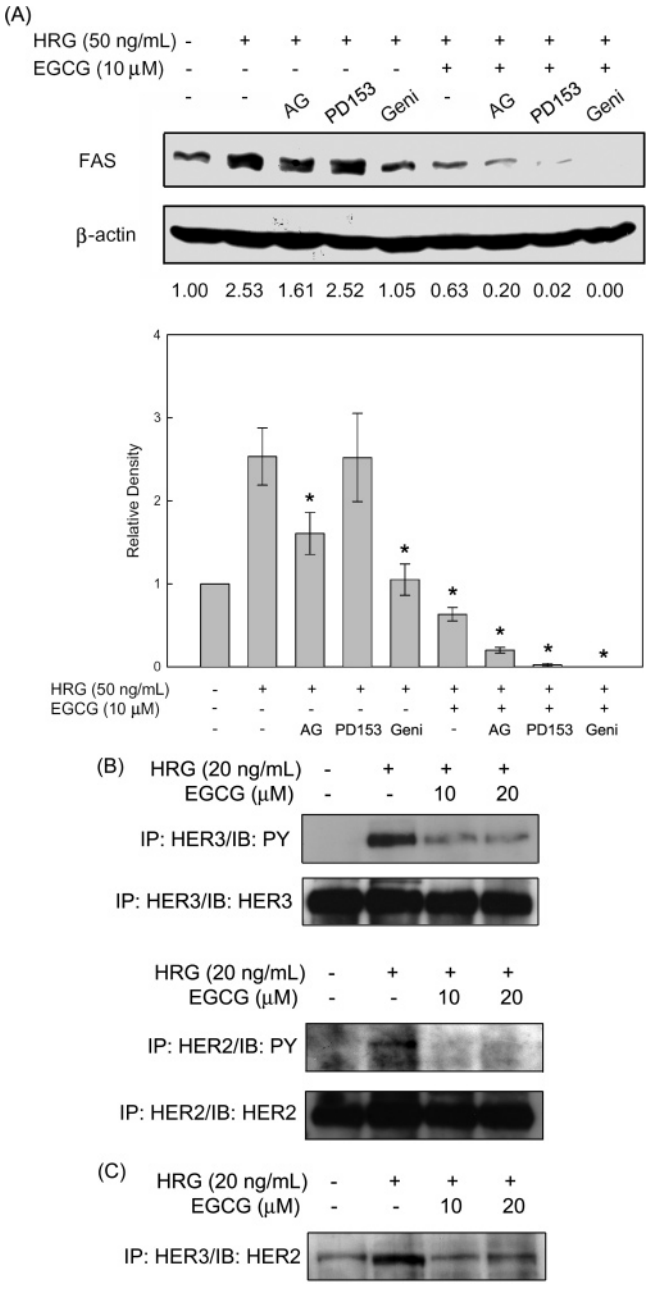

Figure 5. FAS induction by HRG-β1 requires HER2 activation. (**A**) MCF-7 cells were serum-starved for 24 h and then treated with 50 ng/mL HRG-β1 for 9 h in the presence or absence of the HER2 inhibitor AG825 (AG) (1 μM), the EGFR inhibitor PD153035 (PD153) (1 μM), or the tyrosine kinase inhibitor genistein (Geni) (10 μM) (1 h of preincubation with or without 10 μM EGCG). Immunoblotting was performed using specific antibodies to FAS or β-actin. Relative density values shown below the β-actin lane are normalized ratios of the intensity of the FAS band to the corresponding actin band. The means of the relative density are plotted in the bottom panel. * $p < 0.05$, compared with the HRG-β1-treated control. (**B**) Either HER2 or HER3 activation (phosphorylation) was inhibited by treatment of EGCG. Serum-starved MCF-7 cells were incubated with or without various doses of EGCG as indicated for 30 min and then activated with 20 ng/mL HRG-β1 for 10 min. After incubation, cell lysates were prepared, and HER2 and HER3 proteins were immunoprecipitated (IP). The tyrosine phosphorylation of HER2 and HER3 was determined by immunoblotting using anti-phosphotyrosine antibody (4G10), and the HER2 and HER3 native proteins were monitored to ensure consistent loading. (**C**) Down-regulation of the HER2–HER3 heterodimer by EGCG treatment. HRG-β1-stimulated cell lysates were immunoprecipitated with HER3 and then immunoblotted for HER2.

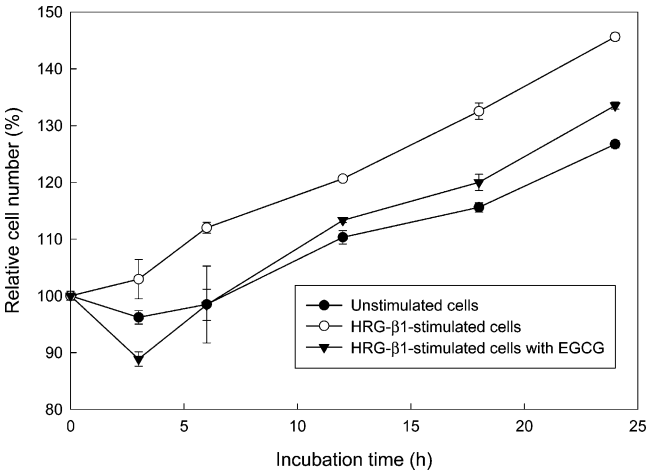

Figure 6. Effect of EGCG on growth of HRG-β1-stimulated MCF-7 cells. Serum-starved MCF-7 cells were treated with 0 or 20 μM EGCG for 30 min before 20 ng/mL HRG-β1 was added into the culture medium. After 0, 3, 6, 12, 18, and 24 h of incubation, the cell growth rate was then determined by the MTT assay as described in the Materials and Methods, and the results are shown as the cell number relative to that of the unstimulated cells at 0 h. Each experiment was independently performed three times and expressed as the mean ± SE.

treatment (**Figure 5C**). These results indicate that inhibition of HER2−HER3 heterodimer function may be a crucial mechanism for blocking HRG-β1-induced FAS expression by EGCG.

EGCG Inhibits Growth of HRG-β1-Stimulated MCF-7 Cells. Suppression of FAS expression may cause growth inhibition of cancer cells (*9*). It was therefore of interest to determine whether EGCG would suppress the growth of MCF-7 cells driven by HRG-β1 stimulation accompanied by down-regulation of FAS. Serum-starved MCF-7 cells were pretreated with 20 μM EGCG for 30 min and then incubated with 20 ng/mL HRG-β1 for the indicated periods. Then the cell growth rate was assessed by means of the MTT method. As shown in **Figure 6**, HRG-β1 markedly increased the proliferation rate of MCF-7 cells compared with unstimulated cells. Notably, pretreatment of HRG-β1-stimulated cells with EGCG decreased their rate of proliferation in culture, indicating the ability of EGCG in growth inhibition of HRG-β1-stimulated cells. Combined with the findings in **Figure 4,B**, we suggest that suppression of FAS expression by EGCG may be one of the key factors to lead to growth inhibition of MCF-7 cells.

DISCUSSION

In human normal tissues, FAS is suppressed by the presence of fatty acids in the daily diet; however, tumor-associated FAS is overexpressed despite high levels of ambient fatty acids. How does tumor-associated FAS constitutively maintain? The involvement of PI3K/Akt and MAPK signaling cascades, two pathways frequently activated in response to cancer-related overexpression of growth factors (e.g., EGF, heregulin) or growth factor receptors (e.g., EGFR, HER2, or HER3), have been demonstrated to constitutively mediate FAS up-regulation in cancer cells (*23*, *26*, *27*). Consistent with these findings, our current data exhibit that PI3K inhibitors, dominant-negative Akt, and the MEK inhibitor strongly inhibit HRG-β1-mediated FAS up-regulation (**Figure 3**), indicating that FAS expression in response to HRG-β1 stimulation in MCF-7 cells occurs through

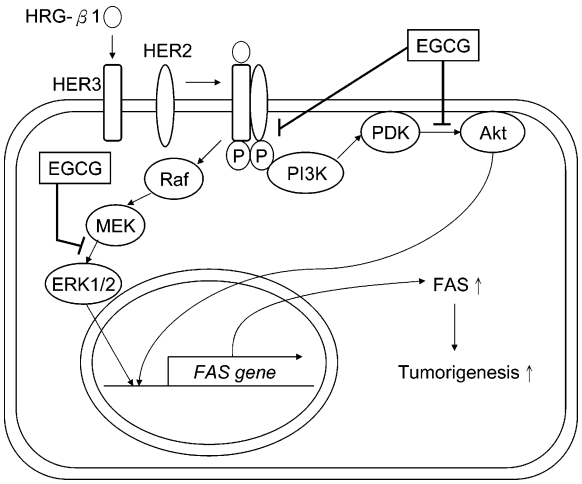

Figure 7. Proposed model for the cancer-preventive action of EGCG at multiple steps in HER2- or/and HER3-overexpressed breast cancer cells. HRG-β1, an HER3 ligand, transactivates HER2 and consequently stimulates tumor-associated FAS expression via major alternative pathways of downstream HER2 signaling. One is mediated by PI3K/Akt signaling, and the other is modulated by MAPK cascade signaling. However, EGCG inhibits tyrosine phosphorylation and heterodimerization of HER3 and HER2 and prevents the activation of Akt and ERK1/2 that finally lead to down-regulation of HRG-β1-elicited FAS induction. These effects could contribute to the anticancer activity of EGCG. PDK = phosphotidylinositol-3,4,5-triphophate-dependent kinase, and Raf = MEK kinase.

a modulation of PI3K/Akt and MAPK oncogenic cascades. This is the first report of a link between HRG-β1 signaling and FAS pathways.

HRG-β1 belongs to a member of the EGF-like ligand family, is highly expressed in breast cancer tissues (*14*), and is thought to be responsible for breast cancer development and progression. Here we demonstrated that HRG-β1 can regulate HER2−HER3 heterodimerization and thereby activate downstream signaling including PI3K/Akt and MAPK cascade pathways which finally lead to FAS expression in MCF-7 human breast cancer cells (**Figure 7**). These findings imply that suppression of the route responsible for HRG-β1-elicited FAS expression may be an effective method for the cancer-preventive action of tea. On the basis of this assumption, we found that the green tea polyphenol EGCG can block HRG-β1-mediated FAS induction by inhibiting the activation of the HER2−HER3 heterodimer and suppressing the activation of PI3K/Akt and ERK1/2 (as illustrated in **Figure 7**).

Using the specific inhibitors for the erbB family, we found that HRG-β1-induced FAS expression was significantly inhibited by the HER2 inhibitor AG825 and tyrosine kinase inhibitor genistein (**Figure 5A**) but not the EGFR inhibitor PD153035, implying that HER2 protein and tyrosine kinase activity are essential for HRG-β1 signaling. Since HER3 is the intrinsic tyrosine kinase-defective protein (*28*), we suggest that HRG-β1-mediated up-regulation of FAS in MCF-7 cells requires combinatorial receptor interactions between HER3 and HER2. In fact, the HER2−HER3 heterodimer is the most transforming and mitogenic receptor complex (*29*). On the basis of a gene expression survey and proteomic analysis in breast cancer cells (*23*, *24*), HER2 mediates the up-regulation of several genes including FAS whose elevation is associated with more advanced disease and portends a poor prognosis. Consistent with these observations, the results in **Figure 5B,C** show HRG-β1-induced tyrosine phosphorylation of HER3 and HER2 and formation of the HER2−HER3 heterodimer, respectively, implicating that HER3 ligand binding may recruit HER2 to form the HER2−HER3 heterodimer, where HER2 helps in HER3 transphosphorylation and activation. Collectively, we suggest that HRG-β1-associated FAS expression in MCF-7 cells is mediated by the HER2−HER3 heterodimer, but this issue requires further study.

Inhibition of growth factor signaling may be one of the possible cancer-preventive mechanisms of EGCG on the basis of several investigations. In our previous study, a modulation between EGCG and EGFR has been demonstrated by using in vitro kinase assays (*30*). We found that EGCG blocks EGF binding to EGFR and suppresses EGF-elicited activation and autophosphorylation of EGFR intrinsic tyrosine kinase (*30*). Accumulating studies have demonstrated that EGCG inhibits basal HER2 receptor tyrosine phosphorylation in human head, neck, and breast carcinoma cells and blocks the activation of HER2 in HER2-overexpressed cancer cell lines (*19*−*21*). In light of these observations, we suggest that EGCG might compete with HRG-β1 to bind to HER3, interrupt the formation of the HER2−HER3 heterodimer, or inhibit the protein kinase activity of HER2, which transphosphorylates HER3 (**Figure 7**). Supporting this assumption, we found that tyrosine phosphorylation of HER2 in response to HRG-β1 was fully suppressed by EGCG treatment, and a similar inhibitory result was also observed on HER3 phosphorylation (**Figure 5B**). As demonstrated in **Figure 5C**, the inhibition of HER2 and HER3 interaction by EGCG may provide an opportunity to effectively decrease the tyrosine phosphorylation of HER2 and HER3. Totally, we inferred three possible mechanisms by which EGCG prevents HRG-β1-driven HER3 activation: (i) EGCG could prevent HRG-β1 binding to HER3, (ii) EGCG could block the recruitment of HER2 to HER3, or (iii) EGCG could inhibit the intrinsic tyrosine kinase activity of HER2. The details of how these effects occur remain to be deciphered.

The data given here provide clear evidence that the expression of FAS in MCF-7 and AU565 human breast cancer cell lines in response to HRG-β1 was blocked by EGCG treatment. Using transcription inhibitor actinomycin D and RT-PCR analysis reveal that the inhibition of transcriptional regulation might participate in EGCG-mediated FAS suppression in HRG-β1-stimulated MCF-7 cells, implicating that HRG-β1 signaling might activate certain transcription factor(s) which mediate(s) *FAS* gene expression. One possible transcription factor candidate is sterol-responsive element-binding proteins (SREBPs), the major factors involved in the regulation of FAS (*31*), which are known to be regulated by PI3K/Akt and MAPK cascade pathways (*32*, *33*). Moreover, several studies have shown that the transcription factor Sp-1 is essential for sterol regulation of human FAS promoter I (*34*) and full activation of rat SREBP-1c promoter by insulin (*35*). Our previous study also reveals that EGCG inhibits EGF-stimulated Sp-1 DNA binding activity, which is linked to EGF-induced *FAS* gene expression (*18*). Taken together, we suggest that HRG-β1 induces the activation of PI3K/Akt and MAPK signal transduction pathways, which might drive SREBP-1c or/and Sp-1 to transactivate *FAS* gene expression. Further studies are needed to determine whether EGCG could directly or indirectly influence the DNA binding ability or/and the transactivity of SREBP-1c and Sp-1 that might lead to *FAS* gene expression in response to HRG-β1.

The findings of this study reveal the molecular mechanisms of tumor-associated FAS up-regulation by HRG-β1 and suggest that EGCG provides a natural compound that may be useful in the treatment of cases of breast carcinoma in which overexpression of FAS in response to oncogenic changes, including

overexpression of growth factors or growth factor receptors, plays a critical role in tumor growth and survival.

LITERATURE CITED

(1) Witkowski, A.; Rangan, V. S.; Randhawa, Z. I.; Amy, C. M.; Smith, S. Structural organization of the multifunctional animal fatty-acid synthase. *Eur. J. Biochem.* **1991**, *198* (3), 571−9.

(2) Wakil, S. J. Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* **1989**, *28* (11), 4523−30.

(3) Medes, G.; Thomas, A.; Weinhouse, S. Metabolism, of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. *Cancer Res.* **1953**, *13* (1), 27−9.

(4) Alo, P. L.; Visca, P.; Trombetta, G.; Mangoni, A.; Lenti, L.; Monaco, S.; Botti, C.; Serpieri, D. E.; Di Tondo, U. Fatty acid synthase (FAS) predictive strength in poorly differentiated early breast carcinomas. *Tumori* **1999**, *85* (1), 35−40.

(5) Rossi, S.; Graner, E.; Febbo, P.; Weinstein, L.; Bhattacharya, N.; Onody, T.; Bubley, G.; Balk, S.; Loda, M. Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol. Cancer Res.* **2003**, *1* (10), 707−15.

(6) Rashid, A.; Pizer, E. S.; Moga, M.; Milgraum, L. Z.; Zahurak, M.; Pasternack, G. R.; Kuhajda, F. P.; Hamilton, S. R. Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am. J. Pathol.* **1997**, *150* (1), 201−8.

(7) Pizer, E. S.; Kurman, R. J.; Pasternack, G. R.; Kuhajda, F. P. Expression of fatty acid synthase is closely linked to proliferation and stromal decidualization in cycling endometrium. *Int. J. Gynecol. Pathol.* **1997**, *16* (1), 45−51.

(8) Gansler, T. S.; Hardman, W., 3rd; Hunt, D. A.; Schaffel, S.; Hennigar, R. A. Increased expression of fatty acid synthase (OA-519) in ovarian neoplasms predicts shorter survival. *Hum. Pathol.* **1997**, *28* (6), 686−92.

(9) De Schrijver, E.; Brusselmans, K.; Heyns, W.; Verhoeven, G.; Swinnen, J. V. RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. *Cancer Res.* **2003**, *63* (13), 3799−804.

(10) Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **1987**, *235* (4785), 177−82.

(11) Holbro, T.; Hynes, N. E. ErbB receptors: directing key signaling networks throughout life. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 195−217.

(12) Yarden, Y.; Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2* (2), 127−37.

(13) Peles, E.; Yarden, Y. Neu and its ligands: from an oncogene to neural factors. *Bioessays* **1993**, *15* (12), 815−24.

(14) Dunn, M.; Sinha, P.; Campbell, R.; Blackburn, E.; Levinson, N.; Rampaul, R.; Bates, T.; Humphreys, S.; Gullick, W. J. Co-expression of neuregulins 1, 2, 3 and 4 in human breast cancer. *J. Pathol.* **2004**, *203* (2), 672−80.

(15) Sliwkowski, M. X.; Schaefer, G.; Akita, R. W.; Lofgren, J. A.; Fitzpatrick, V. D.; Nuijens, A.; Fendly, B. M.; Cerione, R. A.; Vandlen, R. L.; Carraway, K. L., 3rd. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* **1994**, *269* (20), 14661−5.

(16) Atlas, E.; Cardillo, M.; Mehmi, I.; Zahedkargaran, H.; Tang, C.; Lupu, R. Heregulin is sufficient for the promotion of tumorigenicity and metastasis of breast cancer cells in vivo. *Mol. Cancer Res.* **2003**, *1* (3), 165−75.

(17) Lin, J. K.; Liang, Y. C.; Lin-Shiau, S. Y. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.* **1999**, *58* (6), 911−5.

(18) Yeh, C. W.; Chen, W. J.; Chiang, C. T.; Lin-Shiau, S. Y.; Lin, J. K. Suppression of fatty acid synthase in MCF-7 breast cancer cells by tea and tea polyphenols: a possible mechanism for their hypolipidemic effects. *Pharmacogenomics J.* **2003**, *3* (5), 267−76.

(19) Masuda, M.; Suzui, M.; Lim, J. T.; Weinstein, I. B. Epigallocatechin-3-gallate inhibits activation of HER-2/neu and downstream signaling pathways in human head and neck and breast carcinoma cells. *Clin. Cancer Res.* **2003**, *9* (9), 3486−91.

(20) Pianetti, S.; Guo, S.; Kavanagh, K. T.; Sonenshein, G. E. Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells. *Cancer Res.* **2002**, *62* (3), 652−5.

(21) Shimizu, M.; Deguchi, A.; Lim, J. T.; Moriwaki, H.; Kopelovich, L.; Weinstein, I. B. (-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin. Cancer Res.* **2005**, *11* (7), 2735−46.

(22) Shimizu, M.; Deguchi, A.; Joe, A. K.; McKoy, J. F.; Moriwaki, H.; Weinstein, I. B. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. *J. Exp. Ther. Oncol.* **2005**, *5* (1), 69−78.

(23) Kumar-Sinha, C.; Ignatoski, K. W.; Lippman, M. E.; Ethier, S. P.; Chinnaiyan, A. M. Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. *Cancer Res.* **2003**, *63* (1), 132−9.

(24) Zhang, D.; Tai, L. K.; Wong, L. L.; Chiu, L. L.; Sethi, S. K.; Koay, E. S. Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. *Mol. Cell. Proteomics* **2005**, *4* (11), 1686−96.

(25) Sweeney, C.; Fambrough, D.; Huard, C.; Diamonti, A. J.; Lander, E. S.; Cantley, L. C.; Carraway, K. L., 3rd. Growth factor-specific signaling pathway stimulation and gene expression mediated by ErbB receptors. *J. Biol. Chem.* **2001**, *276* (25), 22685−98.

(26) Yang, Y. A.; Han, W. F.; Morin, P. J.; Chrest, F. J.; Pizer, E. S. Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Exp. Cell Res.* **2002**, *279* (1), 80−90.

(27) Van de Sande, T.; De Schrijver, E.; Heyns, W.; Verhoeven, G.; Swinnen, J. V. Role of the phosphatidylinositol 3′-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. *Cancer Res.* **2002**, *62* (3), 642−6.

(28) Citri, A.; Skaria, K. B.; Yarden, Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp. Cell Res.* **2003**, *284* (1), 54−65.

(29) Wallasch, C.; Weiss, F. U.; Niederfellner, G.; Jallal, B.; Issing, W.; Ullrich, A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J.* **1995**, *14* (17), 4267−75.

(30) Liang, Y. C.; Lin-shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. *J. Cell. Biochem.* **1997**, *67* (1), 55−65.

(31) Kim, J. B.; Sarraf, P.; Wright, M.; Yao, K. M.; Mueller, E.; Solanes, G.; Lowell, B. B.; Spiegelman, B. M. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J. Clin. Invest.* **1998**, *101* (1), 1−9.

(32) Fleischmann, M.; Iynedjian, P. B. Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin

and protein kinase B/cAkt. *Biochem. J.* **2000**, *349* (Pt 1), 13−7.

(33) Kotzka, J.; Muller-Wieland, D.; Roth, G.; Kremer, L.; Munck, M.; Schurmann, S.; Knebel, B.; Krone, W. Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade. *J. Lipid Res.* **2000**, *41* (1), 99−108.

(34) Xiong, S.; Chirala, S. S.; Wakil, S. J. Sterol regulation of human fatty acid synthase promoter I requires nuclear factor-Y- and Sp-1-binding sites. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (8), 3948−53.

(35) Cagen, L. M.; Deng, X.; Wilcox, H. G.; Park, E. A.; Raghow, R.; Elam, M. B. Insulin activates the rat sterol-regulatory--element-binding protein 1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements. *Biochem. J.* **2005**, *385* (Pt 1), 207−16.

Received for review February 5, 2007. Revised manuscript received April 27, 2007. Accepted April 30, 2007. This study was supported by the National Science Council (Grants NSC 94-2312-B-040-001 and NSC 95-2320-B-040-037).

JF070316R