行政院國家科學委員會專題研究計畫　成果報告

兒茶素 EGCG 抑制腫瘤相關性脂肪酸合成酵素機制之研究

計畫類別：個別型計畫
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計畫主持人：陳威仁

計畫參與人員：陳政廷，林鉦展

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附件一

行政院國家科學委員會補助專題研究計畫 ☑成果報告 □期中進度報告

兒茶素EGCG抑制腫瘤相關性脂肪酸合成酵素機制之研究

Study on the mechanism by which EGCG inhibits tumor-associated fatty acid synthase

計畫類別：☑個別型計畫　□整合型計畫
計畫編號：NSC 94－2312－B－040－001－
執行期間：94 年 10 月 1 日至　95 年 7 月 31 日

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共同主持人：
計畫參與人員：陳政廷、林鉦展

成果報告類型(依經費核定清單規定繳交)：☑精簡報告　□完整報告

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□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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執行單位：中山醫學大學生物醫學科學學系

中　華　民　國　　95 年　10 月　30 日

1. 中文摘要

(-)-表沒食子酸兒茶素-3-沒食子酸酯（(-)-Epigallocatechin 3-gallate (EGCG)）為綠茶中含量最豐且生物活性最強之兒茶素 (catechin)，根據美國、日本、台灣等專家學者之研究，指出 EGCG 富含多種生物活性，包括降血脂、抗氧化、抗發炎等作用，其中引人注目的是：EGCG 會透過各種的機制，來抑制癌症的生長、運動與轉移，因此 EGCG 被認為具有發展成化學癌症預防或治療藥劑之潛力。

脂肪酸合成酶（Fatty acid synthase (FAS)）為體內將過剩能量經由 acetyl-coA 轉變為 palmitate，之後形成長鏈脂肪酸，儲存於體內的關鍵酵素。近年來研究發現 FAS 在細胞中的角色，不僅僅止於代謝，而是它會藉由控制脂肪酸的合成來調控整個細胞的生理機能。比如說，抑制 FAS 在細胞中的活性會導致細胞凋亡（apoptosis）的發生，暗示 FAS 與細胞存活有關；另外，在一些癌化的細胞中，FAS 的表現量有大量增加的情形；而對一些癌細胞（如乳癌細胞等）中總體基因表現（microarray）的研究發現，FAS 在細胞中的表現量，與致癌基因 *HER2/neu* 的表現呈高度正相關。而根據我們之前的研究也發現生長激素 EGF 會刺激乳癌細胞 MCF-7 中 FAS 的表現與活性，且 EGCG 會降低 EGF 刺激 FAS 表現的過程。

據統計，約 30% 的乳癌個案為致癌基因 *HER2/neu* overexpression，且癒後極差，基於此，本研究的出發點是要瞭解生長激素 heregulin-β1 (HRG-β1：為 HER2 間接的 ligand)，是否會透過活化 HER2 來提昇 FAS 的表現，其促進 FAS 表現的機制為何？而 EGCG 是否會抑制此活化的過程？藉此研究，我們得到兩項主要的研究成果：一是瞭解 FAS 的表現會參與 HER2 過量表現的乳組織細胞的癌化，其作用的機制可能是透過 PI3K/Akt 與 MAPK cascade 兩種訊號路徑來調控；二是 EGCG 能有效抑制此過程，由此證明 EGCG 的預防癌症能力。

關鍵字：脂肪酸合成酶；(-)-表沒食子酸兒茶素-3-沒食子酸酯；Heregulin-β1；*HER2/neu*；Akt；PI3-K

2. 英文摘要

Tumor-associated fatty acid synthase (FAS) is implicated in tumorigenesis and is known to be connection to HER2 by systemic analyses (*1, 2*). Suppression of FAS in cancer cells may lead to growth inhibition and cell apoptosis. Our previous study (*3*) demonstrated that (-)-Epigallocatechin 3-gallate (EGCG), the green tea catechin, could suppress FAS expression by suppressing EGFR signaling and downstream PI3K/Akt activation in MCF-7 breast cancer cell line. Herein, we examined the effects of EGCG on FAS expression modulated by other member of 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, 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 that 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 ERK 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 heterodimer between HER2 and HER3, and their tyrosine kinase activities are essential for HRG-β1-mediated elevation of FAS. Take together, these findings extend our previous study indicate EGCG may be useful in the chemoprevention of breast carcinoma in which FAS overexpression results from HER2 or/and HER3 signaling.

Keywords: EGCG; heregulin-β1; FAS; HER; erbB; Akt; ERK

3. **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 (*4, 5*). 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 FAS shows more active and highly expressed (*6*). Numerous clinical and basic studies disclose that high levels of FAS are present in many varieties of common human cancers such as human breast (*7, 8*), prostate (*9, 10*), colon (*11*), endometrium (*12*), and ovary (*13*) cancers. FAS is implicated in tumorigenesis through its role in cell proliferation and membrane lipid incorporation of neoplastic cells. Suppression of FAS in cancer cells may lead to growth inhibition and cell apoptosis. This differential expression of FAS between normal tissues and cancers suggests that FAS may be the target for anticancer drug development.

The *HER2/neu* (or *c-erbB2*) oncogene, which is the second member of the erbB family, encodes a transmembrane tyrosine receptor kinase, is amplified in 20–30 % of human breast cancers (*14*) and is overexpressed in other cancers. Overexpression of HER2 is often associated with poor prognosis. Systemic analyses have revealed a molecular connection between HER2 and FAS (*1, 2*). Unlike the other erbB family members, HER2 is an orphan receptor and its natural ligand has yet been not characterized; however, HER2 can be activated by its own overexpression or be transactivated by heregulins (HRGs) (*15, 16*). HRGs are signaling proteins that are ligands for the erbB family. It exerts 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 (*17, 18*).

(-)-Epigallocatechin 3-gallate (EGCG) is the most abundant and biologically active catechin extracted from green tea and exerts its anti-mutagenic activity through blockade of mitotic signal transduction (*19, 20*). Our previous report has demonstrated that EGCG could suppress FAS expression by down-regulating EGF receptor/PI3K/Akt/Sp-1 signal transduction pathway in MCF-7 breast cancer cell line (*3*). We postulate that EGCG would inhibit FAS expression through other erbB family pathway such as HER2 or HER3. In fact, it has been reported that EGCG down-regulates HER2 signaling in human breast, head, neck, and colon cancer cells (*21-23*) and inhibits HER3 activation in human colon cancer cells (*24*), implying that EGCG may inhibits the downstream phenotype controlled by HER2 or HER3.

This study was undertaken to investigate whether EGCG would down-regulate HRG-β1-stimulated FAS expression in human breast cancer cells. We demonstrated that EGCG inhibited HRG-β1-induced FAS expression in different breast cancer cell lines. Using transcription inhibitor actinomycin D and RT-PCR analysis revealed that the inhibition of transcriptional regulation might participate in the EGCG-mediated FAS suppression in HRG-β1-stimulated MCF-7 cells. The suppression of HER2 inhibitor AG825 on the FAS induction indicates that HRG-β1 up-regulated the *FAS* gene through HER2 and HER3 coordination. Searching the downstream kinase regulators involved in HER2 signaling, we found that EGCG inhibited the activation of Akt and ERK (data not shown) that were required for

HRG-β1-dependent Fas induction. Our current data implicate a new therapeutic insight for the cancer chemopreventive activity of EGCG on HER2 or/and HER3 positive breast cancers.

4. **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, USA). The HER2 inhibitor AG825 was purchased from Calbiochem (La Jolla, CA). The antibody to β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK (Thr42/Tyr44), and anti-ERK were from Cell Signaling Technology (Beverly, MA). RT-PCR reagents were from Promega (Madison, WI, USA). 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).

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 of penicillin, 100 μg/mL of streptomycin, and kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Western Blot Analysis

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 phenylmethylsulfonyl 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, USA), and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham, UK).

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), 1X reaction buffer, 1 μM 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. 5 μl 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 (*25*). p38 dominant-negative mutant (dnp38) generously provided by Dr. C. C. Chen (Department of Pharmacolgy, College of Medicine, National Taiwan University, Taipei, Taiwan). MCF-7 cells were transiently transfected with dnAkt or dnp38 or pcDNA3 using Lipofectin® Reagent (GIBCO, Invitrogen; Grand Island, NY) according to the manufacturer’s instruction. After transient transfection, cells were serum-starved for 18 h and then stimulated with 50 ng/mL of HRG-β1 for 9 h, and then cells were lysed for analysis.

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.

5. 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. Fig. 1A shows that HRG-β1 increased FAS expression dramatically in MCF-7 and AU565 cells and this induction was dosage-dependent. However, MDA-MB-453 cells did not show significant Fas induction by HRG-β1 stimulation. As shown in Fig. 1B, the time-dependent induction of Fas appeared after HRG-β1 stimulation and occurred as early as 6h 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 basal level of FAS (Fig. 1A).

EGCG Inhibits HRG-β1-Induced FAS Expression in MCF-7 Cells.

In our previous study, we demonstrate that EGCG suppresses FAS expression by down-regulating EGFR signaling pathway (*3*). Herein, the effect of EGCG on FAS expression stimulated by HRG-β1 was investigated. As shown in Fig. 2A, the protein level of FAS induced by HRG-β1 was gradually decreased following the appearance of EGCG, and the decrease in protein level of FAS was dosage-dependent no matter in MCF-7 cells. Next, to elucidate whether the decreased FAS induction by EGCG treatment was due to the down-regulation of mRNA, reverse transcription-polymerase chain reaction (RT-PCR) was performed on HRG-β1-treated

MCF-7 cells with or without EGCG exposure. The data in Fig. 2B exhibit that EGCG lowered HRG-β1-mediated FAS mRNA expression in MCF-7 cells both. The present data suggest that EGCG inhibits the HRG-β1-induced FAS expression through transcriptional regulation.

HRG-β1 Induces FAS mRNA Expression in MCF-7 Cells.

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), a 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. Fig. 3A shows that ActD completely abolished HRG-β1-mediated FAS mRNA up-regulation, whereas CHX had no effect on FAS mRNA induction by HRG-β1. However, CHX treatment suppressed FAS protein expression (Fig. 3B) even though FAS mRNA induction by HRG-β1 occurred (Fig. 3A). 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 (*26, 27*). 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 (Fig. 4A). On the other hand, MEK inhibitor PD98059 also blocked FAS up-regulation by HRG-β1 (Fig. 4A). However, p38 MAPK inhibitor SB203580 did not induce a remarkable change in FAS expression in HRG-β1-treated cells (Fig. 4A). To further confirm the importance of Akt in the FAS induction by HRG-β1, we established a MCF-7 cell line transient expressing dominant-negative Akt or p38 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 p38 (Fig. 4B). Collectively, the data obtained here strongly suggests that Akt and ERK may act as up-stream kinases to activate FAS up-regulation in response to HRG-β1.

HER2 is Essential for FAS Induction by HRG-β1.

To ascertain whether HER2 involves 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 (Fig. 5). 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 the effect of HRG-β1. Fig. 5 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.

6. Discussion

In this study, we demonstrated that HRG-β1 regulates FAS expression in MCF-7 human breast cancer cells via the PI3K/Akt and MAPK cascade pathways, and the green tea polyphenol, EGCG, could block this induction by suppressing Akt and ERK activation. The expression of HRG-β1-induced FAS was significantly inhibited by HER2 inhibitor AG825 and tyrosine kinase inhibitor genistein (Fig. 5), indicating that tyrosine kinase activity is essential for HRG-β1 signal transduction. Since HER3, which is driven by HRG-β1, possesses an impaired intrinsic tyrosine kinase activity (*28, 29*), we suggest that HRG-β1-mediated up-regulation of FAS in MCF-7 cells requires combinatorial receptor interactions between HER3 and HER2. In fact, HER2/HER3 heterodimer is the most transforming and mitogenic receptor complex (*30-32*). Based on gene expression survey and proteomic analysis in breast cancer cells (*1, 2*), HER2 mediates the up-regulation of several genes including FAS whose elevation is associated with more advanced disease and portends a poor prognosis. Collectively, we suggest that HRG-β1-associated FAS expression in MCF-7 cells is mediated by HER2/HER3 heterodimer, but this issue requires further study.

In human normal tissues, FAS is suppressed by the presence of fatty acids in the 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 activating in response to cancer-related overexpression of growth factors (e.g., EGF, heregulin) or growth factor receptors (e.g., EGFR, HER2), have been demonstrated to mediate constitute FAS up-regulation in cancer cells (*1, 33-37*). Consistent with these findings, our current data exhibit that PI3K inhibitors, dominant-negative Akt, and MEK inhibitor strongly inhibit HRG-β1-mediated FAS up-regulation, respectively (Fig. 4A and 4B), indicating that FAS expression in response to HRG-β1 stimulation in MCF-7 cells occurs through a modulation of PI3K/Akt and MAPK oncogenic cascades. This is the first report of a link between HRG-β1 signaling and FAS pathways.

Inhibition of growth factor signaling may be one of the possible cancer preventive mechanisms of EGCG based on several investigations. In our previous study, a modulation between EGCG and EGFR has been demonstrated by using in vitro kinase assays (*38*). We observed that EGCG blocks EGF binding to EGFR and inhibits the protein tyrosine kinase activity and autophosphorylation of EGFR by EGF (*38*). Additionally, the inhibition of basal HER2 receptor tyrosine phosphorylation by EGCG has been demonstrated in human head and neck and breast carcinoma cells Other investigations have showed that EGCG inhibits the activation of HER2 in HER2-overexpressed cancer cell lines (*21-23*). In light of these observations, we suggest that EGCG might reduce HRG-β1-induced FAS expression through

inhibiting HER2 tyrosine kinase activity. EGCG might compete against HRG-β1 to bind to HER3 or inhibit protein kinase activity of HER2 to trans-phosphorylate HER3. Supporting this assumption, we found that HER2 is necessary for FAS induction by HRG-β1 as demonstrated by a specific HER2 tyrosine kinase inhibitor, AG825 (Fig. 5), enhancing the possibility that EGCG down-regulates FAS induction by HRG-β1 because of inhibition of HER2 tyrosine kinase activity.

The data given here provide clear evidence that expression of FAS in MCF-7 human breast cancer cell lines was induced by HRG-β1 and that this induction was because of up-regulation of FAS mRNA, suggesting that HRG-β1-induced expression of FAS may be mediated by certain transcription factor(s). On possible candidate is sterol-responsive element-binding proteins (SREBPs), the major factors involving in the regulation of FAS (*39*), which are known to be regulated by PI3K/Akt and MAPK cascade pathways (*40, 41*). Moreover, studies showed that the transcription factor Sp-1 is essential for sterol regulation of human FAS promoter I (*42*) and fully activation of rat SREBP-1c promoter by insulin (*43*). Our recent study demonstrated that EGCG inhibits EGF-stimulated Sp-1 DNA binding activity (*3*). Take together; we suggest that SREBP-1c and Sp-1, which are driven by activation of PI3K/Akt and MAPK signaling pathways in response to HRG-β1, are required for *FAS* gene expression. Additional studies are needed to determine whether EGCG directly or indirectly influences SREBP-1c and Sp-1 activities that might be required for *FAS* gene expression in response to HRG-β1.

The findings of this present study reveal the molecular mechanisms of tumor-associated FAS up-regulation by HRG-β1 and suggest that EGCG provides a nature 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, which plays critical roles in tumor growth and survival.

7. Acknowledgments

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8. References

1. 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.

2. 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.

3. 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.

4. 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.
5. Wakil, S. J., Fatty acid synthase, a proficient multifunctional enzyme. *Biochemistry* **1989,** 28, (11), 4523-30.
6. 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.
7. 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.
8. Milgraum, L. Z.; Witters, L. A.; Pasternack, G. R.; Kuhajda, F. P., Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. *Clin Cancer Res* **1997,** 3, (11), 2115-20.
9. 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.
10. Swinnen, J. V.; Roskams, T.; Joniau, S.; Van Poppel, H.; Oyen, R.; Baert, L.; Heyns, W.; Verhoeven, G., Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. *Int J Cancer* **2002,** 98, (1), 19-22.
11. 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.
12. 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.
13. 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.
14. Slamon, D. J.; Godolphin, W.; Jones, L. A.; Holt, J. A.; Wong, S. G.; Keith, D. E.; Levin, W. J.; Stuart, S. G.; Udove, J.; Ullrich, A.; et al., Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **1989,** 244, (4905), 707-12.
15. Holmes, W. E.; Sliwkowski, M. X.; Akita, R. W.; Henzel, W. J.; Lee, J.; Park, J. W.; Yansura, D.; Abadi, N.; Raab, H.; Lewis, G. D.; et al., Identification of heregulin, a specific activator of p185erbB2. *Science* **1992,** 256, (5060), 1205-10.
16. Peles, E.; Yarden, Y., Neu and its ligands: from an oncogene to neural factors. *Bioessays* **1993,** 15, (12), 815-24.
17. 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.
18. Carraway, K. L., 3rd; Cantley, L. C., A neu acquaintance for erbB3 and erbB4: a role for

receptor heterodimerization in growth signaling. *Cell* **1994,** 78, (1), 5-8.
19. 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.
20. Lin, J. K.; Liang, Y. C., Cancer chemoprevention by tea polyphenols. *Proc Natl Sci Counc Repub China B* **2000,** 24, (1), 1-13.
21. 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.
22. 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.
23. 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.
24. 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.
25. Villanueva, A.; Garcia, C.; Paules, A. B.; Vicente, M.; Megias, M.; Reyes, G.; de Villalonga, P.; Agell, N.; Lluis, F.; Bachs, O.; Capella, G., Disruption of the antiproliferative TGF-beta signaling pathways in human pancreatic cancer cells. *Oncogene* **1998,** 17, (15), 1969-78.
26. Fambrough, D.; McClure, K.; Kazlauskas, A.; Lander, E. S., Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. *Cell* **1999,** 97, (6), 727-41.
27. 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.
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. Guy, P. M.; Platko, J. V.; Cantley, L. C.; Cerione, R. A.; Carraway, K. L., 3rd, Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* **1994,** 91, (17), 8132-6.
30. Pinkas-Kramarski, R.; Soussan, L.; Waterman, H.; Levkowitz, G.; Alroy, I.; Klapper, L.; Lavi, S.; Seger, R.; Ratzkin, B. J.; Sela, M.; Yarden, Y., Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *Embo J* **1996,** 15, (10), 2452-67.
31. Alimandi, M.; Romano, A.; Curia, M. C.; Muraro, R.; Fedi, P.; Aaronson, S. A.; Di Fiore, P. P.; Kraus, M. H., Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* **1995,** 10, (9), 1813-21.
32. 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.
33. 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.
34. Swinnen, J. V.; Heemers, H.; Deboel, L.; Foufelle, F.; Heyns, W.; Verhoeven, G., Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. *Oncogene* **2000,** 19, (45), 5173-81.
35. Wang, D.; Sul, H. S., Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt. *J Biol Chem* **1998,** 273, (39), 25420-6.
36. 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.
37. Zhang, D. H.; Tai, L. K.; Wong, L. L.; Sethi, S. K.; Koay, E. S., Proteomics of breast cancer: enhanced expression of cytokeratin19 in human epidermal growth factor receptor type 2 positive breast tumors. *Proteomics* **2005,** 5, (7), 1797-805.
38. 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.
39. 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.
40. 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.
41. 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.
42. 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.
43. 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.

Figures

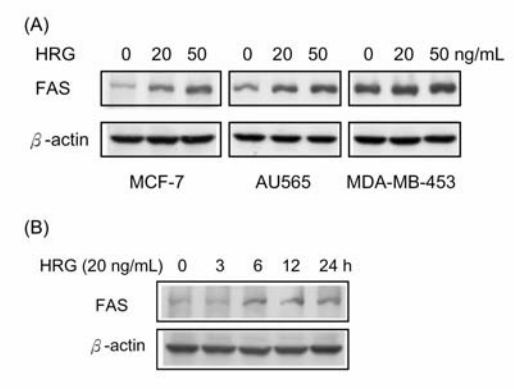

Fig. 1. HRG-β1 up-regulates FAS expression in various breast cancer cell lines. (A) Dose effects of heregulin-β1 on FAS expression in breast cancer cell lines. Cell lysates were collected from serum-starved MCF-7, AU565, or MDA-MD-453 cells treated with 0, 20, or 50 ng/mL HRG-β1 for 24 h; (B) Time-dependent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

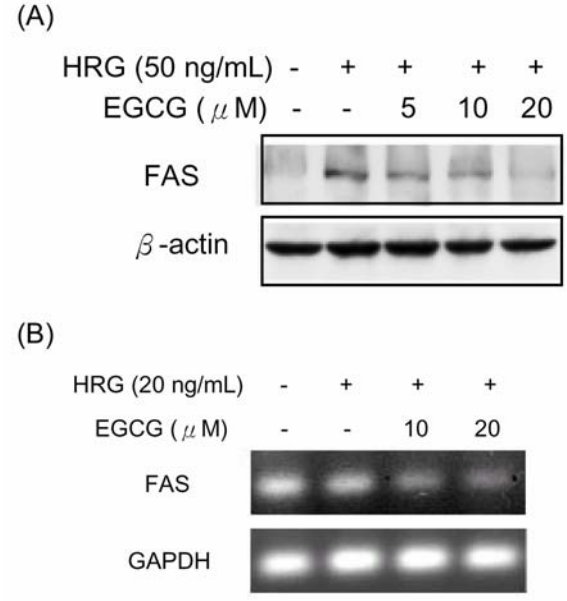

Fig. 2. EGCG inhibits HRG-β1-induced FAS expression in MCF-7 cells. (A) EGCG reduces the protein level of FAS induction by HRG-β1 in a dose-dependent manner. Serum-starved MCF-7 cells were pre-incubated with various dosage of EGCG for 30 min, and then stimulated with 50 ng/mL of HRG-β1 for 9 h. Western blotting were performed using specific antibodies to FAS or β-actin, respectively; (B) EGCG down-regulates mRNA expression of FAS. Serum-starved MCF-7 cells were pretreated with EGCG at indicated doses for 30 min before HRG-β1 (50 ng/mL) treatment. After incubation for 6 h, total RNA was isolated and the RNA expression was analyzed by RT-PCR as described in "Materials and Methods". GAPDH cDNA was used as an internal control.

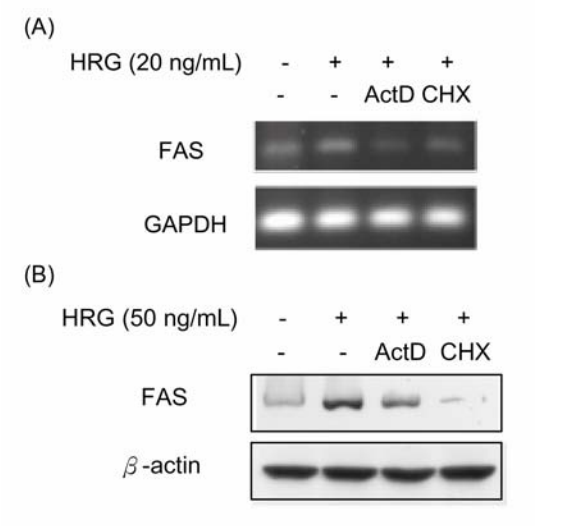

Fig. 3. Transcriptional regulation of FAS expression by HRG-β1 in MCF-7 cells. (A) RT-PCR analysis of cDNA from MCF-7 cells. Cells were pretreated with 4 μg/mL of actinomycin D (ActD) or 30μg/mL of cycloheximide (CHX) for 1 h before HRG-β1 (20 ng/mL). After 6 h incubation, total RNA was isolated and analyzed by RT-PCR. (B) Western blotting analysis of FAS protein in MCF-7 cells. 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 Western blotting using FAS or β-actin antibody.

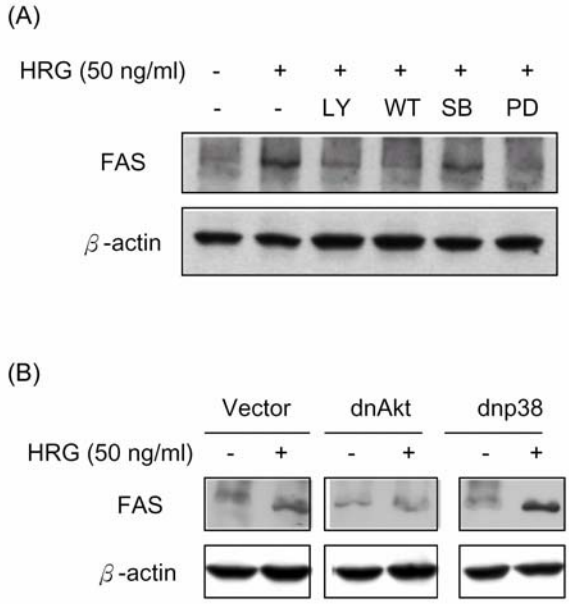

Fig. 4. FAS up-regulation by HRG-β1 requires Akt and ERK. (A) PI3K and MEK inhibitors suppress FAS expression induced by HRG-β1. Serum-starved MCF-7 were pretreated with 10 μM LY294002, 1 μM wortmannin, 20 μM SB203580, or 50 μM PD98059 for 1 h, and then stimulated with 50 ng/mL of HRG-β1. The FAS and β-actin protein levels were determined by Western blotting; (B) Dominant-negative Akt inhibits HRG-β1-regulated FAS expression. MCF-7 cells were transfected with dominant-negative Akt (dnAkt), dominant-negative p38 (dnp38), or control vector (pcDNA3) and subsequently treated with HRG-β1 (50 ng/mL) for 9 h. Cell lysates were analyzed by Western blotting using anti-FAS antibody. β-actin was used as a loading control.

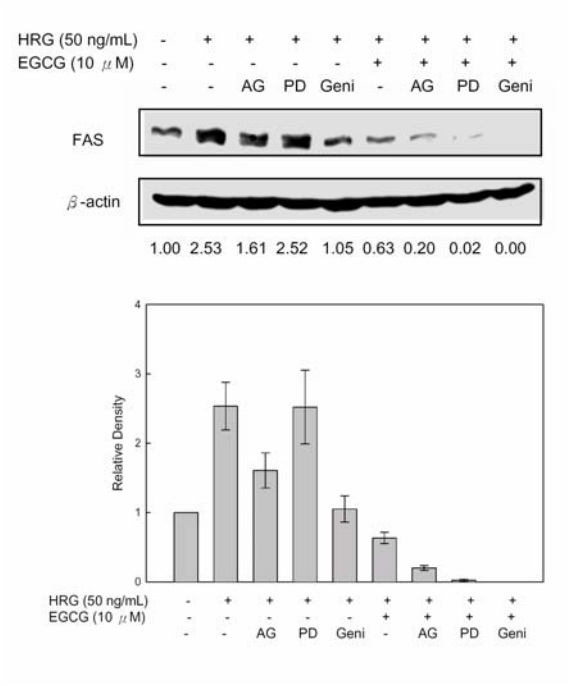

Fig. 5. The FAS induction by HRG-β1 requires HER2. MCF-7 cells were serum-starved for 24 h, and then treated with 50 ng/mL of HRG-β1 for 9 h in the presence or absence of HER2 inhibitor AG825 (AG) (1 μM), or EGFR inhibitor PD153035 (PD) (1 μM), or tyrosine kinase inhibitor genistein (Geni) (10 μM) (1 h pre-incubation with or without 10 μM of EGCG). Western blotting were performed using specific antibodies to FAS or β-actin, respectively. Relative density values shown below the β-actin lane are normalized ratio of the intensity of FAS band to the corresponding actin band. The means of the relative density are plotted in the bottom panel. **P*<0.05, ***P*<0.01, compared with the HRG-β1-treated control.

9. 計畫成果自評

(1) 瞭解 FAS 的表現會參與 HER2 過量表現的乳組織細胞的癌化，其作用的機制可能是透過 PI3K/Akt 與 MAPK cascade 兩種訊號路徑來調控。

(2) EGCG 能有效抑制 HER2/HER3 活化 FAS 表現，由此證明 EGCG 的預防癌症能力。

(3) 本計劃目前已達預定之成效。

(4) 我們已將研究成果彙整，將投稿至 SCI 排名期刊。