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Research Article

EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK in p53 positive and negative human hepatoma cells

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In the previous studies, (−)-epigallocatechin-3-gallate (EGCG) has been shown to have anticarcinogenic effects *via* modulation in protein expression of p53. Using p53 positive Hep G2 and p53 negative Hep 3B cells, we found that treatment of EGCG resulted in dose-dependent inhibition of cellular proliferation, which suggests that the interaction of EGCG with p53 may not fully explain its inhibitory effect on proliferation. Caloric restriction (CR) reduces the incidence and progression of spontaneous and induced tumors in laboratory rodents. EGCG has multiple beneficial activities similar to those associated with CR. One key enzyme thought to be activated during CR is AMP-activated kinase (AMPK), a sensor of cellular energy levels. Here, we showed that EGCG activated AMPK in both p53 positive and negative human hepatoma cells. The activation of AMPK suppressed downstream substrates, such as mammalian target of rapamycin (mTOR) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and a general decrease in mRNA translation. Moreover, EGCG activated AMPK decreases the activity and/or expression of lipogenic enzymes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). Interestingly, the decision between apoptosis and growth arrest following AMPK activation is greatly influenced by p53 status. In p53 positive Hep G2 cells, EGCG blocked the progression of cell cycle at G1 phase by inducing p53 expression and further upregulating p21 expression. However, EGCG inducted apoptosis in p53 negative Hep 3B cells. Based on these results, we have demonstrated that EGCG has a potential to be a chemoprevention and antilipogenesis agent for human hepatoma cells.

Keywords: AMPK / EGCG / FASN / mTOR / p53

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1 Introduction

Hepatocellular carcinoma (HCC) is a major health care problem worldwide, and is also one of the four most prevalent malignant diseases of adults in China, Taiwan, Korea, and sub-Africa [1, 2]. The prognosis of patients with HCC is poor because even in the early stages when surgical treatment might be expected to be curative, the incidence of recurrence in patients with underlying cirrhosis is very high due to multicentric carcinogenesis. Therefore, strategies to

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Abbreviations: ACC, acetyl-CoA carboxylase; **AMPK,** AMP-activated protein kinase; **CR,** caloric restriction; **4E-BP1,** eukaryotic initiation factor 4E-binding protein-1; **EGCG,** (−)-epigallocatechin gallate; **FASN,** fatty acid synthase; **HCC,** hepatocellular carcinoma; **mTOR,** mammalian target of rapamycin; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **shRNA,** short hairpin RNA

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prevent recurrence and second primary HCC are required to improve the prognosis.

Cancer chemoprevention by natural compounds has been investigated by many investigators. Tea is one of the most widely consumed beverages, second only to pure water. The possible cancer preventive activity of tea has received much attention in recent years. The inhibitory activities of tea and tea constituents against carcinogenesis at different organ sites have been demonstrated in many animal models. The effect of tea polyphenols, especially (−)-epigallocatechin gallate (EGCG), on human cancers, however, remains inconclusive [3]. Recent studies have revealed that EGCG triggers cancer cells undergoing apoptosis through p53-dependent pathway [4–9]. However, Huh *et al.* [10] indicates that EGCG suppressed cell viability and proliferation in p53 positive and negative ovarian carcinoma cell lines. Therefore, the mechanism by which EGCG inhibits cell proliferation is not clearly understood.

It has been suggested that caloric restriction (CR) extends longevity and reduces age-related pathologies by reducing the levels of DNA damage and mutations that accumulate with age [11]. Cancer is a disease of aging, as the incidence of most cancers increases with age following an accumulation of mutation. CR reduces spontaneous tumor progression in which a tumor suppressor is activated or an oncogene is repressed [12]. Many of the activities of EGCG are similar to the beneficial effects offered by CR, including slowed aging and delaying the onset of chronic disease [13, 14].

One key enzyme thought to be activated during CR is AMP-activated protein kinase (AMPK). The AMPK system plays a role as an energy sensor in eukaryotic cells [15–17], AMPK controls cell switches of ATP-consuming processes such as glycogen synthesis, protein synthesis, sterol synthesis, and up-regulates processes that increase ATP such as glycolysis, mitochondrial biogenesis, and glucose uptake [16]. The AMPK pathway is linked to tumor growth and proliferation through the regulation of the mammalian target of rapamycin (mTOR) pathway. There is currently a high level of interest in signaling through the mTOR. Rapamycin is an anti-cancer drug. It inhibits the growth of a broad spectrum of cancers *via* mTOR, reduces the proliferation of certain tumor cells, and can cooperate with other agents to induce apoptosis. The best-understood roles of mTOR in mammalian cells are related to the control of mRNA translation by the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) [18–20]. In the hypophosphorylation form, 4E-BP1 by mTOR ultimately results in the initiation of translation of certain mRNAs, including those that are needed for cell cycle progression and are involved in cell cycle regulation [21].

Fatty acids (FAs) are important for functions of cell including energy storage, signal transduction cascades, and membrane structure [22]. Past studies showed that FA synthesis is significantly elevated in a variety of cancer cells [23]. FA synthase (FASN) is a key enzyme for lipogenesis. FASN provides the best opportunity for therapeutic applications because of its tissue distribution and unusual enzymatic activity. FASN is down-regulated in most normal human tissues because of the fat in our diet, with the exception of lactating breast and cycling endometrium. In contrast, FASN is often highly expressed in human cancers, including breast, colorectum, prostate, bladder, ovary, oesophagus, stomach, lung, oral tongue, oral cavity, head and neck, thyroid and endometrium, and also in mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas, Paget's disease of the vulva, cutaneous melanocytic neoplasms including melanoma, and HCC [23]. This differential tissue distribution makes FASN an attractive target for cancer cells. Moreover, acetyl CoA carboxylase (ACC), the rate-limiting enzyme for the long-chain FA synthesis that catalyses the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, has been shown to be overexpressed both at the mRNA and protein levels, not only in advanced breast carcinomas but also in preneoplastic lesions associated with increased risk for the development of infiltrating breast cancer [24]. Knockdown of ACC by RNAi impairs tumor cell proliferation and evokes a decrease in cell viability [25, 26].

The mechanism by which EGCG inhibits p53 positive and negative cell proliferation is not clearly understood. The present studies have shown that treatment of EGCG increased AMPK signaling is associated with cancer, suggesting that an attenuation of AMPK signaling might be beneficial for treating cancer. The underlying mechanistic aspects have not been explored.

2 Materials and methods

2.1 Materials

EGCG, C75, compound c, oil red o staining, MTT, PI, and antibodies for β-Actin were purchased from Sigma (St. Louis, MO). Antibodies for FASN, phospho-ACC (Ser 79), phospho-mTOR (Ser 2448), pohspho-4E-BP1 (Thr 37/46), phosphor-AMPK (Thr 172), AMPK, PARP, p21, and p53 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for mouse and rabbit conjugated with horseradish peroxidase were purchased from Chemicon (Temecula, CA). Lipofectamin 2000 Reagent was purchased from Invitrogen (Carlsbad, CA). Western chemiluminescent HRP substrate was from Millipore Corporation (Billerica, MA). The other chemicals and reagents were from Sigma (St. Louis, MO).

2.2 Cell culture

Human hepatoblastoma cells, Hep G2 (p53 wild type) and Hep 3B (p53 deletions) cell lines, were grown in DMEM (Invitrogen Carlsbad, CA) containing 10% fetal bovine

serum (FBS) (Invitrogen Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO_2.

2.3 Short hairpin RNA

RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/ Genomic Research Center, Academia Sinica, supported by the National Research Program for Genomic Medicine Grants of NSC (NSC 97-3112-B-001-016). Short hairpin RNAs (shRNAs) were designed to target specific sequences of human AMPK (Clone ID: TRCN0000010436; Target sequence: 5′-GCATAATAAGTC ACAGCCAAA3′). One day before transfection, cells were seeded without antibiotics with the density of 30–40%. 20 nM AMPK shRNAs were transfected into cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated for an additional 24 h, and EGCG was added as previously described. The cells been lysed were analyzed by Western blot as previously described.

2.4 MTT assay

Cells (1×10^4) were seeded on the 24-well cell culture cluster overnight and then treated with different concentrations of EGCG and incubated for 24 h. Next, 40 μL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT (stock con. 2 mg/mL, Sigma Chemical Corporation) was added to each well and every volume of wells was 500 μL, then incubated for 2 h at 37°C. The MTT-formazan crystals will be formed, and 250 μL of DMSO was then added to dissolve the crystals. Finally, the absorbance at OD 550 nm was detected by the ELISA) reader.

2.5 Western blot

Cells (1×10^6) were washed twice with PBS and then the gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100mM β-glycerophosphate, 20 mM Tris-HCl, 137 mM NaCl, 5 mM EDTA, 0.1% SDS and 10 μg/mL aprotinin; adjusted pH to 7.9) was added to lyse the cells. After the cells lysed, the suspension solution were centrifuged, and the Bio-Rad protein assay kit (Bio-Rad Laboratories) was used to determine the protein contents. 50 μg proteins was taken to be resolved with SDS-PAGE, and transferred to PVDF membrane (polyvinylidene Fluoride Transfer Membrane) (BioTrace, UK). The membrane was blocked by blocking buffer (non-fat milk (5%), NaN_3 (0.2%), and Tween 20 (0.2% v/v) in TBS). Then the PVDF membrane was incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 dilution, Roche Applied Science, Indianapolis, IN). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with a Phosphor-Image system.

2.6 Oil red o staining

Cells (2×10^5) were seeded on the 6-well cell culture cluster overnight. After being treated with EGCG (80 μM), C75 (20 μM), and compound c (15 μM), then incubated for 24 h. Cells were washed with PBS twice and fixed with 10% formaldehyde for 1 h, then they were washed with PBS and 50% isopropanol. Next, the oil red o staining working buffer (stock solution: 3 mg/ml in isopropanol, working solution: 60% Oil Red O stock solution) was added and kept for 1 h, then washed with PBS and 70% ethanol. Finally, 250 μL of isopropanol was added to dissolve the oil red o and the absorbance at OD 510 nm was detected by the ELISA reader.

2.7 Cell cycle analysis

Cells (5×10^5) were cultured in 6 mm cell culture dish and incubated for 24 h. Then cells were harvested in 15 mL tubes, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100% ethanol at –20°C overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at RT for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

2.8 Statistical analysis

All values were expressed as mean ± S.E. Each value is the mean of at least three separate experiments in each groups, Student's *t*-test was used for statistical comparison. * indicates that the values are significantly different from that of the control. (*, $p < 0.05$;**, $p < 0.01$;***, $p < 0.001$)

3 Results

3.1 EGCG up-regulates AMPK activity in p53 positive and negative hepatoma cells

To investigate the bioactivity of EGCG in p53 positive and negative hepatoma cells, we treated Hep G2 cells (p53 positive) and Hep 3B cells (p53 negative) with different concentrations of EGCG at 37°C for 24 h, and assessed cell proliferation by MTT assay. As shown in Fig. 1A, Hep G2 and Hep 3B cells were also inhibited by EGCG in a dose-dependent manner. As the metabolic actions of EGCG require AMPK [27, 28], we hypothesized that the antiproli-

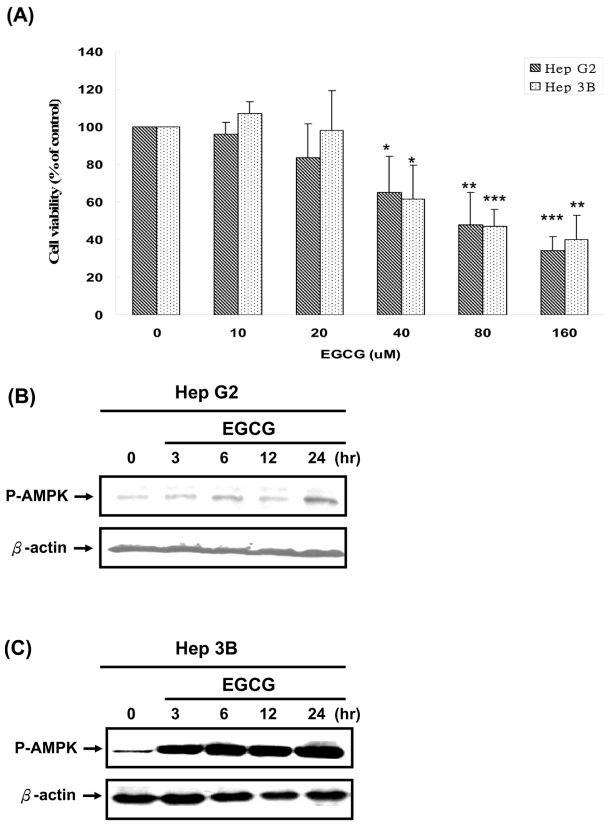

Figure 1. EGCG activates AMPK and a general decrease in cell proliferation in both p53 positive and negative hepatoma cells: (A) Hep G2 and Hep 3B cells were seeded into 24-well plates in the presence of 10% FBS and after 24 h treated with various concentrations of EGCG at 37°C for 24 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without EGCG as 100%. This experiment was repeated three times. Bars represent the S.E, (B) Hep G2, and (C) Hep 3B cells were treated with 80 μM of EGCG for indicated duration, and extracts were analyzed for levels of phosphorylated AMPK (Thr 172) and β-actin by Western blotting. Western blot data presented are representative of those obtained in at least three separate experiments.

ferative effects of EGCG involve the same pathway in p53 positive and negative hepatoma cells. Hep G2 and Hep 3B cells were treated with 80 μM EGCG at 37°C for different durations. Western blot analysis indicated that EGCG stimulates AMPK phosphorylation in a time-dependent manner (Fig. 1B and C). Those results showed that EGCG up-regulated AMPK activity and suppressed cell proliferation in p53 positive and negative hepatoma cells.

3.2 EGCG suppress the protein synthesis by activating AMPK to inbibit mTOR pathway

mTOR/4E-BP1 pathway controls the protein translation/synthesis in various types of cells. 4E-BP1 is phosphorylated by mTOR upon growth factor stimulation, and then

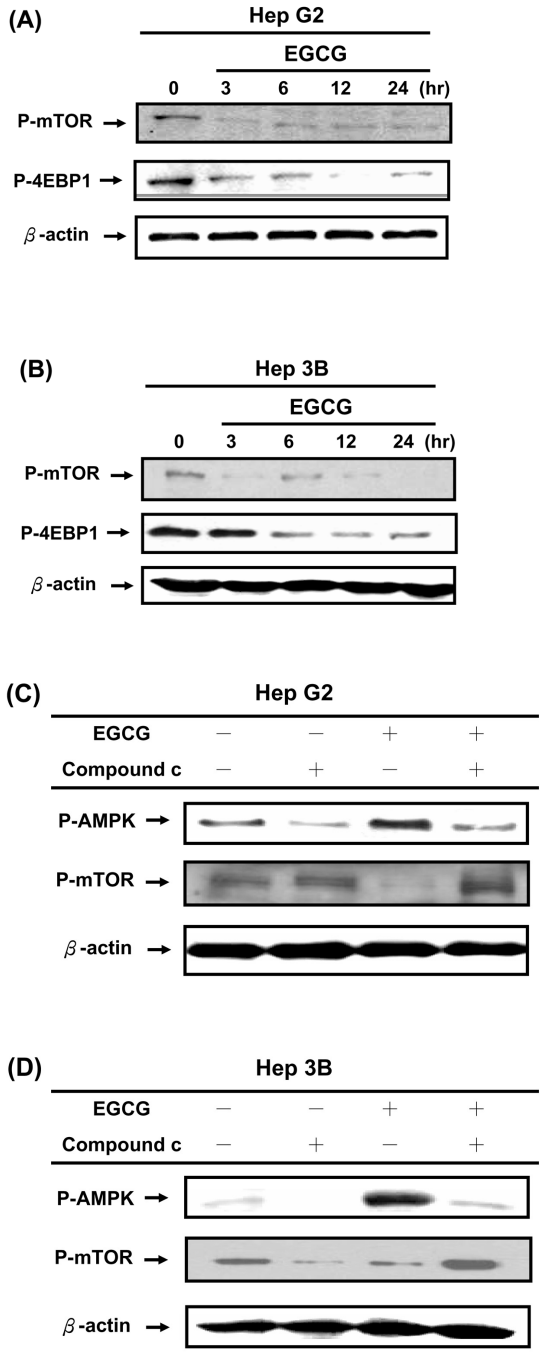

Figure 2. EGCG decreases the protein synthesis by inhibiting mTOR pathway: (A) Hep G2 and (B) Hep 3B cells were treated with 80 μM EGCG for the indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-mTOR (Ser2448), phospho-4E-BP1 (Thr37/46), and β-actin. (C) Hep G2 and (D) Hep 3B were incubated with 15 μM of compound c in the absence and in the presence of 80 μM EGCG at 37°C for 24 h. Phospho-mTOR (Ser2448) and phospho-AMPK (Thr172) protein levels were detected by Western blot. Western blot data presented are representative of those obtained in at least three separate experiments.

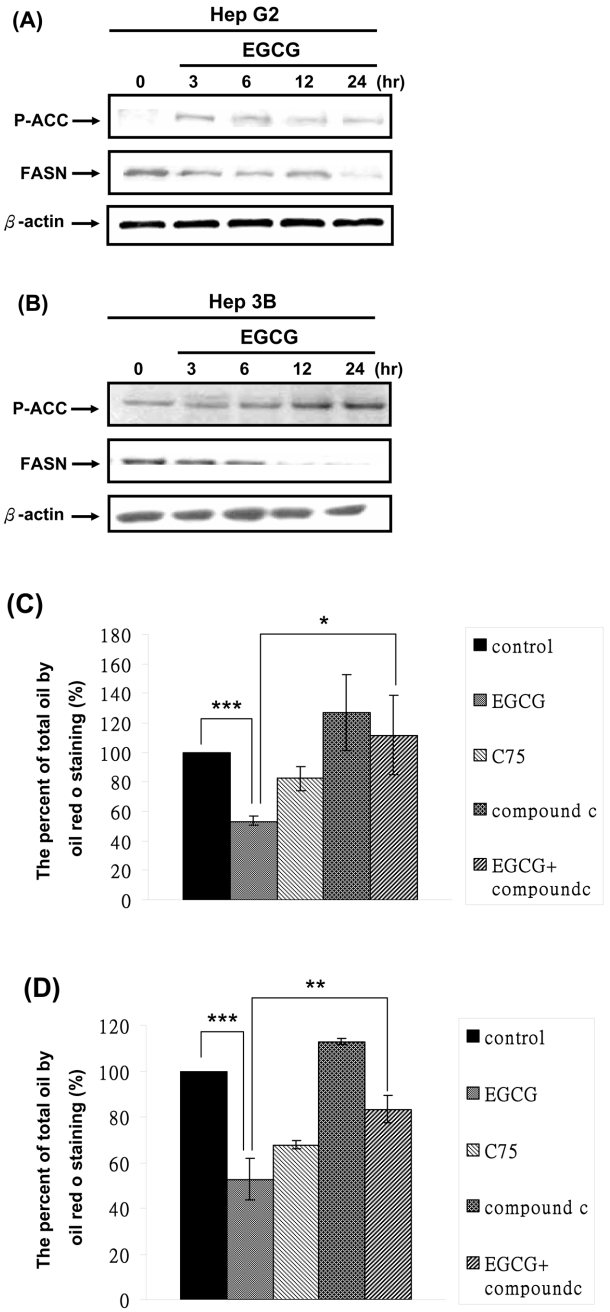

Figure 3. EGCG decreases the activity of FA synthesis by inhibiting the expression of FASN and the activity of ACC. (A) Hep G2 and (B) Hep 3B cells were incubated with 80 μM EGCG for the indicated duration. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against FASN, pospho-ACC (Ser79), and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. To measure the total of lipids, Hep G2 (C) and Hep 3B (D) cells were treated with 80 μM EGCG, 20 μM C75, and 15 μM compound c. The total lipids were measured by oil red o staining, and the percentage of total lipids was calculated by defining the absorption of cells without EGCG as 100%. This experiment was repeated three times. Bars represent the S.E.

the cells undergo cell cycle progression and proliferation [21]. In our study, the hepatoma cells were treated with 80 μM EGCG at 37°C for different durations. phospho-mTOR and 4E-BP1 were detected by Western blotting. The phosphorylation of mTOR and 4E-BP1 were decreased after EGCG treatment for 3 h in Hep G2 (Fig. 2A) and Hep 3B cells (Fig. 2B). To determine EGCG suppressed protein synthesis by activating AMPK to inhibit mTOR pathway, we added the compound c, an AMPK inhibitor, in the absence and in the presence of EGCG. The AMPK activity was suppressed by compound c in the presence of EGCG, and the mTOR activity was recovered in Hep G2 (Fig. 2C) and Hep 3B cells (Fig. 2D). We hypothesized that EGCG, by up-regulating AMPK activity, would inhibit mTOR activation and downstream events in p53 positive and negative hepatoma cells.

3.3 EGCG decreases lipid synthesis by decreasing FASN expression and inhibiting ACC activity

The activity of FASN and ACC are known to be negatively regulated by AMPK [29]. In the present study, the FASN protein level was decreased and ACC was phosphorylated in a time dependent fashion when Hep G2 (Fig. 3A) and Hep 3B (Fig. 3B) cells were treated with 80 μM EGCG. To examine whether EGCG influences total FA content in cells, we treated Hep G2 or Hep 3B cells with 80 μM EGCG in the absence and in the presence of 15 μM of compound c and 20 μM of C75, a FASN inhibitor, and examined the total lipids by oil red o staining. Results show that the lipid contents were decreased in Hep G2 (Fig. 3C) and Hep 3B (Fig. 3D) cells treated with EGCG, but increased in cells treated with EGCG and compound c, showing that the lipid synthesis is restored when compound c is added. These results may suggest that EGCG decreases lipid synthesis in p53 positive and negative hepatoma cells.

3.4 EGCG suppress lipid synthesis by activating AMPK

To further study the effect of AMPK in regulating the activity of FA synthesis enzymes, we added the compound c to the cell culture in the absence and in the presence of EGCG. After the treatment of EGCG, the protein levels of FASN were decreased and phospho-ACC was increased. However, the activities of enzymes of FA synthesis were restored in the presence of compound c in Hep G2 (Fig. 4A) and Hep 3B (Fig. 4B) cells. Besides, we also treated AMPK shRNA to silence the AMPK. The AMPK shRNA rescued cells from the inhibitory effect of EGCG. The activities of FA synthesis enzymes were not inhibited by EGCG when transfected with AMPK shRNA in Hep G2 (Fig. 4C) and Hep 3B (Fig. 4D) cells. These results demonstrate that EGCG inhibits the activity of FA synthesis enzymes and decreases total lipid content through the activation of the AMPK pathway.

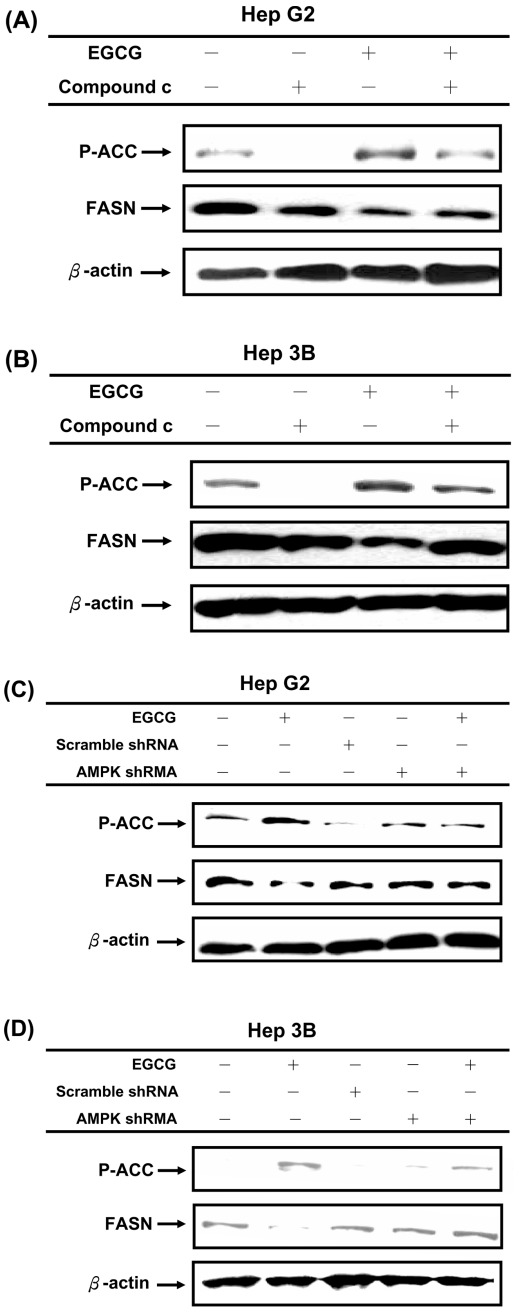

Figure 4. EGCG decreases the activity of FA synthesis *via* activation of AMPK. (A) Hep G2 and (B) Hep 3B cells were stimulated for 24 h with 80 μM EGCG only, or 15 μM compound c in the absence and in the presence of EGCG. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-ACC (Ser79), FASN, and β-actin. (C) Hep G2 and (D) Hep 3B were transfected with 50nmol/L AMPKα1-shRNA or scramble shRNA using lipofectamine 2000. 24 h after transfection, cells were treated with EGCG for another 24 h. After harvesting, cells were lysed and prepared for Western blotting analysis using antibodies against phospho-ACC (Ser79), FASN, and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments.

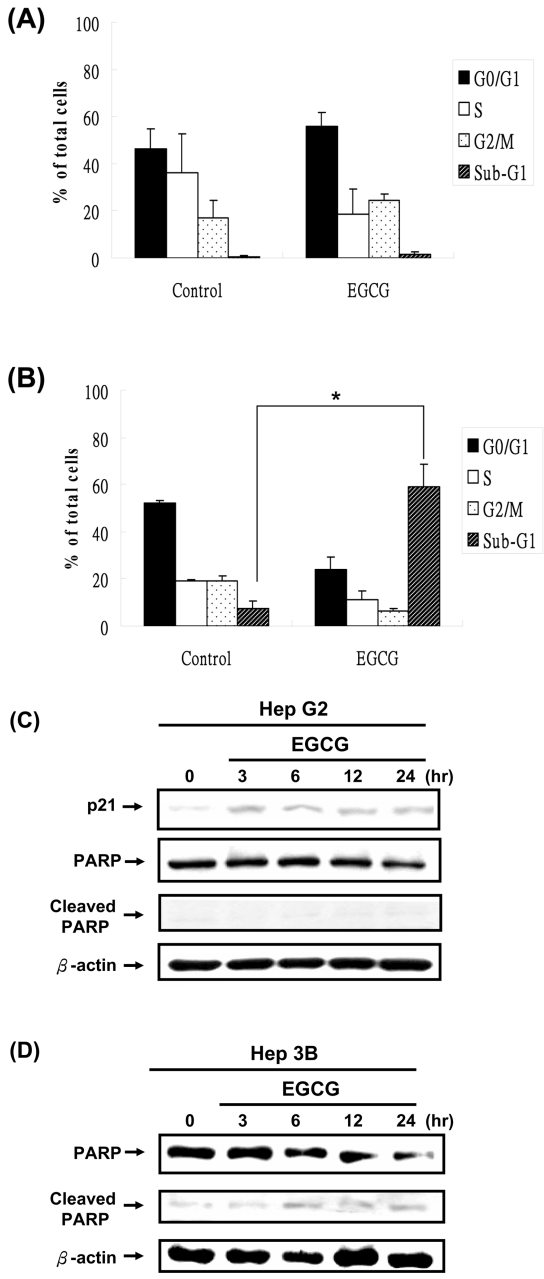

Figure 5. EGCG induces cell cycle arrest in p53 positive cells, but apoptosis in p53 negative cells. (A) Hep G2 and (B) Hep 3B cells were treated with 80 μM of EGCG for 24 h and analyzed for propidium iodide-stained DNA content by flow cytometry. The indicated percentages are the mean of three independent experiments, each in duplicate. Bars represent the S.E. (C) Hep G2 cells were treated with vehicle (DMSO), EGCG (80 μM) for the indicated time. Cells were then harvested and lysed for the detection of p21, PARP, cleaved PARP, and β-actin protein expression. (D) Hep 3B cells were treated with vehicle (DMSO), EGCG (80 μM) for the indicated time. Cells were then harvested and lysed for the detection of PARP, cleaved PARP, and β-actin protein expression. Western blot data presented are representatives of those obtained in at least three separate experiments.

3.5 EGCG induces Hep G2 growth arrested, but induces Hep 3B apoptosis

The decision between apoptosis and growth arrest following FASN inhibition is greatly influenced by p53 status [29, 30]. To examine whether EGCG produces both cytostatic and cytotoxic effects modulated by p53, we used propidium-iodide stain to measure in flow cytometry. Cell cycle of Hep G2 (Fig. 5A) was arrested in G_1 phase. In contrast, Hep 3B cells underwent apoptosis. Moreover, we also examined the expression of G1 related cell cycle control proteins and apoptosis related proteins using Western blot analysis. Hep G2 and Hep 3B cells were treated with 80 μM of EGCG with indicated durations (hours) and used 50 μg of whole-cell extracts for Western blot analyses. After 3 h of EGCG treatment, we found an increased level of p21 in Hep G2 cells (Fig. 5C). However, using 80 μM of EGCG showed a clear apoptosis in Hep 3B cells within 24 h, showing cleavages for PARP in Western blot analyses (Fig. 5D). EGCG, a FASN inhibitor, was more effective at initiating apoptosis in tumor cells with non-functioning p53, whereas cells with intact p53 function tend to exhibit cytostatic responses.

4 Discussion

Most studies of the effects of EGCG on cell signaling networks have been carried out in the context of cancer research. Having noticed that EGCG mimic CR induces metabolic changes in response to AMPK activation [27, 28]. Here, we focus on the effect of AMPK activation on protein synthesis, lipogenesis, cell cycle progression, and discuss the possibility that AMPK might be a therapeutic target for cancer treatment.

There is currently a high level of interest in signaling through mTOR. The role of mTOR in tumor acts as a sensor for energy, growth factors, and nutrients, all of which are required for protein translation. Activation of AMPK results in decreased mTOR signaling. AMPK signal system contains some tumor suppressing genes including LKB1, TSC1, TSC2, and p53 and suppresses tumor growth by inhibiting the activity of various proto-oncogenes such as PI3K, Akt, and ERK [31]. Both TSC1 (also named hamartin) and TSC2 (also named tuberin), tumor suppressing proteins, control the protein synthesis of cells. Activation of AMPK induces activation of the TSC2–TSC1 complex to inhibit mTOR [32]. Eukaryotic translation initiation factor 4E-BP1 is the downstream effector of mTOR. Through this effector mTOR controls the protein translation [33, 34]. Data presented here show that the inhibition of protein translation *via* AMPK–mTOR pathway by EGCG also works whether cancer cells are p53 positive or negative.

AMPK is a key cellular fuel gauge that is exquisitely sensitive to change in the levels of the low-energy indicator AMP [35–37]. This master regulator of metabolism exerts its functions, at least in part, by specifically regulating both the phosphorylation and dephosphorylation cycles of ACC and the expression levels of FASN. Acutely activated AMPK phosphorylates and inhibits ACC. Chronically activated AMPK decreases the expression of SREBP1c, thus suppressing the synthesis of ACC, FASN, and other lipogenic enzymes [38–40]. Recently, Swinnen *et al.* [41] provided evidence for an AMPK-regulated link between energy status, tumor-associated lipogenic metabolism, and the malignant phenotype. By pharmacologically inducing a "low-energy status" in tumor cells, which results in AMPK-induced ACC phosphorylation, FASN downregulation, and a marked decrease in endogenous lipogenesis, cancer cells stopped proliferating and lost their invasive and tumorigenic properties *in vitro* and *in vivo*. Based on more observations we have found that EGCG has multiple beneficial activities similar to those associated with low-energy mimickers, and these might be related to uncharacterized direct actions of EGCG on cancer cells. In this study, we show that AMPK is activated by EGCG, and is required for EGCG suppression of hepatic lipogenesis. From a clinical perspective, these findings justify further work exploring the ability of "low-energy mimickers" to therapeutically manage lipogenic carcinomas.

In cell cycle, AMPK also plays a role as an energy sensor [42, 43]. It seems rational to view AMPK as a survival factor for cancer cells, on the basis of our knowledge of the probable role of AMPK in the augmentation of energy production *via* the activation of glucose uptake, glycolysis, and FA oxidation in response to ATP-depleting stresses [44]. Solid tumors that outgrow the existing vasculature are continuously exposed to a microenvironment in which the supply of both oxygen and nutrition are quite limited. Recent studies show that AMPK is critical for cancer cell adaptation in response to hypoxia or glucose deprivation [45–47]. Moreover, the potential of AMPK as a survival factor has also been well documented in other tissues; AMPK has been clearly shown to protect the heart during ischemia [48]. In accordance with the aforementioned reports and the data documented herein, it seems reasonable to conclude that the inhibition of AMPK in cancer cells may prove useful as an approach for the increased induction of apoptosis in tumor cells after EGCG treatment. However, some have concluded that AMPK activation may be employed as a component of an anti-cancer therapy [49, 50]. The logic of this approach is predicated on recent observations that AMPK also strongly suppresses cell proliferation. This effect is mediated, in part, by several tumor suppressor proteins associated with the AMPK signaling network, including LKB1 and the tuberous sclerosis complex (TSC2) [51]. Moreover, Jones *et al.* [52] recently reported that the activation of AMPK induces p53-Ser15 phosphorylation in response to glucose deprivation, resulting in replicative senescence. The ability of AMPK to promote senescence or to inhibit cell proliferation in response to energy starvation

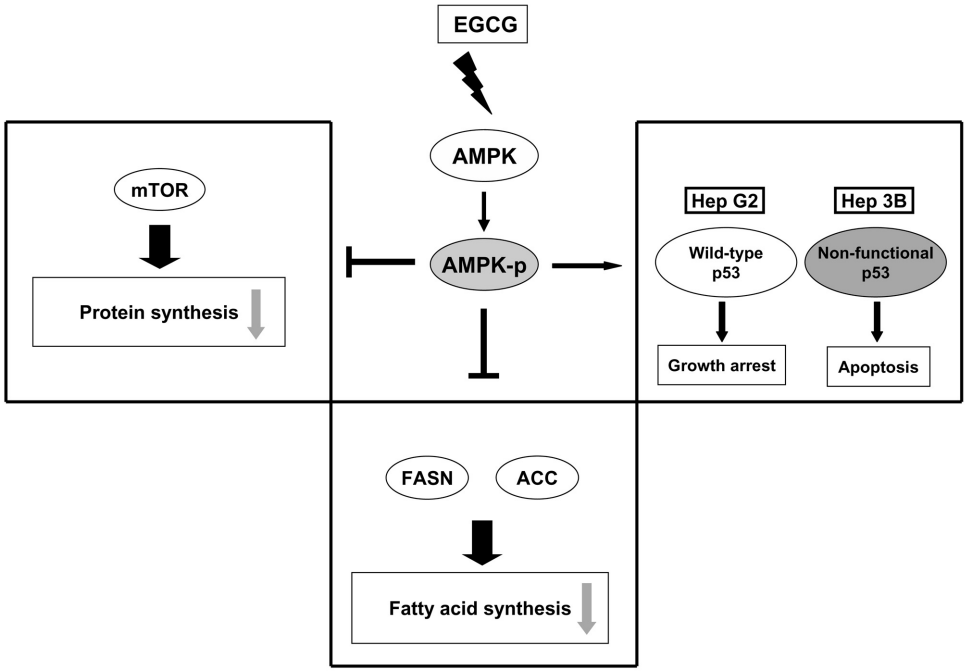

Figure 6. A schematic summary for the anti-hepatoma cell mechanisms of EGCG shown in the present study. EGCG activates AMPK in both p53 positive and negative human hepatoma cells. When this occurs, a key enzyme involved in protein synthesis, mTOR is inhibited. The activity and/or expression of lipogenic enzymes, such as FASN and ACC are also decreased. Moreover, the decision between apoptosis and growth arrest following AMPK activation is greatly influenced by p53 status.

has been interpreted as a check point that couples glucose availability to the progression of the cell cycle; it has been implied that the activation of AMPK might promote the conservation of the remaining energy to support the survival and physiological functions of the cell during cell cycle arrest. The decision between apoptosis and growth arrest after AMPK activation is clearly influenced by p53 function. Our results indicated that EGCG inhibited the proliferation of Hep G2 and Hep 3B cells *via* the activation of AMPK. Hep G2 cells have a normal functional p53, whereas Hep 3B cells have p53 mutation. In the Hep G2 cells, EGCG treatment inhibited the progression of cell cycle in the G1 phase. EGCG increased the expression level of p53 and subsequently enhanced the expression level of p21 resulting in cell cycle arrest in Hep G2 cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after AMPK activation. In contrast, EGCG was able to induce the apoptotic cell death, but it did not inhibit cell cycle progression in Hep 3B cells. The expression level of p21 was not affected in EGCG-treated Hep 3B cells.

In conclusion, AMPK is activated in both p53 positive and negative hepatoma cells. When this occurs, a key enzyme involved in protein synthesis, mTOR is inhibited. In addition, the activity and/or expression of lipogenic enzymes, such as FASN and ACC are also decreased. Interestingly, the decision between apoptosis and growth arrest following AMPK activation is greatly influenced by p53 status. EGCG blocked the progression of cell cycle at G1 phase in p53 positive Hep G2 cells. In contrast, EGCG inducted apoptosis in p53 negative Hep 3B cells (Fig. 6). Taken together, our study suggests that EGCG may be useful as a liver cancer chemopreventive and therapeutic agent. Nevertheless, additional studies are required to evaluate the efficacy of EGCG in suitable experimental animal systems.

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The authors have declared no conflict of interest.

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