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The phyto-chemical (−)-epigallocatechin gallate suppresses gene expression of epidermal growth factor receptor in rat hepatic stellate cells *in vitro* by reducing the activity of Egr-1

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Abbreviations:
DMEM, Dulbecco's modified Eagle's medium
EBS, Egr-1 binding sites
ECM, extracellular matrix
EGCG, (−)-epigallocatechin gallate
EGFR, epidermal growth factor receptor
EMSA, electrophoretic mobility shift assays
ERK, extracellular signal-regulated kinase
FBS, fetal bovine serum
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
HSC, hepatic stellate cells

ABSTRACT

Hepatic stellate cells (HSC) are the major effectors in hepatic fibrogenesis. During liver injury, HSC become activated and proliferative. Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) are the potent mitogens for many cell types. We previously demonstrated that (−)-epigallocatechin gallate (EGCG), the major and active component in green tea extracts, inhibited HSC growth, including reducing cell proliferation, and inducing apoptosis. We have reported that EGCG interrupts PDGF signaling by reducing receptor tyrosine phosphorylation and gene expression of PDGF-β receptor. Additional experiments are necessary to elucidate the effect of EGCG on EGF signaling in activated HSC. The aims of this study are to evaluate the effect of EGCG on the expression of EGFR and to elucidate the underlying molecular mechanisms in activated HSC. We hypothesize that EGCG might interrupt EGF signaling by suppressing gene expression of EGF receptor (EGFR) in activated HSC, which, together with the interruption of PDGF signaling, might collectively result in the inhibition of HSC growth. The present report demonstrates that the phyto-chemical dose-dependently suppresses gene expression of EGFR in activated HSC *in vitro*. The Egr-1 binding site located in the *egfr* promoter is found to be cis-activating element in regulating the promoter activity of the gene. EGCG inhibits the *trans*-activation activity of Egr-1 in activated HSC by suppressing gene expression of the transcription factor. The interruption of the ERK signaling pathway by EGCG reduces the *trans*-activation activity of Egr-1 and the promoter activity of EGFR gene in HSC. Taken together, our results demonstrate that EGCG suppresses gene expression of EGFR in rat activated HSC *in vitro* mediated by reducing the *trans*-activation activity of Egr-1.

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LDH, lactate dehydrogenase
MAPK, the mitogen-activated protein kinases
PDGF, platelet-derived growth factor

1. Introduction

Hepatic fibrosis is a wound healing response to chronic insults in the liver with excessive production of extracellular matrix (ECM). Hepatic stellate cells (HSC), a Vitamin A-storage cell type located in the subendothelial space of Disse, play a central role in hepatic fibrogenesis [1]. Upon liver injury, normal and quiescent HSC become activated and undergo profound phenotypic changes, including enhanced cell proliferation, *de novo* expression of α-smooth muscle actin (α-SMA) and excessive production of ECM. *This process is called HSC activation*, which is mainly triggered by growth factors and cytokines. In addition to platelet-derived growth factor (PDGF), a major mitogen for HSC proliferation [2,3], epidermal growth factor (EGF) is another potent mitogen for many cell types [4]. High expression of EGF and its receptor is a characteristic of cirrhosis [5]. Although the correlation between EGF and the development of hepatic fibrosis was recognized decades ago [6], it remains largely unknown how EGFR gene expression is regulated in HSC during hepatic fibrogenesis.

EGF signaling starts from binding of EGF or TGFα to EGF receptor (EGFR/erbB1/HER1), which belongs to the receptor tyrosine kinase family. Upon binding to EGF, EGFR dimerizes, and auto-phosphorylates its tyrosine residues, which is followed by recruitment of adapter proteins and propagation of the signal into the nucleus. Several downstream signaling pathways are activated upon EGF stimulation, including the mitogen-activated protein Kinases (MAPK). MAPK are members of a three-kinase phospho-relay system. MAPK kinase kinases (MKKK) phosphorylate and activate MAPK kinases (MKK), which, in turn, phosphorylate and activate MAPK, including extracellular regulated kinases (ERK), leading to the activation of selected transcription factors and the ultimate regulation of expression of target genes [7]. Activation of the EGF signaling cascade results in diverse effects, including stimulation of cell proliferation and inhibition of apoptosis [4].

In the last decade, advances in the understanding of genes promoting HSC activation are impressive. There are, however, few breakthroughs in the therapeutic intervention of hepatic fibrogenesis. Therefore, research identifying anti-fibrotic agents that are innocuous is of high priority and urgently needed. Most evolving anti-fibrotic therapies will be aimed at inhibiting HSC activation. Substantial evidence has shown the role of green tea in liver protection [8,9]. We previously demonstrated that (−)-epigallocatechin gallate (EGCG), the major active component in green tea extracts, inhibited cell growth of activated HSC *in vitro*, including reducing cell proliferation and inducing apoptosis [10,11]. The underlying mechanisms remain largely to be defined. We assumed that the inhibition of HSC growth by EGCG might result, at least partially, from the interruption of PDGF signaling and EGF signaling. We recently reported that EGCG interrupted PDGF signaling by blocking receptor tyrosine phosphorylation and reducing gene expression of PDGF-βR [11].

The aims of this study are to evaluate the effect of EGCG on regulating gene expression of EGFR and to elucidate the underlying molecular mechanisms in activated HSC. We hypothesize that EGCG might interrupt EGF signaling by suppressing gene expression of EGF receptor (EGFR) in activated HSC, which, together with the interruption of PDGF signaling, might collectively result in the inhibition of HSC growth. Results from the current report support our hypothesis. The underlying molecular mechanisms are further investigated.

2. Methods and materials

2.1. Hepatic stellate cells isolation and culture

Isolation of rat HSC was previously described [10]. Briefly, the liver of male Sprague–Dawley rats (200–250 g) was perfused *in situ* with collagenase, pronase and DNAase. HSC were isolated using density gradient centrifugation with OptiPrep (Oslo, Norway) and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with fetal bovine serum (FBS) (10%). HSC with four to eight passages were used for experiments. EGCG (purity > 95%) was purchased from Sigma (St. Louis, MO). EGCG toxicity to HSC was carefully studied by examining lactate dehydrogenase (LDH) release [10]. It was shown that EGCG up to 100 μM was not toxic to cultured HSC. EGCG at 50 μM was chosen for most of experiments in this study. In some experiments as indicated, HSC were starved in serum-free DMEM for 48 h prior to the stimulation with FBS (10%) in the presence or absence of EGCG at the indicated concentrations. Serum starvation makes cells more sensitive to serum stimulation [12,13].

2.2. Immuno-cytochemical analyses

Serum-starved HSC in six-well plates were stimulated with FBS (10%) in the presence or absence of EGCG (50 μM) for 24 h. Cells were fixed with methanol (100%). After rinsing and blocking with PBS/BSA (1%), cells were labeled with anti-EGFR serum (1:100, Santa Cruz). As a negative control, cells were labeled with normal (non-immune) rabbit IgG. The cells were immuno-stained using biotinylated secondary antibodies and the ABC kit from Vector laboratories, Inc. (Burlingame, CA), as we previously described [14].

2.3. Western blotting analyses

Whole cell lysates were prepared using radio-immunoprecipitation analyses buffer (RIPA) supplemented with

protease inhibitors. Cell lysates were subjected to SDS-PAGE. Target proteins were detected by using antibodies against EGFR (1:500), or Egr-1 (1:1000), or p-ERK1/2 (1:500), or total ERK1/2 (1:3000) (Santa Cruz Biotechnology). Protein bands were visualized by using chemiluminescence reagent (Kirkegaard & Perry Laboratories). β-Actin was used as an internal control for equal loading in the Western blotting analyses detected by anti-β-actin antibodies (Sigma, 1:10,000). After normalization with the internal control β-actin, the level of target protein bands was densitometrically determined by using Quantity One® 4.4.1 (Bio-Rad). The variation in the density was expressed as fold changes compared to the control in the blot.

2.4. RNA isolation and real-time PCR

Total RNA was extracted using TRI-reagent (Sigma) according to the protocol provided by the manufacturer. Real-time PCR was carried out as we described previously [10]. The endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers were the followings: *EGFR*: (F), 5′-TGC ACC ATC GAC GTC TAC AT-3′, (R), 5′-AAC TTT GGG CGG CTA TCA G-3′; *Egr-1*: (F), 5′-GCC TGC GAC ATC TGT GGA A-3′, (R), 5′-GCC GCA AGT GGA TCT TGG TA-3′; *GAPDH*: (F), 5′-GGC AAA TTC AAC GGC ACA GT-3′, (R), 5′-AGA TGG TGA TGG GCT TCC C-3′. mRNA fold changes of target genes relative to the GAPDH control were calculated as suggested by Schmittgen et al. [15].

2.5. Plasmid constructs

The luciferase reporter constructs with various lengths of the *egfr* promoter were kindly provided by Dr. Alfred C. Johnson (National Cancer Institute, NIH), including pER1-Luc, pER8-Luc, pER9-Luc, and pER10-Luc [16]. The pER484-389-Luc and pER484-389mut-Luc were also kindly provided by Dr. Alfred C. Johnson, in which the *egfr* promoter region from −484 to −389 with or without site-directed mutation in the Egr-1 binding site was inserted into a luciferase reporter plasmid [16]. The cDNA expression plasmids pdn-ERK and pa-ERK, respectively, contain a fragment of cDNA encoding the dominant negative form of ERK, or the constitutively active MEK1, an immediate upstream kinase of ERK [17,18]. The cDNA expression plasmid pEgr-1cDNA, containing Egr-1 cDNA in a CMV-driven expression vector, was kindly provided by Dr. Alfred C. Johnson, as well. The minimal Egr-1-responsive luciferase reporter plasmid pEgr-1-Luc (i.e. pEBS1^4-Luc), containing four Egr-1 binding sites (EBS), was a gift from Dr. G. Thiel [19]. The cDNA expression plasmid pdn-Egr-1cDNA (i.e. pCMVFLAG-Egr-1DN) was generously provided by Dr. G. Thiel, as well [20]. This plasmid of dominant negative Egr-1 generates a *trans*-dominant negative mutant Egr-1, which competitively binds to Egr-1 binding sites, but lacks the *trans*-activation activity [20].

2.6. Transient transfection assays

Transient transfection assays were performed using LipofectAMINE® (Life Technologies) following the protocol provided by the manufacturer. In brief, semi-confluent HSC in six-well culture plates were transiently transfected with reporter plasmids (3–4 μg DNA/well). Transfection efficiency was controlled by co-transfection of the β-galactosidase reporter plasmid pSV-β (1 μg/well) (Promega). Luciferase activity was measured using an automated luminometer (Turner Designs, Sunnyvale, CA) according to the protocol provided by the manufacturer. β-Galactosidase assays were performed using an assay kit from Promega Corp. Each treatment had a triplicate in every experiment. Each experiment was repeated for at least three times. Luciferase activity was expressed as relative unit after normalization with β-galactosidase activity.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted as previously described [10]. The P(egfr) of 5′-GAC TAG GCC CGC GGG GGC CAC CGC TG-3′ was used in EMSA, which contained the Egr-1 binding site observed in the *egfr* promoter. The double-stranded oligonucleotide probe was prepared by mixing two single-strand of complementary oligonucleotides in the annealing buffer containing Tris–HCl (10 mM) (pH 7.5), EDTA (1mM) (pH 7.5), and NaCl (100 mM). The mixture was heated at 95 °C for 10 min and allowed to slowly cool to room temperature. The double-stranded probe was labeled with ^{32}P-γ-dATP using T_4 polynucleotide kinase. For EMSA, nuclear extracts were incubated with the probe in the binding buffer for 30 min at room temperature. Poly dI:dC was used to eliminate non-specific binding. For competition assays, nuclear extracts were incubated with 10× or 25× excess of the unlabeled probe for 10 min, followed by incubation with the ^{32}P-labeled probe for an additional 20 min at room temperature. For supershift assays, nuclear extracts were incubated with 2 μl of anti-Egr-1 antibodies (Santa Cruz Technology) for 30 min at 37 °C prior to the addition of the ^{32}P-labeled probe. Non-denatured polyacrylamide gel electrophoresis (6%) was performed to separate probe-protein complexes in 0.5× Tris–borate-EDTA (TBE) buffer with 150 V constant voltage for 3.5–4 h. After electrophoresis, the gel was transferred to a piece of filter paper and exposed to a Kodak X-ray film at −80 °C.

2.8. Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's *t*-test ($p < 0.05$ was considered significant). Where appropriate, comparisons of multiple treatment conditions with control were analyzed by ANOVA with the Dunnett's test for post hoc analysis.

3. Results

3.1. EGCG suppresses expression of EGFR gene and reduces its promoter activity in HSC

EGF is an important growth factor that promotes HSC proliferation [3,6]. High expression of EGF and its receptor is a characteristic of cirrhosis [5]. It was assumed that EGCG might have inhibitory effect on EGFR gene expression, collectively leading to the inhibition of cell growth of activated HSC. To test the assumption, the effect of EGCG on regulation of EGFR gene expression was evaluated. Serum-starved HSC

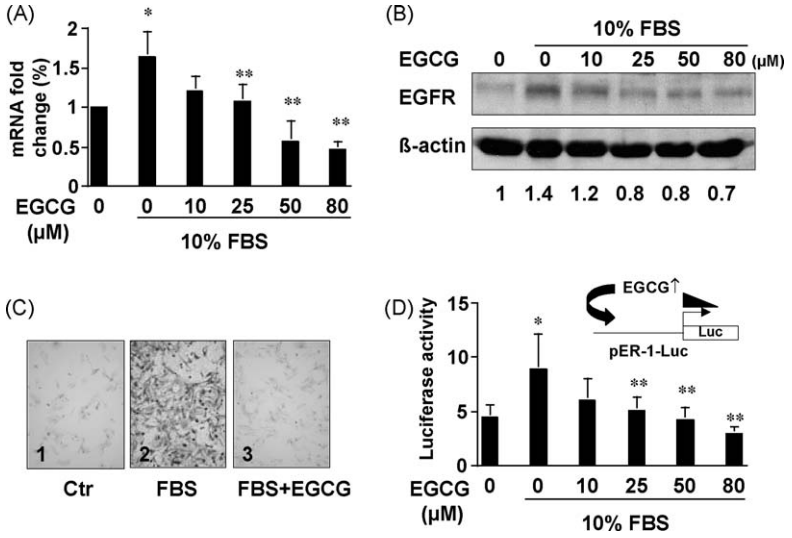

Fig. 1 – EGCG suppresses expression of EGFR in activated HSC in vitro. After serum-starvation for 48 h, passaged HSC were either maintained in serum-free DMEM, or stimulated with 10% of FBS in the absence or presence of various concentrations of EGCG as indicated for an additional 24 h. (A) Real-time PCR assays of EGFR mRNA. GAPDH was used as an invariant internal control for calculating mRNA fold changes ($n = 3$). $^{*}p < 0.05$, vs. cells maintained in serum-free medium; $^{**}p < 0.05$, vs. cells stimulated with 10% of FBS without EGCG; (B) Western blotting analyses of EGFR. β-actin was probed as an internal control for equal loading. After normalization with β-actin, the level of EGFR was densitometrically determined. The numbers beneath the blots were the fold changes in the density compared to the control without FBS nor EGCG ($n = 3$); (C) immuno-cytochemical analyses of the abundance of EGFR in cells maintained in serum-free medium (1), or stimulated with FBS (10%) in the absence (2) or presence (3) of EGCG (50 μM) for 24 h (100×); (D) transfection assays of cells with the *egfr* promoter luciferase reporter plasmid pER1-luc, treated as described above. Luciferase activities were normalized with β-galactosidase activities. Values were means ± S.D. ($n = 3$). $^{*}p < 0.05$, vs. cells maintained in serum-free medium; $^{**}p < 0.05$, vs. cells stimulated with 10% of FBS without EGCG.

were maintained in serum-free medium or stimulated with FBS (10%) with EGCG at the indicated concentrations for 24 h. As shown in Fig. 1A and B, FBS dramatically increased the level of EGFR in serum-starved HSC. EGCG caused a dose-dependent reduction in EGFR at the levels of transcription and translation demonstrated by real-time PCR and Western blotting analyses, respectively. Further immuno-cytochemical analyses confirmed the inhibitory effect of EGCG on the abundance of available EGFR in activated HSC *in vitro* (Fig. 1C).

To begin to elucidate the mechanisms of the inhibitory effect, HSC were transfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc, in which a fragment of 5′ flanking promoter region (1109 bp) of EGFR gene was inserted into the luciferase reporter plasmid pGL3. Transfection assays in Fig. 1D demonstrated that EGCG dose-dependently reduced luciferase activity in cells, suggesting that EGCG reduced the promoter activity of EGFR gene in activated HSC *in vitro*. Taken together, these results demonstrated that EGCG suppressed gene expression of EGFR in activated HSC *in vitro*.

3.2. *Localization of EGCG-response element(s) in the promoter of EGFR gene*

To explore the underlying molecular mechanisms by which EGCG reduced the promoter activity of EGFR gene in activated HSC, promoter deletion analyses were performed to locate cis-response elements in the *egfr* promoter in response to EGCG. HSC were transfected with a group of luciferase reporter constructs with different lengths of the *egfr* promoter (Fig. 2A). Cells were subsequently treated with or without EGCG at 50 μM for 24 h. As shown in Fig. 2A, EGCG significantly reduced luciferase activity in cells transfected with pER1-Luc and pER8-Luc by 54 and 60%, respectively. In great contrast, deletion of the DNA fragment of −481 to −384 in the plasmid pER9-Luc resulted in the failure in response to EGCG and the loss of the basal promoter activity, suggesting that the DNA fragment of −481 to −384 in the *egfr* promoter might contain EGCG response element(s). In addition, the DNA fragment might play a critical role in maintaining the basal promoter activity of the gene in activated HSC.

Computer-aided search revealed a consensus Egr-1 binding site (EBS) in the DNA fragment of −481 to −384. To evaluate the role of the Egr-1 binding site in the inhibition of the *egfr* promoter activity by EGCG, site-directed mutageneses of the EBS were generated in the plasmids pER1-Luc and pER484-389-Luc [16]. The latter was created by inserting the DNA fragment of −484 to −389 into the luciferase reporter vector pGL3. As shown in Fig. 2B, luciferase activities in cells transfected with pER1-Luc and pER484-389-Luc were reduced by 51 and 37%, respectively, in response to EGCG. However, site-directed mutageneses of the EBS in both plasmids resulted in the failure in the response to EGCG, indicating that the presence of the wild-type Egr-1 binding site was required for EGCG to reduce the *egfr* promoter activity. However, because of the existence of the basal promoter activity in cells transfected with the mutant in pER1(Egr-1mut)-Luc, the Egr-1 binding site

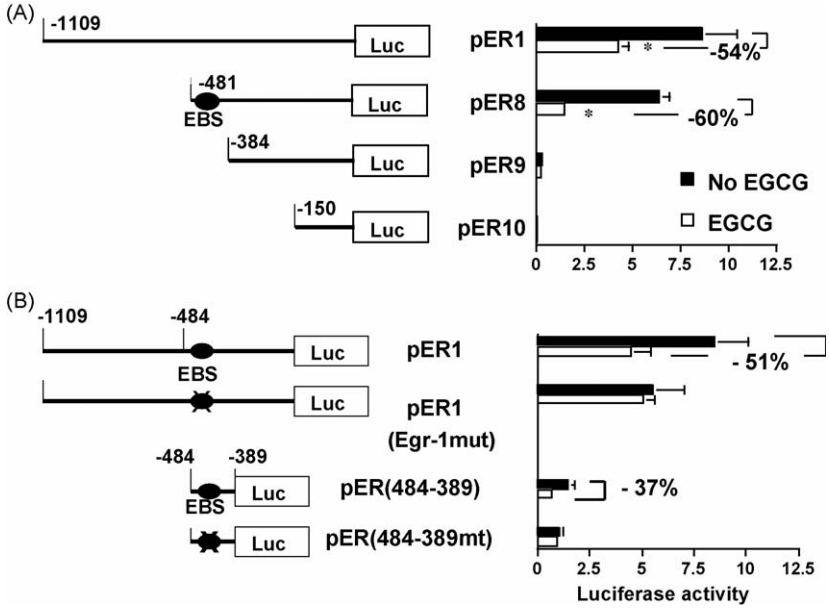

Fig. 2 – Localization of EGCG-response element(s) in the promoter of EGFR gene. Passaged HSC were transfected with a group of luciferase reporter plasmids containing different lengths of the *egfr* promoter as described in Section 2. The cells were subsequently treated with or without EGCG at 50 μM for 24 h. Luciferase activities were normalized with β-galactosidase activities. Values were expressed as means ± S.D. ($n = 3$). The numbers of percentage were the reduction in luciferase activity caused by EGCG. $^{*}p < 0.05$, vs. cells without EGCG. (A) Transfection assays of cells transfected with the reporter plasmids containing different sizes of the *egfr* promoter; (B) transfection assays of cells transfected with two pairs of plasmids, i.e. the parental plasmids and their mutants with site-directed mutations in the Egr-1 binding site.

was unlikely to be the key to maintain the basal promoter activity of the gene in activated HSC (Fig. 2B). Taken together, these results suggested that the Egr-1 binding site in the *egfr* proximal promoter might be the EGCG response element acting as a cis-activating element in regulating the promoter activity. In addition, these results implicated the involvement of the transcription factor Egr-1 in regulating the *egfr* promoter activity by EGCG.

3.3. EGCG reduces the DNA binding activity of the transcription factor Egr-1

To clarify the *trans*-activating factor that binds to the EBS, electrophoretic mobility shift assays (EMSA) were performed using the ^{32}P-labeled probe P(egfr) containing the EBS found in the *egfr* proximal promoter. Serum-starved HSC were stimulated with or without FBS (10%) in the presence or absence of EGCG (50 μM) for 24 h. Nuclear protein extracts were prepared for EMSA. As shown in Fig. 3, compared with the untreated control (lane 1), FBS dramatically stimulated the DNA binding activity of a factor to the probe (lane 2). The DNA binding activity of the factor was significantly suppressed by EGCG (lane 3). Competition assays with 10- or 25-fold excess of the unlabeled probe (Pegfr) demonstrated that the unlabeled probe dose-dependently reduced the level of the complex of protein-^{32}P-labeled probe, suggesting that this protein specifically bound to the probe P(egfr) (lanes 4–5). Supershift assays were performed to determine the property of the binding protein (lane 6). Addition of anti-Egr-1 antibodies resulted in the reduction in the level of the protein-probe complex and caused an apparent super-shifting band (lane 6), indicating that the factor that binds to the probe was the transcription factor Egr-1. Taken together, the gel shift assays demonstrated that Egr-1 was the factor binding to the EBS in the *egfr* promoter, and that its DNA binding activity was significantly reduced by EGCG in activated HSC.

3.4. EGCG significantly reduces the trans-activation activity of Egr-1 in HSC

To determine the effect of EGCG on the *trans*-activation activity of the transcription factor Egr-1, HSC were transfected with the Egr-1 luciferase reporter plasmid pEgr-1-Luc, in which a DNA fragment containing four Egr-1 binding sites was subcloned in the luciferase reporter plasmid pGL3 [19]. After serum starvation for 24 h, cells were stimulated with or without FBS (10%) in the absence or presence of EGCG at the indicated concentrations for an additional 24 h. As shown in Fig. 4A by luciferase assays, EGCG dose-dependently reduced luciferase activity in the cells, indicating that EGCG reduced the Egr-1 *trans*-activation activity in HSC.

To further confirm the inhibitory effect, pre-confluent HSC in six-well culture plates were co-transfected with a total of 4.5 μg of plasmid DNA per well, including 2 μg of the Egr-1 luciferase reporter plasmid pEgr-1-Luc, 0.5 μg of pSV-β-gal, pEgr-1cDNA at the indicated doses and the empty vector pcDNA. The amount of DNA of pEgr-1cDNA plus the empty vector was equal to 2 μg. The cDNA expression plasmid

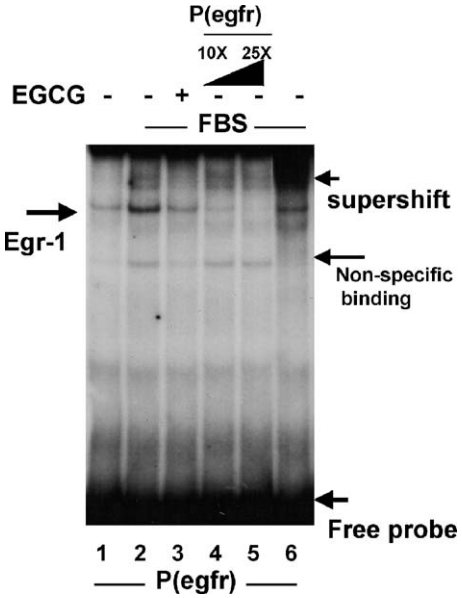

Fig. 3 – EGCG reduces the DNA binding activity of Egr-1 in HSC. Serum-starved HSC were stimulated with or without FBS (10%) in the presence or absence of EGCG (50 μM) for 24 h. Nuclear protein extracts were prepared for EMSA using the ^{32}P-labeled probe P(egfr) containing the Egr-1 binding site found in the promoter of EGFR gene. Competition assays were performed using 10- or 25-fold excess of the unlabeled probe (Pegfr). Anti-Egr-1 antibodies (2 μl) were used for supershift assays. A representative EMSA was shown from three independent experiments.

pEgr-1cDNA contains Egr-1 cDNA in a CMV-driven expression vector. The empty vector was used to ensure an equal amount of total DNA in each well. After transfection, cells were subsequently treated with or without EGCG at 50 μM for 24 h. As demonstrated in Fig. 4B, compared with the control (the first column on the left), forced expression of Egr-1 cDNA, which increased the abundance of cellular Egr-1, significantly increased luciferase activity (the second column on the left). EGCG reduced, as expected, luciferase activity in cells (the third column on the left). Further experiments indicated that forced expression of Egr-1 cDNA dose-dependently eliminated the inhibitory effect of EGCG on luciferase activity. Taken together, these results collectively demonstrated that EGCG significantly reduced the *trans*-activation activity of Egr-1 in activated HSC *in vitro*. The dose-dependent elimination of the inhibitory effect of EGCG by introducing exogenous Egr-1 implicated the effect of EGCG on reduction of the level of cellular Egr-1.

3.5. *EGCG reduces the level of Egr-1 in HSC by suppressing its gene expression*

To explore the mechanisms of EGCG in the inhibition of the *trans*-activation activity of Egr-1 in activated HSC, we hypothesized, based on the above observations, that EGCG might reduce the level of cellular Egr-1 by suppressing gene expression of Egr-1 in HSC. To test the hypothesis, serum-starved HSC were maintained in serum-free DMEM, or stimulated with 10% of FBS in the absence or presence of EGCG at the indicated concentrations for 24 h. Real-time PCR and Western blotting analyses demonstrated that EGCG dose-dependently reduced the abundance of Egr-1 at the levels of transcription and translation (Fig. 5A and B), suggesting that EGCG reduced the abundance of cellular Egr-1 in activated HSC by suppressing expression of the gene.

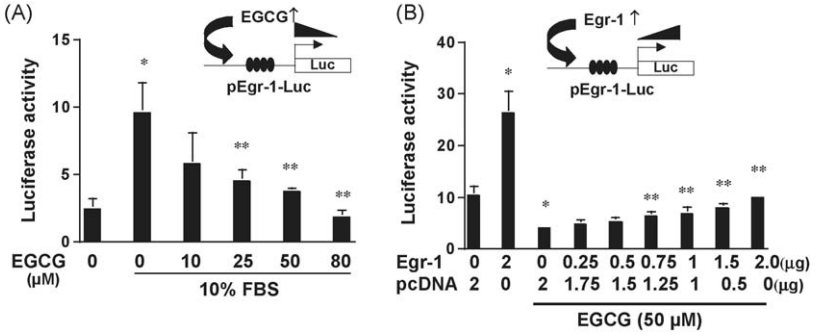

Fig. 4 – EGCG significantly reduces the *trans*-activation activity of Egr-1 in HSC. (A) HSC were transfected with the Egr-1 luciferase reporter plasmid pEgr-1-Luc, containing four Egr-1 binding sites. After serum starvation for 24 h, cells were stimulated with or without FBS (10%) in the absence or presence of EGCG at the indicated concentrations for 24 h. Luciferase activities were normalized with β-galactosidase activities. Values were expressed as means ± S.D. (n = 3). $^{*}p < 0.05$, vs. cells with the untreated control; $^{**}p < 0.05$, vs. cells stimulated with FBS, but no EGCG. (B) HSC in six-well culture plates were co-transfected with a total of 4.5 μg of plasmid DNA per well, including 2 μg of pEgr-1-Luc, 0.5 μg of pSV-β-gal, pEgr-1cDNA at the indicated concentrations and the empty vector pcDNA. The amount of DNA of pEgr-1cDNA plus the empty vector was equal to 2 μg. The empty vector was used to ensure an equal amount of total DNA in each well. After transfection, cells were subsequently treated with or without EGCG at 50 μM for 24 h. Luciferase activities were normalized with β-galactosidase activities. Values were expressed as means ± S.D. (n = 3). The co-delivery of pEgr-1-Luc and Egr-1 cDNA without EGCG treatment served as a positive control (the second column on the left). $^{*}p < 0.05$, vs. cells co-transfected with no pEgr-1cDNA, and no EGCG treatment (the first column on the left); $^{**}p < 0.05$, vs. cells co-transfected with no pEgr-1cDNA, but treated with EGCG (the third column on the left).

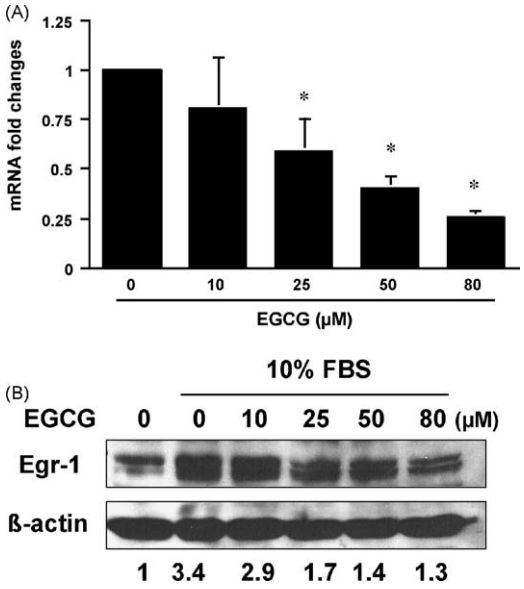

Fig. 5 – EGCG suppresses gene expression of Egr-1 in HSC. Serum-starved HSC were stimulated with or without FBS (10%) in the presence or absence of EGCG (50 μM) for 24 h. (A) Real-time PCR analyses of Egr-1 mRNA. GAPDH was used as an invariant control for calculating mRNA fold changes ($n = 3$). $^{*}p < 0.05$, vs. cells without EGCG; (B) Western blotting analyses of Egr-1. β-Actin was probed as an internal control for equal loading. A representative was shown from three independent experiments. After normalization with β-actin, the level of Egr-1 was densitometrically determined. The numbers beneath the blots were the fold changes in the density compared to the control without FBS nor EGCG ($n = 3$).

3.6. *The reduction in the trans-activation activity of Egr-1 results in the decline in the egfr promoter activity in HSC*

To verify the role of Egr-1 in regulating the *egfr* promoter activity by EGCG, HSC were co-transfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc and the Egr-1 cDNA expression plasmid pEgr-1cDNA at the indicated concentrations. As shown in Fig. 6A, compared with the untreated control (the first column on the left), forced expression of Egr-1 cDNA significantly increased luciferase activity (the second column on the left). EGCG significantly reduced, as expected, luciferase activity in the cells (the third column on the left). Forced expression of Egr-1 cDNA dose-dependently eliminated the inhibitory effect, suggesting that the increase in the abundance of cellular Egr-1 abolished the inhibitory effect of EGCG on the *egfr* promoter activity in HSC.

The extent of Egr-1 involvement in regulating the *egfr* promoter activity was further addressed by utilizing a *trans*-dominant negative mutant of Egr-1, in which dominant mutations result in the loss of the *trans*-activation activity of the DNA binding factor [20]. HSC were co-transfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc and the dominant negative Egr-1 cDNA expression plasmid pdn-Egr-1 at the indicated concentrations. As shown in Fig. 6B by luciferase assays, forced expression of dominant negative Egr-1 cDNA dose-dependently reduced luciferase activity in the cells, indicating that dn-Egr-1 without the *trans*-activation activity mimicked the inhibitory effect of EGCG and reduced the *egfr* promoter activity. Taken together, these results demonstrated that the reduction in the *trans*-activation activity of Egr-1 resulted in the decline in the *egfr* promoter activity in HSC.

3.7. *Interruption of the ERK signaling pathway reduces the trans-activation activity of Egr-1 and suppresses the egfr promoter activity in HSC*

We demonstrated that EGCG reduced the *trans*-activation activity of Egr-1, leading to the decline in the *egfr* promoter

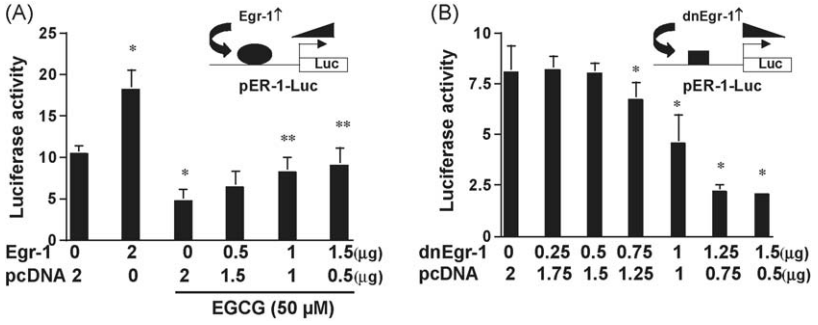

Fig. 6 – The reduction of the *trans*-activation activity of Egr-1 results in the decline in the *egfr* promoter activity in HSC. Passaged HSC in six-well culture plates were co-transfected with a total of 4.5 μg of plasmid DNA per well, including 2 μg of pER1-Luc, 0.5 μg of pSV-β gal, a cDNA expression plasmid at the indicated doses and the empty vector pcDNA. The amount of the cDNA expression plasmid plus pcDNA was 2 μg. Cells were subsequently treated with or without EGCG at 50 μM for 24 h. Luciferase activities were normalized to β-galactosidase activities. Values were means ± S.D. ($n = 3$). (A) Cells were co-transfected with the Egr-1 cDNA expression plasmid pEgr-1cDNA. The co-delivery of pER-1-Luc and Egr-1 cDNA without EGCG treatment served as a positive control (the second column on the left). $^{*}p < 0.05$, vs. cells co-transfected with no pEgr-1, with no EGCG treatment (the first column on the left); $^{**}p < 0.05$, vs. cells co-transfected with no pEgr-1, but treated with EGCG (the third column on the left). (B) Cells were co-transfected with the dominant negative Egr-1 cDNA expression plasmid pdn-Egr-1. $^{*}p < 0.05$, vs. cells co-transfected with no pdnEgr-1 (the first column on the left).

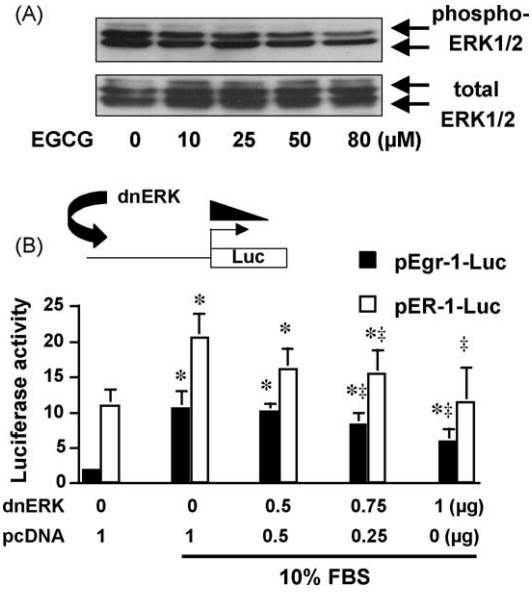

Fig. 7 – Interruption of the ERK signaling pathway reduces the *trans*-activation activity of Egr-1 and suppresses the *egfr* promoter activity in HSC. (A) HSC were serum-starved for 24 h. The cells were pretreated with EGCG at various concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 20 min. Phosphorylated ERK1/2 were detected by Western blotting analyses using anti-phospho-ERK antibodies (1:500). Total ERK1/2 were detected as an internal control for equal loading. A representative was shown from three independent experiments. (B) Passaged HSC were co-transfected with the *egfr* promoter reporter plasmid pER1-Luc, or the Egr-1 activity reporter plasmid pEgr-1-Luc, plus pdn-ERK, containing dominant negative ERK cDNA, at indicated doses. After transfection, cells were serum-starved for 24 h prior to the stimulation with or without FBS (10%) for an additional 24 h. Luciferase activities were normalized with β-galactosidase activities. Values were means ± S.D. ($n = 3$). $^{*}p < 0.05$, vs. cells without FBS stimulation (the first columns on the left); $^{‡}p < 0.05$, verse cells co-transfected with no pdnERK, but treated with FBS (the second columns on the left).

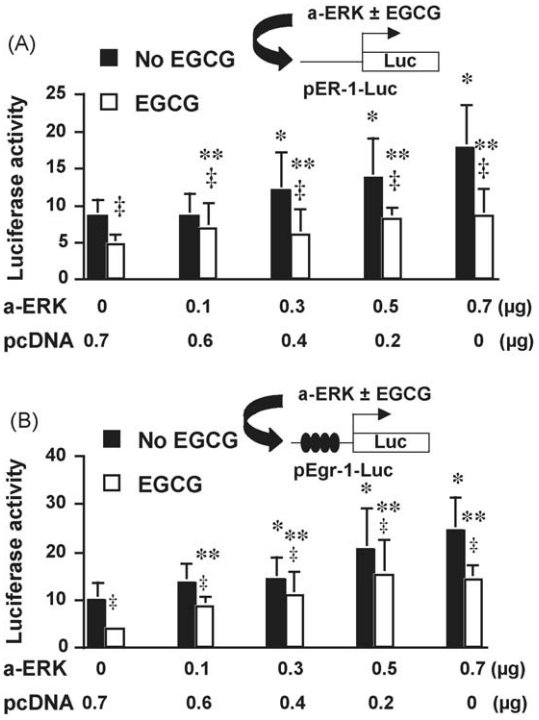

Fig. 8 – The interruption of the ERK signaling pathway is required for EGCG to reduce the *trans*-activation activity of Egr-1 and to suppress the *egfr* promoter activity in HSC. Passaged HSC were co-transfected with a luciferase reporter plasmid and pa-ERK, containing cDNA encoding constitutively active MEK1, an immediate upstream kinase of ERK. Cells were subsequently treated with or without EGCG at 50 μM for 24 h. Luciferase activities were normalized with β-galactosidase activities. Values were means ± S.D. ($n = 3$). $^{*}p < 0.05$, vs. cells without EGCG (the first black column on the left); $^{**}p < 0.05$, vs. cells treated with EGCG (the first white column on the left); $^{‡}p < 0.05$, vs. cells co-transfected with the same amount of pa-ERK, but without EGCG treatment (the black columns in both panels). (A) Cells were co-transfected with the *egfr* promoter reporter plasmid pER1-Luc and pa-ERK, (B) cells were co-transfected with the Egr-1 activity reporter plasmid pEgr-1-Luc and pa-ERK.

activity in HSC (Figs. 4–6). To examine the role of ERK in the inhibitory effects, we hypothesized that the interruption of the ERK signaling pathway by EGCG result in the reduction in the *trans*-activation activity of Egr-1, leading to the inhibition of the *egfr* promoter activity in HSC. Our pilot study observed that EGCG reduced receptor tyrosine phosphorylation of EGFR (data not shown). To test our hypothesis, we need to verify the inhibitory effect of EGCG on ERK. Serum-starved HSC were pretreated with EGCG at various concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 20 min. As shown in Fig. 7A by Western blotting analyses, EGCG dose-dependently reduced the level of phosphorylated ERK, suggesting that EGCG interrupted the ERK signaling pathway, which is consistent with other prior reports that EGCG inhibits the activity of ERK in other cell types [21–23].

To evaluate the effect of the interruption of the ERK signaling pathway on the activity of Egr-1, or the *egfr* promoter, HSC were respectively co-transfected with the Egr-1 activity luciferase reporter plasmid pEgr-1-Luc, or the *egfr* promoter luciferase reporter plasmid pER1-Luc, plus the plasmid pdn-ERK at the indicated concentrations (Fig. 7B). The cDNA expression plasmid pdn-ERK contains a fragment of cDNA encoding the dominant negative form of ERK (dn-ERK) [17]. After transfection, cells were serum-starved for 24 h prior to the stimulation with or without FBS (10%) for an additional 24 h. Serum starvation made cells more sensitive to serum stimulation [12,13]. As shown in Fig. 7B, compared to the controls (the first columns on the left), FBS significantly increased luciferase activity in cells transfected with no dn-ERK (the second columns on the left). The blockade of the ERK

signaling pathway by forced expression of dn-ERK mimicked the inhibitory roles of EGCG observed in Figs. 1D and 4A, respectively, and resulted in a dose-dependent reduction in luciferase activity in the cells. These results suggested that the interruption of the ERK signaling pathway by EGCG might play a critical role in the inhibition of the activity of Egr-1 and the *egfr* promoter in HSC.

3.8. *The interruption of the ERK signaling pathway is required for EGCG to reduce the trans-activation activity of Egr-1 and to suppress the egfr promoter activity in HSC*

To further evaluate the role of the interruption of the ERK signaling pathway by EGCG in the inhibition of the activity of Egr-1 and the *egfr* promoter in HSC, passaged HSC were respectively co-transfected with the plasmid pER1-Luc (Fig. 8A), or the plasmid pEgr-1-Luc (Fig. 8B), plus the plasmid pa-ERK at the indicated concentrations. The cDNA expression plasmid pa-ERK contains a fragment of cDNA encoding the constitutively active form of MEK1 (a-ERK), an immediate upstream activator of ERK [18]. After transfection, cells were treated with or without EGCG at 50 μM for 24 h. As shown in Fig. 8A and B by luciferase assays, forced expression of a-ERK dose-dependently increased luciferase activity in the cells, including those with EGCG treatment (the white columns). These results suggested that the introduction of exogenous a-ERK could eliminate the inhibitory effects of EGCG and that the interruption of the ERK signaling pathway might be required and play a critical role in the inhibitory effects. In addition, compared to cells co-transfected with the same amount of pa-ERK, but without EGCG treatment (the black columns), EGCG, which inhibited ERK activity (Fig. 7A), significantly reduced luciferase activity (the white columns) (Fig. 8A and B). These results demonstrated that activation of the ERK signaling pathway stimulated the *trans*-activation activity of Egr-1 and increased the *egfr* promoter activity. EGCG showed its inhibitory effects on the ERK-dependent activity of Egr-1 and the EGFR gene promoter. Taken together, our results demonstrated that the interruption of the ERK signaling pathway was required for EGCG to reduce the *trans*-activation activity of Egr-1 and to suppress the *egfr* promoter activity in HSC.

4. Discussion

In the last decade, the role of PDGF and its signaling in stimulating cell growth of HSC was extensively studied [2,3]. In addition to PDGF, evidence has shown the mitogenic role and the stimulatory effect of EGF on cell proliferation of many cell types [24]. However, activation of EGF signaling and its role in HSC activation remain largely undefined. This report evaluated the effect of EGCG on regulation of EGFR gene expression and elucidated the underlying molecular mechanisms. Our results demonstrated that EGCG suppressed gene expression of EGFR in HSC by reducing the *trans*-activation activity of Egr-1. The interruption of the ERK signaling pathway by EGCG reduced the *trans*-activation activity of Egr-1 and inhibited the *egfr* promoter activity.

EGF is a potent mitogen for numerous cell types, including hepatocytes [25,26]. EGF was suggested to play a critical role in maintenance of hepatocytes cell mass in a rat model with cirrhosis caused by bile duct ligation [27]. It was demonstrated that EGF and its receptors were highly expressed in human cirrhotic liver [5]. It was suggested that persistent *de novo* ligand synthesis and its signaling might contribute to an autocrine-mediated hepatocyte proliferation within the regenerative nodule [5]. In addition, EGF has been shown to enhance the proliferation of bile duct epithelial cells and HSC *in vitro* [6,28,29]. A recent report demonstrated that bile acid stimulated HSC proliferation via the activation of EGFR signaling *in vitro* [30]. However, compared with the knowledge of effects of EGF on hepatocytes regeneration in fibrosis and cirrhosis, the effect of EGF on HSC remains largely to be defined.

Others and we have observed that EGCG rapidly reduces the level of receptor tyrosine phosphorylation of EGFR (data not shown here). Compared with the rapid impact on the level of the phosphorylation, it takes several additional steps for EGCG to show its effect on the reduction in the abundance of EGFR. These steps include translocation of phosphorylated ERK, expression of intermediators at levels of transcription and translation. It is, therefore, understandable that, regarding the regulation of gene expression of EGFR by EGCG, cells might show a delayed response (Fig. 1), compared to the rapid phosphorylation. We previously demonstrated that treatment of HSC with EGCG for 24 h significantly inhibited HSC growth by regulating expression of growth related genes, including Cyclin D1, p21, p27, and PDGF-β receptor [10,11]. In addition, 24 h time point was also used in other prior studies of effects of EGCG on regulation of gene expression [21,31]. A recent study reported that treatment of EGCG for 24 h reduced the level of EGFR protein and its signaling in esophageal adenocarcinoma OE19 cells [32]. Our results in this study demonstrated that the treatment with EGCG for 24 h was long enough to show its effect on inhibition of EGFR gene expression in HSC. However, it bears indicated that the 24 h time point may not be optimal to show its maximal inhibitory effect.

EGCG suppressed EGFR gene expression and inhibited the promoter activity of the gene in HSC (Fig. 1), which was mediated by reducing the *trans*-activation activity of Egr-1 (Figs. 4–6). The gene of Egr-1 belongs to a group of early growth response genes, which are rapidly induced by growth factors, including EGF [33]. Substantial evidence has demonstrated its critical role in controlling cell proliferation [34]. Prior studies suggested that EGFR might be a potential target of Egr-1 under hypoxia [16]. We further demonstrated that EGCG reduced the *trans*-activation activity of Egr-1 in HSC by suppressing Egr-1 gene expression (Figs. 4 and 5). Many studies have implicated a causal relationship between the activation of ERK and the induction of Egr-1 gene expression [33,35]. Activation of ERK induces gene expression of Egr-1 mediated by the transcription factor Elk-1, a substrate of ERK [35]. Elk-1 binds to the multiple serum response elements and their adjacent Ets motifs located in the promoter of Egr-1 gene and stimulates the promoter activity, leading to the activation of Egr-1 transcription [35]. The role of the ERK signaling pathway in the EGCG inhibition of the EGFR gene expression was evaluated in the current study. The interruption of the ERK signaling pathway by dominant negative ERK mimicked the effects of EGCG on the reduction of the *trans*-activation activity

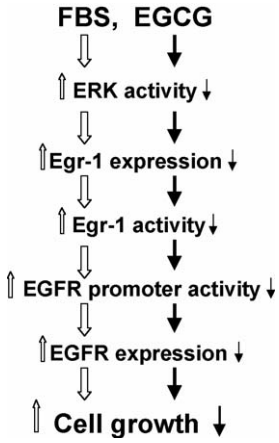

Fig. 9 – The sequential events in the inhibition of EGCG on FBS-stimulated EGFR expression, leading to the reduction of growth of activated HSC in vitro. Schematic denoting proposed steps for the inhibitory effect of EGCG on regulation of EGFR gene expression stimulated by FBS, which contributes to the inhibition of cell growth of activated HSC. Explanation is given in the text.

of Egr-1 (Fig. 7B), and on the suppression of the *egfr* promoter activity in HSC (Fig. 7B). In addition, forced expression of constitutively activated ERK abolished the inhibitory effects of EGCG in a dose-dependent manner (Fig. 8). These results collectively indicated that the interruption of the ERK signaling pathway by EGCG played a critical role in the reduction of the *trans*-activation activity of Egr-1 and the suppression of the promoter activity of EGFR gene (Figs. 7 and 8). On the other hand, EGCG has been shown to modulate multiple cell-growth related signaling pathways, including the ERK signaling pathway [36]. Therefore, the EGCG inhibitory effects could be considered ''non-specific''. It has been demonstrated that EGCG non-specifically binds proteins and modulates the activity of enzymes, leading to inhibition of cell-cycle-related kinases, MAPK and the activity of receptor tyrosine kinases [37,38]. Our results do not exclude the roles of other signaling pathways in the EGCG suppression of EGFR gene expression.

Compelling evidence has shown that HSC activation and hepatic fibrogenesis are induced by oxidative stress [39,40]. Although the antioxidant Vitamin E inhibits HSC activation [39] and hepatic fibrogenesis [41], currently well-known antioxidants in protecting the liver from fibrogenesis are not very effective [42]. Green tea is the most consumed beverage in the world [43,44]. Of the polyphenols purified from green tea, EGCG is the major constituent and the most potent antioxidant [45]. The antioxidant potential of EGCG is far greater than that of Vitamin E and/or C [46], which might allow it to succeed where other antioxidants have failed in preventing oxidative stress-related diseases. Our preliminary results demonstrated that EGCG attenuated oxidative stress by increasing *de novo* synthesis of glutathione, the major cellular antioxidant system [47]. The antioxidant property of EGCG is required for the polyphenol to inhibit EGFR gene expression and HSC growth. The underlying mechanisms are under further investigation.

To delineate the sequential events in the inhibition of EGFR gene expression by EGCG and in the inhibition of cell growth of activated HSC *in vitro*, a model is proposed in Fig. 9. FBS induces gene expression of EGFR and stimulates cell growth of HSC. The suppression of FBS-stimulated EGFR gene expression by EGCG is initiated by blocking the transient activation of the ERK signaling pathway. The inhibition of ERK activation suppresses the gene expression and reduces the *trans*-activation activity of the transcription factor Egr-1, leading to the suppression of gene expression of EGFR by reducing the promoter activity. These inhibitory actions, together with others, ultimately result in the inhibition of cell growth of activated HSC *in vitro*. Additional experiments are ongoing to evaluate the effect of EGCG on other cell survival signaling, including PI3K/AKT, in activated HSC. It bears emphasis that this model does not rule out the involvement of any other mechanisms in the inhibition of EGFR gene expression and in the inhibition of cell growth of activated HSC by EGCG. Our results provide novel mechanisms by which EGCG inhibits HSC growth and potential therapeutic approaches for the prevention and treatment of hepatic fibrosis.

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