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Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis

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RUNNING TITLE: Elevated GSH by EGCG inhibits HSC growth

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ABBREVIATIONS

BSO, buthionine sulfoximine, **DMEM**, Dulbecco's modified Eagle's medium; **ECM**, extracellular matrix**; EGCG**, (-)-epigallocatechin-3-gallate; **EGFR,** epidermal growth factor receptor; **GCL**, glutamate-cysteine ligase; **FBS**, fetal bovine serum; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **HSC**, hepatic stellate cells; **NAC**, N-acetyl-cysteine; **PDGF-ßR,** platelet-derived growth factor-beta receptor; **TGF-ßR**, transforming growth factor-beta receptor**.**

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ABSTRACT

Activation of hepatic stellate cells (HSC), the key effectors in hepatic fibrogenesis, is characterized by enhanced cell proliferation and overproduction of extracellular matrix (ECM). Oxidative stress promotes HSC activation. Glutathione (GSH) is the most important intracellular antioxidant, whose synthesis is mainly regulated by glutamate-cysteine ligase (GCL). We previously reported that (-)-epigallocatechin-3-gallate (EGCG), the major and most active component in green tea extracts, inhibited HSC activation. The aim of this study is to elucidate the underlying mechanisms. We hypothesize that this inhibitory effect of EGCG might mainly result from its antioxidant capability by increasing *de novo* synthesis of GSH. In this report, we observe that EGCG enhances the levels of cytoplasmic and mitochondrial GSH and increases GCL activity by inducing gene expression of the catalytic subunit GCLc, leading to *de novo* synthesis of GSH. Real-time PCR and Western blotting analyses show that *de novo* synthesis of GSH is required for EGCG to regulate expression of genes relevant to apoptosis and to cell proliferation. Additional experiments demonstrate that exogenous TGF-β1 suppresses GCLc gene expression and reduces the level of GSH in cultured HSC. Transient transfection assays and Western blotting analyses further display that EGCG interrupts TGF-β signaling by reducing gene expression of TGF-β receptors and Smad4, leading to increased expression of GCLc. These results support our hypothesis and collectively demonstrate that EGCG increases the level of cellular GSH in HSC by stimulating gene expression of GCLc, leading to the inhibition of cell proliferation of activated HSC *in vitro*.

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INTRODUCTION

Hepatic stellate cells (HSC) are the major players during liver fibrogenesis. Upon liver injury, normally quiescent HSC become activated, undergo profound morphological changes and *trans*-differentiate into myofibroblast-like cells. This process is termed as "HSC activation", in which, loss of vitamin A droplets, *de novo* expression of α-smooth muscle actin (α-SMA), enhanced cell proliferation and excessive production of extracellular matrix (ECM) are the most characteristic features (Friedman, 2004; Kisseleva and Brenner, 2006). HSC activation is triggered by release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSC and fibrogenic transforming growth factor-beta1 (TGF-ß1) mainly from Kupffer cells (Win et al., 1993). This process is coupled with sequential up-expression of PDGF-beta receptor (PDGF-ßR) (Wong et al., 1994), type I & II receptors for TGF-ß (Friedman et al., 1994) and EGF receptor (EGFR) (Komuves et al., 2000). It is very important to note that culturing quiescent HSC on plastic plates causes spontaneous activation, mimicking the process seen *in vivo*, which provides a good model for elucidating underlying mechanisms of HSC activation and for studying possible therapeutic intervention of the process (Friedman, 2004; Kisseleva and Brenner, 2006).

Oxidative stress reflects the imbalance of prooxidants and antioxidants. Oxidative stress-related molecules include reactive oxygen species and lipid peroxidation end products. Increasing evidence has demonstrated that oxidative stress promotes HSC activation and collagen production and plays an important role in the pathogenesis of liver fibrosis (Greenwel et al., 2000; Lee et al., 1995; Tsukamoto et al., 1995). Mammalian cells respond to oxidative stress through antioxidant defense, which

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includes antioxidant enzymes and non-enzyme molecules. Glutathione (GSH) is the predominant low-molecular weight thiol and the most important non-enzyme antioxidant. GSH in cells is located in both cytoplasm and mitochondria. The mitochondrial pool of GSH in cells is critical for regulation of thiol and redox status (Kroemer et al., 1998). GSH is sequentially synthesized from glutamate, cysteine and glycine, which is mainly controlled by the rate-limiting enzyme glutamate-cysteine ligase (GCL). GCL is composed of two subunits, *i.e.* the heavy catalytic subunit GCLc (73kDa) and the light regulatory subunit GCLm (31kDa). As an antioxidant, GSH effectively protects cells against damage caused by oxidative stress, including scavenging free radicals, removing hydrogen peroxide (H_2O_2) and lipid peroxides and preventing oxidation of molecules in cells.

Application of antioxidants is rational in treatment and prevention of diseases closely associated with oxidative stress (Sueoka et al., 2001). Green tea has been consumed for thousands of years and has displayed numerous beneficial effects to human health (Sueoka et al., 2001). (-)-epigallocatechin-3-gallate (EGCG), the major constitute in green tea, possess potent antioxidant capability (Rice-Evans, 1999). Recent studies have demonstrated the effects of green tea extracts on the protection of the liver against early alcoholic injury in rats (Arteel et al., 2003). We previously reported that EGCG inhibited HSC activation by inhibiting cell proliferation and suppressing gene expression of ECM components (Chen and Zhang, 2003; Chen et al., 2002). In addition, we reported that EGCG inhibited ECM gene expression in activated HSC by interrupting TGF-β signaling through attenuating oxidative stress (Yumei et al., 2006). The aim of this study is to elucidate mechanisms of EGCG in the inhibition of growth of activated

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HSC. We hypothesize that the inhibitory effect of EGCG on HSC growth might mainly result from its antioxidant capability by increasing *de novo* synthesis of GSH. Results presented in the current study support our hypothesis and provide novel insights into the mechanisms of EGCG in the inhibition of HSC activation.

METHODS AND MATERIALS

Isolation and culture of hepatic stellate cells: HSC were isolated using density gradient centrifugation with OptiPrep (Oslo, Norway) from livers of male Sprague-Dawley rats (200-250 grams) as we previously described (Chen and Davis, 1999). HSC were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). HSC with 4~8 passages were used for experiments. EGCG (purity>95%), N-acetyl-cysteine (NAC) and buthionine sulfoximine (BSO) were purchased from Sigma (St. Louis, MO). Glutathione monoethyl ester (GSH-MEE) was purchased from CALBIOCHEM® (San Diego, CA).

Western blotting analyses: Whole cell extracts were prepared from cultured HSC. Protein concentrations were determined using the BCA™ Protein Assay Kit according to the protocol provided by the manufacturer (Pierce, Rockford, IL). Thirty micrograms of total proteins were subjected to SDS-PAGE (10%). Target proteins were detected by primary antibodies and secondary antibodies conjugated with horseradish peroxidase purchased from Santa Cruz Biotechnology. β-actin or β-tubulin was probed as an internal control. Protein bands were visualized by using chemiluminescence reagent (Amersham).

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RNA isolation and real-time PCR: Total RNA was extracted using TRI-reagent according to the protocol provided by the manufacturer (Sigma). Real-time PCR was performed as we described previously (Chen, 2002). mRNA fold changes of target genes relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control were calculated as suggested by Schmittgen et. al. (Schmittgen et al., 2000). Following are the primers used in these studied: **EGFR** (F), 5'-TGC ACC ATC GAC GTC TAC AT-3', (R), 5'-AAC TTT GGG CGG CTA TCA G-3'; **PDGF-βR** (F), 5'-CTG CCA CAG CAT GAT GAG GAT TGA T-3', (R), 5'-GCC AGG ATG GCT GAG ATC ACC AC-3'; **Bax** (F), 5'-GGG TGG TTG CCC TTT TCT ACT-3', (R), 5'-CCC GGA GGA AGT CCA GTG TC-3'; **Bcl-2** (F), 5'-ATG GGG TGA ACT GGG GGA TTG-3', (R), 5'-TTC CGA ATT TGT TTG GGG CAG GTC-3'; **GCLc** (F), 5'-GTC TTC AGG TGA CAT TCC AAG C-3', (R), 5'-TGT TCT TCA GGG GCT CCA GTC-3'; **GCLm** (F), 5'-CTG CTA AAC TGT TCA TTG TAG G-3', (R), 5'-CTA TGG GTT TTA CCT GTG-3' ;**GAPDH** (F), 5'-GGC AAA TTC AAC GGC ACA GT-3', (R), 5'-AGA TGG TGA TGG GCT TCC C-3'.

Determination of cell proliferation: Cell proliferation was determined by using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay Kit, following the protocol provided by the manufacturer (Promega, Madison, WI).

Plasmid constructs and transient transfection*:* The GCLc promoter luciferase reporter plasmid pGCLc-luc was generated by inserting a promoter region (-1758/+2bp) of rat *GCLc* into the pGL3-enhancer luciferase reporter plasmid (Yang et al., 2001). The cDNA expression plasmid pdn-Tβ-RII was a gift from Dr. Robert J. Lechleider (National

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Cancer Institute, Bethesda, MD), containing a full length of cDNA encoding the dominant negative form of Tβ-RII (de Caestecker et al., 1998). The cDNA expression plasmid psmad4-cDNA encodes a full length of constitutively active form of Smad4, which was kindly provided by Dr. Lechleider, as well (de Caestecker et al., 1998). Transient transfection assays were performed using LipofectAMINE® (Life Technologies) following the protocol provided by the manufacturer. Transfection efficiency was controlled by co-transfection of the ß-galactosidase reporter plasmid pSV-ß (0.5~0.8μg/well) (Promega). Luciferase activities were expressed as relative unit after normalization with ß-galactosidase activity. Results were combined from multiple independent experiments (n≥6).

Isolation of cytoplasmic and mitochondrial fractions for GSH determination Cytosol and mitochondria from cultured rat HSC were prepared by using a Mitochondria Isolation Kit for Cultured Cells purchased from Pierce (Pierce Biotechnology, Inc. Rockford, IL). Mitochondrial pellets were re-suspended in 1 × phosphate-buffered saline containing 0.1% Triton X-100, disrupted by sonication, and centrifuged at 15,000 × *g* for 30 min. The supernatant was assayed for mitochondrial GSH.

GSH assays: Levels of GSH and GSSG were determined by using the enzyme immune assay kit GSH-400® (Cayman, Ann Arbor, MI), following the protocol provided by the manufacturer. The concentration of total GSH was calculated according to the equation in the protocol.

Analyses of GCL activity: GCL activity was spectrophotometrically determined as described previously with slight modifications (Fraser et al., 2003). Briefly, a sample of

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cell extracts (20μl) was mixed with the reaction solution (0.21ml) containing Tris-HCl (100mM), pH 8.0, KCl (150mM), $MgCl_2$ (20mM), Na_2EDTA (2mM), Na_2ATP (5mM), phosphoenolpyruvate (2mM), L-glutamate (10mM), L-α-aminobutyrate (10mM), NADH (0.27mM), type II rabbit muscle pyruvate (2μg) and lactate dehydrogenase (2μg). The reaction was initiated by the addition of ATP to a final concentration at 5mM. The decrease in the absorbance of NADH at 340nm was monitored for 30 min with interval of 2 min by using a SpectraMax 190 plate reader (Molecular Device, Sunnyvale, CA) and expressed as mmoles of NADH oxidized/min. Protein concentration was quantitated by BCA assay (Pierce). The final GCL activity was calculated and expressed as mmoles of NADH oxidized/min/mg protein.

Statistical analyses: 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.

RESULTS

EGCG elevates the level of cellular GSH and increases the activity of GCL in HSC

EGCG is a potent antioxidant (Rice-Evans, 1999). To evaluate the effect of EGCG on the level of GSH, cultured HSC were treated with EGCG at 50μM for various hours. Cytosol and mitochondria were prepared from these cells for determination of GSH. As shown in Fig. 1A, EGCG enhanced the levels of GSH in both cytoplasm and mitochondria in cultured HSC. It was observed that EGCG had no significant effect on

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the elevation of the cellular GSH content in the first several hours, indicating a delayed response to the natural antioxidant. In addition, the EGCG enhancement of the GSH contents in cytoplasm and in mitochondria was biphasic at beginning. We hypothesized that EGCG might increase the level of cellular GSH in HSC by enhancing the activity of GCL, the rate-limiting enzyme in *de novo* synthesis of GSH. To test the hypothesis, the activity of GCL was analyzed in passaged HSC treated with EGCG at indicated concentrations for 24 hr. As shown in Fig. 1B, EGCG increased the activity of GCL in a dose-dependent manner in these cells. Taken together, these results suggested that EGCG might elevate the level of cellular GSH in HSC by increasing the activity of GCL.

The activity of GCL is required for EGCG to elevate GSH contents in HSC

Prior studies have demonstrated that BSO, a specific inhibitor of GCL, strongly inhibits GCL activity, leading to depletion of cellular GSH (Griffith, 1982). To confirm the role of GCL in the EGCG elevation of the content of cellular GSH in activated HSC, pilot experiments were performed to determine the effective dosage and duration of BSO treatment in passaged HSC. As shown in Fig. 2A & B, treatment of cells with BSO reduced the level of cellular GSH in activated HSC in a dose-dependent (0~0.5mM) and time-dependent (>8hr) manner. Compared to that in the control cells, the level of cellular GSH was significantly reduced by approximately 40% in cells treated with BSO at 0.25mM for 24 hr. BSO at concentrations higher than 0.5mM showed no additional impact on the reduction in the level of GSH in cultured HSC. Exposure of cells to BSO at concentrations lower than 0.5mM for less than 30hr showed no significantly effect on the cell viability (data not shown), which is consistent with other prior observations

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(Nieto et al., 1999). The treatment of HSC with BSO at 0.25mM for 24 hr was, therefore, selected for our further experiments. Passaged HSC were treated with EGCG (50μM), or NAC (5mM), or GSH-MEE (2mM) for 24hr with or without the pre-exposure to BSO (0.25mM) for 1hr. NAC is a precursor in the formation of the antioxidant GSH in cells, while GSH-MEE is a cell-permeable derivative of GSH that undergoes hydrolysis by intracellular esterases to release GSH. The levels of GSH in these cells were analyzed. As expected, EGCG, as well as NAC and GSH-MEE, significantly increased the level of total intracellular GSH in the cells (Fig. 2C). The enhancement of cellular GSH concentration by EGCG and NAC, but not GSH-MEE, was completely blocked by the pretreatment with the specific GCL inhibitor BSO, suggesting that GCL activity was required for EGCG and NAC to enhance the level of cellular GSH in cultured HSC. Prior study has demonstrated that the effect of GSH-MEE on increasing the level of cellular GSH was resistant to BSO due to transport of GSH-MEE followed by intracellular hydrolysis and release of GSH (Tsan et al., 1989). GCL is not directly involved in the elevation of GSH content from GSH-MEE. These results confirmed the role of GCL in the EGCG elevation of the content of cellular GSH in activated HSC. It is noteworthy that compared with the GSH precursors NAC at 5mM and GSH-MEE (2mM), EGCG at 50μM caused a similar, if not greater, effect on increasing the level of cellular GSH (Fig. 2C), suggesting that EGCG might employ a different and more efficient mechanism to boost GSH synthesis.

***de novo* synthesis of GSH plays critical roles in the EGCG inhibition of cell proliferation and in the regulation of expression of genes relevant to cell proliferation**

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To test our assumption that the increase in the level of cellular GSH induced by EGCG might lead to the inhibition of cell proliferation of activated HSC *in vitro*, HSC were treated for 24hr with EGCG (50μM), NAC (5mM), or GSH-MEE (2mM), with or without the pre-exposure of the cells to BSO (0.25mM) for 1hr. As shown in Fig. 3A, EGCG significantly reduced, as expected, the number of viable HSC by approximately 55% compared to the untreated control. The antioxidants NAC and GSH-MEE mimicked the inhibitory effect and also caused an apparent reduction in the number of viable HSC. Blocking *de novo* GSH synthesis by BSO abrogated the inhibitory effects of EGCG and NAC on cell proliferation. However, the pretreatment with BSO could not diminish the role of GSH-MEE in the reduction in the number of viable cells. The production of GSH from GSH-MEE is resistant to BSO due to intracellular hydrolysis by esterases, without the involvement of GCL (Tsan et al., 1989). These results collectively suggested the requirement of *de novo* synthesis and enhanced level of cellular GSH in the EGCG inhibition of HSC growth.

To elucidate the underlying mechanisms of EGCG in the inhibition of cell proliferation of HSC, we hypothesized that the elevation of the level of cellular GSH by EGCG might alter expression of genes relevant to cell proliferation and to apoptosis. To test this hypothesis, passaged HSC were treated with EGCG (50μM), NAC (5mM) or GSH-MEE (2mM) for 24 hr with or without the pre-exposure to BSO (0.25mM) for 1hr. Gene expression was analyzed by real-time PCR and Western blotting analyses, respectively. As shown in Fig. 3B, the steady state mRNA levels of PDGF-ßR and EGFR, both of which mediate the most important mitogenic signaling in promoting HSC proliferation, were significantly reduced by EGCG, NAC, as well as GSH-MEE. Blocking

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de novo synthesis of GSH by the pretreatment with BSO eliminated the inhibitory effect of EGCG and NAC. However, BSO had no effect on GSH-MEE. Further experiments in Fig. 3B demonstrated that EGCG, NAC and GSH-MEE increased the mRNA level of pro-apoptotic Bax and reduced the abundance of anti-apoptotic Bcl-2 in passaged HSC, suggesting their role in the induction of apoptosis. Blocking GSH synthesis by BSO abolished the role of EGCG and NAC, but not GSH-MEE, in the regulation of the expression of the genes relevant to apoptosis. These observations were verified by Western blotting analyses (Fig. 3C). In addition, EGCG, mimicking the role of NAC, reduced the level of cyclin D1, a critical regulator of in G1 and S-phase transition of cell cycle, and enhanced the abundance of $p21^{(WAF1/Cip1)}$ and $p27^{(Kip1)}$, two critical inhibitory proteins in regulating cell cycle progression, in activated HSC *in vitro*, suggesting the importance of the antioxidants in cell cycle progression. Pretreatment with BSO abrogated their regulatory roles. Taken together, the observations of the sensitivity of EGCG and NAC to BSO and the GSH-MEE resistance to BSO in Fig. 3 provided strong evidence that the process of the EGCG inhibition of HSC growth, including inducing cell cycle arrest and apoptosis, was mainly mediated by *de novo* synthesis of cellular GSH.

EGCG increases gene expression of GCLc, but not GCLm, in activated HSC

It is worth noting that, compared to the effects of NAC at 5mM and GSH-MEE at 2mM, EGCG at a much lower concentration (50μM), showed similar, if not greater, impacts on the elevation of cellular GSH contents (Fig. 2C), on the inhibition of cell proliferation of HSC (Fig. 3A) and on the regulation of gene expression (Fig. 3B & C), suggesting that EGCG might employ a different, but more efficient mechanism. We

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showed that EGCG increased the activity of GCL, the rate-liming enzyme in *de novo* synthesis of GSH, in activated HSC *in vitro* (Fig. 1B). To explore underlying mechanisms, it was postulated that EGCG might induce gene expression of GCL, leading to the increase of the enzyme activity and to the elevation of the cellular GSH content in activated HSC. To test the postulation, passaged HSC were treated with EGCG at various concentrations for 24hr. The effects of EGCG on gene expression of the two subunits of GCL, *i.e.* GCLc and GCLm, were analyzed by real-time PCR and Western blotting analyses, respectively. Compared to those in un-treated control cells, EGCG dose-dependently increased the steady-state level of GCLc transcript (Fig. 4A) and the abundance of GCLc protein (Fig. 4B). In great contrast, EGCG had no apparent impact on gene expression of GCLm in passaged HSC (Fig. 4A and 4B, respectively). These findings collectively suggest that EGCG might increase the level of cellular GSH in passaged HSC by inducing gene expression of GCLc, leading to the increased activity of GCL.

Exogenous TGF-β1 reduces cellular GSH level by suppressing GCLc gene expression in passaged HSC

Further studies were performed to explore the mechanisms of EGCG in the induction of GCLc gene expression in activated HSC. Prior reports demonstrated that TGF-ß deplete intracellular GSH content in some cell types, including fibroblast (NIH3T3) (Liu et al., 2004) and alveolar epithelial cells (Jardine et al., 2002). It was suggested that TGF-β signaling might suppress gene expression of GCL (Arsalane et al., 1997; Jardine et al., 2002). We previously demonstrated that EGCG interrupted

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TGF-ß signaling by suppressing gene expression of TGF-ß receptors, leading to the inhibition of gene expression of ECM components in activated HSC (Yumei et al., 2006). We, therefore, hypothesized that the EGCG interruption of TGF-ß signaling might result in the elimination of its suppressive effect and in the induction of gene expression of GCLc in activated HSC. To test the hypothesis, we evaluated the effect of TGF-ß1 on the levels of GSH in cytoplasm and in mitochondria in passaged HSC. Cells were treated with TGF-ß1 at 10ng/ml for various hours. Cytosol and mitochondria were prepared from these cells. The levels of GSH in cytoplasm and in mitochondria were respectively determined. As displayed in Fig. 5, exogenous TGF-β1 significantly reduced the levels of GSH in both cytoplasm and mitochondria in passaged HSC. However, a biphasic response of the GSH content to TGF-ß1 treatment was observed in cytoplasm and mitochondria. The level of GSH in cytoplasm was rapidly reduced in the first 8 hours after the exposure to TGF-β1. However, the level of GSH in mitochondria was gradually reduce and was still significantly delayed after the treatment for 30hr. Taken together, these results supported our hypothesis and demonstrated the inhibitory role of TGF-ß signaling in the level of cellular GSH in passaged HSC.

To further test our hypothesis and explore underlying mechanisms, passaged HSC were divided into two groups. One group of cells was treated with TGF-β1 at various concentrations for 24hr. Another group of cells was treated with TGF-β1 at 10ng/ml for various periods of time. Medium was replaced every 24hr with fresh exogenous TGF-β1. The expression of GCLc and GCLm was analyzed by real-time PCR and Western blotting analyses. As shown in Fig. 6A and 6B, TGF-β1 dose-dependently reduced gene expression of GCLc, but not GCLm, at both levels of transcription and translation. In

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addition, the decrease in gene expression of GCLc lasted no less than 72 hours, as displayed in Fig. 6C and 6D. Taken together, these results demonstrated that the activation of TGF-β signaling by exogenous TGF-ß1 suppressed gene expression of GCLc, but not GCLm, in activated HSC *in vitro*.

EGCG induces GCLc gene expression by interrupting TGF-β signaling

We previously observed strong basal TGF-β signaling in passaged HSC without the addition of exogenous TGF-β (Yumei et al., 2006). It is presumably activated by paracrine and autocrine action of TGF-β present in medium containing FBS (10%) and secreted by passaged HSC, respectively. Forced expression of the dominant negative form of Tβ-RII (dn-Tβ-RII) in transfection assays interrupted TGF-β signaling in passaged HSC (Yumei et al., 2006). To verify the effect of TGF-β signaling on the inhibition of GCLc gene expression, passaged HSC were co-transfected with the plasmids pdn-Tβ-RII and pGCLc-luc. The GCLc promoter luciferase reporter plasmid pGCLc-luc contains a fragment of the rat *GCLc* promoter (-1758/+2bp), subcloned in the luciferase reporter plasmid pGL_3 (Yang et al., 2001). The cDNA expression plasmid pdn-Tβ-RII contains a full length of cDNA encoding the dominant negative form of Tβ-RII (de Caestecker et al., 1998). A total of 4.5μg of plasmid DNA per well was used for co-transfection of HSC in 6-well culture plates. It included 2μg of pGCLc-luc, 0.5μg of pSV-ß-gal, and 2μg of pdn-Tβ-RII at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. As shown by luciferase assays in Fig. 7A, forced expression of exogenous dn-Tβ-RII dose-dependently increased luciferase activity in cells transfected with pGCLc-luc. This

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result suggested that the interruption of TGF-β signaling induced the promoter activity of GCLc gene in activated HSC *in vitro*.

Additional experiments were performed to evaluate the impact of EGCG on the protein abundance of Smad4, a key mediator in TGF-β signaling. As shown in Fig. 7B, EGCG apparently reduced the abundance of Smad4 in a dose-dependent manner, suggesting that EGCG interrupted TGF-β signaling not only by suppressing gene expression of TGF-β receptors (Yumei et al., 2006), but also by reducing the abundance of the key mediator Smad4 (Fig. 7B). To confirm the observation, HSC were co-transfected with the plasmid pGCLc-luc and the plasmid pSmad4, containing a full length of cDNA encoding the constitutively active form of Smad4. After overnight recovery, cells were treated with or without EGCG (50μM) for an additional 24hr. As shown in Fig. 7C, luciferase activity in cells transfected with pSmad4 (the 2nd column on the left) was significantly reduced by approximately 42% compared to that in the control cells (the 1st column on the left). In great contrast, EGCG (50μM) dramatically increased luciferase activity by more than 2.5 fold (3rd column on the left). Forced expression of the constitutively active form of Smad4 dose-dependently eliminated the role of EGCG in the elevation of luciferase activity in cells (Fig. 7C), indicating the inhibitory effect of Smad4 on the promoter activity of GCLc gene in activated HSC. These results collectively demonstrated that EGCG induced GCLc gene expression by suppressing TGF-β signaling and thereby abolished its inhibitory action on expression of GCLc gene in activated HSC *in vitro*.

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DISCUSSION

We previously reported that EGCG inhibited HSC activation *in vitro* by inhibiting cell proliferation and suppressing expression of ECM genes (Chen et al., 2002). Additional experiments revealed that EGCG reduced ECM gene expression by interrupting TGF-β signaling through attenuating oxidative stress (Yumei et al., 2006). The current study examined the mechanisms of EGCG in the inhibition of cell proliferation of activated HSC. Results in this report demonstrated that EGCG elevated the level of cellular GSH in activated HSC by increasing the activity of GCL, leading to the regulation of expression of genes relevant to cell proliferation. Additional experiments indicated that EGCG induced gene expression of GCLc and eliminated the effect of TGF-β signaling on suppressing expression of the gene by interrupting its signaling in activated HSC *in vitro*. It was recently observed that the maximum concentration of EGCG in human plasma was approximately 65 ng/ml within 1–2hr after oral ingestion of 350ml of commercial green tea beverage containing 581mg of total catechins with 0.31mg/ml of EGCG (Masukawa et al., 2006). It was also shown that the absorption rates of catechins in green tea, including EGCG, into human blood were different and very low. Most of them were retained in the blood for only few hours. We used EGCG at 50μM (22.92μg/ml) in most of our *in vitro* experiments, which is higher than that observed in human blood. However, it bears emphasis that because the *in vivo* system is multi-factorial, directly extrapolating *in vitro* conditions and results, *e.g.* effective concentrations, to the *in vivo* system might be misleading.

Oxidative stress represents an important and novel class of "the 3rd messenger", leading to activation of several signal pathways associated with inflammation. Oxidative

stress has been implicated in stimulation of hepatic fibrogenesis. In addition to over-production of pro-oxidants during liver injury, weakening in antioxidant defense synergistically facilitates oxidative stress. Depletion of GSH, the most abundant antioxidant molecule, in chronic liver injury potentiates oxidative stress, which promotes HSC growth and collagen production. Antioxidants have been proposed in prevention and treatment of hepatic fibrosis. In the present report, we demonstrated that EGCG and the GSH precursors of NAC and GSH-MEE significantly increased the level of cellular GSH, inhibited cell proliferation of HSC, and regulated expression of genes relevant to cell proliferation and apoptosis in activated HSC *in vitro*. NAC elevates the level of cellular GSH by *de novo* synthesis of the thiol catalyzed by GCL, which could be specifically inhibited by BSO. However, the increase in the level of cellular GSH from GSH-MEE is resistant to BSO due to intracellular hydrolysis by esterases, without the involvement of GCL (Tsan et al., 1989). Depletion of GSH alone by BSO was not sufficient to stimulate cell proliferation. However, the pretreatment with BSO, inhibiting GCL activity, apparently abolished the roles of EGCG and NAC, but not GSH-MEE, in the elevation of cellular GSH content, in the reduction of the number of viable HSC and in the regulation of expression of genes relevant to cell proliferation and apoptosis. These results collectively suggested that these effects of EGCG might require the GCL activity to stimulate *de novo* synthesis of GSH. However, the capability of EGCG in increasing the level of cellular GSH was, in fact, limited. The concentration of EGCG higher than 80μM showed no such dose-dependent effects (data not shown). It could be explained by the theory of the feedback control (Seelig et al., 1984). GSH is known to be a feedback inhibitor of GCL. EGCG increases the activity of GCL, leading to the *de*

novo synthesis of GSH and the elevation of the level of cellular GSH. The elevated GSH content might suppress, in turn, the activity of GCL in a mechanism of the feedback inhibition, resulting in elimination of the role of EGCG in elevating the level of GSH. The feedback inhibition restricts the ability of EGCG within a limited range of concentrations.

We observed that EGCG enhanced the levels of both cytoplasmic and mitochondrial GSH (Fig. 1A). It was also noticed that the levels of GSH showed no significant response to EGCG within the first several hours. It was a typical delayed response, suggesting the requirement of a rate-limiting enzyme in the process of the *de novo* synthesis of GSH. Additional experiments supported this suggestion and demonstrated that EGCG increased the GCL activity by inducing gene expression of GCL, the key rate-limiting enzyme in GSH synthesis, in activated HSC (Fig. 2-4). In addition, the alterations in the level of mitochondrial GSH by either EGCG (Fig. 1A) or TGF-β1 (Fig. 5) were not in the same step with those in cytoplasm. The increase of the GSH content in mitochondria by EGCG was delayed at beginning and caught up with that in cytoplasm later (Fig. 1A). However, the reduction of the GSH content in mitochondria by exogenous TGF-β1 was still delayed after the treatment for 30hr. Prior work observed the similar biphasic depletion of GSH in cytoplasm and mitochondria after the administration of BSO (Griffith and Meister, 1985). Studies have shown that although free GSH is present at similar millimolar concentrations in both mitochondrial matrix and cytoplasm, no GCL is detectable in mitochondria (Griffith and Meister, 1985). There is little, if any, *de novo* synthesis of GSH within mitochondria. Mitochondrial GSH in cells mainly arises from cytoplasm (Griffith and Meister, 1985). The exchange of free

GSH between mitochondria and cytoplasm is distinct (Griffith and Meister, 1985). GSH is readily transported from cytoplasm into mitochondria. However, it very slowly and difficultly escapes from mitochondria to cytoplasm (Griffith and Meister, 1985). The observed biphasic responses of GSH contents in cytoplasm and in mitochondria to EGCG or to TGF-β1 might result from the different exchanging rates of free GSH between mitochondria and cytoplasm.

The level of cellular GSH is mainly determined by GSH synthesis (GSH supply) and GSH-consuming (GSH demand). It bears emphasis that our results do not exclude any other mechanisms involved in the antioxidant capacity of EGCG and in the EGCG elevation of the level of cellular GSH in HSC, including reducing exporting and consuming GSH. This current report focused on the effect of EGCG on GSH synthesis. Additional experiments are ongoing to evaluate the role of EGCG in regulating gene expression and activity of enzymes involved in consuming GSH, including GSH S-transferase and GSH peroxidase. In addition, we could not exclude the roles of any mechanisms and enzymes in the removal of lipid peroxidation products, which requires additional studies.

The concentration of EGCG (50μM) used in this study is much lower than that of NAC (5mM) and GSH-MEE (2mM). However, EGCG produced similar, if not greater, inhibitory effects on HSC growth. These results suggest that EGCG might increase the level of cellular GSH via a different, but more efficient mechanism. Either NAC or GSH-MEE primarily functions as one molecule of the GSH precursor in *de novo* GSH synthesis (Ruffmann and Wendel, 1991). EGCG increased the level of cellular GSH by inducing gene expression of GCLc and enhancing the activity of GCL. This explained

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why EGCG, compared to NAC and GSH-MEE, was more efficient in the elevation of GSH content in cultured HSC.

GCL is a hetero-dimer consisting of an active catalytic (GCLc) and a modulatory (GCLm) subunit. GCLc contains all substrate binding sites, whereas the modulatory subunit GCLm modulates the affinity of the GCLc for substrates and inhibitors. EGCG significantly induced gene expression of GCLc and had no apparent impact on GCLm in passaged HSC. Studies have shown the simultaneous up-regulation of both GCL subunits (Zhang et al., 2007). However, the regulation of *Gclc* and *Gclm* is not always coordinated. In many cases, induction of one gene is favored over the other (Cai et al., 1997; Cai et al., 1995; Moellering et al., 1998). In fact, in some tissues, including the heart and liver, the ratio of GCLc/GCLm is more than 1.0 (Krzywanski et al., 2004). Regulation of GCLc gene expression is affected by many factors, such as oxidative stress, inflammatory cytokines, antioxidants and insulin (Lu, 1999; Lu, 2000).

Our experiments in this study indicated that exogenous TGF-β1 suppressed gene expression of GCLc and reduced the activity of GCL. Our observations are consistent with other prior reports. TGF-β1 depletes cellular GSH content, resulting from the inhibition of gene expression of GCLc (Arsalane et al., 1997; De Bleser et al., 1999; Franklin et al., 2003; Jardine et al., 2002; Liu et al., 2004). TGF-β signaling induces the activity of Smad3-ATF3, leading to the suppression of genes encoding Phase II detoxifying proteins, including GCLc (Bakin et al., 2005). Ectopic expression of ATF3 is sufficient to reduce the GCL activity (Bakin et al., 2005). We previously demonstrated the presence of basal TGF-β signaling in cultured HSC without the addition of exogenous TGF-β. The basal TGF-β signaling is presumably activated by TGF-β

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derived from FBS (10%), as well as secreted by passaged HSC (Yumei et al., 2006). p3xTP-Lux is a TGF-β-inducible luciferase reporter plasmid, containing the plasminogen activator inhibitor gene promoter (Massague, 1998). This plasmid is often used for studying TGF-β *trans*-activation activity in cells and TGF-β signaling. We demonstrated that the luciferase activity in HSC transfected with the p3xTP-Lux was significantly high (Yumei et al., 2006). EGCG caused a dose-dependent reduction in luciferase activity in these cells. EGCG suppressed gene expression of TGF-β receptors in activated HSC *in vitro*, leading to the interruption of TGF-β signaling (Yumei et al., 2006). We, therefore, assumed that the EGCG interruption of TGF-ß signaling might eliminate its suppressive effect on gene expression of GCLc, leading to the induction of GCLc gene expression in activated HSC. This assumption was supported by our further observations. The interrupting TGF-β signaling by forced expression of the dominant negative form of Tβ-RII increased the promoter activity of GCLc, while expression of the constitutively active form of Smad4 abrogated the impact of EGCG on the increase in the promoter activity of GCLc gene. It bears emphasis that we could not exclude any alternative mechanisms in the ECGC induction of GCLc gene expression in activated HSC. In addition, our results could not determine the relationship between the EGCG reduction of the inhibitory effect of TGF-β signaling and the EGCG enhancement of the promoter activity in the induction of GCLc gene expression in activated HSC *in vitro*. The two could be a parallel, or a causal, relationship. The two might play additive or synergistic roles in the up-regulation of gene expression of GCLc in passaged HSC. Additional deletion of gene promoter analyses would be helpful to map out key elements and clarify the underlying mechanism. On the other hand, the enhanced level of cellular

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GSH by EGCG might not be exclusively derived from the induction of GCLc gene expression. EGCG also shows its impacts on other enzyme systems involved in the defense against oxidative stress (Li et al., 2007).

In summary, results in the current study support our general hypothesis and demonstrate that the inhibitory effect of EGCG on HSC growth might mainly result from its antioxidant capability by increasing *de novo* synthesis of GSH. These findings provide novel insights into the mechanisms of EGCG in the inhibition of HSC activation and further consolidate the potential application of EGCG as an antifibrotic agent against liver fibrosis.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. EGCG increases GCL activity in activated HSC *in vitro.*

Passaged HSC were treated with EGCG at 50μM for various hours (**A**), or at various concentrations for 24hr (**B**). Values are means ± S.D. (n≥3). *p<0.05, versus cells treated with no EGCG (the 1st column on the left side). (**A**). Determination of the levels of GSH in cytoplasm and in mitochondria. Total GSH was expressed as nmol/μg protein. (**B**). Measurement of GCL activity.

Figure 2. The inhibition of GCL activity by BSO eliminates the role of EGCG in the elevation of total cellular GSH in activated HSC *in vitro.*

The level of total cellular GSH was measured in passaged HSC. The level of cellular GSH was expressed as nmol/μg protein. Values are mean ± S.D. (n=3). *p<0.05 versus the control cells (the 1st bar on the left). (**A**). Cells were treated with BSO at various concentrations for 24hr; (**B**).Cells were treated with BSO at 0.25mM for various hours; (**C**). Cells were treated with EGCG (50μM), NAC (5mM), or GSH-MEE (2mM), for 24hr with or without the pre-exposure to BSO (0.25mM) for 1 hr. ‡ p<0.05, versus cells treated with EGCG, or NAC only (the 2nd, or the 3rd, bar on the left).

Figure 3. *de novo* synthesis of GSH is required for EGCG to inhibit HSC growth and to regulate expression of genes relevant to cell proliferation.

HSC were treated for 24hr with EGCG (50μM), NAC (5mM), or GSH-MEE (2mM) with or without the pre-exposure to BSO (0.25mM) for 1hr. Values are means ± S.D. (n≥3). *p<0.05, versus cells with no treatment (the 1st column on the left); ‡ p<0.05,

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versus cells treated with EGCG, or NAC only (the 2nd, or the 3rd, column(s) on the left). (**A**). Determination of viable cells. Values are expressed as fold changes compared to the control cells; (**B**), real-time PCR analyses of the steady state mRNA levels of genes. GAPDH was used as an invariant control for calculating fold changes of target mRNA (n=3); (**C**). Western blotting analyses of the abundance of proteins. ß-tubulin was used as an invariant control for equal loading. Representative was shown from three independent experiments.

Figure 4. EGCG increases gene expression of GCLc, but not GCLm, in activated HSC *in vitro*.

Passaged HSC were treated with EGCG at various concentrations for 24 hr. Total RNA or whole cell extracts were prepared for real-time PCR (**A**), or for Western blotting analyses (**B**), respectively. GAPDH or β-actin was used as an invariant internal control, respectively. Values are means ± S.D. (n=3). *p<0.05, versus the control cells treated with no EGCG. Representative was shown from three independent experiments.

Figure 5. Exogenous TGF-β1 reduces the level of cellular GSH in cultured HSC.

Passaged HSC were treated with TGF-β1 at 10ng/ml for various hours. The levels of GSH in cytoplasm and in mitochondria were measured as described in Methods and Materials. Total GSH was expressed as nmol/μg protein. Values are means ± S.D. (n≥3). *p<0.05, verse the control cells with no treatment (the 1st point on the left).

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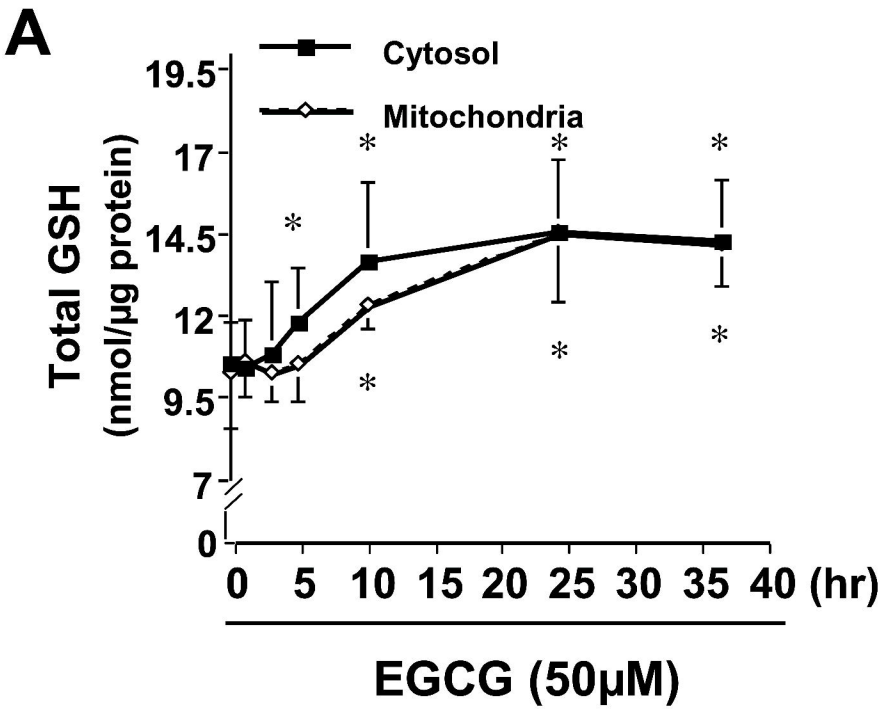
Figure 6. Exogenous TGF-β1 suppresses the expression of GCLc, not GCLm, in a dose- and time-dependent manner in activated HSC *in vitro.*

Passaged HSC were either treated with or without TGF-β1 at indicated concentrations for 24hr (**A** and **B**), or treated with TGF-β1 at 10ng/ml for 24, 48 or 72hr, respectively (**C** and **D**). Total RNA or whole cell extracts were prepared for real-time PCR assays (**A** and **C**), or for Western blotting analyses (**B** and **D**). Values are means ± S.D. (n≥3). GAPDH was used as an invariant internal control for calculating mRNA fold changes (n=3). *p<0.05, versus the control cells with no treatment. ß-actin was used as an internal control for equal loading in Western blotting analyses. Representative was shown from three independent experiments.

Figure 7. Interrupting TGF-β signaling by EGCG results in the induction of GCLc gene expression in activated HSC *in vitro*.

Passaged HSC were co-transfected with the GCLc promoter luciferase reporter plasmid pGCLc-Luc and a cDNA expression plasmid, either pdn-TβRII, encoding the dominant negative form of Tβ-RII **(A)**, or pSmad4, encoding the constitutively active form of Smad4 **(C).** The empty vector pcDNA was used to ensure an equal amount of total DNA in transfection assays. Luciferase activities were normalized to β-galactosidase activity. Values are means ± S.D. (n=3). *p<0.05, versus cells transfected with no pdn-Tβ-RII, or pSmad4, (the 1st column on the left in panel **A**, or **C**, respectively). ‡p<0.05, versus cells treated with EGCG without pSmad4 (the 3rd column on the left in panel **C**). **B.** Western blotting analyses of the abundance of Smad4 in

passaged HSC treated with EGCG at various concentrations for 24 hr. β-actin was used as an internal control for equal loading. Representative was shown from three independent experiments.



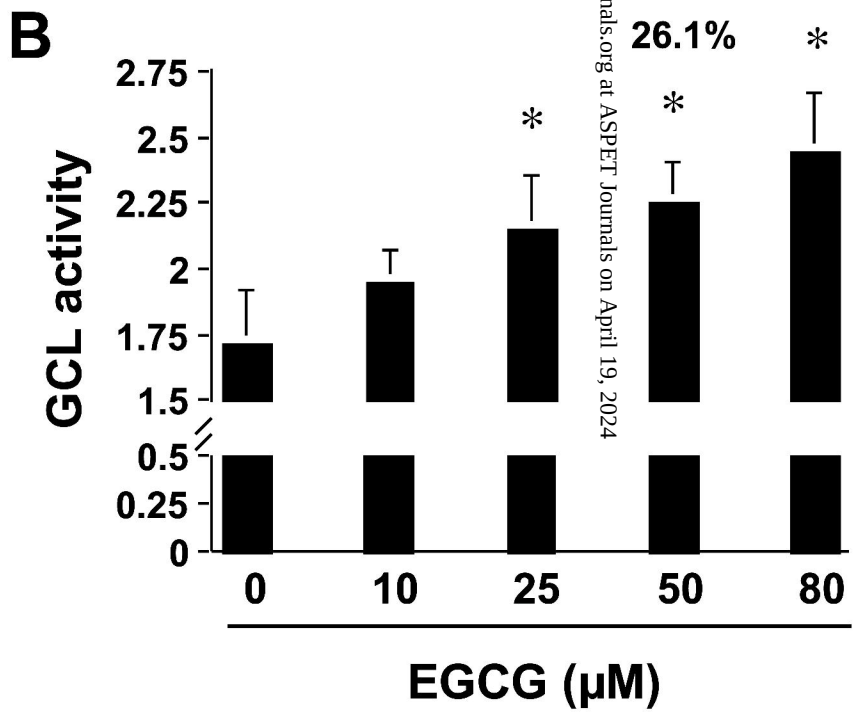


Fig. 1

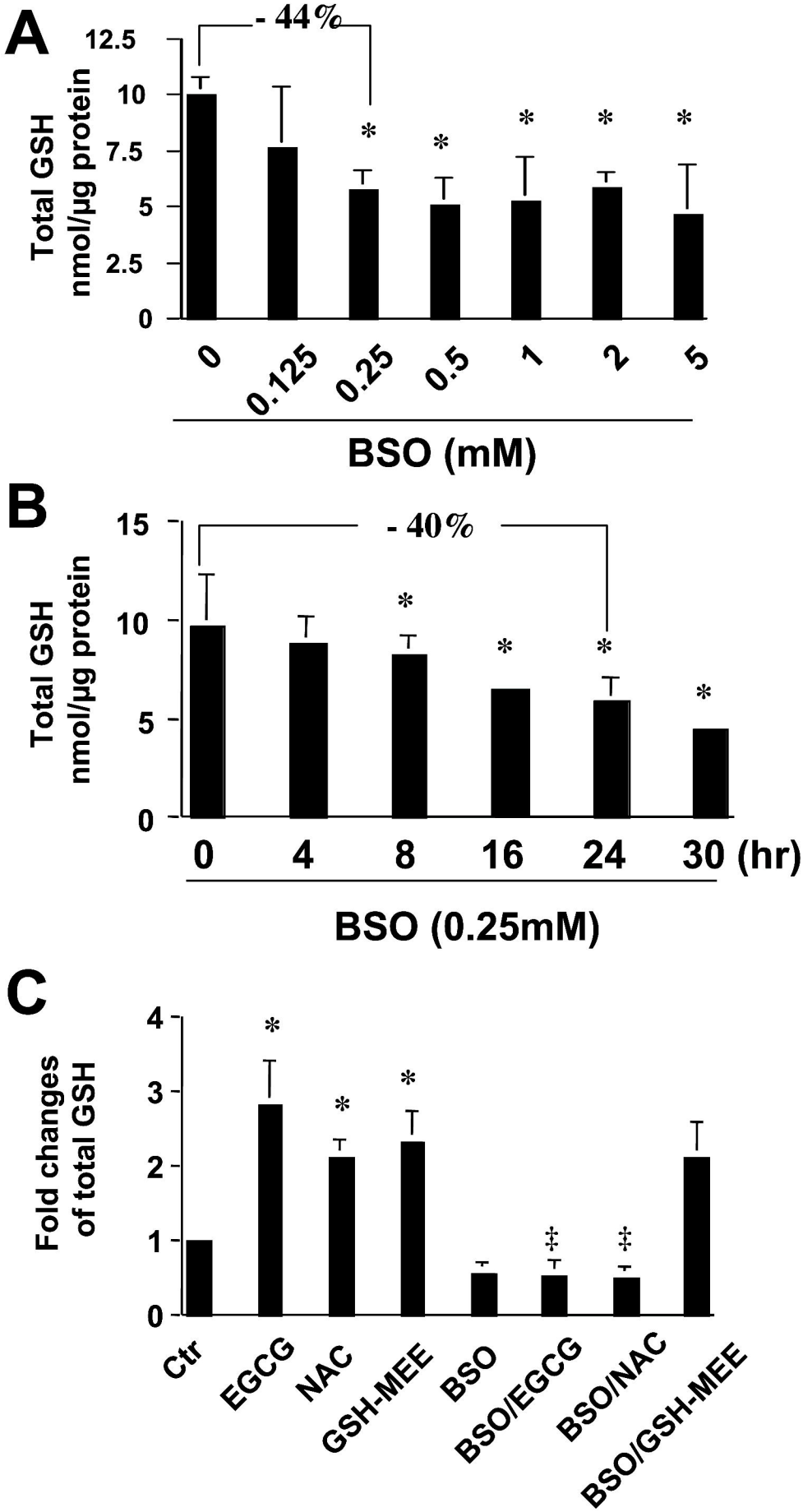
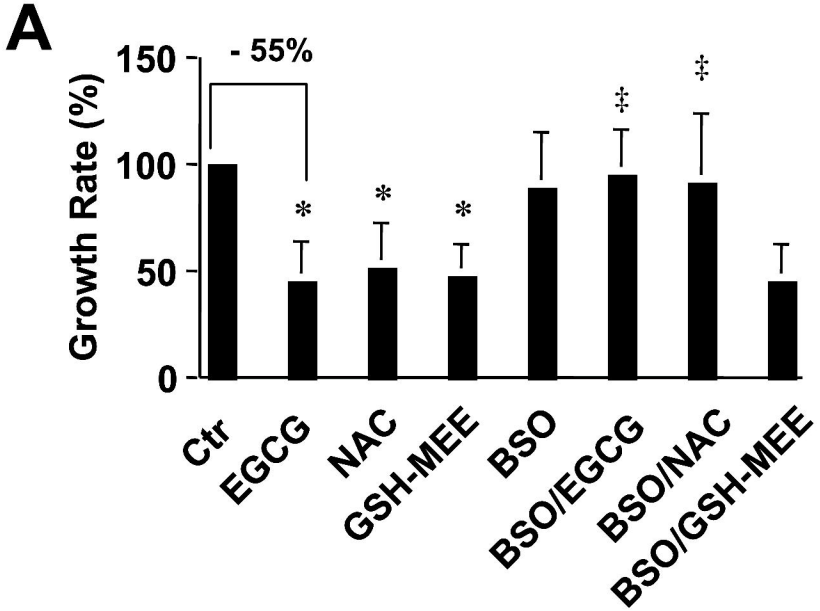
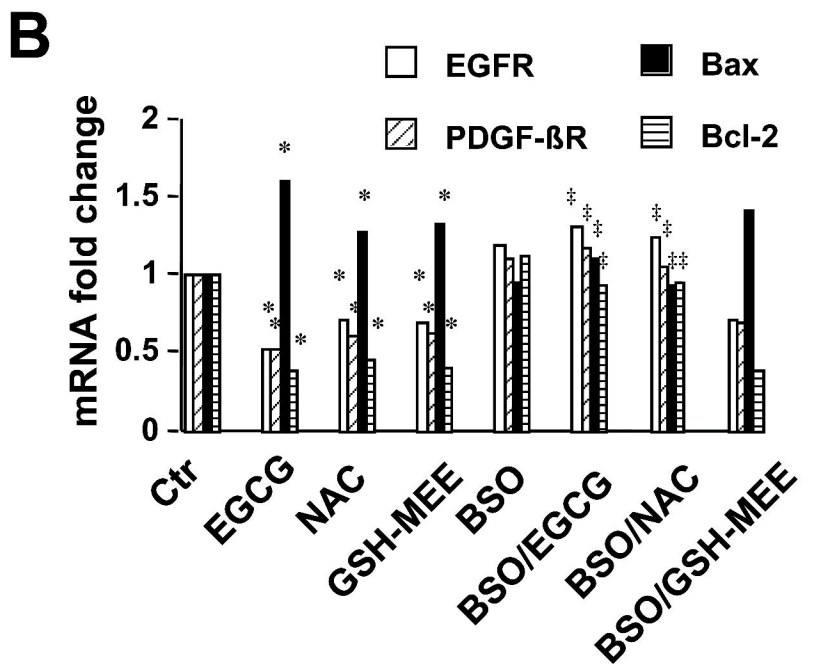

A
- 44%
12.5
10
7.5
5
2.5
0
Total GSH
nmol/μg protein
0
0.125
0.25
0.5
1
2
5
BSO (mM)
B
- 40%
15
10
5
0
Total GSH
nmol/μg protein
0
4
8
16
24
30 (hr)
BSO (0.25mM)
C
4
3
2
1
0
Fold changes
of total GSH
Ctr
EGCG
NAC
GSH-MEE
BSO
BSO/EGCG
BSO/NAC
BSO/GSH-MEE

Fig. 2





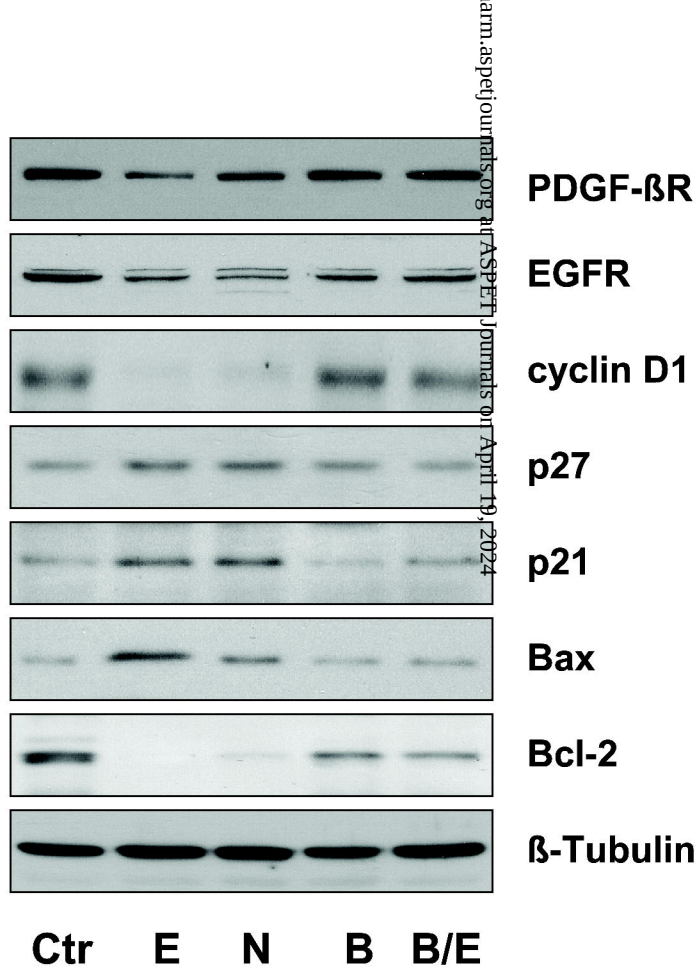


Fig. 3

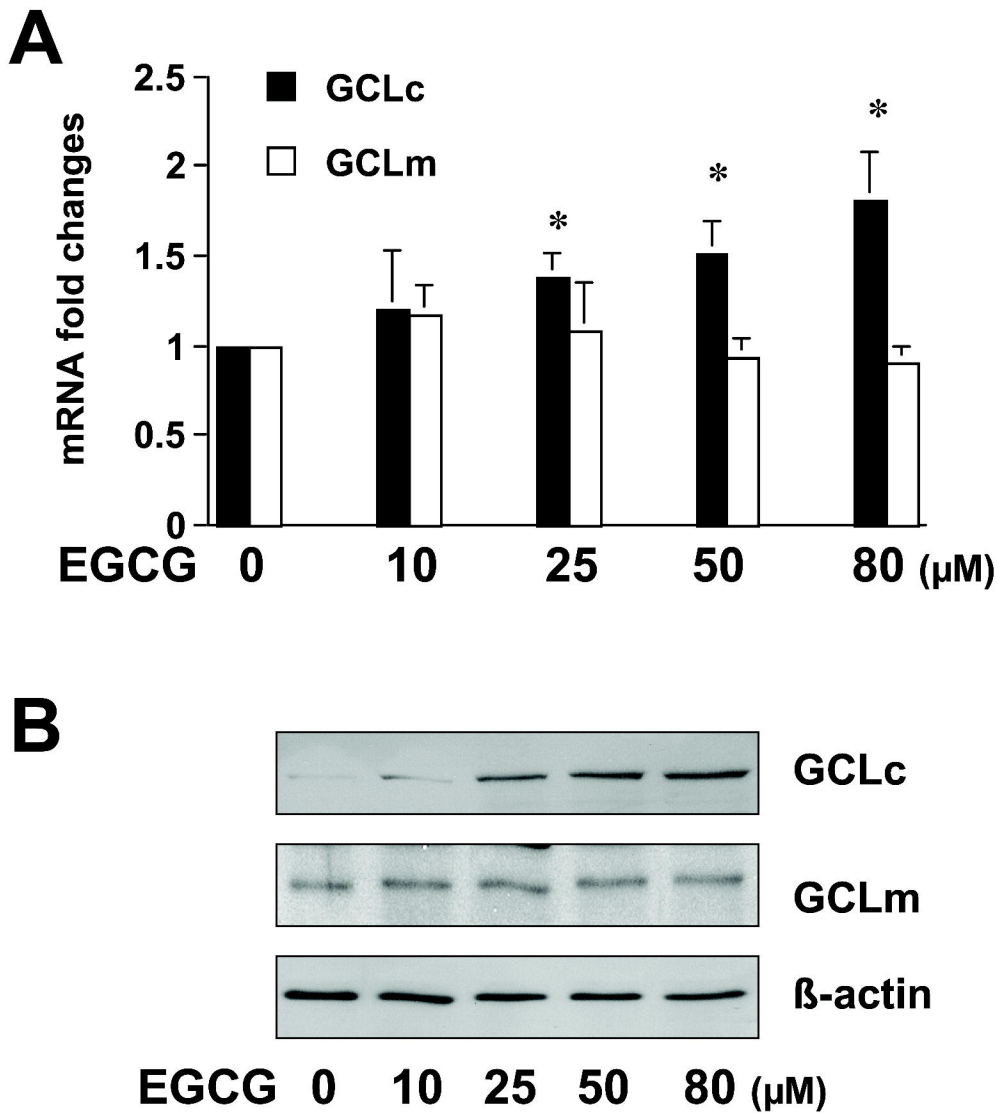


Fig. 4

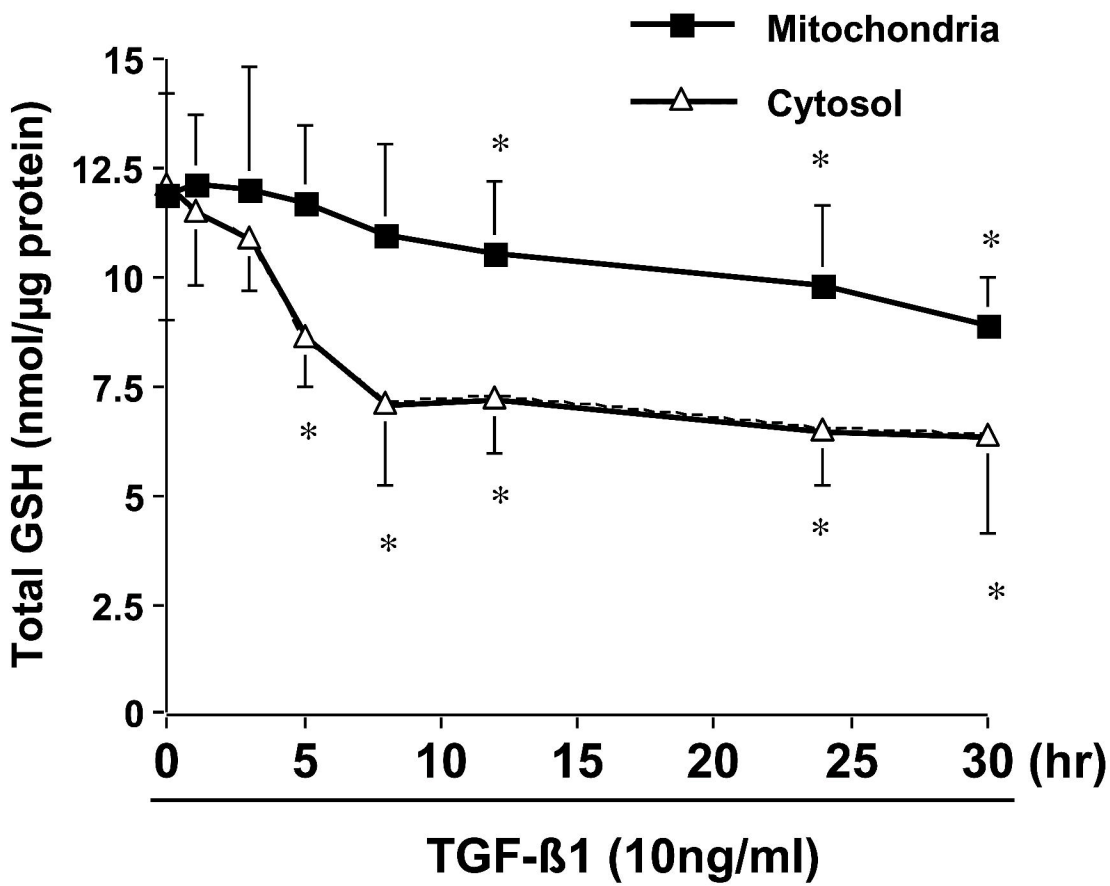


Fig. 5

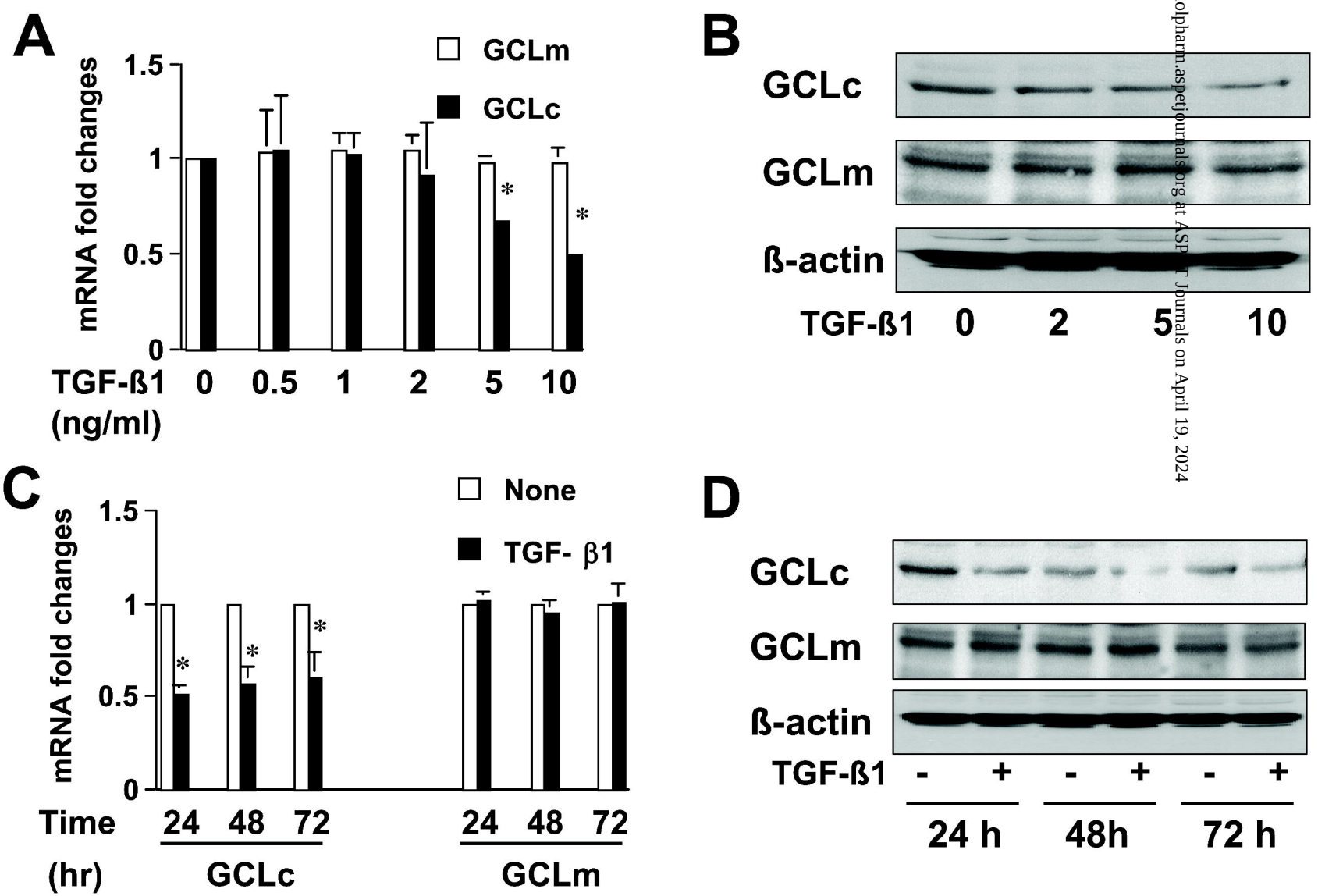


Fig. 6

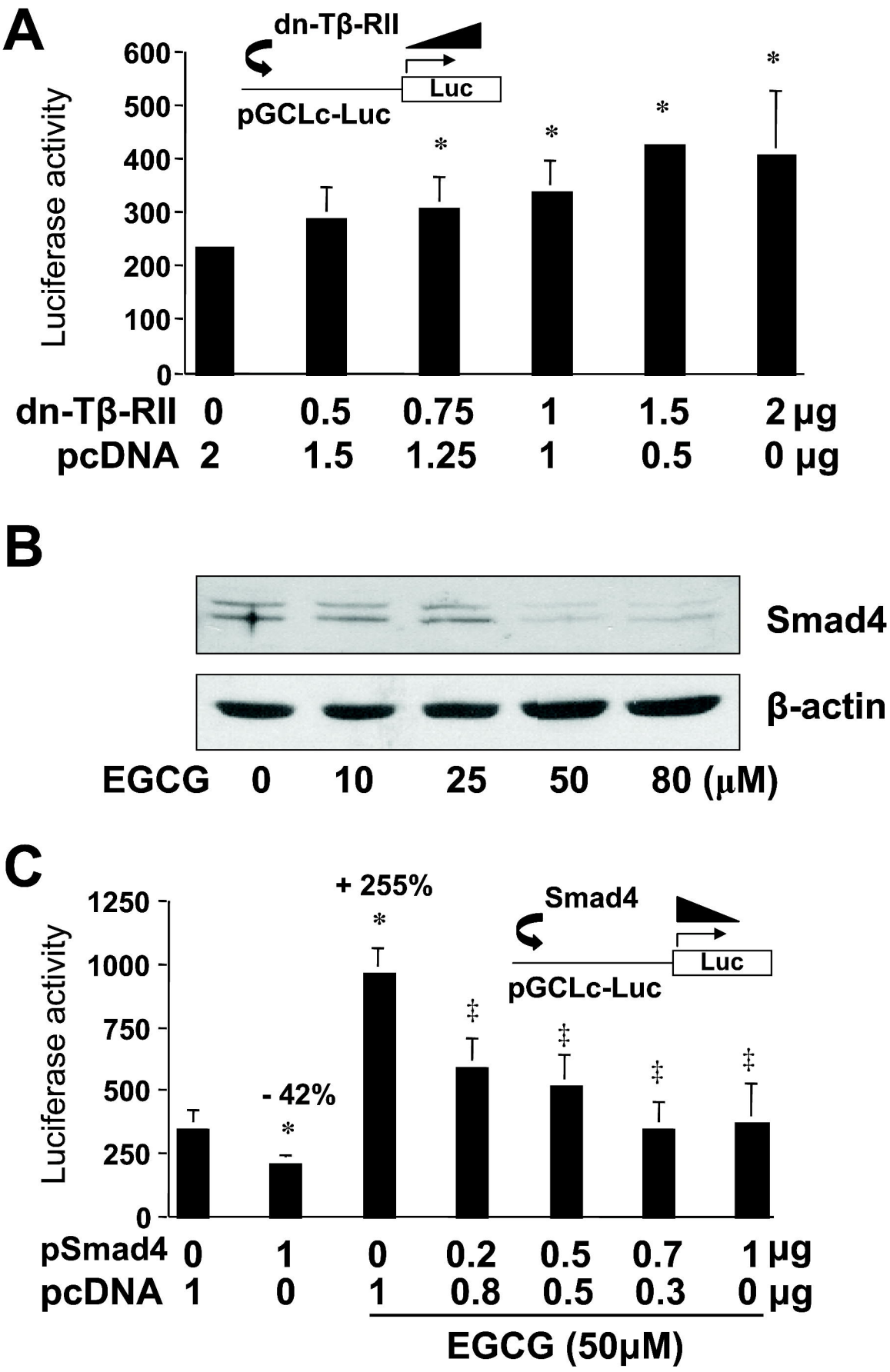

A
dn-Tβ-RII
Luc
pGCLc-Luc
Luciferase activity
600
500
400
300
200
100
0
dn-Tβ-RII 0 0.5 0.75 1 1.5 2 μg
pcDNA 2 1.5 1.25 1 0.5 0 μg
B
Smad4
β-actin
EGCG 0 10 25 50 80 (μM)
C
+ 255%
Smad4
Luc
pGCLc-Luc
Luciferase activity
1250
1000
750
500
250
0
- 42%
pSmad4 0 1 0 0.2 0.5 0.7 1 μg
pcDNA 1 0 1 0.8 0.5 0.3 0 μg
EGCG (50μM)

Fig. 7