(–)-Epigallocatechin-3-Gallate (EGCG) Post-transcriptionally and Post-translationally Suppresses the Cell Proliferative Protein TROP2 in Human Colorectal Cancer Cells

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Abstract. *Background: TROP-2 is a tumor-promoting molecule that has been found to be overexpressed in many cancer cells, making it a plausible biomarker of carcinogenesis. The main aim of this study was to examine the effect of green tea catechins (namely, (–)-epigallocatechin-3-gallate; EGCG) on TROP-2 expression. Materials and Methods: Western blot and RT-PCR were applied to assess TROP2 expression in colorectal cancer cells and tissues. Results: Two different mechanisms were found to operate in diverse cell lines. In SW480 cells, EGCG affected the post-transcriptional processing of the TROP-2 mRNA, as this was quickly and specifically degraded in the presence of EGCG. In HCT-116 cells, EGCG affected TROP-2 expression at the post-translational level. TROP-2 was found to be highly expressed in colorectal tumors compared to adjacent normal tissues. Conclusion: This study provided a novel beneficial activity of green tea as an anti-tumorigenagent causing the suppression of TROP-2 in colorectal cancer.*

TROP-2, as encoded by the tumor-associated calcium signal transducer (*TROP2/TACSTD2*) gene, is a monomeric cell surface glycoprotein that is highly expressed in many human carcinomas (1). It was originally identified in cytotrophoblast and plays a role at specific stages of epithelial cell differentiation (2). TROP2 is known to act as a cell surface receptor recognizing specific ligands and increasing intracellular calcium levels (3). Intracellular calcium regulates the cell cycle in proliferating cells (4) and also plays a role in the regulation of the differentiated function of several nucleated cell types (5). It has also been reported that protein kinase C phosphorylates TROP2 at serine 303 (6). Hence, TROP2 is thought to be a signal transducer playing a regulatory role in the growth of cancer cells (7) and also in morphogenesis (8).

TROP2 has been shown to have an apparent molecular weight of 57 kDa in non-reducing conditions. However, in reducing conditions, it shows two bands of similar intensity around 46 and 51 kDa, and an occasional band of 38 kDa in ovarian cancer cells, due to the disruption by reducing agents of a complex tertiary structure held together by disulphide bridges (1). In addition, Nakashima *et al*. demonstrated multiple polypeptides of TROP2 in esophageal cancer cells, due to post-translational modification or proteolytic processing (9). They also found TROP2 with 47 kDa and 47-62 kDa in esophageal cancer cells and normal cells, respectively. Thus, TROP2 is expressed as different sizes in a cell-specific manner and its biological function needs to be elucidated.

Recently, Guerra *et al*. detected a chimeric *cyclin D1–TROP2* mRNA in human ovarian and mammary cancer cells that was shown to act as a potent oncogene (10). TROP2 expression revealed anchorage-independent growth and tumorigenesis of various colorectal cancer cells (11), and shared homology with TROP1, which is already being used as a target for therapy in colorectal cancer. The antibody used against TROP2 *in vitro* can block the invasiveness of aggressive colon cancer cells (11). Thus, TROP2 is a cell proliferation biomarker and serves a potential target for chemotherapy, as it is present on the cell surface and can be made accessible for therapeutic purposes.

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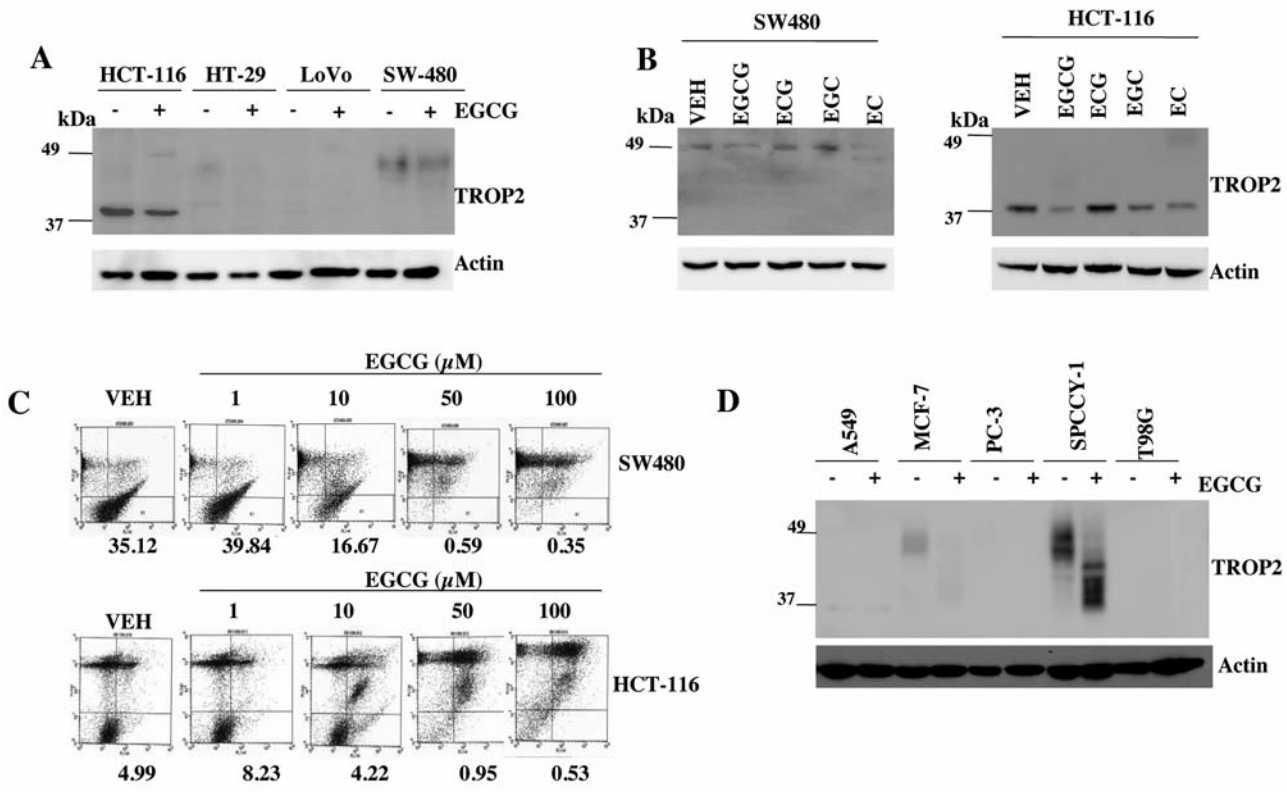

Figure 1. *Suppression of TROP2 by green tea catechins. A: Human colorectal cancer cells were treated with 50 μM of EGCG for 24 h, and Western blot was carried out for TROP2. Actin was used as a loading control. B: SW480 and HCT-116 cells were treated with 50 μM of four different catechins for 24 h, and cell lysates were subjected to TROP2 Western blot. C: SW480 and HCT-116 cells were treated with different doses of EGCG for 24 h, and cell pellets weres incubated with T-16 mAB overnight and subjected to FACS. The Y-axis indicates propium iodine, whereas the X-axis represents florescence. The percentage of positive cells is shown below each graph. D: Western blot analysis for human non-colorectal cancer cells treated with EGCG 50 μM for 24 h.*

Dietary agents such as green tea have long been explored for their medicinal values. (–)-Epigallocatechin-3-gallate (EGCG) is the major catechin found in green tea extract and has very potent antioxidant effects (12). It is becoming increasingly popular as a chemopreventive agent because of its advantage over the classic chemotherapeutic regimen, which produces severe side-effects. Recently, it was shown that EGCG affects angiogenesis by decreasing bFGF expression (13). This study focused on another EGCG target gene, *TROP2*, whose expression was suppressed in two different colorectal cells, HCT-116 and SW480 cells. This is the first study reporting that EGCG suppressed cell proliferator and growth stimulator TROP2.

Materials and Methods

Cell culture and reagents. Human colorectal and non-colorectal cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA), except for the head and neck cancer cell line, SPCCY-1, which was obtained from Dr. Dong M. Shin (Emory University, Atlanta, GA, USA). All cell lines were passaged in a humidified 5% CO_2 incubator at 37˚C. Human colorectal cancer cells were maintained as previously reported (14). The lung and prostate cancer cell lines, A549 and PC-3, respectively, were grown in Ham's F-12 media. The breast cancer and head and neck cancer cell lines, MCF-7 and SPCCY-1, respectively, were grown in DMEM, and the brain cancer cell line, T98G, was grown in EMEM. Primary antibodies for TROP2 and actin were obtained from R & D (Minneapolis, MN, USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), respectively. All the secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ, USA). The chemiluminescence kit for protein detection was obtained from Pierce (Rockford, IL, USA). The catechins EGCG, ECG, EGC, and EC were purchased from Sigma (St. Louis, MO, USA).

Western blot. The indicated cell lines were grown until 60-80% confluency in 6-cm plates, treated with indicated compounds and subjected to Western blot as previously described (15).

RNA isolation and RT-PCR. RNA extraction was carried out using a Qiagen (Valencia, CA, USA) kit, and cDNA was prepared with an I-script synthesis kit (Bio-Rad Laboratories, Hercules CA, USA). RT-PCR was carried out as previously described (15). The PCR primers used were as follows: human *TROP2*, forward (5'-

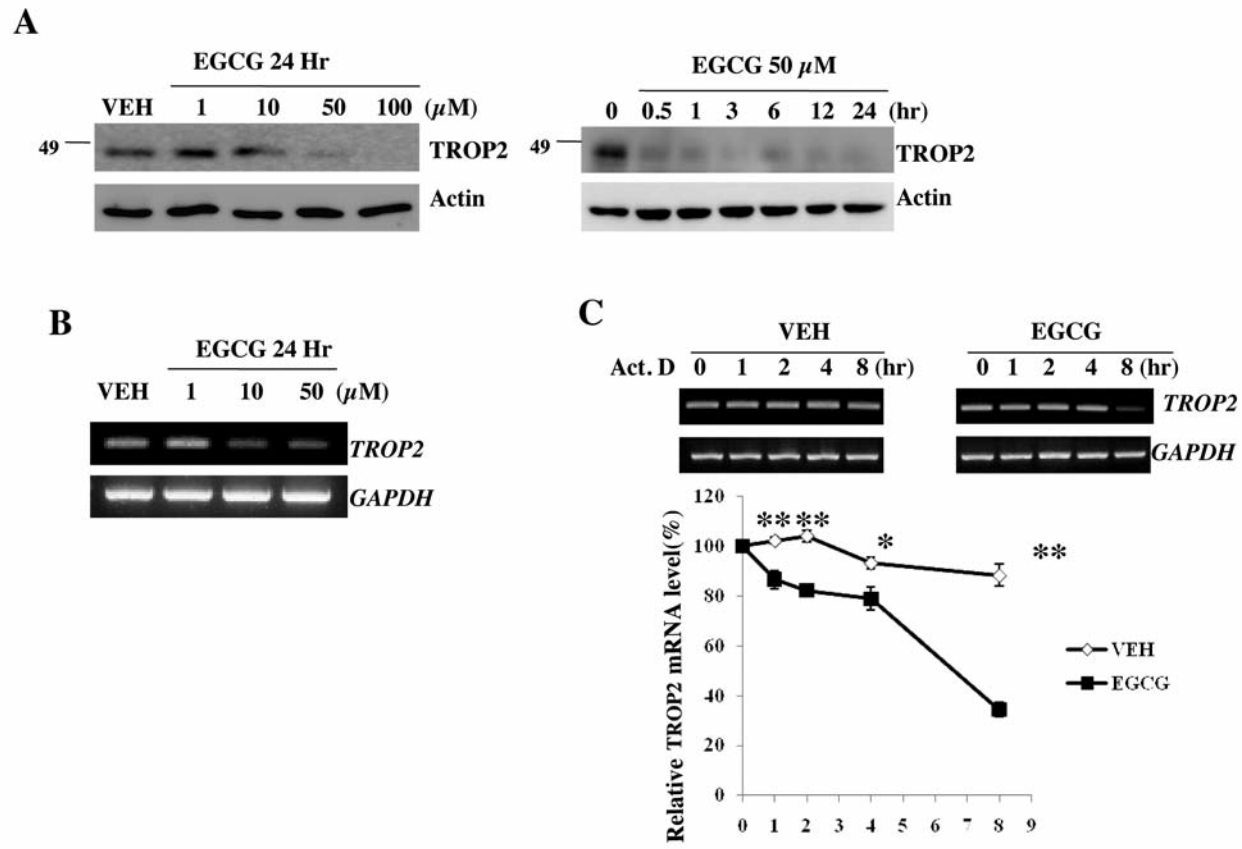

Figure 2. *Post-transcriptional regulation of TROP2 in SW480. A: Left panel: Cells were treated with 1, 10, 50 and 100 μM of EGCG and subjected to Western blot. Right panel: Cells were incubated with 50 μM of EGCG, and samples were harvested at different time points. B: RT-PCR for cells treated with different doses of EGCG. C: Top panel: Cells were incubated for 1 h with vehicle or EGCG at 50 μM and subsequently treated with actinomycin D (5 μM). At indicated time points, total TROP2 RNA was isolated and transcripts were measures by RT-PCR. Bottom panel: The relative level of TROP2 RNA was calculated, and the results were plotted as the percentage of the mRNA level present at time 0 of actinomycin D.*

ccccgcgcctc atccgcccgcgtc -3') and reverse (5'-caagctcggttcctttc tcaactc-3'). The *GAPDH* levels were used for the normalization of RNA using these primers: forward (5'-gggctgcttttaactctggt-3') and reverse (5'- tggcaggtttttctagacgc-3'). Thermal cycle settings were used as previously described (14). The intensity of each band was quantified using Scion Image Software (Scion, Frederick, MD, USA). *GAPDH* was used to normalize the *TROP2* intensity values.

mRNA stability. For the mRNA stability experiment, HCT-116 and SW480 cells were grown in 6-cm plates and then pretreated with DMSO and EGCG for 1 h. Actinomycin D (5 μM; Fisher Bioreagents, Fairlawn, NJ, USA) was added. Total RNA was isolated and RT-PCR was carried out as mentioned above.

Cycloheximide study. HCT-116 and SW480 cells were grown in 6-cm plates until 60-80% confluency, and then pretreated with both DMSO and EGCG for 18 h and 30 min, respectively. The time for pretreatment was decided based on the results obtained for time course, the time at which EGCG starts suppressing the expression of TROP2. Pretreatment was then followed by treatment with cycloheximide (10 μg/ml) for 1, 3, 6, 12 and 24 h. Proteins were harvested using RIPA buffer, and Western blot was carried out as mentioned before (14).

FACS analysis. HCT-116 and SW480 cells were plated in 6-cm plates and treated with EGCG 1, 10, 50 and 100 μM for 24 h. The cells were then trypsinized, and McCoy and RPMI complete media were added. The cells were gently collected and centrifuged at 1,000 rpm for 5 min at 4˚C. The supernatant was removed and cell pellets were washed with FACS buffer containing ice-cold 1× PBS and 1% BSA. FITC-T16 mAb for TROP2, which was previously reported (1), was added to the cell pellets at 5 μg overnight. The next day, the cells were washed with FACS buffer twice and probed with 1 μg FITC-conjugated secondary antibody for 30 min on ice. After washing, the cells were analyzed by flow cytometry after staining with 0.5 μg propidium iodide solution using Becton Dickinson FACs Vantage SE (San Jose, CA, USA), which has an air-cooled 488 Spectra laser and a 647 Innova 70C laser.

Statistical analysis. Statistical analysis was carried out using Student's *t*-test to analyze differences between samples. SAS for Windows (9.1.3) (SAS Institute Inc., Cary, NC, USA) statistical

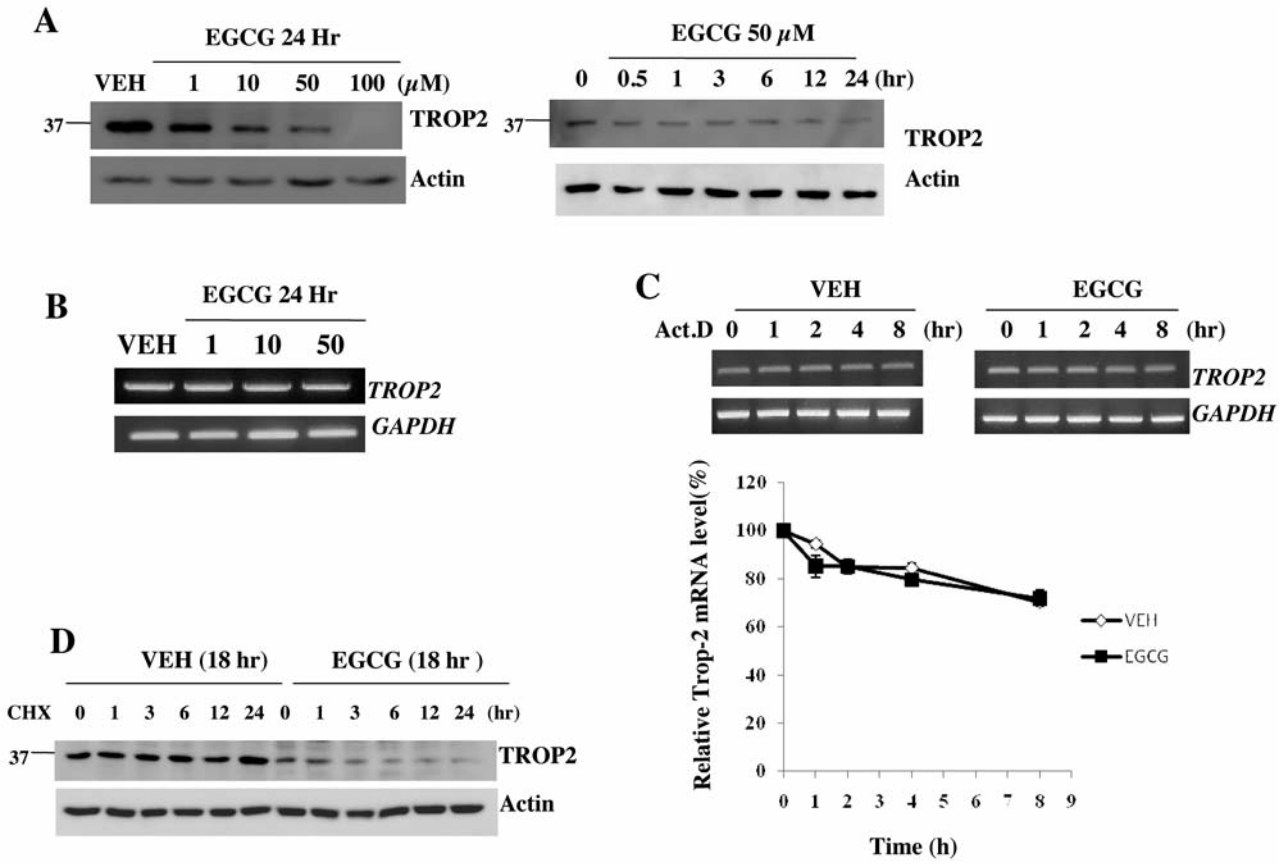

Figure 3. *Post-translational regulation of TROP-2 in HCT-116 cells. A: Cells were treated with EGCG at different doses and for different times. B: Cells were treated with different doses of EGCG for 24 h, and TROP2 transcripts were measured by RT-PCR. C: The mRNA stability assay was carried out using actinomycin D as mentioned above. D: Effect of EGCG on TROP-2 protein degradation. Cells were treated with either vehicle or EGCG for 18 h, followed by cycloheximide treatment, and cell lysates isolated at different time points were subjected to TROP-2 and actin Western blot.*

analysis software was used. A p-value of less than 0.05 was considered significant and represented with one asterisk. Similarly, two and three asterisks were used to show $p<0.01$ and $p<0.001$, respectively.

Results

Effects of EGCG on TROP2 expression in human colorectal cancer cells. Since TROP2 is a tumor-promoting protein, it was first examined whether its expression is affected by EGCG on four different colorectal cancer cells: HCT-116, HT-29, LoVo and SW480. As shown in Figure 1A, HT-29 and LoVo cells had a very low expression of TROP2, whereas HCT-116 and SW480 cells showed significant expression. Interestingly, HCT-116 showed a band around 37 kDa, and SW480 cells had a band around 49 kDa (Figure 1A). These different sizes of TROP2 were previously reported by others in different cells (1, 9). Since HCT-116 and SW480 cells abundantly express TROP2 and could efficiently reveal possible inhibition by EGCG, these two cells were used for further study. The study also examined whether EGCG can lower the expression of TROP1, which is a homolog of TROP2 in human colorectal cancer cells, and found that EGCG had an effect similar to that observed in TROP2 (data not shown). Next, it was investigated whether other catechins affect TROP2 suppression. HCT-116 and SW480 cells were treated with 50 μM of EGCG, ECG, EGC and EC for 24 h, and EGCG was found to be more effective among other catechins in both cell lines (Figure 1B). TROP2 suppression by EGCG was confirmed using FACS analysis. As shown in Figure 1C, EGCG decreased the number of cells in the TROP2-positive population. Finally, the non-colorectal cancer cells A549, MCF-7, PC-3, SPCCY-1 and T98G were investigated in order to examine whether EGCG affects TROP2 expression in these cell types. PC-3 and T98G cells did not show TROP2 expression; however, MCF-7 and SPCCY-1 showed expression similar to SW480 (~49

Figure 4. *A: TROP2 mRNA transcripts in human normal and tumor tissues detected by RT-PCR. Fold tissue change of tumor over normal tissue is given under the figure. B: Western blot analysis for TROP2 protein in normal and tumor human tissue samples. Fold tissue change of tumor over normal tissue is given under the figure. *No value calculated, as there was no band in the normal lane.*

kDa), and A549 showed protein around 37 kDa. Thus, EGCG reduced TROP2 expression not only in colorectal cancer cells but also in lung, breast and oral cancer cells, (Figure 1D).

Post-transcriptional regulation of TROP2 in SW480 cells. SW480 cells were treated with different doses of EGCG for 24 h and at different times with 50 μM EGCG. As shown in Figure 2A, TROP2 was detected around 49 kDa, and its expression was reduced by EGCG at 50 μM and within 30 min. Next, the effect of EGCG on mRNA expression of the *TROP2* gene was examined. EGCG reduced *TROP2* mRNA in a dose-dependent manner (Figure 2B). Further, the potential mechanism responsible for inhibition of *TROP2* mRNA by EGCG was investigated. To determine the effects of EGCG on *TROP2* mRNA stability, the half-life of the mRNAs were first determined by adding EGCG (50 μM) to SW480 cells for 1 h and then treating them with actinomycin D for different time-treated cells. After treatment, mRNA was isolated at 0, 1, 2, 4 and 8 h and subjected to RT-PCR. As shown in Figure 2C, the half-life of *TROP2* mRNA was affected by EGCG, compared to that of the vehicle-treated cells. These results suggested that EGCG may affect *TROP2* mRNA at the post-transcriptional level.

Translational regulation of TROP2 in HCT-116 cells. HCT-116 cells were also treated with EGCG, and TROP2 expression began to decrease with EGCG at 1 μM, but not as quickly as seen in SW480 cells (Figure 3A). Interestingly, the *TROP2* mRNA was not affected by EGCG in HCT-116 cells (Figure 3B). This was supported by the mRNA stability experiments as shown in Figure 3C. Thus, it was postulated that EGCG may affect the protein degradation of TROP2. It is known that EGCG affects the protein degradation pathway in human colorectal cancer LoVo cells (13). HCT-116 cells were pre-treated with DMSO (vehicle) or EGCG for 18 h. The time for pretreatment was selected based on the time course experiment (Figure 3A, right panel). Cells were then co-treated with cycloheximide for 1, 3, 6, 12 and 24 h. EGCG caused rapid degradation of TROP2 protein in the presence of EGCG (Figure 3D), compared to vehicle-treated samples. This result suggested that EGCG affects TROP2 expression at the post-translational level in HCT-116 cells.

Expression of TROP2 in human colorectal samples. Finally, *TROP2* expression in human normal and tumor colon tissue samples obtained from patients were examined and it was found that *TROP2* mRNA and protein expression was higher in tumor tissue compared to normal tissue (Figure 4A and B). These data further supported the role of TROP2 in colorectal tumorigenesis and its use as a chemoprevention target in man.

Discussion

TROP2 has recently been exploited as a therapeutic target because it is highly expressed in most tumors (7), and its presence correlates with higher aggressiveness and distant recurrence of colorectal cancer (16). TROP2 contains an EGF-like repeat and a thyroglobulin repeat (17), involved in IGF-II binding by serum proteins (18). However, no regulatory mechanisms by dietary compounds have been reported. Dietary agents are important in the pathogenesis and prevention of cancer (19), especially in gastric and colorectal cancer as their activity is maximized in the gut (20). Dietary agents also have fewer or no side-effects, which make them more desirable than the classic chemotherapeutic regimen (21).

Green tea acts as a potent anticancer agent, and most of the beneficial effects of green tea are attributed to its active and most abundant catechin, EGCG. The green tea component EGCG can induce apoptosis and cell cycle arrest in cancer cells (22, 23). Other effects of EGCG include inhibition of cyclooxygenase and lipoxygenase (24),

inhibition of angiogenesis (13), inhibition of activator protein-1 (25), activation of p53 tumor suppressor (26), and inhibition of telomerase activity (27). This report showed that EGCG suppresses TROP2 expression in colorectal cancer cells. The strongest suppression was found in the most highly expressing cells, *i.e.* HCT-116 and SW480 cells (Figure 1A), supporting the relevance of the EGCG effects in human cancer cells. HCT-116 and SW480 cells showed two different band sizes of ~37 kDa and ~49 kDa for the TROP2 protein, respectively (Figure 1A). Different sizes of TROP2 have been previously reported in other cell types. For example, TROP2 was immunoprecipitated as a glycosylated cell surface protein in human ovarian cancer cell line OVCA-432 with two bands around 50-60 kDa (1). In addition, a 37 kDa band was also detected in TROP2 transfected cells. This led to the speculation that these differently sized proteins are encoded from the same gene, probably due to different post-translational modifications such as proteolytic processing and differential glycosylation. The different sizes of TROP2 expressed are dependent on cell types, since a similar pattern was observed in other cancer cells; lung cancer cell line A549 showed a band at ~37 kDa, and breast cancer MCF-7 and head and neck cancer SPCCY-1 cell lines showed a band at ~49 kDa (Figure 1D). Thus, modification of TROP2 occurs in different cell types and the biological activity of the modified TROP2 remains to be elucidated.

Using the T-16 monoclonal antibody (1), FACS analysis was performed in SW480 and HCT-116 cells and it was found that EGCG can reduce TROP2 expression in a dose-dependent manner (Figure 1C). EGCG reduce TROP2 expression in both dose- and time-dependent manners in SW480 and HCT-116 cells (Figure 2A and 3A, left and right panel). In SW480 cells, the dose of 50 μM showed a reduction of TROP2 protein expression; the decrease was seen as early as 30 min and complete suppression by 12 to 24 h (Figure 2A, left and right panel). However in HCT-116 cells, the dose of 1 μM began to reduce TROP2 expression and abolished it at 100 μM (Figure 3A, left panel). It started showing a decrease in 30 min, but required a longer time of 24 h to abolish its expression (Figure 3A, right panel). It was speculated that this may be related to a differential stability in different cell lines. TROP2 was, indeed, found to be more stable in HCT-116 cells than in SW480 cells. *TROP2* mRNA expression was reduced in SW480 cells by EGCG in a dose-dependent manner (Figure 2B); however, EGCG was unable to reduce the *TROP2* mRNA in HCT-116 cells (Figure 3B). This result led to the speculation that EGCG reduces the expression of TROP2 in SW480 and HCT-116 cells through two different mechanisms. To confirm this, first an mRNA stability assay was checked in SW480 and HCT-116 cells. Indeed, it was recently shown that EGCG affects mRNA stability of the *NUDT6* gene in human colorectal cancer cells (14). Similarly, EGCG affected mRNA stability of *TROP2* in SW480 cells (Figure 2C). However, EGCG did not affect mRNA stability in HCT-116 cells (Figure 3C, top panel) and *TROP2* mRNA was stable even after 50 μM of EGCG treatment as seen in the vehicle treatment (Figure 3C, bottom panel). Then it was examined whether EGCG affects *TROP2* at the translational level in HCT-116 cells by pre-treating HCT-116 cells with EGCG 50 μM for 18 h. The selection of this time point was based on the time course experiment (Figure 3A, right panel). Cells were then treated with the protein synthesis inhibitor cycloheximide, and the time required for EGCG to reduce TROP2 protein was checked in comparison to vehicle treatment. EGCG reduced TROP2 expression as early as 3 h, whereas TROP2 was very stable in the presence of cycloheximide in the vehicle-treated samples (Figure 3D). These data suggested that EGCG affects the TROP2 protein degradation pathway in HCT-116 cells. Finally, normal and tumor human colorectal tissue samples were examined to determine whether TROP2 is expressed differently in normal and tumor tissues. The *TROP2* mRNA and protein were both highly expressed in tumor tissue with little or no expression in normal tissue. TROP2 protein was detected at around 49 kDa (Figure 4A and B, top panels), as shown in SW480 cells. This was a result similar to a previous report demonstrating that transformed keratinocytes show two bands at 42 and 45-55 kDa (28).

In summary, the findings of the present study clearly demonstrated that the green tea component EGCG has a suppressive effect on oncogenic biomarkers, including TROP2. As human colorectal cancers were found to correspondingly express high levels of TROP2 *in vivo*, this study indicates the potential utility of EGCG in colorectal cancer patients with chemopreventive and/or adjuvant purposes.

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