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Cyclin D1 degradation and p21 induction contribute to growth inhibition of colorectal cancer cells induced by epigallocatechin-3-gallate

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Abstract

Purpose—The public has paid attention to green tea due to its health benefits.

Epigallocatechin-3-gallate (EGCG), the major component of green tea, is well documented to induce apoptosis and cell cycle arrest in cancer cells by targeting multiple signal transduction pathways. However, the detailed mechanism(s) of action needs to be determined.

Methods—Cell growth was evaluated by MTT assay, cell cycle analysis, and caspase 3/7 activity. Protein expression was analyzed through Western blotting. Reverse transcription polymerase chain reaction was used for examining mRNA expression of p21 and cyclin D1. The promoter activity of p21 was assessed by the luciferase reporter system.

Results—We identified cyclin D1 and p21 as molecular targets of EGCG in human colorectal cancer cells. We observed that cyclin D1 was down-regulated, while p21 expression was up-regulated by EGCG in dose- and time-dependent manners. Furthermore, we found EGCG decreased cyclin D1 protein stability, therefore triggering ubiquitin-dependent proteasomal degradation. Meanwhile, EGCG increased p21 promoter activity, resulting in up-regulation of p21 mRNA and protein, which was likely dependent on extracellular-signal-regulated kinase (ERK), inhibitor of nuclear factor kappa-B kinase (IKK) and phosphoinositide 3-kinase (PI3 K).

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Conflict of interest None.

Conclusion—The data presented here details a novel mechanism by which EGCG inhibits cell growth of colorectal cancer cells. Namely, EGCG-induced cyclin D1 degradation and p21 transcriptional activation partially contribute to growth suppression in these cells.

Keywords

EGCG; Cyclin D1; Proteasome; p21; Colorectal cancer

Introduction

Green tea is an ancient beverage and one of the most popular in the world. Compared with other teas, green tea has a higher catechin concentration, which is mainly attributed to its health-promoting effect (Cabrera et al. 2006). Green tea catechins have been studied extensively for their potential anti-tumorigenic activity. While catechins reportedly have diverse effects on lung cancer risk (Le Marchand et al. 2000), they have been consistently shown to reduce the risk of colon cancer. One of these catechins, epigallocatechin-3-gallate (EGCG) can be found at substantial levels in the colonic mucosa of patients (Baek et al. 2004; Ju et al. 2005; Yang et al. 2000). As the most abundant and active catechin, EGCG has been documented to induce apoptosis and cell cycle arrest in different types of cancer, including colorectal cancer (Ahmad et al. 2000; Hwang et al. 2007; Thangapazham et al. 2007). Furthermore, EGCG inhibits intestinal tumorigenesis in *Apc^{min/+}* mice (Ju et al. 2005; Sukhthankar et al. 2008). Potential mechanisms by which EGCG exerts anti-cancer effects include inhibition of receptor tyrosine kinase activity (Shimizu et al. 2005a, b, c), down-regulation of cyclooxygenase-2 and activator protein-1 (Jeong et al. 2004; Shimizu et al. 2005b), activation of p53 tumor suppressor (Hastak et al. 2003), and suppression of telomerase activity (Berletch et al. 2008; Yang et al. 2007, 2009). In addition, EGCG might play anti-angiogenic roles by inhibiting the expression of vascular endothelial growth factor (VEGF) (Shimizu et al. 2010) and basic fibroblast growth factor (bFGF) (Sukhthankar et al. 2008). Thus, EGCG has been considered a potential chemopreventive/therapeutic agent because it targets multiple signaling pathways.

Components of cell cycle machinery are frequently deregulated in the development of a tumor, and this deregulation is mainly attributed to overexpression of cyclins and loss of expression of cyclin-dependent kinases (CDKs) inhibitors in malignant cells. Cyclin D, a well-characterized oncogenic protein related to the cell cycle (review in Musgrove et al. 2011), forms an active complex with CDK4/6, and this complex phosphorylates and inactivates retinoblastoma protein (pRb), therefore serving as a key regulator of progression from the G1 to S phase of the cell cycle. More recently, cyclin D1 has also been identified as a component of DNA repair machinery in human cancer cells, which provides novel evidence for cyclin D1-oriented therapy, especially in retinoblastoma-negative cancers (Jirawatnotai et al. 2011). On the other hand, p21 (also known as p21^{WAF1/Cip1}), a key transcriptional target of p53, causes G1 cell cycle arrest (Waldman et al. 1995) and inhibits the activity of CDK2 and CDK1 (Satyanarayana et al. 2008). In addition, p21 binds to and inhibits proliferative cell nuclear antigen (PCNA), thereby suppressing progressive DNA replication and modulating PCNA-dependent DNA repair system (Waga et al. 1994). Given the important roles in cell cycle regulation and DNA repair pathways, targeting cyclin D1 and p21 expression by cancer chemopreventive agents seems promising for cancer therapy.

EGCG down-regulated cyclin D1 and up-regulated p21 expression has been reported in several different cancer cells (Ahmad et al. 2000; Gupta et al. 2003; Liang et al. 1999). However, the underlying molecular mechanism remains to be investigated. A number of chemotherapeutic agents have also been identified to facilitate cyclin D1 degradation by the ubiquitin-dependent proteasome pathway (review in Alao 2007). Moreover, recent evidence

shows that p21 plays a vital role in the anti-proliferative effect of EGCG in HCT116 cells (Thakur et al. 2010).

In the present study, we assessed the effect of EGCG on cell cycle regulatory protein expression in different colorectal cancer cells and found cyclin D1 and p21 as major targets of EGCG. We found that EGCG facilitated the proteasomal degradation of cyclin D1 at the post-translation level, and up-regulated p21 mRNA and protein expression, by increasing its promoter activity in SW480 cells. Furthermore, extracellular-signal-regulated kinases (ERKs), inhibitor of nuclear factor kappa-B kinase (IKK), and phosphoinositide 3 kinase (PI3 K) might be involved in p21 up-regulation by EGCG. Our data suggest that cyclin D1 degradation and up-regulation of p21 contribute to the anti-proliferative effect of EGCG on colorectal cancer cells.

Materials and methods

Cell lines and reagents

All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). HCT-116 and HT-29 were cultured in McCoy's 5A medium (Mediatech, Manassas, VA). SW480 and Caco-2 cells were maintained in RPMI1640 medium (Mediatech) and DMEM (Invitrogen, Carlsbad, CA, USA), respectively. Cells were cultured at 37 °C under a humidified atmosphere of 5 % CO₂. All media were supplemented with 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. EGCG and epicatechin gallate (ECG) were generously provided by Mitsui Norin (Tokyo, Japan). Epigallocatechin (EGC), epicatechin (EC), and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). Antibodies for cyclin D1, cyclin D3, cyclin E, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); p21 primary antibodies were from Cell Signaling Technology (Beverly, MA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Plasmid construction, mutagenesis, and transient transfection

The pRcCMV-cyclin D1-HA plasmid (wild type form and K33-269R mutation) was provided by Dr. E. Dmitrovsky (Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH). GSK3β tagged by V5 epitope expression vector was previously described (Lee et al. 2010). Cyclin D1 transversions from threonine to alanine at 286 amino acid position, constitutively active mutation (S9A), and kinase-inactive mutation (K85 M) of GSK3β were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). SW480 cells were transfected with indicated vectors using PolyJet reagent (SignaGen, Rockville, MD, USA) according to the manufacturer's protocol.

Cell proliferation assay

Cell proliferation assay was performed according to the manufacturer's instruction (Promega, Madison, WI). Briefly, SW480 cells were seeded in 96-well culture plates and then treated with 0, 1, 10, or 50 µM EGCG in media with 1 % FBS for 0, 2, or 4 days. To measure cell proliferation, 20 µL CellTiter 96 Aqueous One Solution was added to each well, followed by incubation for 1 h at 37 °C. Absorbance (490 nm) was compared with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT).

Cell cycle analysis

SW480 cells were plated in 60-mm tissue culture dishes overnight and treated with 0, 1, 10, and 50 µM EGCG for 48 h in the presence of media containing 10 % FBS. The attached and floating cells were harvested together and washed with phosphate-buffered saline (PBS) solution. Cells were fixed in 70 % ethanol at -20 °C overnight and centrifuged for 5 min at

1000×g. After removing supernatant, cells were washed with PBS with decreased gradient ethanol (50, 20, and 0 %) and then stained with propidium iodide (PI, 70 μM) solution containing RNase A (1 mg/mL). Cell cycle phase distribution was determined on a Beckman Coulter Epixs XL flow cytometer equipped with ModFit LT software.

Caspase 3/7 activity assay

Enzyme activity of caspase 3/7 was analyzed by Apo-ONE Homogenous Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's protocol. Briefly, cells were harvested into RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS) with a 1 % protease inhibitor mixture (EMD Biosciences, La Jolla, CA, USA) and phosphatase inhibitor (1 mM Na₃VO₄, 1 mM NaF). Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA) using BSA as a standard. Cell lysates from SW480 cells (30 μg protein) were mixed with the same volume of caspase-Glo 3/7 reagent in 96-well plates and incubated for 1 h at room temperature in the dark. Luminescence was measured using a FLX800 microplate reader (BioTek).

Promoter activity assay

SW480 cells were plated in 12-well plates (2 × 10⁵ cells/well) for 24 h. Then, the human full-length p21 promoter-luciferase reporter construct pWWP and 0.01 μg of pRL-null vector were co-transfected by PolyJet transfection reagent (SignaGen) according to the manufacturer's protocol. After transfection overnight, cells were treated with DMSO or EGCG (50 μM) in serum-free media for 24 h and then harvested in 1 × passive lysis buffer (Promega). Luciferase activity was measured using DualGlo Luciferase Assay Kit (Promega) according to the manufacturer's instruction.

Reverse transcription polymerase chain reaction

Total RNA of SW480 cells treated by DMSO and EGCG was isolated by E.Z.N.A Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. One μg RNA was reverse transcribed using Verso cDNA synthesis Kit (Fisher Scientific, Pittsburgh, PA). PCR was performed using GoTaq Green Master Mix PCR Mixture (Promega) with primers for human *cyclinD1*, *p21*, and *GAPDH* as follows: cyclin D1, forward 5'-ATGGAACACCAGCTCCTGTGCTGC-3' and reverse 5'-TCAGATGTCCACGTCCCGCACGT-3'; p21, forward 5'-GCGACTGTGATGCGCTAAT-3' and reverse 5'-TAGGGCTTCCTCTTGGAGAA-3'; GAPDH, forward 5'-GGGCTGCTTTTA ACTCTGGT-3' and reverse 5'-TGGCAGGTTTTCTAGACGG-3'.

Western blot analysis

Cells were washed with PBS, and cell lysates were obtained as mentioned above. Protein (30 μg) was combined with an equal amount of 2XSDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and boiled for 5–10 min. After electrophoresis, proteins were transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN) which were subsequently blocked with TBS containing 0.1 % Tween 20 (TBST) and 5 % nonfat milk for 1 h at room temperature. Then, the membranes were incubated with primary antibodies diluted with TBST-nonfat milk (1:1000) at 4 °C overnight. After three washes with TBST, the membranes were incubated with peroxidase-conjugated IgG in TBST-nonfat milk (1:5000) for 1 h at room temperature and then washed with TBST three times for 10 min each. The immunoblots were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and quantified by Scion Image Software (Scion, Frederick, MD, USA).

Statistical analysis

Statistical analysis was performed with the Student's unpaired *t* test. Results were considered statistically significant with a *P* value below 0.05.

Results

EGCG inhibits cell growth and induces cell cycle arrest

The most abundant catechin in green tea is EGCG (Fig. 1a). To investigate the effect of EGCG on colorectal cancer cell growth, SW480 cells were treated with EGCG at different doses and time points. As shown in Fig. 1b, cell growth was significantly inhibited by EGCG in a dose-dependent manner. Since cell cycle arrest and apoptosis are closely related to cell growth, we next measured cell cycle and caspase 3/7 activity in the presence of EGCG. Consistent with cell growth inhibition, EGCG dose-dependently induced G1-phase arrest (Fig. 1c) and significant activation of caspase 3/7 (Fig. 1d).

Effect of EGCG on the expression of protein related to cell cycle

To elucidate the molecular mechanism of cell cycle arrest induced by EGCG, we analyzed the expression level of known cell cycle regulators in colorectal cancer cells. As shown in Fig. 2a, cyclin D1 was decreased following EGCG treatment in a dose- and time-dependent manner; down-regulation was observed in both cytoplasm and nucleus (Fig. S1). A similar result was obtained for cyclin D3, but not cyclin E, in SW480 cells. On the other hand, p21 level was dramatically elevated after exposing cells to EGCG for 24 h. To investigate whether EGCG alters the expression of these proteins in other colorectal cancer cells, HCT-116, HT-29, and Caco-2 cells were treated with 50 μ M EGCG for 24 h. EGCG consistently down-regulated cyclin D1 and up-regulated p21 expression in all the colorectal cancer cells tested (Fig. 2b); however, other proteins tested showed inconsistent expression patterns. Moreover, compared with the other catechins, EGCG maximized the down-regulation of cyclin D1 and up-regulation of p21 expression in both HCT116 and SW480 cells (Fig. 2c). These data suggested that the reduction of cyclin D1 and p21 expression could play a key role in growth inhibition of human colorectal cancer cells induced by EGCG and prompted us to study the detailed mechanism.

EGCG suppresses post-translational expression of cyclin D1

To further understand the underlying mechanism by which EGCG decreased cyclin D1 protein expression, the mRNA level of cyclin D1 was measured at different time points in SW480 cells. No difference was observed in cyclin D1 transcripts up to 24 h after treatment (Fig. 3a). We then asked whether cyclin D1 protein stability was affected by exposure to EGCG. As shown in Fig. 3b, a shortened half-life of cyclin D1 protein in EGCG-treated cells was observed compared to that in DMSO-treated cells. These results indicate that EGCG down-regulates cyclin D1 protein expression at the post-translational level. We therefore asked whether EGCG triggered 26S proteasome-mediated degradation of cyclin D1. Fig. 3c shows that both MG132 and epoxomicin, two well-known proteasome inhibitors, completely blocked cyclin D1 down-regulation in the presence of EGCG, which was dose-dependent (Fig. S2). The same results were also observed in HCT116 cells (data not shown).

EGCG-induced cyclin D1 degradation is threonine-286 phosphorylation independent and requires ubiquitination

It has been well characterized that GSK3 β mediates cyclin D1 phosphorylation at threonine 286 position, which plays a key role in the nuclear export of cyclin D1 and the subsequent ubiquitin-dependent proteolysis (Diehl et al. 1998). However, we showed that GSK3 β

inhibitor LiCl did not restore cyclin D1 expression in the presence of EGCG (Fig. 4a). Further, overexpression of GSK3 β constructs (active and inactive form) did not alter cyclin D1 degradation (Fig. 4b). Thus, EGCG-induced cyclin D1 degradation is independent of GSK3 β . As expected, expression of exogenous wild type cyclin D1 was decreased by EGCG; however, the construct containing T286A did not block cyclin D1 degradation (Fig. 4c). However, another mutant construct (lysine-less mutation, K33-269R) (Feng et al. 2007) restores cyclin D1 expression in the presence of EGCG, indicating that EGCG-induced cyclin D1 degradation requires ubiquitination, independent of GSK3 β .

Up-regulation of p21 expression induced by EGCG involves ERK and IKK

In contrast to cyclin D1 regulation, EGCG dramatically up-regulated and then stabilized the mRNA expression of p21 from 6 to 24 h (Fig. 3a). Furthermore, we examined the promoter activity of p21 after 24 h exposure to EGCG. As shown in Fig. 5a, compared to DMSO treatment, there was a 1.5-fold increase in luciferase activity induced by EGCG. To further investigate the kinase pathways involving in the effect of EGCG on p21 expression, we treated SW480 cells with EGCG and different kinase inhibitors. Compared with vehicle control, ERK (PD98059) and IKK (BAY11-7082) inhibitor completely blocked the up-regulation of p21 expression by EGCG, and PI3 K inhibitor (LY294002) had a marginal effect on p21 expression (Fig. 5b).

Discussion

Cancer can develop from stabilization of oncogenic proteins or, alternatively, from destabilization of tumor suppressors. Cyclin D1, a well-known cell cycle regulatory protein serving as an allosteric activator of CDK4/6, is demonstrated to be aberrantly overexpressed in cancer cells or tumor (Musgrove et al. 2011). Although overexpression of cyclin D1 alone is not sufficient to induce transformation, existing evidence indicates that the nuclear retention of cyclin D1 possesses an oncogenic capacity (Lu et al. 2003). On the other hand, p21 functions as a main mediator of p53 tumor suppressor activity (reviewed in Abbas and Dutta 2009). Loss of p21 expression promotes genomic instability, therefore contributing to human malignancy. In addition, either cyclin D1 down-regulation or the increase in p21 expression contributes to the inhibition of cell cycle progression, resulting in growth suppression of colorectal cancer cells (Arber et al. 1997, 1998).

Here, we observed that EGCG halted SW480 cell growth based on the reduction of cyclin D1 and induction of p21 expression, which was also confirmed in the other colorectal cancer cell lines. These data are consistent with previous reports, indicating that EGCG alters the expression of cyclin D1 and p21 in different cancer cells (Ahmad et al. 2000; Hwang et al. 2007). Although caspase activity was dose-dependently induced by EGCG (Fig. 1d), we did not observe significant apoptotic cells or cleaved poly (ADP-ribose) polymerase (PARP) (a well-known marker of apoptosis) after 24 h treatment (data not show). One explanation is that p21-mediated cell cycle arrest could pro-empt and protect colorectal cancer cells from EGCG-induced apoptosis (Thakur et al. 2010). A similar mechanism could apply to SW480 cells, since we observed p21 induction at the early time points. Furthermore, we compared the effect of different catechins on cyclin D1 and p21 in both HCT116 and SW480 cells and observed a more pronounced decrease in cyclin D1 and increase in p21 expression in the presence of EGCG (Fig. 2c). These results provided extra evidence for the anti-cancer effect of EGCG in colorectal cancer.

The ubiquitin–proteasome pathway plays a central role in the degradation of cell cycle regulators, including cyclin D1. A number of compounds have been reported to induce cyclin D1 degradation (Alao 2007). Although there is no report of EGCG's effects on cyclin D1 degradation by the ubiquitination–proteasome pathway, there are several reports

regarding how EGCG affects the 20S proteasome (Baumeister et al. 1998; Nam et al. 2001; Pettinari et al. 2006). Nam et al. (2001) showed that an ester bond in EGCG potently and specifically inhibits the chymotrypsinlike activity of the proteasome in vitro and in vivo, resulting in the accumulation of p27^{Kip1} and I κ B- α . On the other hand, we recently identified that the bFGF angiogenic factor was down-regulated by proteasome pathways in the presence of EGCG due to increased trypsin-like activity of the 20S proteasome (Sukhthankar et al. 2008). These data indicate that EGCG may affect the 20S proteasome complex depending on the substrate and cell context, thereby leading to oncogenic protein degradation or stabilization of potential tumor suppressor.

Findings reported here first revealed that EGCG regulated cyclin D1 expression at the post-translational level. EGCG-induced cyclin D1 degradation is dependent on proteasome activity, as seen in the restoration of cyclin D1 by MG132 and epoxomicin (Fig. 3c). Cyclin D1 down-regulation may be not from the caspase activity induced by EGCG, since caspase inhibitor did not restore cyclin D1 down-regulation in the presence of EGCG (Fig. S3). It is not clear why all the proteasomal inhibitors do not exhibit cyclin D1 restoration. YU102 inhibitor was developed to selectively target peptidyl-glutamyl peptide-hydrolyzing (PGPH) activities of proteasome (Myung et al. 2001), and lactacystin exhibited less efficiency compared with MG132 and epoxomicin in terms of inhibiting protein degradation (Alao et al. 2006; Meng et al. 1999). Since EGCG could simultaneously increase and decrease specific proteasomal activity (Pettinari et al. 2006; Sukhthankar et al. 2008), the molecular mechanism by which specific proteasomal inhibitors affect EGCG-induced cyclin D1 regulation needs to be elucidated. It is well known that phosphorylation of threonine-286 within the PEST domain of cyclin D1 triggers its nuclear export and subsequently ubiquitination and degradation (Diehl et al. 1997). GSK-3 β has been well documented to phosphorylate threonine-286 of cyclin D1, therefore facilitating its degradation (Diehl et al. 1998). Additionally, there are some other kinases reported to phosphorylate and then degrade cyclin D1, such as p38 (Casanovas et al. 2000), ERK (Okabe et al. 2006), and IKK (Wei et al. 2008). Through using kinase inhibitors and overexpression of the dominant negative forms of kinases, we conclude that EGCG-induced cyclin D1 degradation requires ubiquitination; however, GSK3 β kinase is not involved in EGCG-induced cyclin D1 degradation (Fig. 4).

Another cell cycle regulator, p21 protein, is usually deregulated in cancer due to the loss of function of transcriptional activators, including p53, KLF4, and Smads (Abbas and Dutta 2009). As shown in Fig. 3a, EGCG down-regulates p21 expression at the transcriptional level. Similar data were obtained using the p21 promoter reporter construct (Fig. 5a). However, fold induction was not as much as seen in RT-PCR. This is due to the limitation of the p21 construct, which contains only the 2326 region of the p21 promoter, and to the more stabilized luciferase mRNA, compared to p21 mRNA. In addition, the reporter system could not account for the epigenetic regulation of p21. Indeed, EGCG has been identified as a histone deacetylase (HDAC) inhibitor in skin cancer cells and contributes to the activation of *CDKN1A* gene encoding p21 protein (Nandakumar et al. 2011). HDAC inhibitors function as anti-cancer agents, at least partly, through their ability to promote the induction of p21 (Ocker and Schneider-Stock 2007). Our results show that EGCG up-regulates mRNA and protein expression of p21 through increasing its promoter activity, which is in agreement with previous reports. However, another report showed that EGCG inhibited histone acetyltransferase (HAT) activity (Choi et al. 2009), instead of HDAC. Thus, whether EGCG increased p21 expression in colorectal cancer cells through inhibiting HDAC activity remained to be further investigated.

Our report is the first to show that EGCG facilitates cyclin D1 degradation in colorectal cancer cells through the proteasome pathway. We also identified cyclin D1 and p21 as major

targets of EGCG in colorectal cancer cells, compared to other catechins. Fig. 5c summarizes the molecular mechanisms by which EGCG increases transcriptional activity of p21 and induces cyclin D1 turnover. Thus, our data provide an alternate avenue to understanding the effect of EGCG on colorectal cancer.

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Abbreviations

EGCG	Epigallocatechin-3-gallate
ECG	Epicatechin gallate
EGC	Epigallocatechin
EC	Epicatechin
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
BSA	Bovine serum albumin
PBS	Phosphate-buffered saline
TBS	Tris-buffered saline

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Fig. 1. Cell growth inhibition of SW480 cells treated with EGCG. **a** Chemical structure of EGCG. **b** Cells were treated with DMSO, 1, 10, and 50 μM of EGCG for 0, 2, and 4 days. Cell growth was measured using CellTiter96 Aqueous One Solution Cell Proliferation Assay. **c** Cells were treated with DMSO, 1, 10, and 50 μM of EGCG for 24 h. Cell cycle was analyzed by PI staining as described in “Materials and methods”. **d** Cells were treated with 1, 10, and 50 μM of EGCG for 24 h. Thirty μg cell lysate was employed for caspase 3/7 activity analysis using Apo-ONE Homogenous Caspase-Glo 3/7 Assay. For all figures, values are expressed as mean ± SD of three replicates. * $P < 0.05$, ** $P < 0.01$ versus DMSO-treated cells

Fig. 2.

Effect of EGCG on cell cycle regulatory proteins in colorectal cancer cells. **a** Western blot analysis of the dose- and time-dependent effect of EGCG on cyclin D1, D3, cyclin E, and p21 expression in SW480 cells. **b** Cell lysates from HCT-116, HT29, SW480, and Caco-2 cells treated with DMSO and 50 μ M EGCG for 24 h were subjected to Western blot, using cyclin D1, D3, E, p21, and actin antibodies (SE, short exposure; LE, long exposure). **c** Protein lysates were harvested from HCT116 and SW480 cells treated with different catechins (50 μ M) then subjected to Western blot analysis using cyclin D1, p21, and actin antibodies

Fig. 3.

Post-translational down-regulation of cyclin D1 induced by EGCG. **a** SW480 cells were treated with 50 μ M of EGCG, and total RNA was isolated at 0, 6, 12, and 24 h. After cDNA synthesis, 1 μ g cDNA was used for PCR as described in "Materials and methods". **b** SW480 cells were pretreated with DMSO and 50 μ M EGCG for 12 h, followed by exposure to 10 μ g/mL cycloheximide (CHX). Cells were harvested at the indicated time point and then subjected to immunoblotting using cyclin D1 antibody (*upper panel*). Quantitative analysis of three independent experiments was carried out by Scion Image Software (*lower panel*). **c** SW480 cells were pretreated with different proteasome inhibitors: MG132 (10 μ M), epoxomicin (1 μ M), lactacystin (5 μ M), AW9155 (1 μ M), and YU102 (1 μ M) for 1 h, followed by DMSO and 50 μ M EGCG for 24 h. Cell lysates were subjected to Western blot using cyclin D1 and β -actin antibody. Statistical significance was measured with the Student's unpaired *t* test (* P < 0.05, ** P < 0.01)

Fig. 4.

EGCG-induced cyclin D1 degradation is threonine-286 phosphorylation independent and requires ubiquitination. **a** SW480 cells were pretreated with LiCl at indicated dose for 1 h then co-incubated with 50 μM EGCG as indicated for 24 h. Cell lysates were subjected to Western blot analysis using cyclin D1 and actin antibody. **b** Empty vector (EV), GSK3β expression vector (WT), constitutively active GSK3β mutation (CAT), and kinase-inactive or dominant negative form of GSK3β (DN) were transiently transfected into SW480 cells. After 24 h, cells were incubated with DMSO and 50 μM EGCG for 24 h, as indicated. Cells were harvested, and the lysates were analyzed by Western blot using V5 epitope, cyclin D1, and actin antibodies. **c** Cyclin D1-HA wild type and mutation species (T286A and K33-269R) expression vectors were transfected into SW480 cells for 24 h, followed by incubation with DMSO and EGCG for 48 h as indicated. Immunoblot analysis was performed using anti-HA antibody. Actin was loading control.

Fig. 5.

EGCG up-regulates p21 expression by increasing its promoter activity and involving in ERK and NF- κ B pathways. **a** The p21 promoter-luciferase reporter construct was transiently transfected into SW480 cells as mentioned in "Materials and methods". Cells were exposed to DMSO or 50 μ M of EGCG for 24 h, luciferase activities were analyzed. **b** The following kinase inhibitors were added into SW480 cells for 1 h followed by DMSO or 50 μ M EGCG treatment for 24 h: 10 μ M SB203580 (p38), 40 μ M PD98059 (ERK), 10 μ M BAY11-7082 (IKK), 1 μ M Gö6983 (PKC), and 10 μ M LY294002 (PI3 K). All cell lysates were subjected to Western blot analysis using p21 and actin antibody. **c** Schematic diagram of colorectal cancer cells in response to EGCG treatment. When cells were exposed to EGCG, cyclin D1 degradation was triggered. EGCG treatment resulted in p21 transactivation, resulting in increased mRNA and protein expression. EGCG-induced cyclin D1 degradation and p21 up-regulation lead to cell cycle arrest and subsequent growth inhibition