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The green tea component EGCG inhibits RNA polymerase III transcription

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Abstract

RNA polymerase III (RNA pol III) transcribes many small structural RNA molecules involved in RNA processing and translation, and thus regulates the growth rate of a cell. Accurate initiation by RNA pol III requires the initiation factor TFIIB. TFIIB has been demonstrated to be regulated by tumor suppressors, including ARF, p53, RB, and the RB-related pocket proteins, and is a target of the oncogene c-myc and the mitogen-activated protein kinase ERK. EGCG has been demonstrated to inhibit the growth of a variety of cancer cells, induce apoptosis and regulate the expression of p53, myc, and ERK. Thus, we hypothesized that EGCG may regulate RNA pol III transcription *in cells*. Here, we report that EGCG (1) inhibits RNA pol III transcription from gene internal and gene external promoters (2) EGCG inhibits protein expression of the TFIIB subunits Brf1 and Brf2, and (3) EGCG inhibits Brf2 promoter activity in cervical carcinoma cells.

Keywords

RNA polymerase III; TFIIB; Brf1; Brf2; EGCG

Catechins are flavonoids found in many plant-derived food products including fruits, berries, chocolate, wine, and green tea (*Camellia sinensis*)[1]. (–)-Epigallocatechin gallate (EGCG) (Figure 1) constitutes the largest percentage of catechins found in green tea, accounting for 65% of the total catechin content[2]. Epidemiological and animal studies have demonstrated that EGCG provides protection against a variety of cancers including those of the skin, lung, prostate and breast [3–6]. EGCG also possess anti-proteolytic [7,8], anti-mutagenic [9,10], and anti-proliferative [5,11–13] activity, thereby regulating the growth of a cell.

RNA polymerase III (RNA pol III) is the largest of the eukaryotic DNA dependent RNA polymerases, with 17 subunits, and transcribes many of the genes involved in processing (U6 snRNA) and translation (tRNA), thereby dictating the growth rate of a cell [14]. RNA pol III activity has been shown to be deregulated in a variety of cancers, reviewed in [15–

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17]. Like all other eukaryotic RNA polymerases, RNA pol III cannot recognize its target promoters directly. Proper initiation by RNA pol III is dependent on the transcription factor TFIIB [14,18]. In higher eukaryotes, at least two forms of TFIIB have been identified [19–21]. TFIIB required for initiation from gene internal RNA pol III promoters is comprised of TBP, Bdp1, and Brf1 [22,23]. Initiation from gene external RNA pol III promoters requires TBP, Bdp1, and Brf2 [19–21].

TFIIB is a molecular target of regulation by tumor suppressors, including ARF [24], p53 [25–29], RB [30,31], and the RB-related pocket proteins [32], the oncogene c-myc [30,33] and the mitogen-activated protein kinase ERK [34]. RNA pol III and TFIIB activity have been demonstrated to be negatively regulated by other proteins, such as Maf1 [35–37]. Deregulation of TFIIB-mediated transcription could be an important step in tumor development. Hence, we speculate that TFIIB-mediated transcription may be a novel target of regulation by chemopreventive agents.

EGCG and RNA polymerase III have both been demonstrated to regulate cell growth and proliferation. We hypothesized that EGCG may regulate cell growth through modulation of RNA pol III transcription in the cervical carcinoma cell line HeLa. Here, we report that: (1) EGCG treatment inhibits transcription by RNA pol III, from both gene internal (tRNA) and gene external (U6 snRNA) promoters, (2) EGCG inhibits expression of the TFIIB subunits Brf1 and Brf2, and (3) activity from the human Brf2 promoter is inhibited by EGCG.

MATERIALS AND METHODS

Cell culture and luciferase assays

HeLa cells were maintained in 5% FBS DMEM (Biowhittaker). RNA pol III luciferase vectors and assays have been previously described [37]. For EGCG experiments, cells were treated with increasing concentrations of EGCG (Alexis Corp.), in 1% DMSO, 24 hours post transfection, and harvested at times indicated in the figure legends. Experiments were performed in triplicate, repeated three independent times, and the data presented are representative. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (GraphpadPrism3.03, San Diego California USA).

Western blot analysis

Untransfected and HeLa cells transiently transfected with an empty Flag vector or 3XFlagMaf1 [37] were treated with 0 uM or 25 uM EGCG, harvested, and nuclear extracts were prepared [20], and western blot were performed as previously described [20,37].

Cloning of the Brf2 promoter

Gene2Promoter analysis software program of Genomatix Suite (www.genomatixsuite.de) was utilized to identify a putative promoter for human Brf2. Putative transcription factor sites within the human Brf2 promoter were identified using MatInspector [38] of the Genomatix Suite. PCR primers flanking the Brf2 promoter sequence were designed with Kpn I and Bgl II restriction sites and used to PCR amplify the promoter sequence from human genomic DNA and cloned into the Kpn I and Bgl II sites of the promoterless pGL3 Basic vector.

RESULTS AND DISCUSSION

EGCG inhibits RNA polymerase III transcription in HeLa cells

The human cervical carcinoma cell line HeLa was treated with 10uM, 25uM, and 50uM EGCG (Figure 1B) for 24, 48 and 72 hours. As demonstrated in Figure 1B, treatment with

increasing concentrations of EGCG inhibited HeLa cell proliferation in a dose dependent manner, consistent with previously published reports [12,39]. We then determined if EGCG could modulate RNA pol III transcription in these cells. Asynchronous HeLa cells were transiently transfected with either pGL3-U6 (gene external RNA pol III U6 promoter), pGL3-VAI (prototypical gene internal RNA pol III tRNA-like promoter), or a promoterless pGL3 vector and treated with increasing concentrations of EGCG (5uM, 10uM, 25uM, 50uM) and analyzed using our recently characterized RNA pol III luciferase assay [37]. Treatment of HeLa cells with increasing concentrations of EGCG inhibits transcription from the U6 (Figure 2A) promoter in a dose-dependent manner. At the lowest dose tested, 5uM, the data was highly statistically significant, $p < 0.001$. Interestingly, U6 transcription appears to be more sensitive to treatment with EGCG at lower concentrations than VAI transcription, compare Figure 2A to 2B. Inhibition of VAI transcription was statistically significant only at higher EGCG doses of 25uM and 50uM. The observed inhibition of RNA pol III transcription in Figure 2 is a direct consequence of EGCG treatment, as the total cellular protein concentrations remained unchanged despite treatment with increasing concentrations of EGCG (Figures 2 and 2B, lower panels).

Inhibition of RNA pol III transcription does not appear to be due to the direct inhibition of the enzymatic activity of RNA pol III by EGCG, as the observed inhibition from gene internal and gene external promoters was not equivalent. We therefore investigated the effect of EGCG on the RNA pol III initiation factor TFIIB.

EGCG inhibits expression of the TFIIB subunits Brf1 and Brf2

To elucidate the mechanism by which EGCG inhibits RNA pol III transcription, we determined if EGCG affects expression of TFIIB. We examined the expression of the TFIIB subunits Brf1, specific for gene internal RNA pol III promoters, and Brf2, specific for expression from gene external RNA pol III promoters in response to EGCG treatment. Nuclear extract was prepared from untransfected HeLa cells and HeLa cells transiently transfected with FlagMaf1 treated with 0 or 25uM EGCG for 72 hours. A 25uM dose of EGCG was used since this dose inhibited both U6 and VAI transcription and was statistically significant (Figure 2). The nuclear extracts were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose, immunoblotted with anti-Flag, or a previously characterized CSH1043 antibody known to cross react with both Brf1 and Brf2 [20]. Expression of the TFIIB subunits Brf1 and Brf2 was severely diminished with 25uM EGCG treatment (Figure 3A). Interestingly, expression of the Maf1 protein, a negative regulator RNA pol III transcription in response to stress *in vitro* [35,36] and *in vivo* [37], was unaffected by EGCG (Figure 3A), suggesting the mechanism by which EGCG inhibits RNA pol III transcription may occur via a pathway independent of Maf1. Maf1 is a common component of at least three signaling pathways leading to RNA pol III transcription repression: the secretory defect signaling pathway, the target of rapamycin (TOR) signaling pathway, and the DNA damage signaling pathway ([40], reviewed in [41,42]). Although the protein levels of exogenously Flag-tagged Maf1 remain constant in response to EGCG, we cannot speculate if this is true for endogenous Maf1 expression.

The human Brf2 promoter activity is inhibited by EGCG

The observed decrease in Brf1 and Brf2 protein expression in Figure 3 raises the possibilities that EGCG may inhibit RNA pol III transcription by: (1) increasing the rate of Brf1 and Brf2 protein turnover, (2) decreasing the stability of Brf1 and Brf2 mRNA, or (3) decreasing expression of Brf1 and Brf2 genes. Previous results have demonstrated that EGCG inhibits proteasome activity, reviewed in [43]. Therefore, to determine the mechanism(s) by which Brf1 and Brf2 expression is inhibited by EGCG we cloned the human Brf2 promoter. Using Gene2Promoter software of the Genomatix Suite, we identified

a putative promoter for Brf2, Figure 4A. We amplified this putative Brf2 promoter by PCR and sub-cloned it into the promoterless pGL3-Basic vector, generating Brf2-pGL3. As expected, Brf2-pGL3 was able to drive luciferase expression in a DNA concentration-dependent manner in HeLa cells (Figure 4B). Treatment of Brf2-pGL3 with increasing concentrations of EGCG showed a statistically significant ($p<0.01$) decrease in luciferase expression (Figure 4C). These data suggest that the observed decrease in Brf2 expression (Figure 3) may be a direct result of inhibition of Brf2 promoter activity by EGCG.

Analysis of the Brf2 promoter (Figure 4A) reveals a single binding site for nuclear factor kappa B (NF- κ B). Hence, we speculate that regulation of the human Brf2 promoter may involve NF- κ B which has been previously demonstrated to be regulated by EGCG [11, 44, 45]. NF- κ B is an oxidative stress-sensitive transcription factor regulating genes involved in inflammation, immunity, growth and cell death [43]. The ROS scavenging property of EGCG inhibits NF- κ B activation, leading to decreased expression of pro-inflammatory and survival genes [46]. The Brf2 promoter also has a putative binding site for the HMG-Box containing protein 1 (HBP1) (Figure 4A) a known transcription repressor which has been demonstrated to be induced upon EGCG treatment [12]. Also, activity from the Brf2 promoter could be inhibited by the activity of HBP1 in response to EGCG, leading to the inhibition of RNA pol III transcription. Additionally, it has been demonstrated that EGCG can inhibit DNA methyltransferases (DNMT) *in vitro* [47]. We speculate that demethylation of the hypermethylated in cancer (HIC1) promoter allows for expression of this tumor suppressor, and ultimately leading to inhibition of Brf2 expression (Figure 4A). Future mutation analysis of the Brf2 promoter will be paramount in determining the mechanism of EGCG regulation.

To the best of our knowledge, this is the first report of the modulation of the activity of RNA polymerase III by a natural product with known anticancer activity. Our data demonstrates that EGCG negatively regulates RNA pol III transcription in HeLa cells. This observed RNA pol III inhibition may be a direct result of the inhibition of Brf1 and Brf2 expression by EGCG. We further demonstrate, in the case of U6 RNA pol III transcription, Brf2 expression is regulated at the transcriptional level by EGCG. Future studies to determine the effect of EGCG on the expression of other TFIIIB subunits Bdp1 and TBP will provide more detailed insight into the mechanism(s) by which EGCG regulates RNA pol III transcription from both gene internal and gene external promoters.

RNA pol III regulates cell proliferation and has been shown to be de-regulated by oncogenic signals [15–17]. Polyphenolic antioxidants such as EGCG have been shown to possess cancer chemopreventative activity, mediated by several mechanisms that are postulated to stem either from its antioxidant effects as a ROS (reactive oxygen species) scavenger [6] or by direct inhibition of molecular targets [47–49]. The antioxidant activity of EGCG has been shown to regulate multiple signal transduction pathways [43,50] including the activities of several oxidative stress-responsive transcription factors such as NF- κ B, AP-1 and Nrf2. However, low concentrations of EGCG has also been shown to be pro-oxidative and to increase the concentrations of H₂O₂ in the culture medium, and postulated to be responsible for the pro-apoptotic and antiproliferative effects of EGCG [51,52]. In our studies, we tested concentrations of EGCG (up to 25 μ M), previously demonstrated to inhibit cell proliferation and intracellular oncogenic signaling networks [53]. Whether the inhibition of Brf2 expression and RNA pol III transcription is due to the direct antioxidant effect of EGCG, or via the generation of H₂O₂ can be determined by repeating the experiments in Figure 2 in the presence of catalase, previously shown to decompose hydrogen peroxide in culture systems [51,52]. Further investigation of the transcriptional control of Brf2 expression via kinase-dependent signaling networks will also shed light into the mechanism of inhibition of

RNA polymerase III by EGCG. These studies may reveal novel mechanisms of the anti-tumor properties of this natural product.

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Figure 1. EGCG inhibits HeLa cell proliferation

(A) Structure of (–) - epigallocatechin gallate (EGCG). (B) HeLa cells treated with increasing concentrations of EGCG as indicated and counted using a hemocytometer at the time points indicated. Each dose and time point was performed in triplicate.

Figure 2. EGCG inhibits RNA pol III transcription in HeLa cells

(A) HeLa cells transiently transfected with pGL3-U6 (100ng), or empty pGL3 vector (100ng), treated with increasing concentrations of EGCG (0 μM , 5 μM , 10 μM , 25 μM , 50 μM). Lower panel corresponds to total cellular protein concentrations used in luciferase assay as determined by Lowry assay. (B) HeLa cells transiently transfected with pGL3-VAI (100ng), or empty pGL3 vector (100ng), treated with increasing concentrations of EGCG. Lower panel corresponds to total cellular protein concentrations used in luciferase assay as determined by Lowry assay. All luciferase assay results are expressed as relative light units (RLU): the average of the *Photinus pyralis* firefly activity observed divided by the average of the activity recorded from the *Renilla* luciferase vector. Experiments were done in triplicate, repeated three times, and representative experiments are depicted. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 3. EGCG inhibits expression of the TFIIB subunits Brf1 and Brf2

(A) Nuclear extract prepared from HeLa cells transiently transfected with FlagMaf1 and untransfected HeLa cells treated with 0μM or 25μM EGCG and immunoblotted with an anti-flag and CSH1043 antibodies. Arrows depict migration of Brf1, Brf2 and Maf1. (B) Same immunoblot from 2A reprobed using anti-actin antibody as loading control.

Figure 4. EGCG inhibits activity from the human Brf2 promoter

(A) Schematic representation of the human Brf2 promoter as determined by Gene2Promoter software of Genomatix Suite. Putative transcription factor binding sites are depicted as identified by MapInspector [38]. Bolded ATG indicates start of translation, and italicized sequence denotes 5' untranslated sequence. (B) HeLa cells transiently transfected with pGL3 (200ng), pGL3-U6 (200ng), Brf2-pGL3 (50ng, 100ng, 200ng). (3) HeLa cells transiently transfected with pGL3 (100ng), Brf2-pGL3 (100ng) and treated with increasing concentrations of EGCG. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (GraphpadPrism3.03);**= $p < 0.01$.