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The Bmi-1 polycomb protein antagonizes the (−)-epigallocatechin-3-gallate-dependent suppression of skin cancer cell survival

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The polycomb group (PcG) proteins are epigenetic regulators of gene expression that enhance cell survival. This regulation is achieved via action of two multiprotein PcG complexes—PRC2 (EED) and PRC1 [B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1)]. These complexes modulate gene expression by increasing histone methylation and reducing acetylation—leading to a closed chromatin conformation. Activity of these proteins is associated with increased cell proliferation and survival. We show increased expression of key PcG proteins in immortalized keratinocytes and skin cancer cell lines. We examine the role of two key PcG proteins, Bmi-1 and enhancer of zeste homolog 2 (Ezh2), and the impact of the active agent in green tea, (−)-epigallocatechin-3-gallate (EGCG), on the function of these regulators. EGCG treatment of SCC-13 cells reduces Bmi-1 and Ezh2 level and this is associated with reduced cell survival. The reduction in survival is associated with a global reduction in histone H3 lysine 27 trimethylation, a hallmark of PRC2 complex action. This change in PcG protein expression is associated with reduced expression of key proteins that enhance progression through the cell cycle [cyclin-dependent kinase (cdk)1, cdk2, cdk4, cyclin D1, cyclin E, cyclin A and cyclin B1] and increased expression of proteins that inhibit cell cycle progression (p21 and p27). Apoptosis is also enhanced, as evidenced by increased caspase 9, 8 and 3 cleavage and increased poly(adenosine diphosphate ribose) polymerase cleavage. EGCG treatment also increases Bax and suppresses Bcl-xL expression. Vector-mediated enhanced Bmi-1 expression reverses these EGCG-dependent changes. These findings suggest that green tea polyphenols reduce skin tumor cell survival by influencing PcG-mediated epigenetic regulatory mechanisms.

Introduction

Polycomb group (PcG) proteins play an important role in regulating gene repression via epigenetic modification of chromatin structure including effects on histone acetylation and methylation (1–6). PcG proteins operate as two classes of multimeric chromatin-binding complexes—polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (3). The PRC1 multiprotein complex includes B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1), HPC, HPL, Mel18, SCML and Ring 1A/B, whereas the PRC2 multiprotein complex contains enhancer of zeste homolog 2 (Ezh2), EED, Suz12 and RbAp46. As an initial step in regulation, the PRC2 complex recruits histone deacetylase to chromatin to catalyze local histone deacetylation. This is followed by trimethylation of histone H3 K27 via the action of Ezh2 to create histone H3 lysine 27 trimethylation (H3 K27-3M) (5,7). H3 K27-3M serves as a binding site for the chromodomain of the Bmi-1 PcG protein of the PRC1 complex (7). Once bound to chromatin, the Bmi-1–PRC1 complex catalyses histone H2A ubiquitination at K119 (1,7). These sequential trimethylation and ubiquitination events are required for PcG protein-dependent gene silencing (3,5).

Bmi-1 is thought to function as an oncogene in which levels are elevated in primary myeloid leukemia and leukemic cell lines (8) and enhanced expression of Bmi-1 causes neoplastic transformation in lymphocytes (9). Recent studies suggest that Bmi-1 plays a vital role in various human epithelial cancers including breast (10,11), prostate (12), colon (13), pancreas (14) and non-small-cell lung cancer (15,16). Bmi-1 overexpression is reported to increase cell proliferation and tumorigenesis through repressing the expression of cell cycle cyclin-dependent kinase inhibitors such as $p16^{ink4a}$, $p19^{arf}$ and $p21^{cip1}$ (4). We previously reported that Bmi-1 is overexpressed in the human squamous skin cancer and HaCaT cells compared with normal epidermal keratinocytes (17). However, there is no information available regarding the mechanism whereby Bmi-1 may enhance skin cancer cell survival nor is there information assessing the likelihood that chemopreventive agents may suppress skin cancer cell survival by altering PcG protein function.

Green tea polyphenols have been shown to prevent carcinogenesis in a number of experimental cell culture and animal-based models of cancer (18–20). (−)-Epigallocatechin-3-gallate (EGCG) is the major bioactive polyphenol present in green tea. A host of mechanisms have been described that may account for the efficacy of these compounds (21,22); however, little attention has been paid to the impact that these polyphenols may have on PcG function. We recently showed that EGCG treatment promotes normal human epidermal keratinocyte differentiation (23,24). The mechanism is strictly associated with differentiation and we did not detect activation of apoptosis (25). Other groups report that EGCG treatment suppresses growth, causes cell cycle arrest and increases apoptosis in skin cancer cells (26–28). In the present study, we examined the ability of EGCG to inhibit PcG gene function in skin cancer cells. We show that expression of pro-survival PcG proteins is increased in skin cancer cells as compared with normal and that EGCG treatment of the cancer cells suppresses PcG protein expression and histone methylation leading to reduced cell survival. This is associated with EGCG-dependent changes in cell cycle regulator and apoptosis proteins that is consistent with reduced cell proliferation and increased apoptosis. We further show that enhanced expression of selected PcG proteins antagonizes the EGCG biological action and the EGCG impact on these endpoints.

Materials and methods

Chemicals and reagents

EGCG, dimethyl sulfoxide (DMSO) and anti-mouse monoclonal β-actin (A5441) antibody were purchased from Sigma (St. Louis, MO). The EGCG is >95% pure as characterized by high-performance liquid chromatography. EGCG was prepared in DMSO as a 1000-fold concentrate and stored at −80°C. Trypsin–ethylenediaminetetraacetic acid (EDTA), Hanks' balanced salt solution, keratinocyte serum-free medium, gentamicin and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-Ezh2 was procured from BD Transduction Labs (San Jose, CA) (612667). Mouse monoclonal anti-Suz12 (04-046) and a rabbit polyclonal anti-histone H3 K27-3M were from Upstate (Lake Placid, NY) (07-449). Mouse monoclonal antibodies for cyclin D1 (554180) and poly(adenosine diphosphate ribose) polymerase (55494) and rabbit polyclonal antibody for Bcl-X (610211) were purchased from BD Pharmingen (San Diego, CA) (55494). Rabbit polyclonal anti-cyclin E (sc-481), anti-cyclin-dependent kinase (cdk)2 (sc-163), anti-cdk4 (sc-601), Bax (sc-493) and mouse monoclonal anti-cyclin A (sc-239), cyclin B1 (sc-245), anti-cdk1 (sc-54) (anti-p21/Waf1

Abbreviations: Bmi-1, B-cell-specific Moloney murine leukemia virus integration site 1; BrdU, bromodeoxyuridine; cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGCG, (−)-epigallocatechin-3-gallate; Ezh2, enhancer of zeste homolog 2; hBmi-1, human Bmi-1; H3 K27-3M, histone H3 lysine 27 trimethylation; PBS, phosphate-buffered saline; PcG, polycomb group; PFU, plaque forming units; siRNA, small interfering RNA.

(sc-6246) and anti-p27/Kip1 (sc-1641) were obtained from Santa Cruz Biotechnology (Santa cruz, CA). Mouse monoclonal anti-Bmi-1 (ab14389) was from Abcam (Cambridge, MA). Rabbit polyclonal antibodies specific against human Bmi-1 (92830), caspase 9 (9502), caspase 3 (9665) and mouse monoclonal caspase 8 (9746) were from Cell signaling (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies including sheep anti-mouse IgG (NA931) and donkey anti-rabbit IgG (NA934) were from GE Healthcare (Piscataway, NJ).

Bmi-1 encoding adenovirus

The human Bmi-1 (hBmi-1) complementary DNA was amplified using reverse transcription–polymerase chain reaction from total RNA isolated from normal human epidermal keratinocytes (17). The resulting 1060 bp product was cloned into pΔE1sp1Btet to create pΔE1sp1Btet-hBmi-1. pΔE1sp1Btet-hBmi-1 was then recombined with the pJM17 adenovirus backbone plasmid using A293 packaging cells to form tAd5-hBmi-1. Empty virus, tAd5-EV, was also prepared. For expression, cells were coinfected with these viruses in the presence of the Ad5-TA helper virus, which encodes the tetracycline activator (17).

Cell culture

SCC-13, A431 and HaCaT cells were obtained from American Type Culture Collection (Rockville, MD). These cells were grown and maintained in DMEM supplemented with D-glucose, L-glutamine, 110 mg/l sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal calf serum. Normal keratinocytes, obtained from human foreskins, were cultured in keratinocyte serum-free medium and utilized at the passage third.

Cell proliferation studies

SCC-13 cells were plated in 35 mm dishes in DMEM (supplemented as above) and allowed to attach overnight. Cells were washed with serum-free medium and then infected with 10 plaque forming units (PFU) of tAd5-EV or tAd5-hBmi-1 in the presence of 10 PFU of Ad5-TA. After 3 h, serum-supplemented medium was restored and at 24 h postinfection, the cells were treated with medium containing either DMSO or EGCG until 72 h postinfection. The cells were then harvested in 0.025% trypsin containing 1 mM EDTA and counted using a coulter counter.

Cell cycle analysis

Subconfluent SCC-13 cells were infected with empty or Bmi-1-expressing virus in the presence of Ad5-TA helper virus. After 24 h, the cells were treated with 0.1% DMSO or 60 μM EGCG for an additional 48 h. The cells were then trypsinized, washed twice and resuspended in phosphate-buffered saline (PBS) and fixed in methanol (29). For cell cycle analysis, the cells were washed two times and suspended in PBS, incubated with 20 μg/ml RNAse for 30 min and stained with propidium iodide at 50 μg/ml for 1 h followed by flow cytometry analysis (29).

BrdU uptake analysis

Subconfluent SCC-13 cells were plated on coverslips and infected with 10 PFU of empty or Bmi-1-expressing virus in the presence of 10 PFU of Ad5-TA helper virus. After 24 h, the cells were treated with 0.1% DMSO or 60 μM EGCG for an additional 48 h. At this point, the cells, which were ∼80% confluent, were incubated for 2 h in medium containing 10 μM bromodeoxyuridine (BrdU). The cells were then washed with PBS, fixed with 4% formaldehyde in PBS for 30 min at room temperature and permeablized for 20 min in PBS containing 0.2% Triton X-100. The cells were then washed with PBS and treated with 2 M hydrochloric acid for 20 min at room temperature to denature the DNA. After an additional PBS wash, the cells were treated with 0.1% trypsin for 5 min at 37°C to retrieve antigen and then blocked with PBS containing 5% goat serum. The cells were then incubated with murine anti-BrdU (Sigma, B8434) followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Molecular Probes, Carlsbad, CA; A21424) for 1 h at room temperature. The cells were washed with PBS, co-stained with 4′,6-diamidino-2-phenylindole and 10 fields of a minimum of 100 cells each were counted to determine percent of BrdU-positive cells.

Immunoblot analysis

Subconfluent cultures were infected with adenovirus and then treated with or without EGCG. After treatment, both attached and floating cells were collected and washed in PBS and lysates were prepared in cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM sodium vanadate, 1 μg of leupeptin and 1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by Bradford Bio-Rad protein assay and equal amounts of protein were electrophoresed on a reducing and denaturing polyacrylamide gel and transferred to nitrocellulose membrane for detection of proteins using appropriate antibodies.

Electroporation of SCC-13 cells

SCC-13 cells, growing in DMEM, were harvested in 0.025% trypsin containing 1 mM EDTA and washed with PBS. The cells (1.5×10^6) were resuspended in serum-free medium and electroporated with Bmi-1, Ezh2 or scrambled control small interfering RNA (siRNA) (Santa Cruz Biotechnology) using the AMAXA electroporation system and the T-018 protocol. The cells were then plated into 35 mm dishes in serum-containing medium. At 48 and 72 h post-electroporation, the cells were harvested and assayed for counting and immunoblot analysis.

Results

Trimethylation of histone H3 lysine-27 by the Ezh2 protein of the PRC2 complex is a key event in the PcG protein-dependent silencing of gene expression. H3 K27-3M constitutes a binding site for the Bmi-1 protein and other proteins of the PRC1 complex, and this binding is required for gene silencing (30–32). We initiated the present studies by examining the level of H3 K27-3M in several immortalized and transformed keratinocyte cell lines. SCC-13 cells are facial epidermis-derived squamous cell carcinoma cells (33), A431 are epidermoid carcinoma cells derived from the epidermis of an 85-year-old female (34) and HaCaT cells are SV40 T-antigen-immortalized quasi-normal human epidermal keratinocytes (35). As shown in Figure 1A, the level of H3 K27-3M is markedly elevated in SCC-13, HaCaT and A431 cells as compared with normal human keratinocytes. This finding predicts that these cell lines may have elevated levels of proteins comprising the PRC2 complex. Indeed, Figure 1A confirms that these cells express increased levels of two components of the PRC2 complex, Ezh2 and Suz12. We also confirm our previous finding that Bmi-1, a PRC1 component, is overexpressed in skin cancer cells (17). We next examined the impact of EGCG treatment on PcG protein level in A431, HaCaT and SCC-13 cells. As shown in Figure 1B, EGCG treatment of HaCaT, A431 or SCC-13 cells reduces Ezh2, Suz12 and Bmi-1 level, and the Ezh2 reduction is associated with reduced H3 K27-3M formation. In each case, this reduction is associated with a 60–80% reduction in cell number after treatment for 48 h with EGCG (data not shown).

Since SCC-13 cells are more aggressive than A431 and HaCaT in terms of growth and malignant state, they were selected for detailed

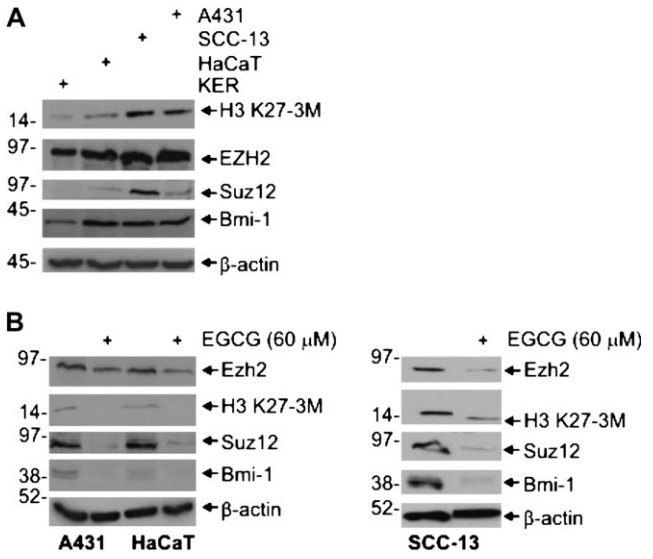

Fig. 1. PcG protein and histone H3 K27-3M levels are increased in skin cancer cell lines. (**A**) Total cell extracts were prepared from subconfluent cultures of normal human epidermal keratinocytes and A431, SCC-13 and HaCaT cells followed by electrophoresis and immunoblot. β-Actin is used as a loading control. Similar results were observed in each of three repeated experiments. (**B**) PcG protein levels are reduced in EGCG-treated A431, HaCaT and SCC-13 cells. Cells were treated with 0 or 60 μM EGCG for 24 h and extracts were prepared for detection of the indicated markers. Similar results were observed in each of three experiments.

study. An EGCG concentration–response curve reveals that Suz12, Ezh2 and Bmi-1 levels are substantially reduced by treatment with 60 μM EGCG (Figure 2A), and a time-course study of 60 μM EGCG-treated cells shows that the reduction in PcG expression and H3 K26-3M formation is substantial at 24 h (Figure 2B). These findings suggest that EGCG treatment interferes with PcG protein function leading to changes in histone methylation and that this is achieved via an EGCG-dependent reduction in PcG protein level.

To assess the biological impact associated with these changes, we examined the effect of EGCG on cell number and determined whether forced Bmi-1 expression could reverse the response. As shown in Figure 3A, treatment with EGCG produces marked changes in SCC-13 cell morphology in empty vector-infected cultures. Moreover, these changes are partially reversed in cells infected with the Bmi-1 expression vector, tAd5-hBmi-1. In addition, Bmi-1 overexpression results in a higher cell density in cells not treated with EGCG (top panels). These findings suggest that Bmi-1 can partially reverse the EGCG-dependent changes in cell morphology and increase cell number. To further test this possibility, SCC-13 cells were infected with empty virus or Bmi-1-expressing virus, and at 24 h, the cells were treated for an additional 72 h with or without EGCG. Figure 3B shows that EGCG treatment reduces cell number and that this reduction is largely reversed by Bmi-1. It should be noted that these changes are less pronounced in more confluent cultures. Figure 3C confirms the reduction of Bmi-1 in the presence of EGCG and the restoration of levels in Bmi-1 vector-infected cells.

We next explored the mechanism responsible for the reduction in cell number in the EGCG-treated cultures. As shown in Figure 4A, treatment with EGCG produces marked changes in cyclin, cdk and cyclin-dependent kinase inhibitor levels. EGCG treatment reduces cdk1, cdk2, cdk4, cdk6, cyclin D1, cyclin E and cyclin A level and increases p21 and p27 level—changes that are generally associated with reduced cell proliferation. We further show that EGCG treatment increases cell cycle inhibitor protein levels (Figure 4B). We next examined the impact of EGCG on apoptosis markers. We show that EGCG treatment increases cleavage of procaspase 9, 8 and 3, and also poly(adenosine diphosphate ribose) polymerase (Figure 4C). In addition, EGCG treatment reduces Bcl-xL level and increases Bax level (Figure 4C). These responses are consistent with a reduction in cell proliferation and an increase in apoptosis. Moreover, in each case, the EGCG-dependent changes are reversed in the presence of forced elevated Bmi-1 expression.

To assess the impact of these biochemical changes on cell cycle progression and apoptosis, we performed BrdU labeling and cell sorting experiments. SCC-13 cells were infected with empty or Bmi-1-encoding vector and the cells were then treated with EGCG. As shown in Figure 5A, Bmi-1 expression slightly increases BrdU uptake in cells grown in the absence of EGCG and, as expected, EGCG treatment reduces BrdU uptake by nearly 70%. Moreover, it is interesting that Bmi-1 expression substantially reverses this EGCG-associated reduction, suggesting that Bmi-1 acts to keep cells progressing through the cell cycle. Figure 5B shows that Bmi-1 expression does not markedly alter the distribution of cells with the cell cycle. However, the EGCG-dependent reduction in cell number is associated with a reduction in the number of G_1 phase cells from 54 to 34%. This is accompanied by a slight increase in S-phase cells and a major increase is in the number of subG_1 cells. These finding suggests that EGCG enhances apoptosis and reduces cell cycle progression.

Thus, we show that overexpression of Bmi-1 partially reverses the EGCG-dependent reduction in SCC-13 cell survival and the biochemical changes (increased apoptosis, reduced cell cycle regulatory protein expression, etc.) associated with EGCG treatment. This is particularly interesting since we show that EGCG reduces Ezh2 level and H3 K27-3M formation. In the absence of Ezh2 and H3 K27-3M, it is difficult to see how forced expression of Bmi-1 could protect cells from EGCG since these cells would lack H3 K27-3M that forms the Bmi-1 chromatin-binding site. We therefore determined whether Bmi-1 expression restores Ezh2 level and H3 K27-3M formation. SCC-13 cells were treated with empty or Bmi-1-encoding adenovirus and after 24 h treated with EGCG for an additional 48 h prior to extract preparation. As shown in Figure 6, constitutive expression of Bmi-1 restores Ezh2 level leading to normal levels of H3 K27-3M formation.

The above findings suggest that Bmi-1 and Ezh2 enhance skin cancer cell proliferation and survival and protect the cells against challenge with stress agents. These findings predict that reducing

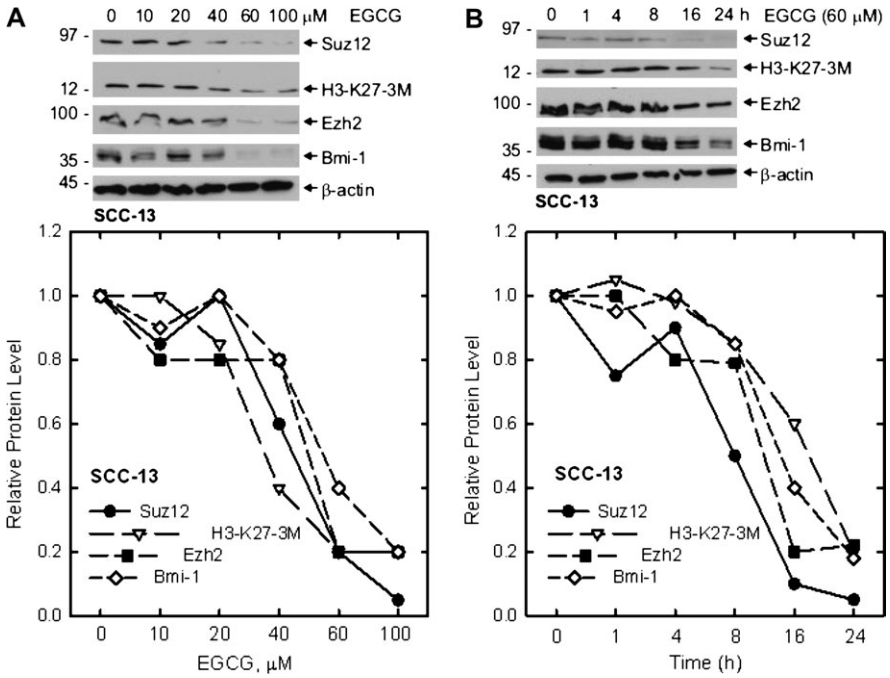

Fig. 2. PcG protein levels are reduced in EGCG-treated SCC-13 cells. (**A** and **B**) SCC-13 cells were treated with 0–100 μM EGCG for 24 h or treated with 60 μM EGCG for 0–24 h. Total cell extracts were prepared for electrophoresis on an 8–10% polyacrylamide gel for immunoblot with the indicated antibodies. β-Actin levels were measured to normalize protein loading. The gels were scanned with a densitometer and the data were plotted relative to the control protein level.

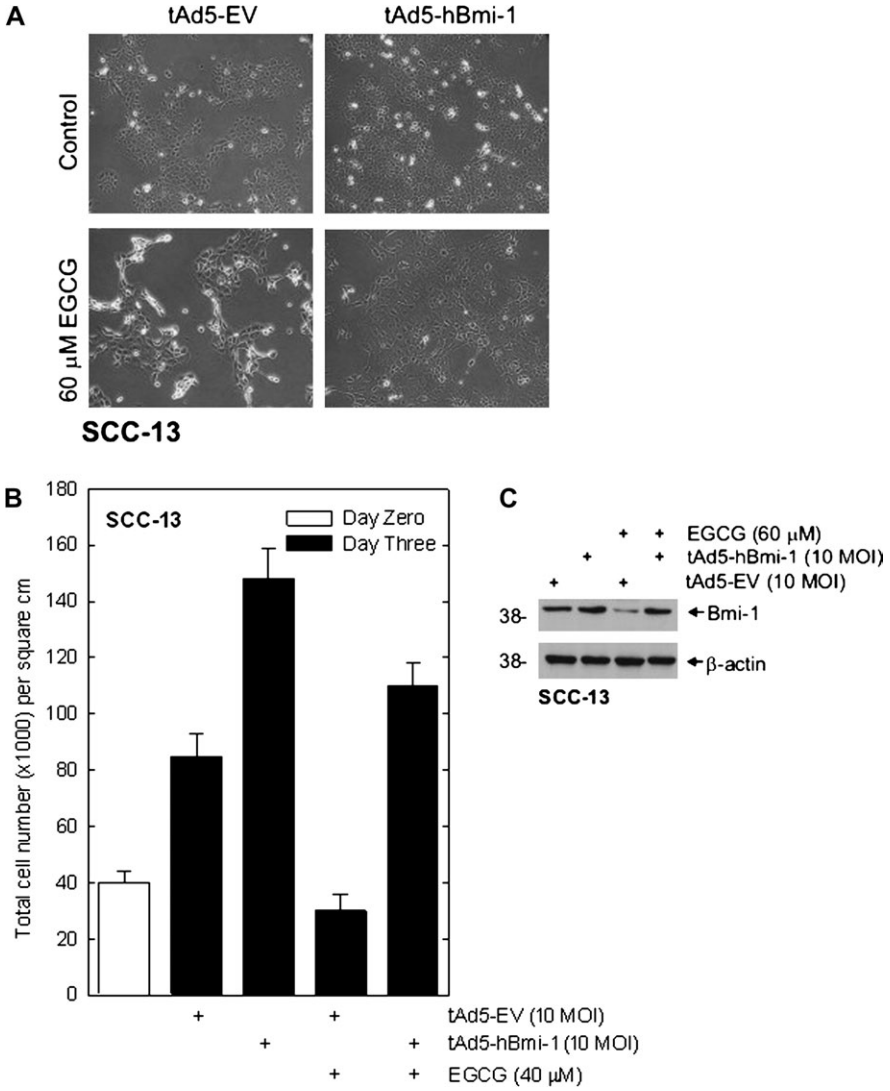

Fig. 3. Impact of Bmi-1 and EGCG on cell number. Thirty percent confluent 35 mm dishes of SCC-13 cells were infected with 10 PFU of tAd5-hBmi-1 or tAd5-EV in the presence of 10 PFU Ad5-TA. After 24 h, fresh virus-free medium containing vehicle (DMSO) or 60 μM EGCG was added and incubation was continued for an additional 72 h. (**A** and **B**) SCC-13 cell morphology and cell counts. The open bar indicates cell number on day zero at the time of virus infection, and the shaded bars are the cell counts after 72 h of EGCG treatment. The bars in the graphical panel represent the mean ± SEM, $n = 3$. Student's unpaired *t*-tests indicate that the tAd5-EV and tAd5-hBmi-1 groups are significantly different ($P < 0.001$) and that the tAd5-EV/EGCG and tAd5-hBmi-1/EGCG groups are significantly different ($P < 0.001$). (**C**) Treatment with EGCG produces a substantial reduction in Bmi-1 level and this is restored by expression of Bmi-1 encoding adenovirus.

the level of Bmi-1 or Ezh2 will result in reduced cell number. To test this, SCC-13 cells were quantitatively electroporated with Bmi-1 and/or Ezh2 siRNA and at 48 and 72 h posttreatment, the cells were harvested and counted. As shown in Figure 7A, treatment with Bmi-1 or Ezh2 siRNA reduces the level of the corresponding protein. These blots also confirm that the knockdown is selective as Bmi-1 siRNA does not reduce Ezh2 level and vice versa. These blots also show that knockdown of Ezh2 is associated with reduced formation of H3 K27-3M. Figure 7B shows that knockdown of either Bmi-1 or Ezh2 results in reduced cell number and that the reduction is most dramatic when both are reduced. At 72 h, the reduction in cell number when both Bmi-1 and Ezh2 are reduced is highly significant. Additional studies indicate that knockdown of Bmi-1 or Ezh2 does not markedly alter expression of the cell cycle control genes or the proteins involved in apoptosis (data not shown).

We next assessed whether EGCG regulates PcG gene expression in normal human keratinocytes. We have shown previously that Bmi-1 overexpression in normal cells enhances survival (17). We therefore monitored whether treatment with EGCG alters PcG gene expression in normal cells. Normal keratinocytes were treated for 24 h with 60 μM EGCG and PcG levels were monitored by immunoblot. As shown in Figure 8, EGCG treatment reduces Suz12, Ezh2 and Bmi-1 level and the reduction in Ezh2 level is associated with reduced H3 K27-3M. In each case, EGCG treatment reduces level by 90%. These findings indicate that the ability of EGCG to reduce PcG protein expression is not unique to immortalized and transformed keratinocytes.

Discussion

PcG proteins influence a range of processes including cell cycle progression, apoptosis, chromosome inactivation and stem cell survival (1,36–39). Bmi-1 was initially described as an oncogene that cooperated with *c-myc* in initiation and development of murine lymphoma (40). Other studies identified Bmi-1 as a key regulator of cell proliferation and senescence, hematopoiesis and self-renewal of normal and transformed stem cells (41,42). Consistent with this role, enhanced expression of Bmi-1 results in transformation and immortalization of normal fibroblasts and epithelial cells through activation of the human telomerase reverse transcriptase (*hTERT*) gene (10).

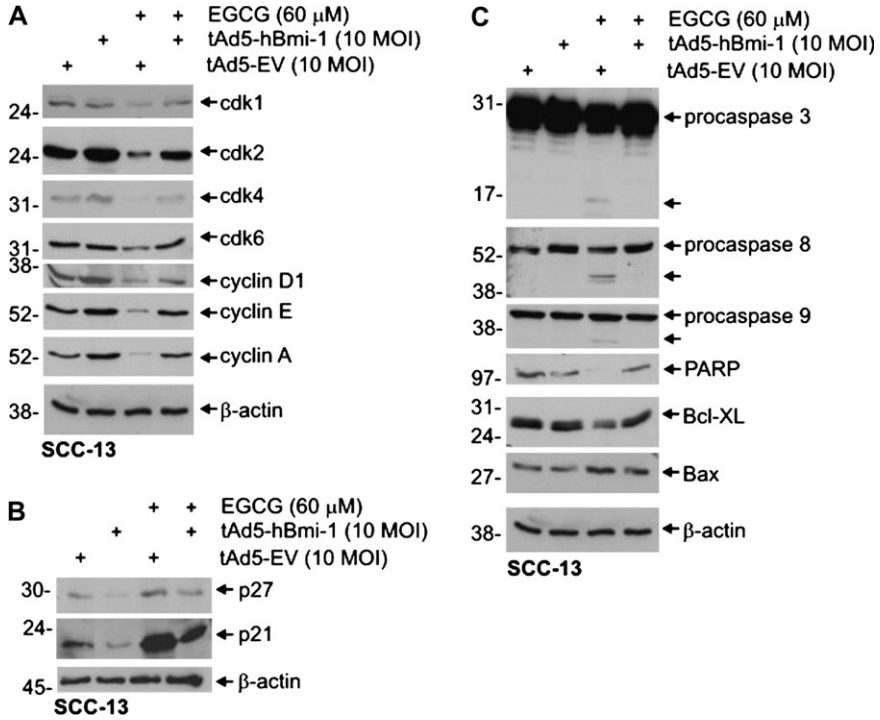

Fig. 4. EGCG and Bmi-1 regulate cell cycle and apoptotic endpoints. Thirty percent confluent cultures of SCC-13 cells, growing in 35 mm dishes, were infected with 10 PFU of tAd5-hBmi-1 or tAd5-EV along with 10 PFU Ad5-TA. After 24 h, the cells were treated for an additional 48 h with or without 60 μM EGCG. Total cell extract was prepared and electrophoresed on a 10% polyacrylamide gel for immunoblot detection of the indicated epitopes (**A**–**C**). β-Actin was included as a loading control. Similar results were observed in three separate experiments. Both surface-attached and floating cells were harvested and included in the immunoblot analysis.

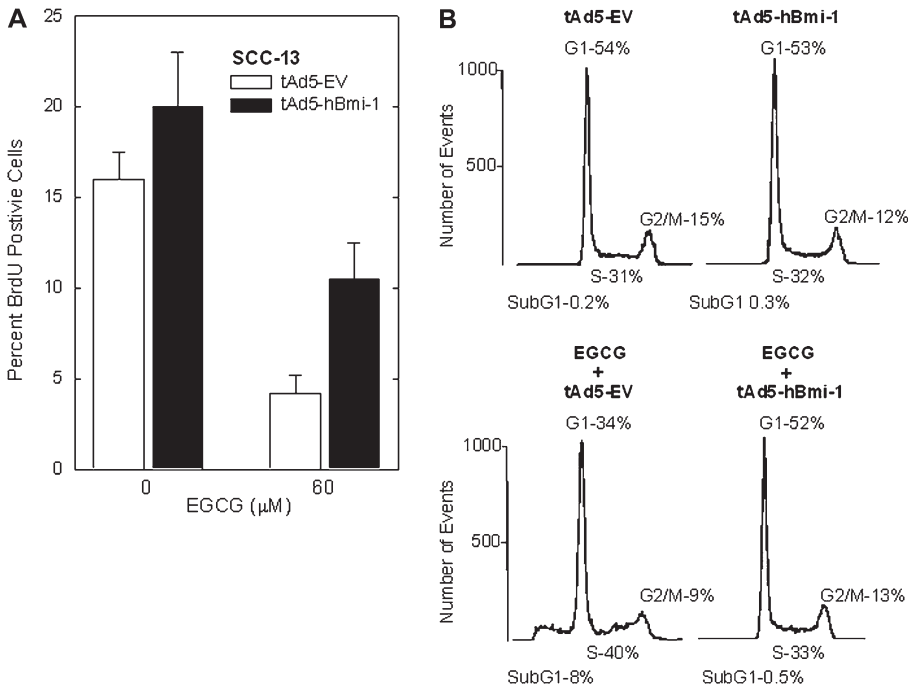

Fig. 5. Impact of EGCG and Bmi-1 on BrdU incorporation and cell cycle distribution in SCC-13 cells. SCC-13 cells were infected with 10 PFU of empty (EV) or hBmi-1-encoding adenovirus. After 24 h, the cells were treated with or without 60 μM EGCG for an additional 48 h. (**A**) EGCG treatment suppresses BrdU incorporation. Cells, treated with the indicated viruses and EGCG as above, were incubated with 10 μM BrdU during the final 2 h of EGCG treatment and then assayed for BrdU incorporation. The data are the mean ± SD of a representative experiment. (**B**) Impact of EGCG and Bmi-1 on SCC-13 cell cycle phase. After infection with virus and EGCG treatment as indicated above, the cells were harvested for cell cytometry analysis. The *x*-axis represents the propidium iodide level (DNA content) and the *y*-axis, the number of events. The percentage of cells in G_1, S, G_2/M and subG_1 is indicated. Three separate experiments yielded similar results.

Increased expression of Bmi-1 is observed in human epithelial tumors and tumor cell lines, suggesting that it may have a role in epithelial tumor formation (39,43–46).

Ezh2 has been established as the important functional member of PRC2 complex in the gene silencing process. Ezh2 is a methyltransferase that methylates histone H3 K27 (31,46,47). This is a key step in forming the interaction site for subsequent interaction of the PRC1 complex with chromatin via interaction with the Bmi-1 protein of the PRC1 complex. Like Bmi-1, increased Ezh2 expression is associated with aggressive tumor formation and poor prognosis in prostate and breast cancers (30,48) and also bladder, melanoma and bronchial squamous cell carcinoma (49–51). In addition, Ezh2 is amplified in a subset of cancers and cancer cell lines leading to increased expression (52,53). For example, Berezovska *et al.* (54) reported that the Bmi-1 and Ezh2 genes are amplified in metastatic prostate cancer. Ezh2 is also increased in mantle cell lymphoma and co-expressed with Bmi-1 in Reed–Sternberg cells in Hodgkin's disease and non-Hodgkin's lymphoma (55,56). These studies indicate that Bmi-1 and Ezh2 function as oncogenes and suggest that they are potential therapy targets.

We recently reported that Bmi-1 messenger RNA and protein are expressed in the cultured human epidermal keratinocytes and in the basal and suprabasal layers of the epidermis (17). Our findings suggest that Bmi-1 may play a role in preventing premature keratinocyte death during differentiation. Consistent with this idea, increased expression of Bmi-1 via adenovirus delivery enhances survival of stress-challenged normal keratinocytes (17). In the present study, we examine the role of the PcG proteins in skin cancer cells. Our results show that Bmi-1, Ezh2 and Suz12 are increased in skin cancer cell lines as compared with normal human keratinocytes and that this increased expression is associated with increased formation of trimethylated H3 K27. Consistent with a role for Ezh2 in this process, knockdown of Ezh2 using siRNA results in a reduction in H3 K27-3M level. The increased H3 K27-3M level indicates that functional activity of the Ezh2-containing PRC2 complex is increased in skin cancer cells. Moreover, expression of the PRC1 complex component Bmi-1, which binds the H3 K27-3M site, is also elevated, suggesting increased PRC1 complex activity. Functional Bmi-1 overexpression studies indicate that increased Bmi-1 is associated with enhanced SCC-13 cell survival in response to challenge with chemopreventive agent.

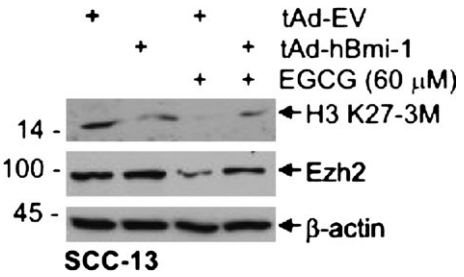

Fig. 6. Bmi-1 expression restores Ezh2 expression and H3 K27-3M formation. SCC-13 cells were infected with 10 multiplicity of infection of empty (EV) or hBmi-1-encoding adenovirus. After 24 h, the cells were treated with or without 60 μM EGCG for an additional 48 h. Extracts were prepared for detection of the indicated epitopes. Similar results were observed in each of three separate experiments.

To gain insight regarding the role of PcG proteins in mediating the response to chemopreventive agent challenge, we examined the impact of EGCG treatment on Bmi-1, Suz12 and Ezh2 expression and function in skin cancer cells. We show that EGCG reduces Bmi-1, Suz12 and Ezh2 levels in three separate immortalized epidermis-derived cell lines, A431, HaCaT and SCC-13. Detailed studies were performed using the most transformed of these lines, SCC-13 (57). Ezh2 is a specific methyltransferase that specifically trimethylates lysine 27 of histone H3 (31,32). An important finding is that EGCG treatment of SCC-13 cells reduces Ezh2 expression in an EGCG concentration- and time-dependent manner and that this is associated with a genome-wide reduction in H3 K27-3M level. A 10-fold reduction in Ezh2 histone methyltransferase level correlates with a corresponding reduction in H3 K27-3M. In addition to Ezh2, the level of a second PRC2 complex protein, Suz12 (58), is reduced. Thus, EGCG attenuates PRC2 complex function by reducing expression. We also examined the impact on the PRC1 complex component, Bmi-1. Bmi-1 level is substantially reduced following treatment with EGCG. Taken together, these results indicate that EGCG treatment reduces functional activity of both the PRC2 and PRC1 complexes.

EGCG treatment also reduces SCC-13 cell number and this reduction is associated with a change in level of key cell cycle regulators. EGCG treatment reduces expression of key cell cycle regulatory proteins that function at multiple cell cycle stages (59,60). In addition, the cyclin–cdk complexes are negatively regulated by cyclin-dependent

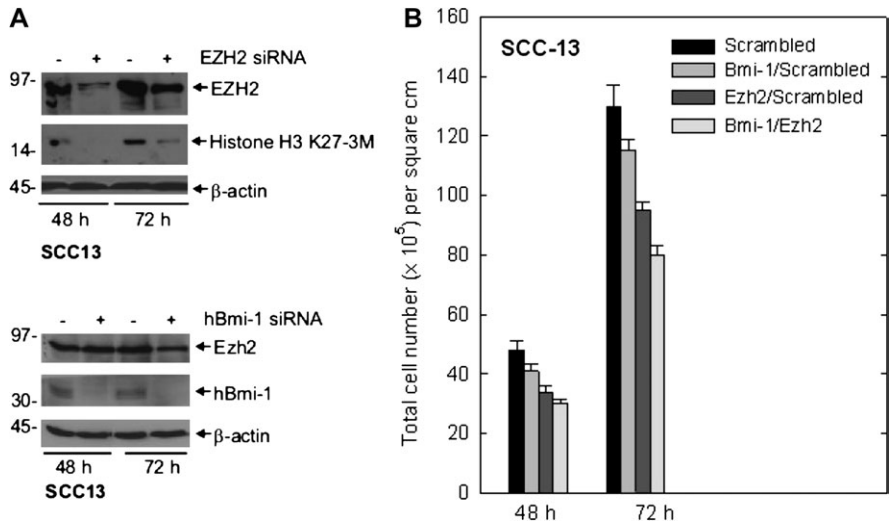

Fig. 7. Bmi-1 and Ezh2 siRNA suppress SCC-13 cell proliferation. SCC-13 cells were electroporated with control (3 μg), Bmi-1/control (1.5 μg each), Ezh2/control siRNAs (1.5 μg each) or Bmi-1/Ezh2 siRNAs (1.5 μg each) using the AMAXA electroporation system and the T-018 electroporation protocol. Cells were then plated in 35 mm dishes and fresh medium was added on alternate days. (**A**) Extracts were prepared at 48 h post-electroporation for detection of Ezh2, H3 K27-3M, Bmi-1 and β-actin. β-Actin was monitored as a loading control. (**B**) At the indicated times post-electroporation, cells were harvested for cell count. The bars represent mean ± SEM, $n = 3$. Student's unpaired t-test indicates that the 72 h scrambled versus Bmi-1/Ezh2 groups are significantly different ($P < 0.002$).

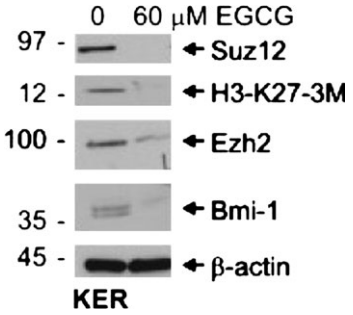

Fig. 8. EGCG suppresses PcG function in normal human keratinocytes. Normal human keratinocytes were treated for 24 h with 60 μM EGCG and extracts were prepared for detection of the indicated proteins. The level of each marker was reduced by >90% in this experiment when normalized to β-actin. This experiment was repeated three times with essentially identical results.

kinase inhibitors including $p21^{cip1}$, $p27^{kip1}$ and $p16^{ink4A}$ (61). In EGCG-treated SCC-13 cells, cdk (cdk1, cdk2, cdk4 and cdk6) and cyclin (cyclin D1 and E) levels are decreased, and p21 and p27 levels are increased. These changes are consistent with the observed EGCG-dependent decrease in cell number and suggest that EGCG impacts cell cycle kinases and kinase inhibitors at multiple stages in the cell cycle. EGCG had been reported to induce G_1 cell cycle arrest via induction of $p21^{cip1}$ and the suppression of G_1-phase-specific markers (cyclin D1, cyclin E, cdk2 and cdk4) in A431 cells (26). Our studies suggest that in SCC-13 cells, the impact on cell cycle is more global in that the EGCG influences cell cycle markers at multiple cell cycle stages, although a modest reduction in the number of cells in G_2/M phase is observed. In addition, EGCG enhances apoptosis as evidenced by an increase in the Bax:Bcl-xL ratio, cleavage of procaspases 3, 8 and 9 and poly(adenosine diphosphate ribose) polymerase and accumulation of $subG_1$ cells. These EGCG-associated apoptotic changes are reversed by high-level Bmi-1 expression, as is the reduction in cell number. Taken together, these results suggest that high levels of Bmi-1 can inhibit EGCG action in skin cancer cells.

We show that EGCG reduces Bmi-1 and Ezh2 level in SCC-13 cells and Ezh2-dependent H3 K27-3M formation. We further show that forced Bmi-1 expression protects SCC-13 cells against these EGCG-dependent changes. However, it is not clear how this could work since the chromatin-binding site that is necessary for Bmi-1 function requires Ezh2 activity. Since EGCG treatment reduces Ezh2 level, it is hard to understand how Bmi-1 could function in EGCG-treated cells. A particularly interesting observation is that forced Bmi-1 expression in EGCG-treated cells restores Ezh2 level and H3 K27-3M formation. Thus, Bmi-1 expression leads to expression of the enzyme responsible for construction of its chromatin-binding site.

We also examined whether PcG protein knockdown (in the absence of EGCG) reproduces the EGCG-dependent reduction in cell number and PcG expression. These studies show that Ezh2 knockdown leads to reduced proliferation and reduced H3 K27-3M formation and that reducing Bmi-1 also reduces cell number. When both are knocked down, there is a significant reduction in cell number compared with cells expressing normal levels of these regulators. However, this response is not as marked as observed following EGCG treatment. This suggests that the reduction in Bmi-1 and Ezh2 observed in EGCG-treated cells is only partially responsible for the EGCG-dependent reduction in cell number. We suspect this is because reducing Bmi-1 and/or Ezh2 does not enhance apoptosis (data not shown). However, although Bmi-1 and Ezh2 do not influence the basal level of apoptotic activity, their overexpression protects the tumor cells against the proapoptotic action of EGCG.

To assess the generality of our observations, we examine the impact of EGCG on PcG protein levels in two additional epidermis-derived cell lines, A431 and HaCaT. A431 are epidermoid carcinoma cells and HaCaT cells are SV40 T-antigen-immortalized quasi-normal human epidermal keratinocytes (35). Both lines display elevated Ezh2 (and H3 K27-3M), Bmi-1 and Suz12 expression and grow more rapidly than normal keratinocytes. Our studies show that EGCG treatment reduces the level of Bmi-1, Ezh2 and Suz12 and H3 K27-3M in these cell lines, suggesting that response to EGCG is generally observed in human epidermis-derived transformed and immortal cells. Interestingly, treatment of normal keratinocytes also results in a reduction in Bmi-1 and Ezh2 level and reduced formation of H3 K27-3M. This suggests that PcG function is suppressed by EGCG in both normal and transformed cell types. This is frequently assumed to mean that EGCG would not be useful for treatment of skin cancer; however, we know that EGCG is less effective in cells that are not proliferating at a high rate, such as differentiating keratinocytes. Moreover, since EGCG enhances keratinocytes differentiation (25), treatment with EGCG is not probably to produce a negative impact on epidermis. Tumor cell survival, in contrast, should be reduced due to EGCG-dependent apoptosis.

The PRC2 and PRC1 PcG protein complexes are thought to act sequentially to regulate expression of a host of genes involved in cell proliferation and survival. In this model, the PRC2 complex binds to chromatin and catalyzes the trimethylation of H3 K27. This trimethylated histone constitutes a binding site for subsequent interaction with the PRC1 complex Bmi-1 protein leading to additional histone modification and modulation of gene expression (3,7). We propose that skin cancer cells are predisposed to survive, in part, because the level of these protein regulators is increased and that this leads to the pro-survival changes in gene expression reported in this study. Our studies further suggest that this regulatory mechanism can be antagonized by the green tea polyphenol, EGCG, via a mechanism that involves an EGCG-dependent reduction in PcG protein level.

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