Green Tea Polyphenols Reverse Cooperation between c-Rel and CK2 that Induces the Aryl Hydrocarbon Receptor, Slug, and an Invasive Phenotype

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Abstract
Exposure to and bioaccumulation of lipophilic environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs), has been implicated in breast cancer. Treatment of female rats with the prototypic xenobiotic PAH 7,12-dimethylbenz(a)anthracene (DMBA) induces mammary tumors with an invasive phenotype. Here, we show that green tea prevents or reverses loss of the epithelial marker E-cadherin on the surface of DMBA-induced in situ cancers. To investigate the mechanism(s) leading to a less invasive phenotype, the effects of the green tea polyphenol epigallocatechin-3 gallate (EGCG) on mammary tumor cells were assessed. EGCG reversed epithelial to mesenchymal transition (EMT) in DMBA-treated NF-κB c-Rel–driven mammary tumor cells and reduced levels of c-Rel and the protein kinase CK2. Ectopic coexpression of c-Rel and CK2α in untransformed mammary epithelial cells was sufficient to induce a mesenchymal gene profile. Mammary tumors and cell lines derived from MMTV-c-Rel transgenic mice expressed serine/threonine kinase, has been shown to enhance NF-κB activity and transformed phenotype of breast cancer cells in vitro (10, 11). CK2 contains two catalytic (α/α or α’/α’) and two regulatory (β/β) subunits. Many human breast cancer tissue samples and derived cell lines express aberrantly high levels of both nuclear c-Rel (12) and CK2 activity (13). Furthermore, elevated levels of NF-κB and CK2 typhied DMBA-induced mouse mammary tumors (14). We have recently engineered transgenic mice with targeted c-Rel or CK2α expression in the mammary gland and observed that ~30% of mouse mammary tumor virus (MMTV)-c-Rel mice or MMTV-CK2α transgenic mice develop late onset mammary tumors (13, 15).

Epithelial to mesenchymal transition or EMT is a process whereby primary in situ tumors progress toward an invasive or metastatic phenotype. During this process, cancer cells typically lose expression of proteins that promote cell-cell contact such as E-cadherin and γ-catenin, display induction of zinc-finger master transcriptional regulators such as Snail or Slug, and acquire mesenchymal markers such as vimentin, fibronectin, and N-cadherin, which promote cell migration and invasion (16). The loss of E-cadherin is frequently considered to be a hallmark of EMT and of a more migratory, invasive carcinoma. Several mechanisms have been implicated in the control of EMT in breast cancer. Estrogen receptor (ER) signaling contributes to maintenance of the epithelial phenotype by regulating MTA3, a component of the histone deacetylase NuRD, which in turn inhibits transcription of the gene encoding Snail, which is a repressor of E-cadherin (17). The AhR, which mediates signaling induced by exposure to PAH such as DMBA, has recently been shown to activate transcription of increased breast cancer to carcinogen exposure (2–4) and from studies showing increased levels of PAHs in sera of breast cancer patients (2) and of AhR in breast carcinomas (5, 6). Treatment of female Sprague-Dawley rats with a single dose of the PAH 7,12-dimethylbenz(a)anthracene (DMBA) induces mammary tumors in 90% to 95% of animals (7). In addition, PAH transform mammary epithelial cells in culture yielding highly malignant cells (8, 9). For example, exposure of the untransformed human breast epithelial cell line MCF-10F to either the PAH DMBA or benzo(a)pyrene led to the development of the highly transformed lines D3-I and BP1, respectively (8). We recently showed that DMBA treatment of rel−3983 cells, established from an NF-κB c-Rel–driven mouse mammary tumor, induced a line termed rel−3983D with a highly invasive phenotype accompanied by elevated NF-κB activity (9).

The c-Rel subunit is a member of the NF-κB family of transcription factors, distinguished by the presence of an NH2-terminal Rel homology domain. The other NF-κB subunits in mammals are RelA, RelB, c-Rel, p50, and p52. CK2, a ubiquitously expressed serine/threonine kinase, has been shown to enhance NF-κB activity and transformed phenotype of breast cancer cells in vitro (10, 11). CK2 contains two catalytic (α/α or α/α’) and two regulatory (β/β) subunits. Many human breast cancer tissue samples and derived cell lines express aberrantly high levels of both nuclear c-Rel (12) and CK2 activity (13). Furthermore, elevated levels of NF-κB and CK2 typified DMBA-induced mouse mammary tumors (14). We have recently engineered transgenic mice with targeted c-Rel or CK2α expression in the mammary gland and observed that ~30% of mouse mammary tumor virus (MMTV)-c-Rel mice or MMTV-CK2α transgenic mice develop late onset mammary tumors (13, 15).

Introduction
The majority of breast cancers (~90%) are late onset and arise from somatic genetic abnormalities, whereas inherited mutations of genes predisposing to early onset breast cancer, such as of BRCA-1 and BRCA-2, account for only ~10% of cases. It has been suggested that the overall increase in late onset breast cancer incidence seen in the United States since the 1980s results, in part, from increased exposure to and bioaccumulation of lipophilic environmental pollutants, such as polycyclic aromatic hydrocarbons (PAH; ref. 1). It has been proposed that PAHs are capable of inducing mutations and directly altering signal transduction pathways through mediators such as the aryl hydrocarbon receptor (AhR). This hypothesis is based on epidemiologic studies relating...
 Slug (18), another repressor of E-cadherin gene transcription (19), suggesting a signaling mechanism by which PAH may contribute to EMT.

Breast cancer incidence in Asia is lower than in Europe and North America, and epidemiologic evidence suggests that this may be due to dietary factors. Tea (Camellia sinensis L.) has chemopreventive effects against various tumors, including breast cancer (20). We showed that green tea reduced mammary gland carcinogenesis by DMBA in the Sprague-Dawley rat model (21). Specifically, we observed that drinking green tea significantly increased mean latency to first tumor and reduced tumor burden by 70%. In addition, an 87% reduction in the number of invasive tumors per tumor-bearing animal was noted. Green tea contains many polyphenols, including epigallocatechin-3 gallate (EGCG), which possess antioxidant qualities (20). EGCG inhibits activation of NF-κB in Her-2/neu overexpressing breast cancer cells (22) and induces expression of FOXO3a (23). Here, we asked whether drinking green tea affects E-cadherin expression in DMBA-induced in situ mammary tumors in Sprague-Dawley rats. We report that the decrease in E-cadherin normally induced by DMBA exposure is ablated by green tea. EGCG treatment also represses the mesenchymal phenotype of rel-3983D cells, reversing the DMBA-mediated induction of c-Rel and CK2. Importantly, coexpression of c-Rel and CK2 is sufficient to activate an AhR to Slug signaling cascade and a more invasive phenotype that can be inhibited by EGCG. Together, these results indicate that activation of CK2 and c-Rel by DMBA induces EMT via an AhR and Slug signaling pathway; whereas inhibition of this cascade by EGCG promotes mesenchymal to epithelial transition.

Materials and Methods

S In Sprague-Dawley rat studies, Virgin female Sprague-Dawley rats were treated according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. Forty Sprague-Dawley female weanling rats (weighing 40–50 g) were treated, as described previously (21). Rats were given either deionized water or 0.3% green tea or sesame oil, respectively) by gastric gavage at age 8 weeks.

Female weanling rats (weighing 40–50 g) were treated, as described previously (21). Rats were given either deionized water or 0.3% green tea as food additive and consumed for 2 weeks. The rats were then killed and sacrificed, and the mammary glands were excised, and samples fixed in ice-cold 4% formaldehyde for histologic examination. The overall staining score was derived from staining intensity (on a scale of 1 to 3) × % stained cells by two trained pathologists (A.E. Rogers and S. Yang) and averaged to give a final staining score for each slide. The mean scores for each different treated group of rats were then compared using a Student’s t test, and the corresponding mean SD and P values were obtained.

Transgenic mice. Creation of the MMTV-c-Rel and MMTV-CK2α transgenic mouse lines was previously described (13, 15). Breeding of MMTV-CK2α mice and MMTV-c-Rel mice created bitransgenic MMTV-c-Rel × CK2α mice (25). Female transgenic mice were continuously bred to induce transgene expression through activation of the hormone-dependent MMTV-LTR promoter. Mice were monitored biweekly for the appearance of tumors. Tumors and tissues were removed at necropsy. Samples of tumors were either sent for histology, used for establishment of cell lines, or as described previously (9), or frozen immediately after extraction in liquid nitrogen and stored at −80°C. All mice were housed in a barrier facility at the Boston University School of Medicine transgenic mouse facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

Mouse histology. Upon necropsy, tumors and other mammary glands, heart, lung, liver, kidney, spleen, and the adrenal gland were removed and immediately fixed in Optimal Fix (American Histology Reagent Co.) and shipped. The tissues were processed, embedded in paraffin and sectioned at 7 μm. The sections were mounted on glass slides and stained with H&E using routine laboratory procedures in the Transgenic Core Pathology Laboratory at the University of California, Davis. Sections were compared with other specimens in the extensive mouse mammary tumor database.

Cell growth and treatment conditions. NMuMG is an untransformed, immortalized mouse mammary epithelial cell line and was cultured as described previously (20). Mammmary tumors from the MMTV-c-Rel mice and MMTV-CK2α mice were derived by explant, as described previously (9). The cell line derived from MMTV-c-Rel line 14 #3983 tumor (rel-3983) was subjected to treatment either with DMBA dissolved in DMSO (rel-3983D) or DMSO vehicle (rel-3983V), as characterized previously (9). The rel-127 line was derived from a mammary adenocarcinoma that developed in the MMTV-c-Rel mouse, which also displayed papillary bronchial adenocarcinomas (15). The cell lines rel/C2-214 (mammary secretory carcinoma with lymphophic lymphoma in the mammary gland and lung), rel/C2-2382 (mammary adenocarcinoma with lymphphocytic lymphoma in lymph nodes), and rel/C2-2881 (adenosquamous cell carcinoma) were derived from a mammary carcinoma in the MMTV-c-Rel × CK2α bitransgenic mouse (25). EGCG were purchased from LKT Laboratories, Inc.

Matrigel assay. Matrigel (BD Biosciences) was diluted with cold serum-free DMEM to a working concentration of 6.3 mg/mL and kept on ice until use. Two hundred microliters of diluted Matrigel was added to each well of 24-well plates at low confluence, and 24 h later, media was replaced with growth medium containing the appropriated concentration of drugs was added. The Matrigel assay was then mixed with 190 μL of Matrigel at 4°C and added to the preset Matrigel layer in 24-well plates, which was again incubated at 37°C for 30 min. Lastly, 500 μL of complete growth medium containing the appropriated concentration of drugs was layered on top of the cells and incubated from 3 to 7 days before the colonies were photographed.

Immunoblot analysis. Whole cell extracts (WCEs) were prepared and subjected to electrophoresis in an 8% or 10% polyacrylamide-SDS gel and immunoblotting, as previously described (15). Antibodies used were as follows: c-Rel (sc-71; Santa Cruz Biotechnology); AhR (BioMol); phospho-Akt (Ser473 and 9271) and phospho-GSK3 from Cell Signaling; vimentin (MS-129-P; NeoMarkers); CK2 (α/β’), E-cadherin, fibronectin, and γ-catenin (all from BD Transduction Laboratories); and Slug (Abgent) and β-actin (A-5451; Sigma).

Plasmid constructs and transfection analysis. The pcDNA3-c-Rel expression vector, containing murine c-Rel, was kindly provided by Thomas D. Gilmore (Boston University, Boston, MA) and the pcDNA3-CCK2α expression vector was as previously described (11). For immunoblot analysis, cells were plated in 100 plates at low confluence, and 24 h later, cells were transfected with the indicated amounts of DNA using FuGene6

6 http://www.mp.ucdavis.edu/igmice/firststop.html
transfection Reagent (Roche Diagnostics Co.). Forty-eight hours after transfection, WCEs were prepared. The AhR repressor-encoding plasmid (pcDNA-FhAhRR) was provided by Mark E. Hahn (Woods Hole Oceanographic Institution, Woods Hole, MA). We have shown previously that transfection of the AhRR plasmid into mammalian mammary tumor lines significantly suppresses AhR activity (6, 26). The human AhR-encoding plasmid (pcDNA-AhR) was kindly provided by Dr. Y. Fujii-Kuriyama (University of Tsukuba, Tsukuba, Japan).

**Invasion assays.** Suspensions of 5 × 10⁵ cells were layered in the upper compartment of a Transwell (Costar) on an 8-μm diameter polycarbonate filter (8-μm pore size) precoated with 30 μg of Matrigel, and incubated at 37°C for the indicated times. Migration of the cells to the lower side of the filter was evaluated with the acid phosphatase enzymatic assay using p-nitrophenyl phosphate and OD₄₁₀nm determination.

**Results**

**Green tea in drinking water preserves the expression of E-cadherin in DMBA-induced rat mammary DCIS in vivo.** Previously, we had shown that green tea resulted in an 87% reduction in tumors invading the ducts in female Sprague-Dawley rats exposed to DMBA by gavage (21). To begin to examine the mechanism by which green tea reduces the invasive phenotype of the resulting mammary tumors, we assessed the effects of DMBA and green tea on E-cadherin expression in DCIS lesions. Paraffin-fixed sections of grossly normal tissue blocks from control Sprague-Dawley rats or from those treated with DMBA and given either water or green tea as drinking fluid (as indicated). B, rel-3983D (untreated) or rel-3983D cells in the presence of either 60 μg/ml EGCG or carrier DMSO as indicated, were grown in Matrigel. After 5 d, the colonies were photographed at a magnification of ×50. C, WCEs (20 μg) from rel-3983V and rel-3983D cells were subjected to immunoblotting for E-cadherin (E-cad), γ-catenin (γ-cat), CK2, Phospho-Akt (P-Akt), and β-actin. D, rel-3983D cells were treated with 60 μg/ml EGCG or carrier DMSO for 72 h, and WCEs (20 μg) were subjected to immunoblotting for E-cadherin, γ-catenin, c-Rel, CK2, P-Akt, Phospho-GSK3 (P-GSK3), and β-actin.

E-cadherin staining was detected (Fig. 1A). In contrast, retention of E-cadherin expression was seen in DCIS lesions in DMBA-treated rats drinking green tea. Slides were scored as described in the Materials and Methods based on the intensity of staining and percentage of positive cells. For the water- or green tea–drinking control animals given vehicle sesame oil, scores were 170 ± 108 and 202 ± 103, respectively (n = 5). DCIS specimens in rats exposed to DMBA and drinking water displayed a profound reduction in E-cadherin staining (14 ± 6; n = 4 samples). However, the E-cadherin staining in DCIS lesions in animals exposed to DMBA and drinking green tea was similar to the controls (194 ± 54; n = 4 samples). The difference in staining between the DMBA-treated animals drinking water and green tea was significant, with a P value of 0.0006 by Student’s t test. Thus, DMBA causes a significant decrease in E-cadherin expression in DCIS and green tea blocks these effects, leading to maintenance of E-cadherin expression, consistent with the dramatic reduction in invasive tumors.

**EGCG inhibits the invasive phenotype induced by exposure to DMBA in vitro.** Because, to a large extent, the cancer preventive effect of green tea seems to be mediated through the polyphenol components, the major tea polyphenol EGCG was assessed for its anti-invasive effects using an in vitro model (9). DMBA-induced rel-3983D cells showed reduced contact inhibition and surface E-cadherin, and increased NF-κB binding, c-Rel expression, and ability to form highly invasive colonies in Matrigel compared with control vehicle (DMSO)-treated rel-3983V cells (ref. 9; see Fig. 1B). To determine whether EGCG could reverse an invasive phenotype, rel-3983D cells were plated in Matrigel in the presence of vehicle alone or 60 μg/ml EGCG. After 5 days of incubation in the presence of vehicle, the DMBA-induced rel-3983D cells formed branching colonies with an invasive phenotype in Matrigel (Fig. 1B). In the presence of EGCG, branching structures were not formed and the rel-3983D cells...
acquired a noninvasive phenotype that was similar to that of the rel-3983V cells (Fig. 1B).

We further characterized the profile of EMT gene products and of regulatory kinases in rel-3983D cells (Fig. 1C). Levels of E-cadherin protein were profoundly lower in rel-3983D cells compared with the control rel-3983V cells, whereas γ-catenin expression was only moderately lower. We also noted a substantial increase in protein kinase CK2 levels in rel-3983D cells, consistent with the high CK2 levels seen in DMBA-induced mouse mammary tumors (14). A robust increase in Akt phosphorylation was also observed in rel-3983D cells (Fig. 1C).

To measure the effects of EGCG on the invasive profile of gene expression in rel-3983D cells, cultures were incubated with 60 μg/ml EGCG or carrier DMSO for 72 h and whole cell protein extracts were analyzed (Fig. 1D). EGCG treatment increased levels of the epithelial markers E-cadherin and γ-catenin. The induction of the c-Rel NF-κB subunits, observed in rel-3983D versus rel-3983V cells (9), was reversed upon EGCG treatment (Fig. 1D). Similarly, EGCG treatment reversed the DMBA-induced up-regulation of CK2 and phosphorylation of Akt and GSK3.

**CK2α and c-Rel cooperate to promote a more invasive gene profile in vitro.** Although exposure to DMBA can activate multiple signaling pathways and mediators in addition to c-Rel and CK2, we sought to test the ability of these two genes to induce an invasive gene profile. Untransformed NMuMG mouse epithelial cells were transfected with vectors expressing either protein alone or in combination and the effects on EMT gene expression profile measured by immunoblot analysis (Fig. 2A). Expression of c-Rel alone did not affect E-cadherin or γ-catenin levels, whereas CK2α expression resulted in modest decreases in both proteins. However, the combination of c-Rel and CK2α expression vectors led to a more profound reduction in E-cadherin and γ-catenin levels (Fig. 2A). Furthermore, the combination of CK2α and c-Rel caused a substantial increase in vimentin and a more moderate induction of fibronectin (Fig. 2A), two markers of invasive mesenchymal tumors. Thus, coexpression of c-Rel and CK2α results in more robust effects on invasive gene profile than either alone, suggesting that they have cooperative effects.

**MMTV-c-Rel × CK2α bitransgenic mouse develop highly invasive mammary tumors.** To investigate whether c-Rel and CK2 cooperate in vivo to promote invasiveness, a bitransgenic MMTV-c-Rel × CK2α mouse model was prepared (25). Mice were subjected to biweekly palpable examinations for mammary tumors. On detection of a tumor, mammary glands and other organs were excised and subjected to histopathologic analysis.

The bitransgenic mice exhibited an increase in the incidence of breast tumor formation (∼50%) with the average age at onset of 20.5 months (Table 1), similar to the age of onset in the MMTV-c-Rel or MMTV-CK2α single transgenic mice (20 and 23 months, respectively; refs. 13, 15). The pathology suggested a highly aggressive phenotype for many of the tumors. More than half were papillary or microcystic adenocarcinomas or adenocarcinomas (11 of 16 tumors; one with a metastasis to the lungs). Thus, c-Rel and CK2α cooperate to generate tumors with a highly invasive phenotype.

Epithelial and mesenchymal phenotypes are usually associated with specific cell morphology. Because most of the cell lines established from MMTV-CK2α mammary tumors displayed

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**Figure 2.** c-Rel and CK2 cooperate to promote a mesenchymal gene profile. A, NMuMG cells were transiently transfected with 3 μg of either c-Rel or CK2α expression vector alone, or in combination with empty vector (EV) to a 6 μg DNA total, as indicated. Forty-eight hours after transfection, WCEs were prepared and samples (20 μg) were subjected to immunoblotting for E-cadherin, γ-catenin, vimentin (vimen), fibronectin (FN), c-Rel, CK2, and β-actin, as loading control. B, WCEs (20 μg) from MMTV-c-Rel (rel-3983 and rel-127) and MMTV-c-Rel × CK2α cell lines (rel/CK2-214, rel/CK2-2282, and rel/CK2-2881) were subjected to immunoblotting for E-cadherin, γ-catenin, vimentin, fibronectin, Phospho-Akt (P-Akt), Phospho-GSK3 (P-GSK3), and β-actin. C, WCEs, prepared either from rel-3983V and rel-3983D cells, were subjected to immunoblotting for AhR (top) and Slug (bottom), and β-actin.

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epithelial characteristics, the morphologies of cell lines derived from MMTV-c-Rel and MMTV-c-Rel × CK2α tumors were compared (Fig. 3). Lines were successfully established from three tumors in MMTV-c-Rel × CK2α mice: rel/CK2-214 (mammary secretory carcinoma), rel/CK2-2282 (mammary adenosquamous carcinoma), and rel/CK2-2881 (mammary adenocarcinoma). These were compared with the two previously established lines from MMTV-c-Rel tumor–derived cell lines. Conversely, MMTV-c-Rel/a-Rel and CK2 cooperate to induce the AhR and Slug. Recently, AhR activation with an environmental ligand has been shown to induce transcription of Slug, which encodes a zinc finger transcriptional repressor of E-cadherin, critical for induction of EMT (18). As we have previously shown that AhR expression is upregulated in breast cancer (5, 6, 14), this led us to hypothesize a role for a potential AhR/Slug pathway in the observed invasive phenotype induced by expression of DMBA or c-Rel/CK2. Consequently, we first tested whether DMBA-mediated transformation of the rel-3983D cells is accompanied by an increase in AhR and Slug expression. Indeed, the more invasive rel-3983D cells expressed higher levels of AhR and Slug protein compared with the vehicle-treated-control rel-3983D cells (Fig. 2B). To confirm the role of AhR in Slug expression, an expression vector encoding a repressor of AhR, termed AhRR, was used. Expression of AhRR in rel-3983D cells potently reduced Slug levels (Fig. 4A, left). Moreover, ectopic expression of AhR could partially restore Slug levels in rel-3983D cells treated with EGCG (Fig. 4A, right), consistent with the finding that AhR can control this master regulator. We next asked whether AhR expression is induced to a higher extent in the more invasive tumors in MMTV-c-Rel × CK2α bitransgenic compared with expression levels in MMTV-c-Rel mouse mammary tumors. As predicted, primary MMTV-c-Rel × CK2α mammary tumors displayed higher levels of AhR than MMTV-c-Rel mammary tumors (Fig. 4B). Unfortunately,

Table 1. Histopathology of tumors in female MMTV-c-Rel × CK2α bitransgenic mice after multiple cycles of pregnancy and regression

<table>
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<tr>
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<th>Age † (mo)</th>
<th>Mouse ‡ no</th>
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<tr>
<td>Mammary secretory carcinoma with lymphocytic lymphoma in mammary gland and lung</td>
<td>20</td>
<td>214</td>
</tr>
<tr>
<td>Mammary adenosquamous carcinoma</td>
<td>20</td>
<td>2881</td>
</tr>
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<td>Mammary adenocarcinoma with lymphocytic lymphoma in lymph nodes and spleen</td>
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<td>2282</td>
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<td>5837</td>
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<td>7068</td>
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<tr>
<td>Mammary papillary and glandular adenocarcinomas and squamous nodules</td>
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<td>5827</td>
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<td>7084</td>
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<td>5885</td>
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*Bitransgenic multiparous female mice were monitored for tumor incidence by biweekly palpable examination. Histopathologic analysis of mammary glands and other organs (heart, lung, liver, kidney, spleen, and adrenal gland) from the same animal was performed when presence of a tumor was detected.
† Age of transgenic mouse (in months) at the time of mammary tumor detection.
‡ An identification number was given to the individual mice of the cohort for further analysis of the tumors.
a nonspecific band precluded detection of Slug in these extracts, and hence, extracts from the derived cell lines were analyzed. Consistent with their more invasive phenotype, the three cell lines established from MMTV-c- Rel × CK2α mice (rel/CK2-214, rel/CK2-2282, and rel/CK2-2881) displayed stronger expression of Slug compared with the two MMTV-c-Rel lines (rel-3983 and rel/CK2-127; Fig. 4C). To determine if c-Rel and CK2α coexpression is sufficient to up-regulate AhR and Slug expression, nonmalignant NMuMG cells were again transfected with c-Rel or CK2α alone, or with both expression plasmids, as described in Fig. 2A. Expression of c-Rel caused only a modest increase in levels of AhR (1.6-fold) and did not change the levels of Slug (Fig. 4D). CK2α expression caused a more substantial increase in AhR (2.1-fold) and a detectable increase in Slug (Fig. 4D). Similar to the results obtained above on EMT gene expression profile, the combination of c-Rel and CK2α led to a larger increase in levels of AhR (2.5-fold) and to a more potent induction of Slug. Thus, DMBA transformation of epithelial cell lines results in up-regulation of c-Rel and CK2α, which we postulate stimulates the expression of AhR and Slug in breast cancer cells.

EGCG represses the mesenchymal phenotype of MMTV-c-Rel × CK2α cells. We next tested whether EGCG could diminish expression of AhR and Slug in rel-3983D cells. Treatment with 60 μg/mL EGCG reduced levels of AhR and profoundly inhibited the expression of Slug in rel-3983D cells (Fig. 5A). Furthermore, EGCG potently inhibited the ability of these cells to invade through Matrigel (Fig. 5B, left), indicating that EGCG reduced/abolished the invasive phenotype of these cells. This led us to ask whether EGCG can inhibit the invasive phenotype of the MMTV-c-Rel × CK2α cell lines. After 3 days of treatment with 60 μg/mL EGCG, rel/CK2-214 and rel/CK2-2881 cells became markedly less elongated and well spread, and adhered more strongly to the plate, suggesting that they were less motile (Fig. 5C, 2-D culture). To study invasive properties, cells were grown in Matrigel in the presence of EGCG or control DMSO. When rel/CK2-214 and rel/CK2-2881 cells were cultured with EGCG, the cells lost their ability to form highly invasive colonies; only colonies with spherical morphology were seen (Fig. 5C, Matrigel). Furthermore, EGCG treatment induced expression of the epithelial marker E-cadherin in rel/CK2-214 and rel/CK2-2881 cells, and decreased the expression of Slug, the repressor of E-cadherin gene transcription (Fig. 5D). Consistent with our previous data, induction of E-cadherin was accompanied by a reduction in c-Rel and CK2 protein levels (Fig. 5D). Lastly, EGCG treatment profoundly inhibited the ability of rel/CK2-214 cells to invade through Matrigel (Fig. 5B, right). Therefore, EGCG suppresses the mesenchymal phenotype of cells transformed by c-Rel/CK2 coexpression.

Discussion

Here, we show for the first time that green tea and its predominant polyphenol EGCG prevent or reverse EMT of breast cancer cells induced by DMBA treatment and implicate activation of the NF-κB c-Rel subunit and protein kinase CK2 and, subsequently, AhR and Slug as downstream mediators in the process by which this xenobiotic PAH promotes a highly invasive phenotype. Drinking green tea prevented or reversed the loss of the epithelial marker E-cadherin on DMBA-induced rat mammary DCIS. Thus, it is tempting to speculate that maintenance of E-cadherin plays an important role in the profound reduction in invasive tumors in rats that drank green tea. In vitro DMBA exposure similarly conferred an invasive phenotype, and ectopic coexpression of c-Rel and CK2 mimicked the effect of DMBA in terms of inducing an invasive gene profile, including the expression of AhR and Slug. Although exposure to genotoxic environmental carcinogens increases levels of oxidative stress within the cell and results in DNA damage, e.g., causing mutations in or overexpression of known oncogenes such as Ras and Her-2/neu (27–30), the mechanisms mediating activation of CK2 and c-Rel remain to be determined. Mammary tumors and derived cell lines
from MMTV-c-Rel \times \text{CK}2\alpha\text{a} bitransgenic mice displayed a highly invasive phenotype, which was dependent on activation of AhR and Slug, suggesting these are downstream targets of c-Rel and CK2 activity. EGCG treatment of DMBA-transformed or bitransgenic mouse tumor lines reduced the levels of c-Rel and CK2, and, concomitantly, AhR and Slug. EGCG similarly reduced CK2 activity in prostate cancer cells (31). Thus, the invasive phenotype induced by carcinogen exposure, which can be mimicked by coexpression of c-Rel and CK2, is susceptible to inhibition by the predominant green tea polyphenol EGCG.

Work from several laboratories has indicated the relevance of these results to human disease. A recent study has identified c\text{ck2a}1, the CK2\alpha\text{a} gene, as one of the 186 genes forming an "invasiveness gene signature" predictive of metastasis and poor survival in breast cancer (32). Aberrant activation of c-Rel and AhR was observed in the vast majority of breast cancer specimens (5, 12), suggesting the possibility that activation of CK2 permits cooperation with c-Rel. Furthermore, constitutive overexpression of c-Rel in activated B-cell-like diffuse large B-cell lymphomas has been associated with poorer prognosis and enhanced cancer cell survival (33). AhR expression also seems to correlate with tumor progression. For example, although significant AhR levels were noted in primary tumors from MMTV-c-Rel transgenic mice, higher levels were detected in MMTV-c-Rel \times \text{CK}2\alpha\text{a} bitransgenic mice. Similarly, higher AhR levels were found in the DMBA-transformed version of rel-3983 (rel-3983D) than in the noninvasive rel-3983V counterpart. Transformation upon exposure to DMBA or the related PAH, benzo(a)pyrene, increases AhR levels in the human breast epithelial MCF-10F cells (data not shown). Of note, higher AhR levels are associated with up-regulation of Slug, a master regulator of EMT and increased branching and invasiveness in Matrigel. The significant decrease in Slug protein after repression of AhR activity supports the hypothesis that the AhR is constitutively active in mammary tumors and that Slug is one of its downstream targets. Although the mechanism through which the AhR regulates Slug is still under investigation, the possibility that AhR and c-Rel coregulate the Slug promoter is appealing, given past work showing cooperation between AhR and the p65 NF-kB subunit (34). Lastly, our findings are consistent with results of other investigators suggesting a role for exogenous ligand-activated AhR in regulating factors involved in branching and invasive growth such as Slug (18), epieregulin (35), and Cox II (36) in human mammary tumor lines and in other cell types.

The observation of elevated AhR levels in mammary tumors from MMTV-c-Rel \times \text{CK}2\alpha\text{a} bitransgenic mice is reminiscent of our previous studies in which we noted an increase in AhR mRNA and protein levels in both rat (5) and mouse (14) mammary tumors induced by gastric gavage with DMBA. However, here, elevated AhR levels were also seen in transgenic mice that had not been exposed to an exogenous AhR ligand. This result suggests that elevated AhR levels may not be specific to tumors induced with AhR ligands and may in fact be a more general characteristic of mammary tumors. This is particularly relevant given the finding that elevated levels of CK2, c-Rel, and AhR typifies human breast cancers (5, 12, 13). Furthermore, constitutively active AhR has been reported in several types of tumors and transformed cells (6, 26, 37, 38). This raises the possibility that the AhR may contribute directly to promoting a more invasive phenotype via regulation of genes such as Slug. Furthermore, AhR directly regulates cytochrome P450 enzymes (e.g., CYP1B1) that metabolize PAH into mutagenic intermediates in mammary epithelial cells (39, 40). Thus, the increased expression of AhR in tumors may also lead to heightened susceptibility to environmental PAH during the later stages of tumor progression.

Many cancer epidemiologic studies have similarly shown an inverse association between green tea consumption and cancer incidence (20). Consumption of green tea was closely associated with decreased numbers of axillary lymph node metastases among premenopausal patients with stage I and II breast cancer and with increased expression of progesterone receptor and ER\alpha among postmenopausal women (41). An inverse correlation between regular green tea consumption before breast cancer diagnosis and the subsequent risk of recurrence has also been shown (42). A recently reported prospective study of men in Shanghai showed significant dose-related inverse relationships between colon cancer risk, urinary biomarkers of tea polyphenols, and reported green tea ingestion (43). This is the first prospective study reported on green tea effects on colon cancer risk. It gives strong support to the results of earlier epidemiologic studies that, in a recent meta-analysis, showed a similar inverse relationship between green tea consumption and colon cancer risk (44). Epidemiologic data similarly support the anticarcinogenic effects of green tea in the prostate (45). Consistently, several animal studies have shown protective effects of green tea or green tea polyphenols (GTP). Oral infusion of GTPs significantly inhibited

![Figure 4. c-Rel and CK2 cooperate to induce the AhR/Slug pathway.](image_url)

**Figure 4.** c-Rel and CK2 cooperate to induce the AhR/Slug pathway. **A**, rel-3983D cells were transiently transfected in 6-well plates with 2 \( \mu \text{g} \) AhRR or empty vector (EV) for 48 h (left). WCEs (30 \( \mu \text{g} \)) were subjected to immunoblotting for Slug and \( \beta\text{-actin} \). **B**, WCEs prepared from the indicated MMTV-c-Rel and MMTV-c-Rel \times \text{CK}2\alpha\text{a} mammary tumors were subjected to immunoblotting for AhR and \( \beta\text{-actin} \). C, WCEs were prepared from MMTV-c-Rel (rel-3983 and rel-127) and MMTV-c-Rel \times \text{CK}2\alpha\text{a} cell lines (rel/CK2-214, rel/CK2-2282, and rel/CK2-2881), and 20 \( \mu \text{g} \) of samples were subjected to immunoblotting for Slug and \( \beta\text{-actin} \). D, NMuMG cells were transiently transfected with 3 \( \mu \text{g} \) c-Rel alone, 3 \( \mu \text{g} \) CK2\alpha\text{a} alone, 3 \( \mu \text{g} \) c-Rel plus 3 \( \mu \text{g} \) CK2\alpha\text{a}, or expression vector to a 6 \( \mu \text{g} \) DNA total, as indicated. Forty-eight hours after transfection, WCEs (20 \( \mu \text{g} \)) were subjected to immunoblotting for Slug, AhR, and \( \beta\text{-actin} \).
prostate cancer development and increased survival of transgenic adenocarcinoma of mouse prostate mice (46). Oral or topical application of GTPs resulted in markedly reduced tumor size in UV B–induced DMBA-initiated skin tumors in mice (47). Green tea or EGCG reduced chemically and genetically induced tumorigenesis in the small and large intestines of rodents (reviewed in 20). Lastly, our findings are consistent with several recent studies showing green tea polyphenols reduce invasive phenotype and angiogenesis, as judged by expression of such markers as matrix metalloproteinase (MMP)-2 and MMP-9 (48) and vascular endothelial growth factor (49).

Although many in situ tumors can be treated and cured successfully, invasive and metastatic tumors remain the major challenge for cancer therapy. Extensive effort has been undertaken to understand the genetic events governing the process of invasion and metastasis (reviewed in 50). We have used an animal model where tumors with an invasive phenotype can be induced with a single-dose exposure to the prototypical environmental carcinogen DMBA. Although DMBA exposure induces many genetic and epigenetic alterations in cells, we also show that coexpression of two tumor-promoting genes, c-Rel and CK2, can recapitulate, to some extent, this complex process. Importantly, green tea and its major polyphenol EGCG reduced the levels of c-Rel and CK2 and reversed the invasiveness induced by either DMBA or the combination of c-Rel and CK2. More recently, we have observed that EGCG reduces the stability of both CK2 and c-Rel proteins. Although EGCG inhibits multiple cancer-inducing cellular programs (20), the fact that it profoundly improves the histology of the DMBA-induced tumors argues for its value in being incorporated into the diet for chemoprevention against environmental carcinogens.

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