Estradiol Alters Cell Growth in Nonmalignant Colonocytes and Reduces the Formation of Preneoplastic Lesions in the Colon

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Abstract
Numerous clinical and animal studies show that hormone replacement therapy reduces the risk of colon tumor formation. However, the majority of experiments have shown that estradiol (E2) does not inhibit the growth of malignantly transformed colon epithelia. As such, the presented studies focused on evaluating the effects of E2 in noncancerous colonocytes. E2 treatments (0–10 nmol/L) reduced cell growth and increased apoptotic activity in young adult mouse colonocytes (YAMC), a nonmalignant cell line, in a dose-responsive manner. These effects were lost in the YAMC-Ras cells, an isogenic cell line with a single malignant transformation. Cotreatment with an estrogen receptor (ER) antagonist inhibited the physiologic effects of E2 in YAMC cells, suggesting that the response is ER mediated. To further study the effect of E2 on colonic epithelia, we evaluated the development of preneoplastic lesions in ovariectomized wild-type (WT) and ERβ knockout (ERβKO) mice treated with either vehicle or E2. WT E2-treated animals exhibited significantly fewer aberrant crypt foci and increased apoptotic activity in colonic epithelia when compared with WT control mice or ERβKO animals receiving either treatment. For the first time, we showed that E2 alters the growth of nontransformed colonocytes in vitro and that, through an ERβ-mediated mechanism, E2 influences the physiologic noncancerous colonocytes, resulting in fewer preneoplastic lesions. Collectively, these data show that the protective actions of E2 occur primarily during the initiation/promotion stages of disease development and identify the hormone as an important chemoprotective agent. [Cancer Res 2009;69(23):OF1–7]

Introduction
Colon cancer incidence is higher in men (62.9 in 100,000) than in women (45.8 in 100,000; ref. 1). This suggests a possible protection in women due to hormonal differences. Data from the Women’s Health Initiative study showed a significant decrease in the incidence of colorectal cancer in postmenopausal women receiving hormone replacement therapy (HRT) compared with placebo (2). In fact, the majority of clinical studies have shown that either HRT or estrogen replacement therapy (ERT) can significantly reduce the risk of colon cancer in postmenopausal women (3–7). Hoffmeister and colleagues (8) found that HRT and ERT both significantly reduce colorectal cancer incidence, but there was no significant difference in effect between the two therapies. The level of protection seen from ERT correlates with the degree of use, seen as a 29% reduction in colon cancer risk, and if ERT had ever been used, this increases to 45% for those currently undergoing ERT (3).

Animal study data support the theory of a significant protective effect against colon cancer in animals treated with estradiol (E2). E2 treatment in ovariectomized rats reduced dimethylhydrazine-induced tumor numbers in the colon by 71% (9). Orally administered estrone in ovariectomized wild-type (WT) and estrogen receptor (ER) α knockout mice inhibited formation of azoxymethane (AOM)–induced tumors (10). Data from this study are important because the fact that estrone suppressed tumor formation in both WT and ERα knockout mice shows that ERα is not the primary mediator for estrogenic protection in the colon. However, several studies have shown that ERβ expression is inversely associated with colon tumor incidence (11, 12). Most of these studies have correlated reduced ERβ expression with increased risk of colonic malignancy. However, no studies have definitively shown the necessity of ERβ expression in colonocytes to observe this protection.

In an effort to identify the mechanism by which E2 affects cell growth, numerous different colon cancer cell lines have been used as in vitro models; however, a majority of studies have shown that E2 treatment does not influence cell growth in these cell lines (13–17). Based on these data, we hypothesized that E2 would alter the physiology of nonmalignantly transformed colonocytes and that this response would result in reduced formation of premalignant lesions in mice exposed to carcinogen. Furthermore, we hypothesized that this effect would be modulated through ERβ. To test our hypotheses, we characterized the effects of E2 in young adult mouse colonocytes (YAMC) and in WT and ERβ knockout mice (ERβKO). YAMCs are a well-characterized nonmalignant cell line derived from the Immorto mouse (18). The data presented here show that E2, through an ERβ-mediated response, affects cell growth in nontransformed colonocytes, resulting in reduced incidence of premalignant lesions in the colon.

Materials and Methods
Cells. YAMC and YAMC-Ras cells were graciously provided by Dr. Robert Chapkin (Department of Nutrition and Food Science, Texas A&M University, College Station, TX). For general maintenance, cells were cultured in RPMI 1640 (Sigma) with 5% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); 1% penicillin/streptomycin; and 1% Glutamax-1 (Invitrogen). Cells were maintained under permissive conditions, 33°C with 10 units IFNγ/mL (Roche) medium.

Cell growth assays. Forty-eight hours before plating, YAMC or YAMC-Ras cells were transferred to medium containing 5% charcoal-dextran-stripped FBS, 1% Glutamax, 1% penicillin/streptomycin, and 0.1% ITS. Cells were seeded at 30,000 per well on six-well plates. Twenty-four hours after plating, cells were exposed to individual treatments of 0, 100, 1,000, or 10,000 pmol/L E2 or in combination with 1 μmol/L ICI 182,780 (ICI; fulvestrant, Sigma-Aldrich), and 48 h after the first treatment, the medium was
changed and a second dose of the given treatments was delivered. All treatments were diluted in DMSO as 1,000× stocks and delivered as 1 µL/mL medium to achieve the final dose listed. At the end of the 96-h treatment period, cells were trypsinized and prepared for counting. The experiments at nonpermissive conditions were carried out as above but at 39°C and without IFN-γ. Cell concentration was determined using a Beckman Coulter particle counter. Twenty microliters of sample were diluted in 10 mL Isotone II diluent (Beckman Coulter), and each sample was counted thrice. All experiments were performed at the permissive temperature unless otherwise specified. Three wells per treatment per experiment were used and either three or four replicate experiments were conducted.

**SV40 protein levels.** YAMC cells (250,000) were seeded in 25-mm flasks and grown at 33°C in stripped serum medium. Cells were treated with 1 nmol/L E2 or vehicle for 96 h with medium and treatment was changed after 48 h. Protein was extracted by adding 1.5 mL lysis buffer to the flask for 30 min at room temperature. After incubation, solution was mixed gently with a pipette and the contents were transferred to microcentrifuge tubes. Protein was quantified by UV spectrometry. Protein concentration was determined by Western blot using the Immunoblot Western Chemiluminescent Protein Substrate kit (Millipore); the methodology was previously described (19). Antibodies used were SV40 Large T antigen antibody (Calbiochem) and goat anti-mouse IgG-HRP (Assay Designs).

**Reverse transcription-PCR.** YAMC and YAMC-Ras cells were grown in T-25 flasks under permissive conditions. Cells were trypsinized and centrifuged. The colony from a WT untreated mouse was removed and used for RNA isolation using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen). The Invitrogen protocol was followed for both cell and tissue extractions. Reverse transcription-PCR (RT-PCR) samples contained 10 µL TaqMan One Step RT-PCR Master Mix, 1 µL primers (Mm00433149_m1 ERα, Mm00433149_m1 ERβ, Hs99999901_s1 18s; Applied Biosystems), 8 µL RNase-free water, and 1 µL RNA (20 ng/µL). RT-PCR was run on a Bio-Rad iQ5 thermocycler for 40 cycles.

**Cellular apoptosis.** Cells were grown in stripped serum medium for 48 h before plating. Cells (30,000) were seeded on six-well plates and grown in stripped serum medium under permissive conditions. Cells were treated for 96 h, with 0, 100, 1,000, or 10,000 pmol/L E2 treatments changed every 48 h. At the end of the treatment period, cells were trypsinized and collected. After collection, cells were centrifuged and the medium was replaced with lysis buffer from the EnzChek Caspase-3 Assay kit no. 2 (Invitrogen). The Invitrogen protocol was followed for this procedure. Apoptosis was measured as increased fluorescence measured on a TECAN infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

**Mice.** Heterozygous ERβKO (+−) c57BL6/J mice were obtained from The Jackson Laboratory. Mice were bred and housed at the Laboratory Animal Resources and Research facility at Texas A&M University. ERβKO and WT mice were produced from the original breeding pairs and genotyped from genomic tail DNA. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

**Carcinogen treatment and aberrant crypt foci analysis.** Female mice between the ages of 2 and 8 mo were ovariectomized, and either 20 mg cholesterol (Sigma-Aldrich) or 18 mg cholesterol/2 mg E2 pellet was implanted s.c. on the back of the neck at the time of ovariectomy. Pellets were prepared as described in ref. (20). Pellets were replaced 8 wk later. Mice were transferred to a phytoestrogen-free diet at the time of surgery and allowed food and water ad libitum. Two weeks after surgery, mice received the first of six weekly injections of AOM (Sigma-Aldrich) at 10 mg/kg body weight. Eight weeks post-AOM, animals were killed. Blood was collected through cardiac puncture. The colony was resected and 1-cm sections from the distal end were cassetted and fixed in 4% paraformaldehyde (Mallinckrodt Baker, Inc.). The remainder of the colon was flattened between sheets of filter paper and fixed in 70% ethanol. Ethanol-fixed colon sections were stained with 0.5% methylene blue (Sigma-Aldrich) and aberrant crypt foci (ACF) were counted as previously described (21, 22).

**Plasma E2.** Plasma E2 concentrations were determined using an Estradiol EIA kit (Cayman). Fifty microliters of plasma/sample were added to...
sample wells, and samples from E2-treated animals were diluted 1:10. Fifty microfilters of Tracer and antiserum were added per sample well and the plate was incubated for 1 h. The wells were washed five times with wash buffer. Ellman’s Reagent (200 μL) was added per well and incubated in the dark for 60 to 90 min while shaking. Wells were read on a plate reader at 415 nm.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed using the ApopTag Plus Peroxidase In situ Apoptosis Detection kit (Millipore) according to manufacturer’s instructions with slight modifications. Briefly, distal colon sections were fixed in 4% paraformaldehyde overnight, paraffin embedded, sectioned, and mounted. Tissues were deparaffinized in three washes of xylene and rehydrated. Slides were quenched with hydrogen peroxide for 8 min and counterstained in 0.5% methyl green solution for ∼9 min.

**Statistical analysis.** Statistics were performed using a Student’s t test, one-way ANOVA with Tukey’s correction, or two-way ANOVA, depending on the outcome analyzed. t test analyses were performed in Excel, and ANOVA data were run in Minitab 15. A 99% confidence interval for the mean count difference (estrogen minus control) and a one-sided test of whether mean estrogen counts were less than control counts were calculated for ACF data. Also, for total ACFs and high-multiplicity ACFs, we specifically tested for a nonzero interaction in the effect of estrogen by mouse type (knockout or WT). All values listed are group mean and error bars are presented as SEM.

**Results**

**E2 inhibits nonmalignant cell growth.** First, we measured the effect of E2 treatment on cell growth in nonmalignant colonocytes. YAMC cells treated with 0 to 10,000 pmol/L E2 exhibited reduced cell growth when treated for 96 h under permissive conditions (Fig. 1A). As the E2 dose increased, there was a corresponding decrease in cell growth (P < 0.0001). These data show a growth-inhibitory effect in nonmalignant colonocytes exposed to physiologic levels of E2.

We then treated these cells with the same doses of E2 under nonpermissive conditions (39°C) to see if similar responses will be observed when cells were in a more primordial state. Again, E2 suppressed cell growth under these conditions in a dose-dependent manner (P < 0.0001; Fig. 1B).

Because the conditional immortalization of these cells operates through the expression of the temperature-sensitive SV40 large T antigen, we wanted to rule out a possible interaction between SV40 and E2 as the mechanism of effect. We chose to measure the levels of this protein under permissive conditions with and without a 1 nmol/L E2 treatment. E2 treatment did not alter SV40 protein levels when compared with control at the permissive condition (Fig. 1A, inset).

**Effect of E2 on YAMC-Ras cell growth.** Next, we determined if E2 treatment would result in a similar effect on cell growth in an isogenic cell line with a single malignant transformation. YAMC-Ras cells contain an activated v-Ha-Ras oncogene. These cells, when treated with E2, show no reduction in cell number (Fig. 1C). This finding was in stark contrast to what was seen in nonmalignant cells and suggests that a single malignant transformation could alter the ability of colonocytes to respond to the presence of E2.

**ERβ expression in YAMC, YAMC-Ras, and WT mouse colonic tissues.** To see if the change in E2 responsiveness was a result of altered ERβ expression, RT-PCR was performed to evaluate differences in ERβ expression between YAMC and YAMC-Ras cells. Relative expression of ERβ was not significantly different between the YAMC and YAMC-Ras cell lines and was similar to that seen in colonic tissues collected from a female WT mouse (Fig. 2A), and no detectable ERα expression was observed at the mRNA level (data not shown). When YAMC cells were treated with E2, relative expression of ERβ was enhanced compared with vehicle controls (Supplementary Fig. S1). Collectively, these studies show that ERβ is expressed in YAMC cells and its expression changes in the presence of E2. In addition, these data suggest that the loss of E2 responsiveness in the YAMC-Ras cells is not the result of suppressed ERβ expression.

**Cotreatment of ICI with E2 prevents cell growth inhibition.** Having shown ERβ to be present in both cell lines, we next wanted to determine if the growth-inhibitory effect of E2 treatment was mediated through the ER. YAMC cells were treated with 0 to 10,000 pmol/L E2 concentrations either in the presence or absence of 1 μmol/L ICI, a well-described ER antagonist that inhibits both receptor subtypes (Fig. 2B; refs. 23, 24). With each E2 dose, cotreatment with ICI fully inhibited the growth suppression induced by E2.

**Figure 2.** ERβ expression in YAMC and YAMC-Ras cells and ER function in cell growth inhibition. A, RNA levels were measured in colonic tissue from a WT mouse, YAMC, and YAMC-Ras cells. Columns, mean (n = 12); bars, SEM. Bars without a common letter differ; P < 0.05. B, cotreatment with ICI inhibits the effects of E2 in YAMCs. YAMC cells were transferred to a charcoal dextran–stripped medium 48 h before plating. Cells were seeded at 30,000 per well on six-well plates and treated every 48 h for a total of 96 h. ICI treatment inhibited the effects of E2 on cell growth. Data are expressed as percentage of growth of the DMSO control group. Columns, mean (n = 9) from three replicate experiments; bars, SEM. Bars without a common letter differ; P < 0.01.
In fact, cotreating with ICI resulted in cell numbers that were not significantly different than the untreated control group. ICI antagonism of E2 growth inhibition strongly supports the theory that this is an ER-mediated response.

**Apoptosis in YAMC and YAMC-Ras cells treated with E2.** Next, we wanted to identify the physiologic response involved with estrogenic growth inhibition. Caspase-3 activation was used to determine the relative numbers of apoptotic cells when YAMC and YAMC-Ras cells were treated with 0 to 10,000 pmol/L E2 for 96 hours. YAMC cells exhibited an increase in caspase-3 activity in a dose-responsive manner \((P < 0.001; \text{Fig. 3A})\). In contrast, YAMC-Ras cells showed no significant increase in caspase-3 activity at any concentration of E2 (Fig. 3B). These data point to apoptosis being a mechanism involved in the E2-induced growth inhibition of nonmalignant colonocytes.

**Formation of ACF.** We next wanted to investigate the role of E2 treatment in the colon at a premalignant stage of disease. E2 treatment resulted in a significant reduction (14.9 ACFs per animal; \(P < 0.0001\); Fig. 4A). More importantly, when analysis is narrowed to high-multiplicity ACFs (foci involving at least four crypts), the difference between groups becomes more pronounced (Fig. 4B). WT animals receiving E2 averaged 3.2 high-multiplicity ACFs per animal, whereas WT control mice averaged over 10.6 high-multiplicity ACFs per animal \((P < 0.0001)\). These data show a pronounced reduction in preneoplastic lesions associated with E2 treatment.

ERβKO animals had similar numbers of ACFs in both control (21.1 ACFs per animal) and E2-treated (20.2 ACFs per animal, \(P = 0.2\)) mice (Fig. 4A). There was a slight but significant difference \((P = 0.01)\) in high-multiplicity ACFs in the ERβKO mice between treatments, with E2-treated animals averaging 8.4 and control 10.6 ACFs per animal.
animals averaging 9.4 ACFs per animal (Fig. 4B). Together, these data distinctly show a relationship between ERβ and E2 in the colon.

**Plasma E2 concentrations.** We measured plasma E2 levels in vehicle control and E2-treated animals. Mice receiving cholesterol-only pellets showed plasma E2 levels averaging 22.4 pmol/L in WT and 33.9 pmol/L in ERβKO animals (Fig. 5). Those receiving E2-containing pellets showed average E2 levels of 2.9 nmol/L in WT and 3.0 nmol/L in ERβKO animals (P < 0.0001).

**Apoptosis within the colonic crypt.** Having found a distinct phenotype associated with E2 treatment in WT animals, we analyzed apoptosis to see if there was an association with E2 treatment. The induction of colonocyte apoptosis was determined in tissues collected from each experimental group. Within the distal colon, crypts analyzed from WT mice receiving E2 exhibited a significantly higher degree of apoptotic cells compared with those from the control. ERβKO mice showed a slight but significant difference in apoptosis with E2 treatment with more apoptotic cells per crypt than the ERβKO control mice (Fig. 6A). As can be seen in both images, it is common for cells on the luminal surface outside of the crypt structure to become apoptotic in association with these cells being sloughed off. Therefore, only cells within the crypt were analyzed. Interestingly, the increase in apoptosis in E2-treated WT mice was associated with an increase in apoptotic cells observed in the lower regions of the crypt in WT animals treated with E2 (Fig. 6B and C).

**Discussion**

Limited data have shown that E2 treatment reduces cell growth in three colon cancer cell lines, DLD-1, HCT116, and LoVo (25–27). However, the majority of cell culture studies have shown no reduction or an increase in growth in response to E2 in colon cancer cells that are fully transformed (13–17, 28). Collectively, these data highlight the need to study the effects of E2 in other cell models and specifically in nonmalignantly transformed colonocytes. YAMC cells are morphologically primitive epithelial cells, with no evidence of differentiation (18). For the first time, we show that E2 can alter the growth of nonmalignant colonocytes by showing a significant reduction in cell number in YAMC cells treated with E2. This response was also observed in YAMC cells maintained at nonpermissive conditions, suggesting that E2 has similar actions in colon cells that are more primordial in nature. YAMC-Ras are isogenic cells that were developed from YAMCs and contain an activated v-Ha-Ras oncogene. These cells are fully transformed and will continue to grow under nonpermissive conditions and form colonies in soft agar (29). The Ki-Ras oncogene is present in ~50% of colonic adenomas and carcinomas and was found to be common in tumors from all stages, including villous adenomas and villoglandular polyps that are nonmalignant but often progress to malignancy (30, 31). Data from YAMC-Ras cells show that overexpression of an activated v-Ha-Ras oncogene negates the growth inhibition due to E2 exposure.

Having identified an effect of E2 on cell growth, we wanted to determine if this response was ER mediated. ERβ is the predominant form of ER found in the colon and is expressed in some colon cancer cell lines (13, 25). Cotreatment of E2 with ICI, an ER antagonist, resulted in a loss of the growth inhibition induced by E2. Because ERβ expression levels were not significantly different
between YAMC and YAMC-Ras cell lines, the question of mechanism of activity arises. It is possible that a nonfunctional protein is being produced in the YAMC-Ras cells or that malignant transformation affects the pathway at a point after receptor activation. Future studies will address these important questions.

We next measured apoptosis to begin identifying the cellular responses to E₂ that result in growth inhibition. As with cell growth, some studies have shown apoptosis to be increased with E₂ treatment in colon cancer cell lines (28, 32), but others show no response. We observed a significant induction of caspase-3 activity in YAMC cells and no significant response in YAMC-Ras cells when the cell lines were treated with E₂. We also conducted experiments to determine if E₂ treatment altered the progression of cells through the cell cycle, but no significant differences were seen between treatments (data not shown). These data suggest that E₂ reduces cell growth in noncancerous colonocytes by induction of apoptosis; however, this response is lost following malignant transformation.

To investigate how E₂ affects the development of preneoplastic lesions, we first analyzed colonic tissue from WT animals receiving chemical carcinogen to better define the effects of E₂ at a premalignant stage of colon carcinogenesis. Ovariectomized WT mice exhibited a significant reduction, both in total number of ACFs and high-multiplicity ACFs, when treated with E₂ compared with those treated with vehicle control. The profound reduction of high-multiplicity ACFs in E₂-treated mice is of significant interest because these lesions are most predictive of eventual tumor formation (33). These data support other findings showing E₂ to be protective against tumor formation (34–36). However, our studies were focused on examining premalignant tissue and show a chemoprotective role for E₂ therapy before tumor formation.

We then compared the ACF data from WT animals with that from ERβKO mice. Clinical studies have shown reduced ERβ expression in colonocytes within tumors when compared with adjacent uninvolved tissue (37–39). A functional ERβ was previously determined to be involved with E₂ protection in APC Min mice (11). We have studied premalignant carcinogenesis and have shown that E₂ has a profound effect at this stage. In the presented studies, the reduction in the number of ACFs in WT animals treated with E₂ was pronounced and almost completely lost in ERβKO mice. These data clearly define the necessity for functional ERβ expression for E₂ treatment to exhibit a protective effect against cancer development in the colon.

Plasma E₂ concentrations in E₂-treated mice were marginally higher than those seen in a nonpregnant cycling woman, but well below those seen during pregnancy (40, 41). Mice receiving control pellets were below the threshold associated with menopause in women (42). Collectively, our in vitro and in vivo data show the influence of E₂ on cell growth in noncancerous colonocytes, but additional studies are needed to determine the minimal dose required to observe these effects.

We then sought to determine if apoptosis is induced in the colonocytes of animals treated with E₂. There was a significant induction of apoptosis in colonocytes in sections taken from WT animals treated with E₂ when compared with tissues from control mice. These cells are likely to have chemically induced DNA damage, but are not yet to the point of malignant transformation. Collectively, the YAMC and tissue data show that E₂ treatment induces apoptotic activity in nonmalignant colonocytes. Furthermore, these findings suggest that the YAMC cell line is a suitable model for studying the role of E₂ on nonmalignant colonocytes. One interesting observation from analysis of tissues collected from the mice was that apoptotic cells in the WT E₂-treated animals were found throughout the depth of the crypt, whereas the vast majority of apoptotic cells in the WT control–treated group and both treatment groups in the ERβKO mice were confined to the luminal third of the crypt. Generally, the majority of apoptosis seen within a colonic crypt occurs near the luminal surface, before cells are sloughed off. This increased range of apoptotic activity suggests a specific response to carcinogen-induced damage in WT animals treated with E₂. Preliminary data do not show this increased apoptotic activity in the lower portion of the crypt in non–AOM-exposed WT animals treated with E₂ (data not shown). This phenomenon has been referred to as directed apoptosis and may be similar to the short-term apoptotic induction seen in rats after AOM exposure (43).

In conclusion, E₂ treatment showed a distinct phenotype in YAMC cells and colonocytes within WT mice. Treatment with E₂ reduced cell growth and induced apoptosis in nonmalignantly transformed cells in culture; however, expression of activated Ras in an isogenic cell line nullified this effect. In addition, E₂ treatment in ovariectomized WT mice exhibited a significant protection against preneoplastic lesions. This protection was lost in the absence of ERβ and definitively shows that ERβ is the primary mediator of this protective effect. For the first time, these data present evidence that E₂ treatment protects colonocytes from malignant transformation by increased apoptotic activity and begin to define the role of E₂ in nontransformed colonocytes. These findings are first steps toward identifying ERβ as an important target for potential chemopreventative agents in reducing the risk of tumor formation in the colon.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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