Modulation of tumor formation and intestinal cell migration by estrogens in the Apc<sup>Min</sup>/+ mouse model of colorectal cancer

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Epidemiological studies suggest that post-menopausal hormone replacement therapy (HRT) reduces colorectal cancer (CRC) incidence. Phytoestrogens, including the soy isoflavone genistein and coumestrol, are used by many women as alternatives to HRT. Previous studies showed that ovarioectomy induced a 77% increase in intestinal adenoma number in the C57BL/6J-Min/+ (Min/+) mouse, an animal model of adenomatous polyposis coli (APC)-associated CRC. Replacement of estradiol (E2) in ovarioctomized Min/+ mice reduced tumor number to baseline and up-regulated the expression of estrogen receptor β (ERβ). We hypothesized that the phytoestrogens genistein and coumestrol would inhibit intestinal tumorigenesis in ovarioctomized Min/+ mice. Min/+ and Apc<sup>-/-</sup> (WT) mice were ovarioctomized and assigned to either a control diet or treatment with E2, genistein or coumestrol. Treatment of ovarioctomized Min/+ (Min/+ OX) mice with genistein resulted in a non-significant reduction in tumor number. Min/+ OX mice treated with coumestrol had significantly fewer tumors than untreated Min/+ OX controls and the same number of tumors as non-ovarioctomized Min/+ mice. Bromodeoxyuridine migration assays also demonstrated that treatment with E2 or coumestrol improved enterocyte migration rate. Immunoprecipitation and immunohistochemistry analyses showed that impaired association of the adherens junction proteins E-cadherin and β-catenin in Min/+ mice was improved by treatment with either E2 or coumestrol. Immunoblot analyses also showed that expression of ERβ was elevated in enterocytes of Min/+ OX mice treated with E2 or coumestrol as compared with those of untreated Min/+ OX mice. In conclusion, both coumestrol and E2 prevent intestinal tumorigenesis and ameliorate enterocyte migration and intercellular adhesion in the Apc<sup>Min</sup>/+ mouse model of CRC.

Introduction

Recent epidemiological studies suggested that combined estrogen and progesterone hormone replacement therapy (HRT) reduces the incidence of colorectal cancer (CRC) in post-menopausal women (1–3). Estrogens are used by many women for the alleviation of menopausal symptoms and for the preservation of bone density. However, these benefits are tempered by significant risks, including an increased risk of breast and endometrial cancers, as well as an increased risk of stroke and coronary disease if used in association with progestins (4). Studies of estrogen use alone in hysterectomized women have not yet defined the risk–benefit ratio for HRT in this population.

Phytoestrogens, such as the soy isoflavone genistein and the coumestan coumestrol, are estrogenic plant-derived compounds used by many women as alternatives to HRT. Epidemiological studies have demonstrated a reduction in CRC incidence among women in Asian countries where soy-rich diets are consumed (5–7). In addition, the risk of osteoporosis, cardiovascular disease and breast and endometrial cancers among women consuming soy-rich diets is also reduced. Both isoflavones and coumestans bind the estrogen receptor (ER) and induce transcription of ER-responsive genes, suggesting that mechanisms of CRC prevention may be shared among estrogens and their plant-derived homologs (5,8–12). We sought to examine whether phytoestrogens are effective chemopreventive agents and, if so, to identify the mechanisms by which estrogens and phytoestrogens suppress intestinal tumorigenesis.

The adenomatous polyposis coli (APC) protein plays a role in regulating intestinal cell growth and migration. Loss of APC, resulting from point mutation or deletion of the APC gene, is an initiating event in >80% of sporadic CRCs and in nearly 100% of patients with the familial CRC syndrome, familial adenomatous polyposis (FAP) (13). The APC gene encodes a 310 kDa protein that, in association with GSK-3β, a serine-threonine glycoser synthase kinase, down-regulates intracellular levels of β-catenin (13). The oncogenic effects of APC deficiency are in part attributable to excess levels of β-catenin, an oncoprotein capable of inducing Tcf-mediated transcription of growth-promoting genes such as c-Myc and cyclin D1 (13). The Min/+ mouse is a model for FAP and for sporadic APC-associated CRC. The Min/+ mouse bears a germline mutation in Apc that leads to the development of multiple intestinal adenomas by ~4 months of age (13–15). Loss of the wild-type Apc allele in Apc<sup>Min</sup>−/− adenomas results in increased intracellular expression of β-catenin (16). In addition, heterozygous Apc deficiency in the Min/+ intestinal epithelium is associated with slowed enterocyte migration and reduced adherens junction (AJ) integrity (16–18).

Previous studies in our laboratory demonstrated that Apc<sup>Min</sup>−/− adenomas in the Min/+ mouse are hormonally...
responsive. Ovariecotomy of Min/+ females led to a 77% increase in intestinal tumor number ($P = 0.0004$) (19). Treatment of ovariecotomized mice with 17β-estradiol (E$_2$) resulted in a reduction in tumor number to that found among non-ovariecotomized controls ($P = 0.85$). This response was associated with an increased expression of estrogen receptor (ER) β and decreased expression of ERα in Min/+ enterocytes, suggesting that the protective effect of E$_2$ may be mediated by ERβ.

Estrogens exert variable effects depending upon tissue-specific metabolism and receptor expression profile. Estrogens act via binding to the ER, a ligand-activated nuclear transcription factor. Upon ligand binding, the ER dimerizes and initiates gene transcription at the estrogen-response element, as well as alternative response elements (AP-1 and EpRE) (8,20,21). The two major isoforms of the ER are ERα and ERβ. ERα expression predominates in the breast and uterus and ERβ predominates in the urogenital tract. In human colon ERβ expression exceeds that of ERα. However, in human colon cancer ERβ expression is reduced (22,23).

Based upon our data that E$_2$ suppresses intestinal tumorogenesis, we hypothesized that the phytoestrogens genistein and coumestrol would also prevent $Apc$-associated tumorogenesis in the Min/+ intestinal epithelium. We also sought to determine the effects of E$_2$, genistein and coumestrol on intestinal tumor formation and upon markers of enterocyte differentiation, including crypt-villus migration rate, intercellular adhesion and ER expression profile.

### Materials and methods

**Materials**

Min/+ mice and their wild-type (WT) $Apc^{+/+}$ littermates were purchased from the Jackson Laboratory (Bar Harbor, ME). AIN-76A, AIN-76A + 0.1% genistein and AIN-76A + 0.01% coumestrol pelleted diets were obtained from Research Diets Inc. (New Brunswick, NJ). Controlled release 90 day pellets containing 17β-estradiol (NE-121) were obtained from Innovative Research of America ( Sarasota, FL). Genistein was purchased from the Jackson Laboratory. Coumestrol was purchased from Sigma (St Louis, MO). Primary antibodies and protein standards for ERβ and ERα were obtained from Santa Cruz Biotechnology (ERα, MC-20 rabbit polyclonal antibody sc-542; Santa Cruz, CA) and Affinity Bioreagents (ERα standard mp-310; ERα, PA1-310A rabbit polyclonal antibody; ERβ standard, RP-311; Golden, CO). Antibodies directed against β-catenin (clone 14) and E-cadherin (clone 36), as well as the control cell lysates from HeLa and A313 cells, were obtained from BD Transduction Laboratories (San Diego, CA). Antibody directed against actin and the reagents N-acetyl-Leu-Leu-norleucinal (ALLN), phenylarsine oxide (PAO), 5-bromodeoxyuridine (Brdu) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma. Anti-E-cadherin antibody (clone ECD-1) was obtained from Zymed Laboratories (San Francisco, CA). Anti-Brdu (clone BMC 9318) antibody was obtained from Roche Diagnostics (Indianapolis, IN). Biotinylated goat anti-mouse and goat anti-rat antibodies and the Avidin/Biotin Blocking Kit were purchased from Vector Laboratories (Burlingame, CA). Goat anti-mouse horseradish peroxidase conjugate was purchased from Dako (Carpenteria, CA). Biotinylated SDS-PAGE broad range standards were from Bio-Rad (Hercules, CA). Protein G-agarose and Complete™ protease inhibitor tablets were from Roche Diagnostics (Indianapolis, IN). Determination of protein concentrations was performed using the MicroBCA Protein Assay from Pierce (Rockford, IL). All other reagents and materials were as detailed previously (16,24).

**Animal treatments**

Forty female Min/+ and forty gender-matched WT littermates were obtained at 5 weeks of age. Ovariectomy was performed at the Jackson Laboratory on Min/+ mice at 4 weeks of age. Ten non-ovariecotomized Min/+ female littermates were obtained as controls. At 6 weeks we assigned 10 ovariecotomized Min/+ (Min/+ OX) and 10 ovariecotomized WT (WT OX) mice to receive either control (soy-free) AIN76A diet, AIN76A diet + E$_2$ administered s.c. (1.9 mg/90 day controlled release), AIN76A containing 0.1% genistein or AIN76A containing 0.01% coumestrol. This dose of genistein has been shown to yield serum levels in rats (0.4 μM) similar to those measured in humans after consumption of a soy-rich meal (12). Coumestrol at this dose approximates levels found in foods such as pinto beans and produced E$_2$-like responses in rat uterine tissue (25). Mice were treated for 10 weeks, during which time they were checked for signs of distress or anemia, as determined by measuring weekly body weight and food consumption. The weights of all WT mice and all treated Min/+ mice increased significantly during the experiment. The weight gain over 10 weeks of Min/+ mice fed control AIN76A diet, however, was significantly lower than the weight gain among WT mice.

**Measurement of enterocyte migration**

At 16 weeks, following 10 weeks of treatment, all mice were injected i.p. with 30 mg/kg BrdU, a thymidine analog incorporated into actively proliferating cells. Animals were then killed at 24 h intervals (0–96 h) after injection. At the time of death the intestinal tracts were removed from the duodenum to the distal rectum, flushed with phosphate-buffered saline containing physiological concentrations of MgCl$_2$ and CaCl$_2$ and opened longitudinally. Upon dissection of the Min/+ intestine adenomas were counted and excised by two independent observers. Segments of mid small intestine were fixed in 10% buffered formalin for use in immunohistochemistry (IHC) studies. Enterocytes from the remaining length of each small intestine were isolated by lightly scraping the mucosal surface with the edge of a microscope slide (16). This material was then washed in cold phosphate-buffered saline and centrifuged at low speed. The resulting pellets were flash frozen in liquid nitrogen for use in lysate preparation, immunoprecipitation (IP), and immunoblot (IB) analyses as detailed previously (16).

To detect BrdU incorporation into the newly synthesized DNA of epithelial cells, mid small intestinal specimens of ~5 mm in length were formalin fixed, embedded in paraffin and sectioned at 4 μm. Several serial sections were obtained. Before staining, sections were deparaffinized in xylene and rehydrated through an alcohol series. The first section was stained with hematoxylin and eosin to document normal mucosal histology. The sections were then stained with anti-BrdU as follows. Slides were heated in 10 mM citrate buffer, pH 6.0, for 10 min to perform antigen retrieval. Endogenous peroxidase activity was blocked by incubating the slides in 3% H$_2$O$_2$ for 15 min at room temperature. DNA was then denatured by immersing the slides in 2 N HCl for 30 min at 37°C, followed by neutralization in 0.1 M borate buffer, pH 8.5, for 10 min at room temperature. Specimens were then enzymatically pretreated by immersion in 0.5% pepsin for 30 min at 37°C. Serum block using 5% horse serum was then applied to the slides for 10 min at room temperature, followed by blocking steps of 15 min each for avidin and biotin. Specimens were then incubated for 2 h with anti-BrdU antibody at room temperature. Specimens were incubated with DAKO anti-mouse HRP for 30 min at room temperature, followed by color development in DAB for 5 min. Specimens were then dehydrated in a graded ethanol series and coverslipped.

Eight intact histologically normal crypt–villus units from each animal were examined. We focused our study on small intestinal mucosa, since Min/+ mice develop the majority of tumors in the small intestine and few in the colon or duodenum (14,26). To maintain consistent sampling, all specimens were taken from the mid portion of the small intestine. For each unit the highest labeled cell within the crypt–villus was counted starting from the most inferior cell observed in the crypt. Previous histological studies showed no differences in crypt length, crypt area, nuclear density or number of cells per crypt–villus unit in Min/+ small intestinal epithelium as compared with that of their WT littermates (17,18).

**Determination of E-cadherin localization and expression**

Slides were deparaffinized in xylene followed by rehydration in an alcohol series. After an antigen retrieval (detailed above), endogenous peroxidases were blocked in 3% H$_2$O$_2$ for 15 min at room temperature. Slides were then incubated in a rat monoclonal antibody to mouse E-cadherin for 2 h at room temperature. Secondary biotinylated goat anti-rat antibody was applied for 30 min at room temperature. Specimens were then incubated in Vector Elite ABC reagent for 30 min at room temperature. Slides were then stained with DAB for 10 min at room temperature, followed by counterstaining with Mayer’s hematoxylin and alcohol dehydration.

**Total cell lysate (TCL) preparation, immunoprecipitation (IP) and immunoblot (IB) analysis**

Washed enterocytes obtained from each half of the small intestine were placed in 1 ml of lysis buffer (1% Triton X-100, 0.1% SDS, 10% glycerol, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 μm PMSF, 50 mM NaF, 1 mM NaVO$_4$, 10 mM Na$_2$PO$_4$, 10 mM ALLN, 3 mM H$_2$O$_2$ and 0.4 mM PAO) and homogenized in a Dounce homogenizer with 10 strokes of a pestle. The resulting lysates were clarified by centrifuging at 14 000 r.p.m. for 10 min.
Following protein determinations, normalized aliquots of each lysate were placed in sample buffer and stored as above. In parallel, portions of each lysate were pre-cleared by mixing with protein G beads at 4°C for 1 h. Immune complexes were precipitated for 12 h with protein G beads. The beads were stringently washed and bound proteins were released in 50 μl of Laemmlü buffer. The entire amount of each IP sample and the corresponding TCL samples prepared in parallel (controls) were resolved by SDS–PAGE. Procedures for IB analyses were as described (16). In all IB analyses lanes containing control cultured cell lysates as well as biotinylated molecular weight markers were run adjacent to test samples to serve as protein and size standards, respectively. Previously blotted membranes were washed and stripped by incubating at 65°C for 20 min in denaturing buffer (68 mM Tris–HCl, pH 6.8, 10% SDS, 0.01% β-mercaptoethanol) before re-probing.

All experiments were repeated at least five times with independently prepared lysates.

**Results**

**Coumestrol reduced intestinal tumor number in Min+/OX mice**

Tumor counts obtained from Min+/OX mice were compared with those from non-ovariectomized Min+/mice. Consistent with prior studies, 96.7% of tumors in Min+/mice were found in the small intestine, with the remaining 3.3% found in the colon. Ovariectomy increased the mean total tumor number from 18.9 ± 6.8 (SD) to 35.6 ± 9.4, by 88%, in Min+/ females (P = 0.0005). Ovariectomy resulted in a significant increase in tumor burden in the small intestine (P = 0.0004) but a non-significant increase in the colon (P = 0.60). The distribution of tumors was noted to be similar to that of Min+/+ animals (small intestine 97.8%, colon 2.2%). Although the total number of adenomas observed among our Min+/+ population was low, tumor counts were consistent among the 10 mice in this group. The average total number of tumors in the Min+/+ population was 15. Adenoma multiplicity in Min+/+ mice is known to be highly variable and altered by genetic background, namely by the modifier loci Mom1 and Mom2 (27,28). Although Mom1 is observed in C57BL/6 mice, Mom2 occurred spontaneously in another inbred strain and is not yet routinely tested for. Min+/+ mice heterozygous for Mom1 or Mom2 show ~50% and ~90% reductions, respectively, in small intestinal adenoma number (29).

Tumor counts obtained from Min+/+OX mice following 10 weeks of treatment with either E2, genistein or coumestrol are provided in Table I. As observed previously, treatment with E2 reduced total tumor number to the baseline value found in Min+/+ mice, a mean of 21.4 ± 5.8 tumors (P = 0.42 versus Min+/+) (19). E2 replacement significantly decreased small intestinal (P = 0.001) and marginally decreased large intestinal (P = 0.20) tumor numbers relative to untreated Min+/+OX mice. The distribution of tumors was similar for the two groups (small intestine 98.6%, colon 1.4%). Treatment with 0.1% genistein did not prevent intestinal tumor formation. Although we witnessed an 8.2% reduction in total tumor number (32.7 ± 13.8 tumors) relative to untreated Min+/+OX mice, this difference was non-significant (P = 0.59). In ovariectomized mice genistein failed to prevent tumors in either the small intestine (P = 0.62) or the colon (P = 0.33) and the overall distribution of tumors was unchanged (small intestine 98.2%, colon 1.8%). Treatment of ovariectomized mice with 0.01% coumestrol reduced total tumor number to the baseline value found in Min+/+ mice, to an average of 21.6 ± 12.0 tumors (P = 0.55 versus Min+/+). Treatment with coumestrol significantly (39.3%) decreased total tumor number (P = 0.01), small intestinal tumors (P = 0.01) and large intestinal tumors (P = 0.02) relative to untreated Min+/+OX mice.

**Enterocyte migration is reduced by ovariectomy of WT and Min+/+ mice**

Epithelial cells of the intestine are continuously renewed through a process initiated by stem cell division, producing daughter cells that differentiate and migrate from the crypt to the tips of villi. This process allows for rapid replacement of cells damaged by trauma, infection or mutation and actively preserves the functional and structural integrity of the intestine. Disease states, such as tumor formation, arise when this process is disrupted. In a previous study of the Min+/+ mouse we found that enterocyte migration in the histologically normal small intestinal mucosa was decreased by 25% (17). Furthermore, treatment of Min+/+ mice with chemopreventive doses of the non-steroidal anti-inflammatory drug sulindac restored normal enterocyte migration (18,30,31). Subsequent studies suggested that the migration defect results from a loss of AJ formation in Min+/+ enterocytes, characterized by loss of association between E-cadherin and β-catenin at the lateral cell membrane (16).

In the present study we have performed a similar analysis to examine enterocyte migration in the small intestine of ovariectomized WT and Min+/+ females. Our data thus far suggest that estrogens, through a modulatory effect on the differentiation process, may also play a role in maintaining normal intestinal mucosal architecture. We hypothesized that ovariectomy, which deprives the animal of the major source of estrogens, would result in further slowing of enterocyte migration in the Min+/+ intestine relative to non-ovariectomized control animals.

We injected ovariectomized 16-week-old WT and Min+/+ animals with the thymidine analog BrdU, to label proliferating cells. At 24 h time intervals (0–96 h) we examined the mid small intestine from these animals by IHC using a monoclonal anti-BrdU antibody to detect the location of proliferating cells at the time of death. Eight villi from two WT and two Min+/+ animals were examined at each time point. Differences in migration rates between ovariectomized and non-ovariectomized females were evaluated by analysis of covariance (ANCOVA). The highest labeled cell on a villus was modeled as a linear function of time, treatment and time × treatment interaction. Interestingly, ovariectomy appeared to exert an inhibitory effect on enterocyte crypt–villus migration in both WT and Min+/+ females. The greatest inhibitory effect was observed between 48 and 96 h. Among both WT and Min+/+ females ovariectomy led to a significant reduction in migration rate (−0.185 and −0.187 cells/h, P = 0.002 and

<table>
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<tr>
<th>Table I. Estradiol and coumestrol reduce tumor number in Min+/+OX mice</th>
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<tr>
<td>Min+/+OX</td>
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<td>Min+/+OX estradiol</td>
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<td>Min+/+OX genistein</td>
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<td>Min+/+OX coumestrol</td>
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*Tumor counts are expressed as the mean ± SD. Values shown are for n = 10 animals/group.
*P* result from comparison of total tumor counts in two-tailed *t* tests vs. Min+/+ OX animals.
Enterocyte migration is augmented by treatment of Min/+ OX mice with either estradiol or coumestrol

We next examined whether migration rates of Min/+ OX females were altered by treatment with either E2 or coumestrol. Migration rates in genistein-treated animals were not assessed consistent with its lack of a chemopreventive effect. Using ANCOVA analysis, we demonstrated highly significant time × treatment interactions for both E2 and coumestrol (\(P < 0.0001\)) (Table II). The migration rates differed significantly between untreated Min/+ OX mice and Min/+ OX mice treated with either E2 (Figure 2A) or coumestrol (Figure 2B). Treatment of Min/+ OX mice with either compound resulted in an improvement in enterocyte migration, with rates approaching that observed in control WT OX females. This result is illustrated in Figure 2C, by comparison of the relative heights of BrdU-labeled cells among the various treatment groups. In addition, both E2 and coumestrol restored the migration rate in Min/+ OX mice to the rate observed among non-ovariectomized Min/+ females.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Enterocyte crypt-villus migration in the Min/+ mouse is slowed by ovariectomy. At 16 weeks 10 Min/+ OX mice were injected with 30 mg/kg BrdU. Small intestine segments were examined at the indicated intervals post-injection by staining with anti-BrdU antibody. Eight intact intestinal mucosa. Using enterocyte total cell lysates, we performed IP and IB to measure association of the AJ proteins β-catenin and E-cadherin in Min/+ enterocytes. In earlier studies we found that the association of β-catenin and E-cadherin was reduced in Min/+ enterocytes (16). This observation was confirmed in the current study (Figure 3A). Interestingly, ovariectomy alone led to increased β-catenin/E-cadherin binding. This enhanced association may be attributable to higher expression levels and, hence, binding availability of β-catenin in ovariectomized enterocytes. Both E2 and coumestrol treatment increased the association of E-cadherin with β-catenin in Min/+ OX compared with non-ovariectomized Min/+ enterocytes, without altering expression levels of β-catenin (Figure 3A).

To investigate this further, we used IHC methods to examine the intracellular location of E-cadherin (Figure 3B). We found reduced E-cadherin expression at the lateral cell membrane of Min/+ and Min/+ OX animals. Given the increased β-catenin/E-cadherin association we observed in Min/+ OX enterocytes by IP/IB analyses in Figure 3A, this IHC finding suggests that the β-catenin/E-cadherin complex may undergo aberrant trafficking in these enterocytes. A distinct lateral enterocyte membrane localization of E-cadherin was observed in Min/+ OX enterocytes treated with either E2 or coumestrol. This result suggests that E2 and coumestrol help restore AJ integrity at the lateral membrane.

**Table II.** Enterocyte crypt-villus migration in Min/+ OX mice is improved by treatment with either E2 or coumestrol

<table>
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<tr>
<th>Comparison</th>
<th>Rate difference (\Delta)</th>
<th>t-statistic</th>
<th>P-value</th>
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<tr>
<td>Min/+ OX E2 vs. WT OX</td>
<td>−0.015</td>
<td>−0.39</td>
<td>0.70</td>
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<tr>
<td>Min/+ OX coum vs. WT OX</td>
<td>−0.010</td>
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<td>0.73</td>
<td>0.47</td>
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<tr>
<td>Min/+ OX coum vs. Min/+</td>
<td>0.049</td>
<td>0.87</td>
<td>0.39</td>
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*Differences in migration rate were evaluated by ANCOVA using time as a covariate. Rate difference is expressed as cells/h.

**Discussion**

CRC is one of the leading causes of death in industrialized countries. Although there are no clear differences in CRC incidence between men and women, data from a recent meta-analysis of 18 observational studies of post-menopausal women showed a 20% reduction in CRC among users of HRT (relative risk 0.80, 95% CI 0.74–0.86) (2). Recently, the Women’s Health Initiative (WHI) conducted a randomized controlled trial to examine the health effects of HRT in >16 000 women aged 50–79. In support of the observational studies, the WHI demonstrated a 37% reduction (relative risk 0.63, 95% CI 0.43–0.92) in CRC incidence over 5 years among combined estrogen plus progesterin HRT users (3). Despite this benefit and the additional benefit of reduced osteoporotic fracture risk, HRT use in the WHI trial was associated with risks...
including an increased risk of breast cancer, stroke, coronary heart disease, thromboembolic events and, possibly, dementia (2–4,32). These results were based upon a daily combined regimen of estrogen and progesterone in women with a uterus. Studies of estrogen use alone in hysterectomized women are ongoing and thus far have not demonstrated statistically significant adverse outcomes.

Given the possibility of adverse effects with HRT use, there has been a growing interest in the use of phytoestrogens as potential alternatives for HRT. This interest has been fueled by epidemiological studies demonstrating a reduction in menopausal symptoms, rates of coronary heart disease, various cancers and osteoporotic fractures among populations that consume soy-rich diets (33). Phytoestrogens produce weak ER stimulation, of the order of $10^{-2}$–$10^{-3}$ that of E$_2$ (33), and may also produce receptor-independent effects upon epithelial cell differentiation. Isoflavones, such as genistein, and the coumestan coumestrol are the best known phytoestrogens. Both compounds possess a diphenolic ring common to potent synthetic estrogens such as diethylstilbestrol. The major sources of isoflavones are soy products, such as tofu, tempeh and soybeans, whereas coumestans are derived mainly from alfalfa.

Genistein, in addition to acting as a weak estrogen, is a potent inhibitor of tyrosine kinases, aromatase and DNA topoisomerase. It also possesses antioxidant activity, inhibits cell cycle progression and angiogenesis in endothelial cells. Genistein suppresses the growth of a wide range of cancer cells in vitro, including colon cancer cells, with IC$_{50}$ values ranging from 5 to 100 µM (2–25 µg/ml) (7,10,34,35). For example, Kuo et al. found that genistein inhibited the growth of two human colonic adenocarcinoma-derived cancer cell lines, Caco-2 and HT-29, and induced both differentiation and apoptosis (36). Mechanisms proposed for the growth-inhibitory effects of genistein on colon cancer cells include tyrosine kinase inhibition, inhibition of angiogenesis and activation of ERβ.

**Fig. 2.** Treatment with either E$_2$ or coumestrol restores crypt-villus migration in Min/+ OX mice. After 10 weeks treatment 10 mice from each treatment group were injected with 30 mg/kg BrdU. Small intestine segments were examined at the indicated intervals post-injection by staining with anti-BrdU antibody. Eight intact histologically normal crypt–villus units from each animal were examined by an observer blind to the genetic status and treatment group of the animal. Error bars shown at each time point represent standard deviation values. (A) Treatment of Min/+ OX females with E$_2$ significantly improved enterocyte crypt–villus migration rate. The migration rate found in Min/+ OX E$_2$ females approached that found in WT OX females. (B) Treatment of Min/+ OX females with coumestrol also significantly improved enterocyte crypt–villus migration rate. The migration rate found in Min/+ OX coumestrol females approached that found in WT OX females. (C) Serial 4 µm sections from the mid small intestine were processed for IHC and stained with antibody to BrdU (×40).

Treatment with either E$_2$ or coumestrol led to a visible improvement in crypt–villus migration rate.
Studies of the effects of soy isoflavones on the development of colorectal cancers in vivo are limited and have yielded conflicting results. In one in vivo study male and non-ovariectomized female ApcMin+/+ mice were fed genistein in a high fat diet at a concentration of 0.28 g/kg for 11 weeks. No significant differences in the incidence, multiplicity, size or distribution of intestinal tumors were observed (37). In this study we have shown that genistein fed to ovariectomized Min/−/+ mice at a high concentration (1 g/kg in the diet) led to a small, but non-significant decrease in intestinal tumor number. This concentration was selected because it produces a serum concentration in ovariectomized rats of ~0.4 μM, similar to the concentrations measured in humans after consumption of meals rich in soybeans (38,39). In addition, the concentration of genistein in most soy food materials ranges from 1–2 g/kg protein (33). Such concentrations are 10- to 100-fold greater than the EC50 values of genistein with respect to rat or human ERβ and ERα as measured in vitro (11,12). Therefore, we propose that genistein ingested at this high dietary concentration would activate the ER, especially ERβ, for which genistein has a 20- to 30-fold greater affinity.

Genistein did not exert a chemopreventive effect in this study. This may be explained by the variable metabolism of this compound. The effects of genistein are dependent upon
multiple enzymatic metabolic conversions (conjugation and deconjugation) that occur in the gastrointestinal tract, resulting in the formation of heterocyclic phenols with structural similarity to estrogens (40). The concentrations of different genistean metabolites may vary widely between mice administered equal quantities, due to variable rates of deconjugation by gastrointestinal flora and reabsorption into the enterohepatic circulation (33). Alternatively, the interaction of genistein with tyrosine kinases and/or its anti-angiogenic properties may contribute to its antioxidant properties.

Unlike genistein, little is known about the physiological effects of coumestrol beyond its actions as an ER ligand. Limited data exist regarding possible antioxidant properties of coumestans or their effects on tumor cell differentiation or intercellular adhesion. Therefore, we have demonstrated that coumestrol and E2 act at the earliest stages of intestinal tumorigenesis by normalizing Apc-mediated defects in enterocyte migration and intercellular adhesion.

The effects of ovariectomy alone were particularly interesting. Both association between the AJ proteins β-catenin and E-cadherin and overall β-catenin expression were increased in Min/+ OX mice compared with non-ovariectomized controls. It is likely that the increased association of β-catenin and E-cadherin observed in ovariectomized enterocytes is at least partly attributable to the increased expression level and availability of β-catenin. Despite the increased association of β-catenin and E-cadherin, E-cadherin was not properly localized to the intercellular membrane in Min/+ OX enterocytes. We have previously shown that non-ovariectomized Min/+ enterocytes exhibit decreased membrane localization of E-cadherin (16). In ovariectomized enterocytes aberrant internalization of not only E-cadherin but of the β-catenin- E-cadherin complex could explain the inhibitory effect of ovariectomy on enterocyte migration, since efficient assembly and disassembly of AJs at the intercellular membrane is critical for normal enterocyte migration.

Ovariectomy decreased enterocyte migration in both WT and Min/+ mice, suggesting that endogenous estrogens modulate the frequency of turnover of intestinal epithelial cells. High cell turnover rates are one defense against the genotoxic effects of the intestinal microenvironment. It is therefore possible that, even in the absence of a genetic predisposition to colon cancer, surgical menopause causes tumor-promoting alterations in the intestinal mucosa. In another model E2 protected against dimethylhydrazine-induced colon carcinogenesis in ovariectomized rats (43). This treatment was associated with increased expression of the colonic vitamin D receptor. Diets deficient in vitamin D are associated with colon cancer in human observational studies and rodent models (44) and vitamin D deficiency is one cause of slowed enterocyte migration in mice (45). Vitamin D also decreases cell turnover rates in the intestine, increasing DNA repair and decreasing cell proliferation (46). It is therefore possible that the protective effect of E2 in the colon is mediated through this pathway. Studies to investigate this further in the Min/+ mouse are underway.

Ovariectomy results in altered expression of ER subtypes, namely increased expression of ERβ in the intestinal mucosa (19). ERβ is believed to be the natural receptor in the intestinal mucosa and its increased expression and availability to bind ligand may be sufficient to initiate the restoration of normal intercellular adhesion. Enterocyte migration was not favorably altered by an increase in ERβ expression alone, as observed with ovariectomy, likely because the ER ligand is necessary for the chemopreventive actions of ERβ. The additional up-regulation of ERβ expression in E2- and coumestrol-treated enterocytes supports a role for ERβ-mediated phytoestrogen chemoprevention. ERβ has also been shown to play a key role in modulating migration in other regions, including the brain. ERβ knockout (ERβ−/−) mice exhibit delayed migration into the neocortex during corticogenesis (47). We are currently studying the effects of ERβ knockout in the Min/+ mouse model to further elucidate the mechanisms by which estrogens inhibit Apc-mediated tumorigenesis.

In conclusion, the studies presented here suggest that coumestrol and E2 exert similar chemopreventive effects in vivo in the intestinal mucosa. In addition, both agents act upon two processes targeted early in tumorigenesis,
intercellular adhesion and migration. Further studies are warranted to determine whether coumestrol and other phytoestrogens are safe and effective alternatives to HRT for the chemoprevention of colorectal cancer in humans.

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