ESTRADIOL AND GENISTEIN ALTER CELLULAR PHYSIOLOGY OF NON-MALIGNANT COLONOCYTES

A Thesis

by

AUTUMN RENEE BILLIMEK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Nutrition

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Approved by:

Chair of Committee,	Clinton D. Allred
Committee Members,	Joseph M. Awika
	Joseph M. Sturino
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ABSTRACT

Estradiol and Genistein Alter Cellular Physiology of Non-malignant Colonocytes.

(August 2011)

Autumn Renee Billimek, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Clinton D. Allred

Many studies show that estradiol (E_2) and consumption of soy and its primary phytoestrogen component genistein (GEN) can inhibit the formation of colon tumors. However, the effects of E_2 and GEN at physiologically relevant levels in non-diseased colonocytes have yet to be investigated. We hypothesized that E_2 and GEN could prove to be chemo-protective agents in the colon by moderately increasing apoptosis and decreasing proliferation in a healthy system. Thus, the presented studies focused on evaluating the effects of E_2 and GEN in non-malignant colonocytes *in vitro* and *in vivo* to determine how the compounds influence the physiology of these cells. E_2 (1 nM/L) and GEN treatments (1 and 10 μM/L) decreased cell growth, increased apoptosis, and increased p53 transcriptional activity in young adult mouse colonocytes, a non-malignant cell line. To study further the effects of E_2 and GEN in healthy colonic epithelia, we evaluated physiologic changes in colonic crypts in ovariectomized mice given an E_2 pellet, 1,000 ppm GEN diet, or a phytoestrogen free diet. As seen *in vitro*, E_2 treated animals had significantly higher rates of apoptosis with GEN trending in the same fashion. These data demonstrate that E_2 and GEN alter the physiology of non-malignant colonocytes.

Collectively, with our previous data, this suggests that E_2 and GEN influence colonocyte physiology and this state may partially explain how these compounds decrease risk of colon cancer.

DEDICATION

In honor of my mother,

Karen Billimek,

who taught me to work hard

and enjoy every minute God has given us.

Without your love and support, I could not have become the strong person I am today

and reached for my dreams.

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CHAPTER I

INTRODUCTION

In the United States, colon cancer is one of the leading causes of death. According to the American Cancer Society, it is estimated that in 2010 alone nearly 51,370 people in the United States will die as a result of colon cancer. It is believed that colon cancer is greatly affected by hormones, particularly estrogen, as it is evident that women have a decreased incidence of the disease as compared to men (1). Due to this discovery, the use of hormone replacement therapy (HRT) has been used in an effort to prevent colon cancer. In fact, the majority of clinical studies have demonstrated that either HRT or estrogen replacement therapy can considerably lower the risk of colon cancer in post-menopausal women (2-7). This data is also supported by animal studies showing rats given estradiol (E_2) had significantly fewer colon tumors by 71% (8). However, due to an increased risk of cardiovascular disease and breast cancer, there has been controversy over the use of HRT (9). As an alternative, many women have turned to use of dietary phytoestrogens, particularly soy isoflavones such as genistein (GEN), as these compounds mimic the action of estrogen. In general, epidemiological data has suggested that a diet rich in soy can provide chemo-protective benefits and reduce colo cancer risk (10).

Although GEN has been reported to suppress colon cancer cell growth *in vitro* (11), there has been conflicting data as to whether soy isoflavones can inhibit the formation of colon cancer *in vivo*. Up to this point, all studies have used carcinogen-

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induced tumor formation models to determine the therapeutic effects of E_2 and various concentrations of GEN in the diet. However, no studies to date have been focused specifically to the chemopreventative effects of E_2 and GEN given at dietary concentrations in a non-malignant colon model.

Therefore, advancement of research in the area of evaluating how isoflavones influence the formation of colon cancer has been limited since most studies employ models of tumor progression instead of tumor prevention. With the primary interest of colon cancer prevention, we are thus focused on the effects of E_2 and GEN in normal, healthy colonic tissue given at physiologically relevant concentrations. Data from our laboratory demonstrate that E_2 inhibits cell growth through induction of apoptosis in Young Adult Mouse Colonocytes (YAMC), a non-malignant cell line. This modified physiology is believed to result in fewer preneoplastic lesions and thus decreased risk of colon cancer (12).

Based on these data, we hypothesized that E_2 and dietary concentrations of GEN would alter the physiology of these non-malignant colonocytes by inhibiting cell growth and that this response would lead to decreased risk of colon cancer. Furthermore, we hypothesized that this induction of apoptosis was via the p53 pathway, a pathway known to be induced by E_2 in other tissues (13). To test our hypotheses, we characterized the effects of GEN in YAMCs and in healthy mice.

CHAPTER II

LITERATURE REVIEW

Research Justification

Colon Cancer. Colon cancer involves the formation of malignant lesions in the large bowel and is a multi-step process progressing to an invasive stage over many years. The intestinal colonic epithelium is organized into small u-shaped invaginations called crypts that open into the intestinal lumen. The configuration of these crypts allows for increased surface area and thus increased nutrient absorption capabilities. In normal colonic tissue, stem cells at the bottom of the crypt give rise to epithelial cells (14). A single layer of these epithelial cells line the colonic crypts and provide the absorptive, secretory, and barrier functions attributed to the large intestine. As the epithelial cells differentiate, they travel up the crypt wall towards the lumenal surface away from the area of active proliferation where cell growth occurs. Once they have reached the lumenal surface, the epithelial cells may undergo apoptosis and/or are sloughed off and excreted into the fecal stream (14-16). Apoptosis is the process of programmed cell death and is primarily initiated via two pathways. One pathway in particular, the intrinsic pathway, is often triggered due to DNA damage or other types of cellular stress. This pathway involves the release of intracellular pro-apoptotic proteins that activate caspases, a group of proteases that break down proteins critical to cellular structure and survival (17). In healthy colon tissue, apoptosis is generally localized to the lumenal region of the crypt and occurs in fully differentiated cells (18). However, when DNA damage occurs, the epithelial cells undergo apoptosis closer to the zone of proliferation as a defense mechanism to get rid of the damaged cell before it has a chance to undergo

a malignant transformation (19). Thus, one of the primary functions of these epithelial cells is to sustain a highly-regulated balance of cellular proliferation and apoptosis. When there is any slight alteration of this controlled balance, malignant transformations can occur, possibly leading to tumorigenesis (20).

Carcinogenesis in humans often involves several genetic mutations that transform normal cells into a malignant phenotype. Typically, distinct morphological characteristics lead to cancer being distinguished by a continuum of three stages: initiation, promotion, and progression. The initiation stage of colon carcinogenesis is characterized by a change in the genetic makeup of a cell due to a carcinogen interacting with DNA, causing damage, and thus producing DNA lesions or adducts. These lesions lead to changes in DNA structure which can initiate genetic alterations such as the mutational activation of oncogenes or the silencing of tumor suppressor genes like p53 (20-22). The p53 protein is a primary tumor suppressor found in the nucleus of the cell and initiates many physiological functions within the cell. These include initiation of cellular senescence, induction of apoptosis, and initiation of DNA repair mechanisms in response to varying forms of cellular stress (23). The tumor suppressor protein p53 is a primary mediator of the intrinsic apoptotic pathway. Cancer progression often involves loss of heterozygosity in wild type p53 or dominant negative mutations. Point mutations in p53 occur in approximately 50% of colorectal carcinomas (24-28). These modifications could allow cells to hyper-proliferate and escape apoptosis (21, 29-32), and are thus considered precancerous. However, these mutations are frequently repaired during DNA synthesis and thus do not advance to the promotion stage of carcinogenesis.

Whenever DNA repair or apoptotic removal fails to eliminate mutated colonocytes, then the cells are stimulated to grow and divide faster leading to a population of benign cells. During the progression stage of carcinogenesis, there is clonal expansion of initiated cells that accumulate more DNA damage and consequently gain a selective growth advantage. The crypts of these cells become larger and are abnormally shaped. Their accumulation often results in a malignant phenotype. Groupings of these abnormal crypts are called aberrant crypt foci (ACF), and evidence suggests that these foci are pre-neoplastic lesions of colon cancer (21, 29). Studies also reveal that colonocytes lining ACF have increased rates of proliferation (33-35), and do not undergo apoptosis (33, 34, 36). Therefore, chemo-protective agents that decrease the rates of proliferation and increase the rate of apoptosis of colonocytes could lead to a decrease in colon cancer incidence.

Estrogen. One chemo-protective agent that has gained much attention in relation to colon cancer is estrogen. Estrogens are steroid hormones typically associated with the human female reproductive cycle. Although the oncogenic effects of estrogen have been extensively studied in breast cancer, little is known about estrogen signaling in colorectal cancer. The protective effects observed with estrogen treatment in colon cancer are compelling as it has become evident that men are more susceptible to this cancer than women (37). Similarly, there is a general decrease in risk of colon cancer associated with use of hormone replacement therapy (HRT) in post-menopausal women. Many clinical studies have observed a decrease in coloncancer risk associated with HRT in post-menopausal women (2, 6, 38). In one study, a 30-40% decrease in colon cancer risk has been associated with those currently using HRT. The greatest effect was seen in those

using HRT for five or more years but the effect was lost after HRT use was stopped (5). Estrogen replacement therapy (ERT) has also been shown to decrease risk of colon cancer in some studies (3), but other reports show inconclusive results. Though a significant protection against tumor formation has been demonstrated, these therapies are not currently recommended for chemoprevention of colon cancer in clinical practice due to their potential to increase risk of breast cancer and cardiovascular disease (9). Although these therapies are not currently used for chemoprevention, the studies into their effects are critical to understanding how they work in the colon.

Estrogen functions by diffusing from the serum through the lipid bilayer to the intracellular space where estrogen receptors (ER) reside. These receptors, ERα and ERβ, are maintained in a state of transcriptional inactivity within the cell through their interaction with co-repressor molecules (39). The binding of the primary estrogen, 17β-estradiol (E_2), to these receptors leads to a conformational change in which the receptor disassociates from the co-repressors and may recruit co-activator molecules (40). This newly activated complex then binds to an estrogen-response element on target DNA causing increased transcription of the target genes (41). This in turn leads to estrogen affecting the growth, differentiation, and function of its target tissues.

Animal models have long been used to study colon cancer and other diseases. Animal studies support the theory that E_2 treatment reduces the risk of colon cancer. One such study revealed that E_2 treatment in ovariectomized (OVX) rats exhibited reduced tumor development in a dimethylhydrazine induced model of colon cancer (8). In an effort to determine E_2's role in the reduction of colon cancer, researchers began to focus on the hormone and its primary receptors. The nuclear receptor, ERα, was first identified

in rat uteri in 1966 (42). However, in 1996, Kuiper *et al* found a second estrogen receptor, ERβ, which was first characterized in rat prostate and ovary tissue (43, 44). Structurally, the two estrogen receptors ERα and ERβ have similar DNA-binding and ligand-binding domains, but are otherwise very different. The internal ligand-binding compartment is greatly conserved and only differs in two contact residues (45). Although this may explain the similar affinities of endogenous E_2 to these receptors, many ligand-binding assays and ligand-competition experiments demonstrate that estrogens and phytoestrogens have a greater affinity for ERβ than ERα in the colon (46). In a study performed by Guo *et al*., ovariectomized wild-type (WT) and ERα knockout mice were orally administered estrone, a naturally occurring estrogen. Both WT and ERα knockout mice given estrone had decreased formation of carcinogen induced tumors suggesting that ERα is not the primary mediator for estrogenic protection in the colon (47). However, other studies have shown that ERβ expression is inversely associated with tumor incidence in the colon (48, 49). Research into the protective transcriptional effects of estrogen has typically concentrated on two main ideas: induction of apoptosis and inhibition of cellular proliferation in carcinogen treated models. Studies of ovariectomized mice show that E_2 has an inhibitory effect on colonic epithelial cell mitotic activity (50, 51). Weige *et al.* demonstrated the protective effects of E_2 through ERβ during the initiation/promotion stages of colon carcinogenesis, as protection against preneoplastic lesions was lost in ERβ knock-out mice (12). The possibility that ERβ has anti-proliferative activity independent of ERα inhibition was investigated in a transgenic mouse model (48). Min/+ mice have a germ line mutation in a tumor-suppressor gene and are thus useful in the modeling of intestinal tumorigenesis.

Usually, only about 5% of these mice develop colon tumors. However, 63% of mice that were estrogen-receptor β deficient developed at least one colon tumor. Histological analysis revealed increased rates of proliferation and decreased differentiation of the colonic mucosa. It must be noted however that the described effects of E_2 *in vivo* are protective or treatment effects as these models make use of carcinogen or a mutated gene and do not focus on how a normal colon responds to E_2 treatment.

It is well noted that animal models play a vital role in cancer research. However, *in vitro* cell culture systems provide powerful models to discover and study mechanistic pathways responsible for phenotypic alterations seen *in vivo*. In regards to colon cancer prevention, it is believed that the primary effects of E_2 through interaction with ERβ in the colon are to induce apoptosis and decrease proliferation. However, there has been conflicting data in regards to the true effects of E_2 in colon cancer cell lines. A few studies have shown that E_2 treatment can reduce cell growth in three colon cancer cell lines, DLD-1, HCT116, and LoVo (52-54). In the Martineti study, it was revealed that over-expression of ERβ in human colon tumor 118 cells decreased cellular proliferation when the cells were treated with E_2. These effects were evident in an ERβ concentration-dependent fashion (54). On the other hand, induction of apoptosis by E_2 has been described via differing mechanisms including increased DNA fragmentation in COLO205 colon cancer cells (55). It is believed that one method behind this effect may be due to upregulation of the pro-apoptotic *BAX*α gene (56). However, other researchers have determined that E_2, acting through ERβ, can also induce apoptosis in LoVo colon cancer cells through increased p53 signaling. p53 regulates the cell cycle and can induce apoptosis through upregulation of caspase 8 and 9. It has also been proposed that p53

causes a reduction in β-catenin proteins which inhibit cellular proliferation (57). Regardless of the precise mechanism, The E_2–ERβ complex appears to activate caspase 3, inducing the caspase-dependent pro-apoptotic cascade, thus protecting against disordered cell division and carcinogenesis in the colon (58). However, it must be noted that the majority of cell culture studies have shown that fully transformed colon cancer cell lines are not responsive to E_2 treatments (12, 59-61). Arai *et al.* showed that E_2 did not influence cell growth in various HT29, Colo320, Lovo, SW480, and HCT116 colon cancer cell lines (62). Likewise, Gilad *et al.* demonstrated that E_2 did not affect cellular proliferation of HT29 cells (63, 64). Thus further work in this area using non-ransformed cells could prove valuable.

Soy and Genistein. People from Southeast Asia appear to have much lower incidence of breast, prostate and colon cancer compared to Americans and Western Europeans (64). This trend has lead many scientists to believe that there is a diet association between these cultures and decreased risk of these cancers (65). After critiquing many epidemiological studies, it is believed that soy is the primary component of the Asian diet that is responsible for this decreased cancer effect (66-68). The active compounds in soy are believed to be isoflavonoids, which are polyphenolic secondary metabolites that produce hormonal activity in mammals (69). Many of these isoflavonoids exhibit estrogenic action as they preferentially bind to ERβ, stimulate the receptor and induce an estrogenic effect (70). Thus, these compounds are commonly known as phytoestrogens. In foods, phytoestrogens typically occur as inactive precursor molecules that undergo metabolic conversions by microflora in the intestine, resulting in the formation of absorbable, hormone-like compounds. The particularly active

isoflavones genistein, daidzein, and glycitein are thus formed by hydrolysis of their respective glycosides, called genistin, daidzin, and glycitin, which soybeans are notably rich in (71). With the knowledge of the effects of estrogen in decreased rates of colon cancer, looking into the role of phytoestrogenic soy isoflavones seemed a logical step for researchers to pursue.

During the late 1970s scientists began to look at soy and its effectiveness in reducing cancers in an animal model. Two studies used female F344 rats (72) and male Sprague-Dawley rats (73) to examine the effects of soy protein feeding on DMH-induced colon carcinogenesis. However, both studies failed to find significant differences in tumor number or incidences between the control and soy diet groups. Another study using endoscopic inspection of azoxymethane(AOM)-treated F344 rats reported decreased incidence of colorectal tumors in animals fed diets either containing soybean curd or soy protein digest when compared with those fed using the control casein diet (74). In the Hakkak *et al.* study (75), effects of soy protein isolate containing 430 mg total isoflavones per kilogram diet was investigated against AOM-induced colon tumor formation in male Sprague-Dawley rats. Results showed a prominent decrease in incidences of both benign (5 versus 19%) and invasive (10 versus 31%) tumors when compared with controls. It must be noted however that experimental diets were previously administered to parental animals, suggesting that long-term consumption of soy protein may be required to diminish the risk of colon cancer.

For the most part, these experiments looked at soy as a whole and not the individual isoflavones effects. Unfortunately, only a few *in vivo* studies involving isolated phytoestrogens have been executed, and most of these focused on genistein due

to the discovery in 1987 that pharmacological levels of genistein, the primary isoflavone found in soy, inhibited the activity of the epidermal growth factor receptor tyrosine kinase *in vitro,* thus reducing cell growth (76). Many receptor tyrosine kinases are involved in oncogenesis by gene mutation, chromosomal translocation or over-expression. With about 50% of the oncogene protein products being membrane bound receptors with tyrosine kinase activity, it is believed that genistein is the isoflavone in soy responsible for anti-cancer effects when at concentrations larger than 10 μM (77). Genistein also has a structure very similar to E_2 and thus acts like the hormone through binding with ERβ, possibly exerting chemo-protective effects (46). Furthermore, genistein has been found to have anti-angiogenic effects and may block the uncontrolled cell growth associated with cancer cells (78). Thus since this revelation, genistein has been widely studied in both *in vitro* and *in vivo* models to determine its effect on various cancers.

Clark *et al.* (79) were the first to investigate *in vitro* effects of genistein on colonic cancer cells. This study revealed that 1, 5, and 10 μM of genistein dose-dependently repressed growth of HCT-8 human colon cancer cells by 12, 28, and 100%, respectively. Then in 1993, Yanagihara *et al*. (80) examined possible anti-proliferative effects of genistein on a number of colon cancer cell lines. Genistein given at concentrations of 11.5 μM and 9.5 μM strongly inhibited proliferation in HCC-48 and HCC-50 colon cancer cell lines respectively. It was also noted that at above 150 μM, a pharmacological concentration that could never be achieved via the diet, genistein induced a significant increase of DNA strand breaks believed to be due to increasing the rate of topoisomerase II-mediated DNA cleavage (81). Recently, Plewa *et al*. (82)

measured suppression of growth rate of HT-29 cells by treatment with genistein and daidzein. Genistein at 11.5 μM and daidzein at 25 μM significantly suppressed cell growth due to cell cycle arrest mechanisms. As most of these data point to genistein potentially being an effective chemo-preventive agent, one must consider the fact that the concentrations used above are of supplemental value and are thus difficult to obtain via diet alone.

With *in vitro* data suggesting that genistein could prevent colon carcinogenesis, it became necessary to determine if the same effects could be observed *in vivo*. The first study that investigated an isolated phytoestrogen *in vivo* looked at genistein's effects in an AOM-induced carcinogenesis model using male F344 rats. Genistein proved to be a potent isoflavone against development of aberrant crypt foci (ACF). A diet consisting of 75 and 150 mg/kg genistein was shown to be sufficient to yield a statistically significant reduction of ACF number (83). Helms and Gallaher (84)observed similar results in male Wistar rats. DMH-induced ACF formation was decreased up to 35% with a diet containing up to 372 mg/kg genistein. Finally, Thiagarajan *et al*. (85) found that genistein inhibited ACF formation by 40% compared to a diet containing soy protein concentrate with a very low isoflavone content in AOM-treated rats. As these studies show a correlation between genistein and decreased colon tumor incidence, there are however other studies that do not show such an effect. One such study performed by Gee *et al*. (86) used diets enriched with genistein at 250 mg/kg diet. Consumption of diet before treatment with DMH was coupled with a two to threefold increase in ACF development of the rat colon, but there was no effect observed when the genistein was given after treatment with DMH. In spite of the large number of already performed *in*

vivo and *in vitro* studies on a potential positive effect of phytoestrogens, particularly genistein, against the development of colorectal cancer, much work is still needed. Studies need to begin to focus on the effects of such phytoestrogens in normal, healthy systems as a chemo-preventative model before recommendations with respect to phytoestrogen consumption by humans can be made.

Hypothesis. Estradiol and genistein given at dietary concentrations decrease cellular proliferation and induce apoptosis via the p53 pathway in normal, healthy colon tissue and thus prove to be effective chemo-preventive agents.

Specific Aims. The objectives of this study were to:

1. Determine if estradiol and genistein downregulate proliferation in mouse colonocytes.
2. Determine if mouse colonocyte apoptosis is induced by estradiol or genistein.
3. Determine if estradiol and genistein utilize the p53 pathway to induce apoptotic effects in mouse colonocytes.

CHAPTER III

ESTRADIOL AND GENISTEIN ALTER CELLULAR PHYSIOLOGY OF NON-MALIGNANT COLONOCYTES

Introduction

In the United States, colon cancer is the third most prevalent malignancy and ranks third among cancer related deaths. Women have a decreased incidence of colon cancer (43.6 in 100,000) as compared to men (59.0 in 100,000), suggesting a possible protection against the disease due to hormonal changes (1). Data from the Women's Health Initiative Study showed a significant reduction in the incidence of colorectal cancer in post-menopausal women receiving hormone replacement therapy (HRT) compared to placebo control (9). In fact, the majority of clinical studies have demonstrated that either HRT or estrogen replacement therapy can significantly decrease the risk of colon cancer in post-menopausal women with no significant difference in effect between the two therapies (2-7). Clinical data are also supported by animal studies showing rats given estradiol (E_2) had significantly fewer colon tumors by 71% (8). Data from our laboratory suggest that the protective effect of E_2 in the colon begins at early stages of tumor formation, as ovariectomized mice receiving an E_2 implant had fewer preneoplastic lesions in the colon when compared to control animals (12).

However, there has been controversy over the use of HRT as women receiving therapy had increased risk of invasive breast cancer (9). One alternative to HRT that women have turned to is use of dietary phytoestrogens, with the most common class being soy isoflavones. In general, like HRT, epidemiological data suggests that soy reduces colon cancer risk (10). A recent meta-analysis demonstrated that, in women,

consumption of soy significantly reduced incidence of the disease indicating gender specificity for the protective effects of the dietary component (87). Soy isoflavones which have a chemical structure similar to E_2, such as genistein (GEN), mimic the hormone by binding to estrogen receptors and thus eliciting estrogenic responses in multiple tissue types.

GEN has also been reported to suppress colon cancer cell growth *in vitro* (11). However, there has been conflicting data as to whether soy isoflavones can inhibit the formation of colon cancer *in vivo*. In a study performed by Sorensen *et al*., a soy isoflavone diet did not protect against intestinal tumor development in the Min mouse model (88). Conversely, as seen in E_2-treated models, isoflavone containing diets have been shown to reduce the number of aberrant crypt foci, which are considered to be preneoplastic lesions (89, 90). All studies thus far have used carcinogen-induced tumor formation in combination with continuous exposure to either E_2 or GEN containing diets to determine the chemotherapeutic effects of the compounds. However, no studies to date have reported how E_2 or GEN given at physiologically relevant concentrations might impact colonic epithelial cell function in the absence of carcinogen associated damage and/or eventual malignancy.

Therefore, advancement of research in the area of evaluating how E_2 and isoflavones influence the formation of colon cancer has been limited since most studies use tumor models and focused analysis to later promotion and/or progression stages of the disease. However, to truly elucidate their protective roles in the colon, it is imperative that we understand how these compounds influence cell physiology prior to becoming cancerous. As such, the presented studies objectives were to examine the

effects of E_2 and GEN at physiological concentrations on non-malignant colonic epithelia. To do so, both *in vitro* and *in vivo* analysis were conducted and for the first time we report that these estrogenic compounds can alter the physiology of colonocytes in the absence of disease.

Materials and Methods

Cells. Young Adult Mouse Colonocytes (YAMC) cells were supplied by Dr. Robert Chapkin (Department of Nutrition and Food Science, Texas A&M University, College Station, TX). These cells are a well-characterized non-malignant cell line derived from the Immortomouse, and are morphologically primitive epithelial cells with no evidence of differentiation. YAMC cells are conditionally immortalized by the expression of the temperature-sensitive SV40 large T antigen, and do not form tumors in athymic mice (91). For regular maintenance, cells were cultured in RPMI 1640 media (Sigma-Aldrich; St. Louis, MO) with 5% fetal bovine serum (FBS; HyClone; Logan, UT); 1% penicillin/streptomycin; 1% Glutamax-1 (Invitrogen; Carlsbad, CA); and 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences; Franklin Lakes, NJ). Cells were maintained under permissive conditions, 33˚C with 10 units interferon gamma (IFNγ) (Roche; Indianapolis, IN) per milliliter medium.

Cell Growth Assays. Forty-eight hours before plating, YAMC cells were transferred to medium containing 5% charcoal-dextran-stripped FBS, 1% penicillin/streptomycin, 1% Glutamax, and 0.1% ITS and were maintained at non-permissive conditions, 39˚C and without IFNγ. Cells were then seeded at 30,000 cells per well on a six-well plate. Twenty-four hours after plating, cells were given individual treatments of 1 nM/L E_2, 1 µM/L GEN, or 10 µM/L GEN. All treatments were diluted

in DMSO as 1,000x stocks and delivered as 1 µL/mL medium to achieve the final concentration listed. Forty-eight hours after the first treatment, the medium was changed and a second dose of the given treatments was delivered. At the end of the 96 h treatment period, the cells were trypsinized and prepared for counting. Cell concentrations were determined using a Beckman Coulter particle counter. Twenty µL of sample was diluted in 10 mL Isotone II diluent (Beckman Coulter), and each sample was counted three times. Three wells per treatment per experiment were used and three replicate experiments were conducted.

Cellular Proliferation. YAMC cells were grown under non-permissive conditions in stripped serum medium for 48 h before plating. Cells were then seeded at 2,000 cells per well on a 96-well plate and allowed to adhere overnight. Treatments of 1 nM/L E_2, 1 µM/L GEN, or 10 µM/L GEN were given for 96 h and medium changed every 48 h. Bromodeoxyuridine (BrdU) label, from the CalBiochem BrdU Cell Proliferation Assay (QIA58) kit (Calbiochem; Gibbstown, NJ), was added at hour 78 and allowed to incubate for 18 h. BrdU is a synthetic nucleoside that is analogous to thymidine and is thus incorporated into the DNA of newly synthesized replicated cells. The BrdU kit protocol was then followed for the remainder of the procedure. Proliferation was measured as increased fluorescence measured on a TECAN Infinite M200 plate reader at dual wavelengths of 450 and 595 nm. Three wells per treatment p experiment were used and four replicate experiments were conducted.

Cellular Apoptosis. YAMC cells were grown under non-permissive conditions in stripped serum medium for 48 h before plating. Cells were seeded at 30,000 per well on six-well plates and treated for 96 h with treatments of 1 nM/L E_2, 1 µM/L GEN, or 10

μM/L GEN and medium changed every 48 h. At the end of the treatment period, cells were trypsinized and collected. After collection, the 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. Relative apoptosis was determined by fluorescence intensity measured on a TECAN Infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

***p53 Reporter Assay*.** YAMC cells were seeded at 10,000 cells per well on 12-well plates in stripped medium and grown under non-permissive conditions for 72 hrs. After 48 h, cells were transiently transfected with 0.5 μg β-galactosidase (β-gal) and 4 μg pG-13, a basic luciferase (Luc) reporter construct containing three upstream copies of a synthetic p53 response element, per plate using Effectene Reagent (Qiagen; Valencia, CA). Cells were then treated with 1 nM/L E_2, 1 μM/L GEN, or 10 μM/L GEN for the final 18 h. After the 18 hr treatment period, cells were lysed using 200 μL Reporter Lysis Buffer (Promega; Madison, WI) and Luc and β-gal assays (Promega) were conducted to quantify p53 activity and transfection efficiency, respectively using Luc to β-gal ratios and a TECAN Infinite M200 plate reader. Six wells per treatment per experiment were used and four replicate experiments were conducted.

***Animal Study Design*.** c57BL6/J mice were obtained from The Jackson Laboratory. Mice were housed and bred at the Laboratory Animal Resources and Research facility at Texas A&M University. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University. On day one, 30 female mice were individually housed, randomly assigned to

a control, E_2, or dietary GEN treatment group based on age and weight, and given a semi-purified pellet diet (AIN-76, Harlan; Indianapolis, IN). On day sixteen, all mice were ovariectomized and control and GEN mice were given a 20 mg cholesterol (Sigma) pellet while E_2 mice were given a 19 mg cholesterol/1 mg E_2 pellet implanted s.c. on the back at the base of the neck. On day 30, all mice were switched to a semi-purified powder diet given in individual bowls to allow the animals to acclimate to powder diet. GEN treatment group mice were switched to a 1,000 ppm GEN powder diet on day 44 and sacrifice occurred on day 72. At time of sacrifice, blood was collected through cardiac puncture and the uterus was placed in 4 mL 1X PBS for subsequent weighing. The colon was resected and 1-cm sections from the distal end were cassetted and fixed in 4% paraformaldehyde. Food intake and fecal collection was taken once per week to determine difference in intake. To ensure accurate consumption values, food intake for individual mice was determined by measuring amount of diet given in each bowl minus the amount left in each bowl and spillage. No significant differences in food intake were observed between treatment groups.

Colon Crypt Height. Using photos taken at 400x of sections from the apoptosis analysis described below, colon crypt height was measured for 20 crypts from each mouse. Each measurement was taken from the base of the crypt to the top of the crypt using ImageJ software (National Institute of Health). Height, given in inches (150 pixels=1 inch), was subsequently converted into micrometers.

In vivo *BrdU Proliferation Assay*. Detection of cellular proliferation was determined by using immunohistochemistry with Anti-BrdU (Roche) as a primary antibody and Goat anti-mouse HRP conjugated antibody (Abcam; Cambridge, MA) as a

secondary antibody. Two hours prior to sacrifice, animals were injected with 7.5 µL BrdU/g body weight. 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 30 minutes and washed in distilled (DI) water three times. Slides were prepared for antigen retrieval by submerging in a solution containing 9 mL 0.1 M citric acid, 41 mL 0.1 M trisodium citrate, and 450 mL DI water and heating in a microwave on high for 10 mins and medium for 10 mins. Slides were then washed in PBS and a 1:20 dilution of anti-BrdU antibody in PBS with 1% BSA was applied and left to incubate in a humidified chamber at 4˚C overnight. Next, tissues were washed in DI water, a 1:200 dilution of secondary antibody in PBS with 1% BSA was applied and allowed to incubate in a humidified chamber for 1 h at RT. Tissues were stained in a 1% diaminobenzidine (DAB) solution for 10 mins, washed in DI water five times, and counterstained with Hematoxylin for 15 secs. Tissues were then dehydrated and wet mounted using Permount. To analyze tissues, photos of 20 intact crypts per animal were taken at 400x using a Zeiss Axiovert 200 Microscope with an AxioCAM. Each crypt was then divided into a bottom, middle, and top third and the number of proliferative cells and non-proliferative cells were counted for each section.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were completed using the ApopTag Plus Peroxidase *In situ* Apoptosis Detection kit (Millipore; Billerica, MA) according to manufacturer's instructions with a few 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 using ethanol. Endogenous peroxidase activity was quenched as slides were submerged in hydrogen peroxide and methanol for 30 minutes, placed in a 0.5% DAB solution for 20 secs, washed in DI water, and counterstained in methyl green for 5 secs. Tissues were then dehydrated and wet mounted using Permount. To analyze tissues, photos of 20 intact crypts per animal were taken at 400x using a Zeiss Axiovert 200 Microscope with an AxioCAM. Each crypt was then divided into a bottom, middle, and top third and the number of apoptotic cells and non-apoptotic cells were counted for each section.

Statistics. Statistics were performed using a Student's *t* test or Poisson regression model depending on the outcome analyzed. *t* test analyses was performed in Microsoft Excel for the *in vitro* data. A Poisson regression model was used, with the help of Dr. Alan Dabney, to model our *in vivo* apoptosis data within the colonic crypt. Because many crypts had zero counts, we allowed for overdispersion in the model. This allows for flexibility in the mean: variance relationship otherwise required by the Poisson assumption and, in particular, permits valid statistical inference when there is greater variability in the data than would be expected by a pure Poisson model. Statistical inference was carried out by testing model coefficients corresponding to group comparisons with control for each of the E_2 and GEN diets, using a Wald test.

Results

E_2 and GEN Inhibit Non-Malignant Cell Growth. First, we measured the effects of E_2 and GEN treatment on cell growth in non-malignant colonocytes *in vitro*. YAMC cells treated with 1 nM/L E_2, 1 µM/L GEN, and 10 µM/L GEN showed reduced

growth compared to vehicle control when treated for 96 h under non-permissive conditions (Fig. 1). As the GEN dose increased from 1 to 10 µM/L, there was a corresponding decrease in cell growth ($P < 0.01$). These data demonstrate a growth inhibitory effect in non-malignant colonocytes exposed to physiologic levels of E_2 and dietary levels of GEN.

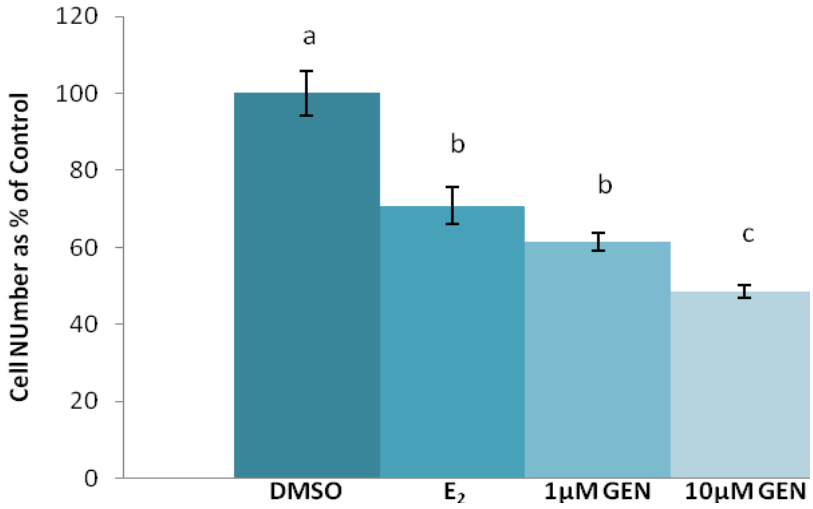


Fig. 1: GEN and E_2 inhibit growth of YAMC cells. YAMC cells were grown at 39°C in the absence of IFNγ. Cells were transferred to a charcoal dextran–stripped medium 48 h before plating. Twenty-four hours after plating, cells were treated with 1 nM/L E_2, 1 µM/L GEN or 10 µM/L GEN, and treatments were replaced 48 h later. Data are expressed as percentage of growth of the DMSO control group. Mean (n = 12) from fo replicate experiments. Bars, SEM, without a common letter differ; $p < 0.01$.

E_2 and GEN Do Not Suppress Proliferation in Non-Malignant Colonocytes in vitro. Next, we wanted to identify the mechanism involved with E_2 and GEN treatment and growth inhibition. To determine if inhibition of cell growth was due to a decrease in cellular proliferation, levels of BrdU incorporation when YAMC cells were treated for 96 h with 1 nM/L E_2, 1 µM/L GEN, and 10 µM/L GEN was measured (Fig.

2). BrdU is a synthetic analog to thymidine and can thus replace the nucleoside during DNA synthesis indicating cellular proliferation (92). No treatment was significantly different from the vehicle control indicating that directly suppressing cellular proliferation is not a mechanism involved in the E_2 or GEN induced growth inhibition of non-malignant colonocytes.

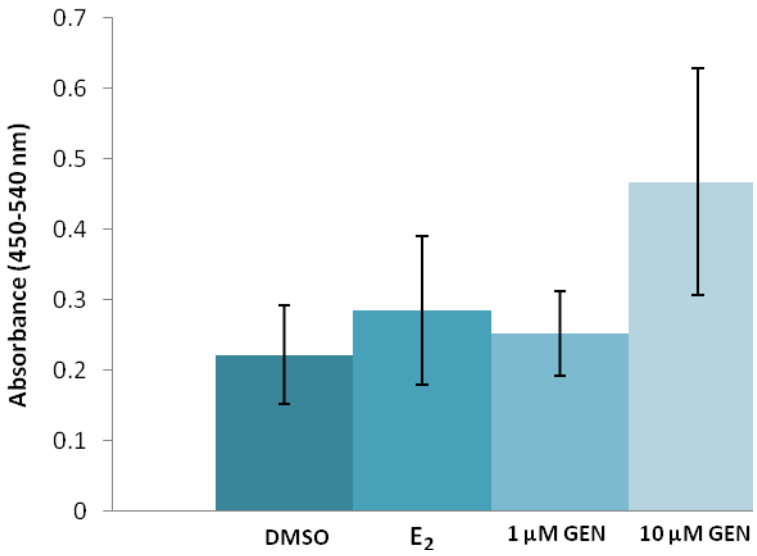


Fig. 2: GEN and E_2 do not suppress proliferation of YAMC cells. YAMC cells were grown at 39°C in the absence of IFNγ. Cells were transferred to a charcoal dextran–stripped medium 48 h before plating. Cells were treated with 1 nM/L E_2, 1 µM/L GEN or 10 µM/L GEN for 96 hours, and treatments were replaced 48 h later. BrdU label was added at hour 78 and allowed to incubate for 18 h. Data are expressed as mean absorbance +/- SEM, which directly correlates to BrdU incorporation. *n* = 12 from four replicate experiments.

Apoptosis in YAMC Cells Treated With E_2 and GEN. We then measured the role of E_2 and GEN on apoptotic activity in the non-malignant colonocytes. Caspase-3 activation was used to determine the relative number of apoptotic cells when YAMC cells were treated with 1 nM/L E_2, 1 µM/L GEN, and 10 µM/L GEN for 96 h (Fig. 3).

YAMC cells showed an increase in caspase-3 activity in E_2-treated cells as well as GEN treated cells in a dose-responsive manner ($p<0.0001$). These data identify induction of apoptosis as a primary mechanism involved in the E_2 and GEN induced growth inhibition of non-malignant colonocytes.

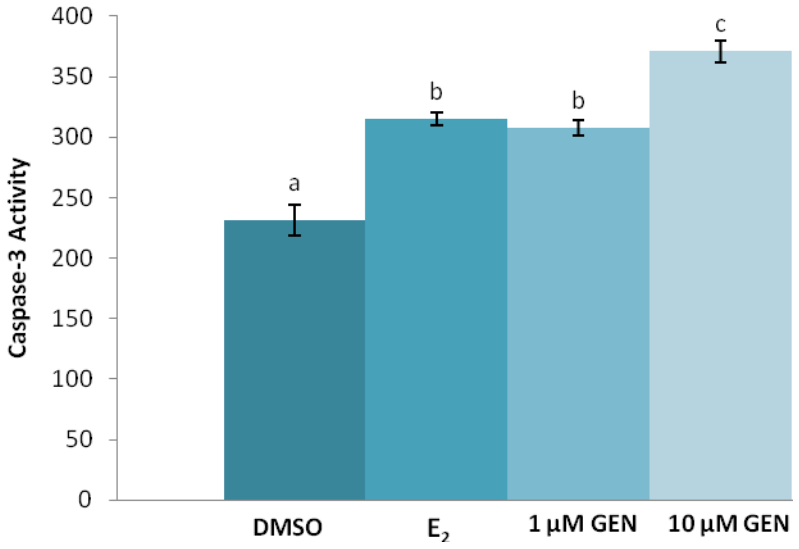


Fig. 3: Apoptosis in YAMC cells treated with GEN and E_2. YAMC cells were grown under non-permissive conditions and transferred to a charcoal dextran–stripped medium 48 h before plating. After 96 h of GEN or E_2 treatment, cells were collected and the EnzChek caspase-3 assay was performed. Data are expressed as increased fluorescence measured when compared with the blank. Mean (n = 9) +/- SEM from three replicate experiments; bars without a common letter differ; $p < 0.001$.

p53 Activity in YAMC Cells Treated With E_2 and GEN. Having shown induction of apoptosis in cells treated with E_2 and GEN, we next wanted to begin to determine pathways involved in this induction. As p53 has been shown to induce apoptosis in response to E_2 and GEN in other cell lines, we measured p53 transcriptional activity in YAMC cells treated with 1 nM/L E_2, 1 µM/L GEN, and 10 µM/L GEN for 18 h (Fig. 4). Both E_2 and GEN increased p53 activity, with GEN doing so in a dose-

responsive manner ($p<0.0001$). This demonstrates that E_2 and GEN upregulate p53 transcriptional activity and suggests that this induction leads to the increased apoptosis previously observed.

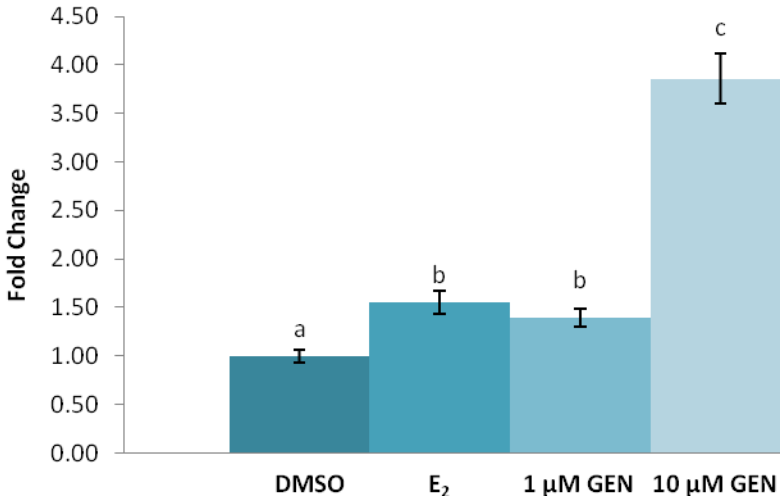


Fig. 4 : GEN and E_2 enhance p53 transcriptional activity in YAMC cells. YAMC cells were grown at 33°C and without IFNγ. Cells were transferred to charcoal dextran stripped media 48 hrs prior to plating. Cells were then grown under non-permissive conditions, at 39 °C, for 72 hrs. After 48 hrs, cells were transfected with pG-13 p53 reporter construct and β-gal construct. Cell lysates were collected and used to perform luciferase and β-galactosidase assays, results were measured on a Tecan Infinite M200 plate reader. Data are presented as percentage of vehicle control. Values are means +/ SEM, n=9. Bars without a common letter differ, $p < 0.01$.

Colon Crypt Height in Animals. With E_2 and GEN treatment resulting in significant changes in YAMC cells, we next wanted to correlate the *in vitro* data to physiological effects of the estrogenic compounds *in vivo*. First, to determine if GEN or E_2 induced any morphological changes in colonic tissue, we measured colon crypt height in mice given an E_2 pellet or on a 1,000 ppm GEN diet. E_2-treated animals had an increase in colon crypt height compared to control animals ($p<0.01$), with GEN animals

trending in the same fashion (Fig. 5). These data suggest that E_2, and potentially GEN, alter colon crypt morphology allowing for the physiologic changes observed.

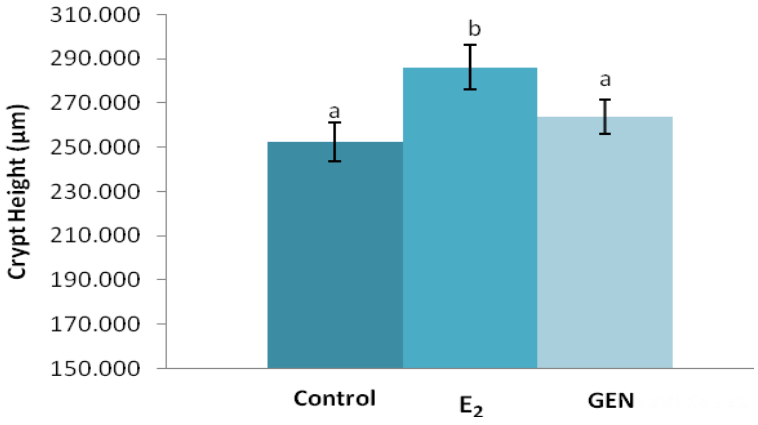


Fig. 5: Colon crypt heights of the distal colon. Sections of distal colon were taken from 7 to 10 mice in each treatment grouped and stained using the TUNEL assay. This data represents 20 intact crypts measured, using Image J software at 400X, from the base of the crypt to the lumen. Bars without a common letter differ, p-values < 0.01.

Proliferation Within the Colonic Crypt. Next, we wanted to investigate the roles of E_2 treatment or GEN diet in non-malignant colonic epithelia. We began by exploring the effects of E_2 and GEN on proliferation in the distal colon of c57BL6/J mice. As seen *in vitro*, there was no significant difference in number of proliferating cells in either E_2-treated animals or those receiving a GEN diet as compared to control mice (Fig. 6). These data indicate that neither E_2 treatment nor a GEN diet influences

proliferation with the colonic crypt of a healthy animal.

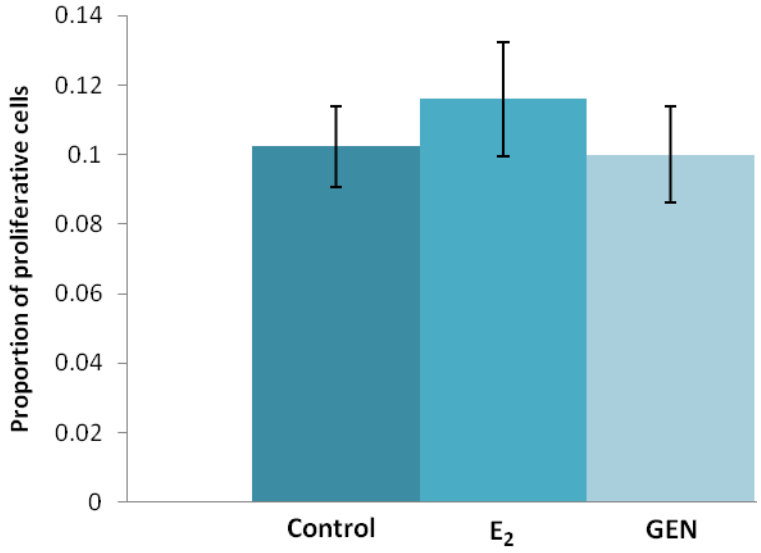
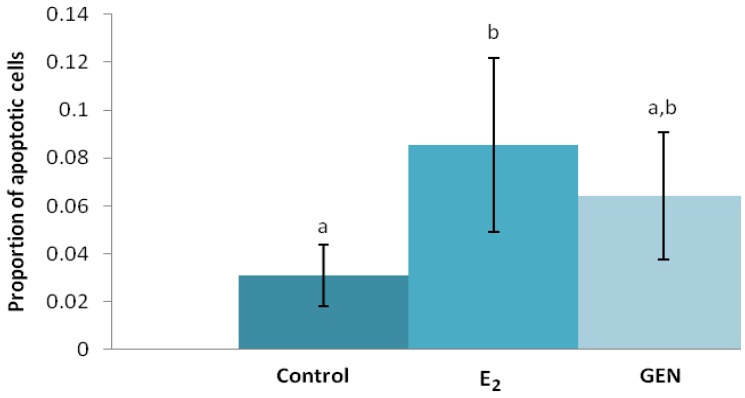
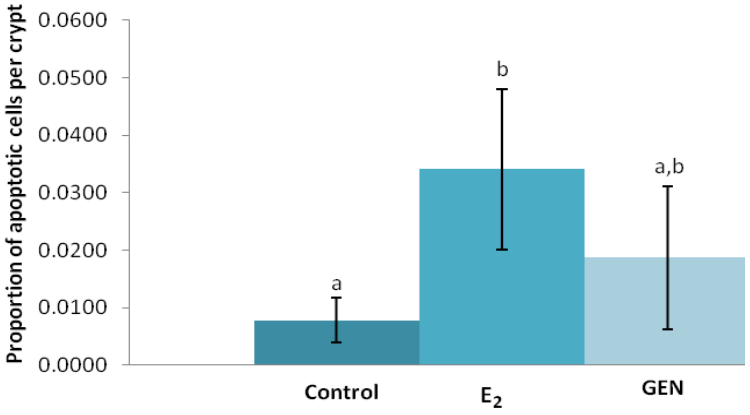


Fig. 6: Proliferation within the colonic crypts of the distal colon. A BrdU immunohistochemistry assay was performed on sectioned tissue from the distal colon. Data are expressed as proportion of proliferative cells found compared with total number of cells found in the crypt. These data are representative of 20 well-oriented crypts per animal and 7 to 10 animals per treatment group.

Apoptosis Within the Colonic Crypt. We then analyzed apoptosis to see if there was a change with E_2 treatment or a GEN diet. Induction of colonocyte apoptosis was determined in distal colon tissues from each experimental group. Crypts analyzed from E_2-treated animals displayed a higher degree of total apoptotic cells compared to the control ($p=0.05$), with a significant increase in the bottom third of the crypt ($p<0.05$). Mice on a GEN diet trended in the same fashion as E_2-treated but did not reach statistical significance (Fig. 7, A and B). These data show a marked increase in apoptotic cells in response to E_2 treatment with dietary GEN trending in the same direction.



A.



B.

Fig. 7: Apoptosis within the total colonic crypts of the distal colon. A, total crypt. A TUNEL assay was performed on sectioned tissue from the distal colon. Data are expressed as proportion of apoptotic cells compared with total number of cells in the crypt. These data are representative of 20 well-oriented crypts per animal and 7 to 10 animals per treatment group. B, bottom section of the colonic crypt. Using same animals and crypts, data are expressed as percentage of apoptotic cells in the bottom third compared with total number of cells in the bottom third of the crypt. Bars without a common letter differ, $p \leq 0.05$.

Discussion

Due to evidence from clinical and animal model experiments, E_2 has become a primary interest in the prevention of colon cancer. Phytoestrogens, such as GEN, appear to exert similar effects as E_2. Several *in vivo* studies have shown reduced risk of colon cancer development when mice and/or rats are treated with E_2 or their diets supplemented with GEN (47, 48, 66, 85). However, these studies utilize colon cancer induction models and since exposure to E_2 and GEN was continuous from before carcinogen introduction until tumor collection, these studies do not distinguish prevention from treatment. Data previously published from our laboratory demonstrates that E_2 inhibits formation of preneoplastic lesions in carcinogen treated animals, demonstrating a protective role of E_2 at a pre-malignant stage of carcinogenesis (12). However, these previous studies also used carcinogen *in vivo* and thus do not allow for interpretation of the effects of E_2 on normal colonic physiology. The primary purpose of the presented studies was to determine the effects of estrogenic compounds on colonocytes in a non-diseased state, with a primary focus on cell growth and apoptosis.

In order to assess E_2 and GEN's role as a chemopreventative agent in the normal colon, it was necessary to study the effects of these compounds in non-malignantly transformed colonocytes *in vitro* and *in vivo*. Although *in vitro* studies have shown that E_2 and GEN can reduce cell number in colon cancer cell lines such as HT-29 and DLD-1 (11, 52, 53, 56, 62, 93), this data is controversial as E_2 data cannot often be reproduced and those using GEN frequently treat with pharmacologic doses. For the first time we reveal that E_2 and dietary relevant concentrations of GEN can change the growth of non-malignant colonocytes by showing a significant reduction in cell number in YAMC cells

treated with E_2 and GEN. This response was observed in YAMC cells maintained at non-permissive conditions (see methods for details), suggesting these compounds can exert their effects in primordial colon cells which have undergone very few population doublings and are therefore more representative of normal colonic epithelia. Having observed reduced cell number in these non-transformed cells, we next wanted to determine if this response was due to suppression of proliferation or increased apoptosis. While some *in vitro* studies show decreased rates of proliferation with increased rates of apoptosis in nasopharyngeal and glioma cell lines, other cell lines such as LoVo and SW620 showed no effect when treated with physiologic levels of E_2 or dietary levels of GEN (11, 55, 94, 95). Once again, the cell lines utilized in these studies were malignant and thus only allow interpretation for chemotherapeutic effects of E_2 and GEN and not protective effects. One study, nonetheless, did show that GEN could inhibit cellular proliferation and induce apoptosis in non-transformed intestinal cells, IEC-6 and IEC-18, which are derived from the ileum (96). In contrast, we observed no significant difference in proliferation rates for colonocytes (YAMCs) treated with E_2 or GEN as compared to vehicle control. Conversely, we did observe significant induction of caspase-3 activity in YAMC cells in response to both E_2 and GEN treatment in a dose-dependent manner. These data demonstrate that both E_2 and GEN reduce cell growth in noncancerous colonocytes by induction of apoptosis and suggest that small and large intestinal epithelia may respond differently to estrogenic compounds.

To begin to investigate the mechanism of action inducing apoptosis, we wanted to determine if E_2 and GEN could induce the p53 activity in YAMC cells. p53 is a commonly known tumor suppressor gene that regulates the cell cycle and can induce

apoptosis (23). Point mutations in p53 have also been linked to colon carcinomas (24, 25, 28). Therefore, it was logical that we begin to look into p53's potential activation by E_2 and GEN. There has been evidence suggesting both E_2 and GEN can upregulate expression of p53 mRNA levels in various cell lines including lung cells and osteoblasts (97-99). Wilson *et al.* also demonstrated that GEN can increase p53 protein levels in HCT-116 cells, a colorectal carcinoma cell line (100). Consequently, we investigated the effects of E_2 and GEN in the YAMC's and observed a significant increase in p53 transcriptional activity following these two treatments in a dose-dependent manner. This data suggests that E_2 and GEN can directly enhance p53 transcriptional activity in non-malignant colonocytes, and this induction is likely the underlying cause of apoptosis observed in YAMC cells. Additional experiments are required to confirm these findings and to determine the relevant downstream targets of p53 that are modulated by E_2 and GEN.

With our *in vitro* data suggesting a change in colon cell physiology due to E_2 and GEN, we next wanted to determine if these effects are apparent, and thus predictive, in a non-carcinogenic *in vivo* model. Methodology was utilized that we have routinely used in the past (12, 101, 102) to deliver physiologically relevant concentrations of E_2. Mice receiving an E_2 pellet had serum levels averaging 0.80 nM/L, which is slightly lower than E_2 levels observed in a nonpregnant cycling woman (103, 104). Control and GEN animals receiving a cholesterol pellet were below the threshold associated with menopause in women (105). For delivery of GEN in the diet, we again used methodology and dietary concentrations routinely used in our laboratory and others (17,41, 106). This GEN containing diet known to produce plasma GEN levels in mice

that are equivalent to circulating levels found in humans consuming a soy diet (101, 107-110).

We first wanted to determine if E_2 and GEN caused gross morphological changes in the colon that could be attributed to an alteration in colonic physiology, we measured colon crypt height. No studies have demonstrated that E_2 or GEN cause any significant change in morphology of the colon. However, in mice treated with the inflammatory chemical dextran sodium sulfate, crypt distortion occurs leading to a decrease in colon crypt height (111). Mice receiving E_2-treatment had increased colon crypt heights compared to control animals with GEN trending in the same fashion. This suggests that E_2 may be providing a pre-emptive, protective mechanism against possible inflammation in the colon while allowing greater nutrient absorption. Further studies are needed to deduce the reason for these morphological changes.

As seen *in vitro,* when examining each sectional third of the crypt (top, middle bottom), there was no difference in overall proliferation rates among the three treatment groups (data not shown). Although studies have shown a decrease in cellular proliferation in mice treated with carcinogen and receiving E_2 or GEN treatments (112) there is no such trend observed in a healthy, non-transformed colonocyte. Likewise, we have demonstrated that the cell cycle is not altered in YAMCs treated with E_2 (12). While confirming what was observed in *vitro,* data from this study suggest the larger finding that altering the rate of cellular proliferation is not a primary mechanism of estrogen suppression of tumor formation.

Finally, knowing that E_2 can elicit apoptotic effects in chemically transformed tissues, we wanted to ascertain if apoptosis is induced in the colonocytes of healthy

animals treated with E_2 or given a GEN diet. As demonstrated *in vitro*, E_2 and GEN treated mice expressed higher rates of apoptosis throughout the depth of the crypt, although not statistically significant. However, E_2 treated animals did have a significant increase in the number of apoptotic cells in the bottom third of the crypt with GEN trending in the same fashion. With a larger number of GEN animals, we believe we could have seen a statistically significant effect from GEN as well. Hall *et al.* noted that in colon tissue not receiving experimental treatments, apoptosis is generally localized to the lumenal region of the crypt as this is the region where differentiating cells migrate and are thus sloughed off (18). However, in response to DNA damage resulting from carcinogen treatment, studies from our laboratory demonstrate that when you treat with E_2, apoptosis is upregulated in the bottom third of the crypt as early as 12 hours post (in press) and up to 8 weeks after carcinogen exposure (12). As the bottom third of the crypt is the zone of actively proliferating cells, these data suggest that E_2 and GEN alter the physiology of this important area even in a non-diseased state. Furthermore, because these tissues are healthy and show no pathological signs of disease, we would not expect large physiological shifts in apoptosis. This relatively modest increase in number of apoptotic cells, primarily in the bottom third of the crypt, indicate that E_2 and potentially GEN might provide protective effects against colon cancer by eliminating damaged cells via apoptosis prior to them becoming fully initiated and transformed.

In conclusion, GEN and E_2 treatments showed unique changes in YAMC cells and non-malignant colonocytes *in vivo*. Treatment with E_2 and GEN diet reduced cell growth, had no effect on cellular proliferation, and induced apoptosis via activation of the p53 pathway in non-malignant colonocytes in culture. Additionally, in

ovariectomized mice receiving no carcinogen treatment, E_2 resulted in no change in cellular proliferation, but apoptosis in colonocytes was induced with dietary GEN trending in the same fashion. For the first time, these data indicate that E_2 treatment and dietary levels of GEN can change the physiology of normal colonic tissue by inducing apoptosis in a non-transformed colonocyte. It is likely that these alterations in cellular physiology contribute to the protective effects against the development of colon cancer by eliminating damaged cells. These findings are primary steps toward identifying the roles of E_2 and GEN in non-malignant colonocytes and provide insight into their potential chemoprotective effects.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Witch colon cancer being the third most prevalent malignancy in the United States, it has become increasingly important to understand the disease and study possible mechanisms of its prevention. Many studies have begun this task by looking into the roles of E_2 and GEN in colon cancer prevention. However, the investigations up to date utilize carcinogen-induced models and thus are truly examining chemo-therapeutic and not chemo-protective effects. Thus, in an effort to elucidate the physiologic effects of E_2 and dietary GEN on healthy colonic tissue, we studied these compounds in non-malignantly transformed colonocytes *in vitro* and *in vivo.* We began by determining the effects these compounds have on cell growth. For the first time, we reveal that E_2 and dietary relevant concentrations of GEN can alter the growth of non-malignant colonocytes by showing a significant decrease in cell number of YAMC cells treated with these compounds. With an evident change in cell number, we sought to determine if this response was due to suppression of proliferation or induction of apoptosis. While a few studies have shown decreased rates of proliferation and increased rates of apoptosis in various colon cancer cell lines treated with E_2 and/or GEN, in regards to healthy colon tissue, we observed no significant difference in proliferation rates for YAMCs treated with E_2 or GEN as compared to vehicle control. However, we did observe a significant increase in induction of apoptotic activity with increased levels of Caspase-3 in YAMCs treated with E_2 and GEN. These results were evident in a dose-dependent manner, suggesting that both E_2 and GEN can reduce cell growth via induction of apoptosis in noncancerous colonocytes.

As induction of apoptosis appeared to be the mechanism involved in reducing colon cell growth, we then investigated the pathway behind this induction. Via use of a p53-reporter assay, it appears as though both E_2 and GEN directly enhance p53 transcriptional activity in non-malignant colonocytes, and that this induction is likely the fundamental cause of the apoptosis observed in the YAMC cells. Finally, with the *in vitro* data suggesting a change in colon cell physiology due to treatment with E_2 and GEN, we utilized a non-diseased state *in vivo* model to determine if we could observe the same effects. The *in vivo* data proved to be consistent with our *in vitro* data as normal, healthy mice receiving E_2 treatment or a GEN diet did not have a significant change in colonocyte proliferation rates. However, E_2 treated mice did exhibit increased rates of apoptosis, particularly in the bottom third of the crypt with GEN trending in the same fashion. Thus, for the first time, these data indicate that E_2 treatment and dietary levels of GEN can alter the physiology of normal, healthy colonic tissue by inducing apoptosis in a non-transformed colonocyte. This physiologic alteration may then give rise to the protective effects observed against colon cancer development as damaged cells are eliminated.

Future Research

Although these data are the first steps toward understanding the chemo-protective roles of E_2 and GEN in non-malignant colonocytes, further investigations should be initiated to determine the precise roles of these compounds in normal colonic tissue. Also, determining optimum concentrations of these compounds to exert beneficial effects should be a primary focus of researchers. Once results are generated in a mouse

models that provide an estimated level of consumption at which chemoprevention is optimized, human trials should follow.

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