ESTROGEN RECEPTOR BETA AND P53 PLAY INTEGRAL ROLES IN ESTRADIOL MEDIATED PROTECTION AGAINST COLON TUMOR DEVELOPMENT

A Dissertation

by

CHARLES CONAN WEIGE

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

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Genetics

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Approved by:

Chair of Committee,	Clinton D. Allred
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ABSTRACT

Estrogen Receptor Beta and p53 Play Integral Roles in Estradiol Mediated Protection Against Colon Tumor Development. (August 2012)

Charles Conan Weige, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Clinton D. Allred

Hormone replacement therapy and estrogen replacement therapy have shown the ability to reduce risk of colon cancer development in clinical and animal studies, but *in vitro* research has been unable to reproduce an estradiol (E_2) induced response in colon cancer cell lines. We demonstrated that young adult mouse colonocytes (YAMC, non-malignant colonocytes) exhibit an anti-proliferative response to E_2 treatment. These cells demonstrate reduced cell culture growth and increased apoptosis in response to E_2. YAMC cells containing an activated Ras mutation are considered to be malignantly transformed, and lose the ability to respond to E_2 treatment. Fulvestrant (ICI) was used as an estrogen receptor antagonist to determine that these results were estrogen receptor mediated. Furthermore, this effect was demonstrated to require the presence of ERβ through the use of a transgenic ERβ knockout mouse. In these mice, the presence of E_2 significantly reduced the formation of azoxymethane induced premalignant lesions.

Since YAMC cells exhibit an anti-proliferative response to E_2 treatment, we utilized isogenic YAMC cell lines with and without a dominant negative p53 mutation to demonstrate that this E_2 induced action involves p53 activity. E_2 treatment results in

increased p53 transcriptional activity and a pro-apoptotic change in expression of p53 downstream targets. Presence of the dominant negative p53 mutant nullifies these effects of E_2 treatment.

The involvement of p53 in the previously described protection against AOM induced premalignant lesions, was investigated using wild type and heterozygous p53 knockout (Het p53KO) mice. The reduction in p53 protein corresponded to reduced effectiveness of E_2 treatment on the prevention of premalignant lesion formation in Het p53KO mice.

In summary, our data indicate that E_2 treatment induces anti-proliferative responses in non-malignant colonocytes and protects against the formation of carcinogen-induced premalignant lesions. These effects require the presence of functional ERβ and p53. Further studies are required to more thoroughly elucidate the specific interactions and downstream effects of ERβ and p53 in response to E_2 stimulation.

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NOMENCLATURE

Ab	Antibody
ACF	Aberrant Crypt Foci
ANOVA	Analysis of Variance
AOM	Azoxymethane
AP-1	Activating Protein-1
APC	Adenomatous Polyposis Coli
BAX	Bcl-2 Associated X protein
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
DAB	Diaminobenzidine
DLD-1	Human colorectal adenocarcinoma cells
DNA	Deoxyribonucleic Acid
E_2	Estradiol
EIA	Enzyme Immunoassay
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
ERβKO	Estrogen Receptor β Knockout mice
ERE	Estrogen Response Element
ERT	Estrogen Replacement Therapy

FBS	Fetal Bovine Serum
HC11	Normal breast epithelial cells
HCT116	Human colorectal carcinoma cells
Hep3B	Human hepatocellular carcinoma cells
HHUA	Endometrial cancer cells
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
HT29	Human colon adenocarcinoma cells
ICI	ICI 182780 (Fulvestrant)
IFNγ	Interferon γ
IgG	Immunoglobulin G
ITS	Insulin/Transferrin/Selenium
LoVo	Human colorectal adenocarcinoma cells
MCF-7	Human estrogen dependent breast cancer cells
MDM2	Murine Double Minute 2
NOXA	Phorbol-12-myristate-13
OVX	Ovariectomy
PBS	Phosphate Buffered Saline
PUMA	p53 Up-regulated Modifier of Apoptosis
RNA	Ribonucleic Acid
SEM	Standard Error of Mean
Sp-1	Stimulating protein-1

SV40	Simian Virus 40 Large T antigen
SW480	Human colon adenocarcinoma cells
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling
WHI	Women's Health Initiative
WT	Wild Type
YAMC	Young Adult Mouse Colonocyte cells
YAMC bleo/neo	YAMC cells containing bleomycin and neomycin
YAMC mp53/neo	YAMC cells containing $p53^{175H}$ mutant
YAMC-Ras	YAMC cells containing activated H-ras

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Colon Cancer Statistics

Colon cancer is the third leading cause of cancer related death in both men and women. In the US, men are at significantly increased risk of colon cancer development (57.2/100,000) compared to women (42.5/100,000) (1). In 2008, colorectal cancer was the third most commonly diagnosed cancer in males and second in females, worldwide (2). Incidence rates are highest in Australia, New Zealand, Europe and North America, with the lowest rates in Africa and South-Central Asia. However, colon cancer incidence rates are increasing in several regions with traditionally low incidence, with rates among males in Japan and the Czech Republic already exceeding the peak incidence seen in the US (2).

Colon Anatomy and Physiology

Colon anatomy and physiology. The human colon is approximately 5-6 feet in length and makes up the distal 20-25% of the alimentary tract. While part of the digestive tract, the colon has a limited role in digestion compared to the small intestine. The primary functions of the colon are the reabsorption of water, salt and metabolites digested by bacteria present in the gut (3, 4).

This dissertation follows the style of Cancer Research.

Anatomically, the colon is divided into eight parts: the cecum; appendix; ascending, transverse, descending, and sigmoid colons; rectum; and anus. The walls of the colon are made up of four layers. The serosal layer is the outermost layer and protects the organ from the external environment. The next layer is the muscularis externa which consists of two thin layers of longitudinal and circular smooth muscle responsible for peristalsis. The submucosal layer is a combination of submucosal glands, blood and lymphatic vessels, and nerves that nourish and remove waste. The mucosal layer is the innermost layer of the intestine surrounding the lumen. The mucosa consists of the muscularis mucosa, lamina propria and epithelium. The epithelial layer of the mucosa is the primary colonic tissue affected by carcinogenesis. It is composed of epithelial cells (colonocytes) that line the luminal surface of the colon. The luminal surface is made up of millions of invaginations referred to as colonic crypts. These crypts appear as small pores or pits when looking at the luminal surface of the colon. This crypt structure allows for a significant increase in available surface area, thus increasing the efficiency at which water, salt, and other metabolites are absorbed.

The colonic crypt is made of several different types of epithelial cells. Colonic stem cells are considered to be present at the base of the crypts (5). As cells approach the luminal surface they become more differentiated, eventually becoming colonocytes, endocrine cells, or goblet cells. The concept that each of these cell types arose from a single progenitor cell type was first proposed by Cheng and Leblond and is known as the Unitarian Hypothesis (6). Epithelial cell renewal within the colonic crypt is driven by many factors such as differentiation, proliferation, and migration towards the lumen (7). As the differentiated cells reach the luminal surface, apoptotic signals are triggered and

these cells are eventually sloughed off (Figure 1.1). This entire process operates on a 3-5 day cycle in most adult mammals (8).

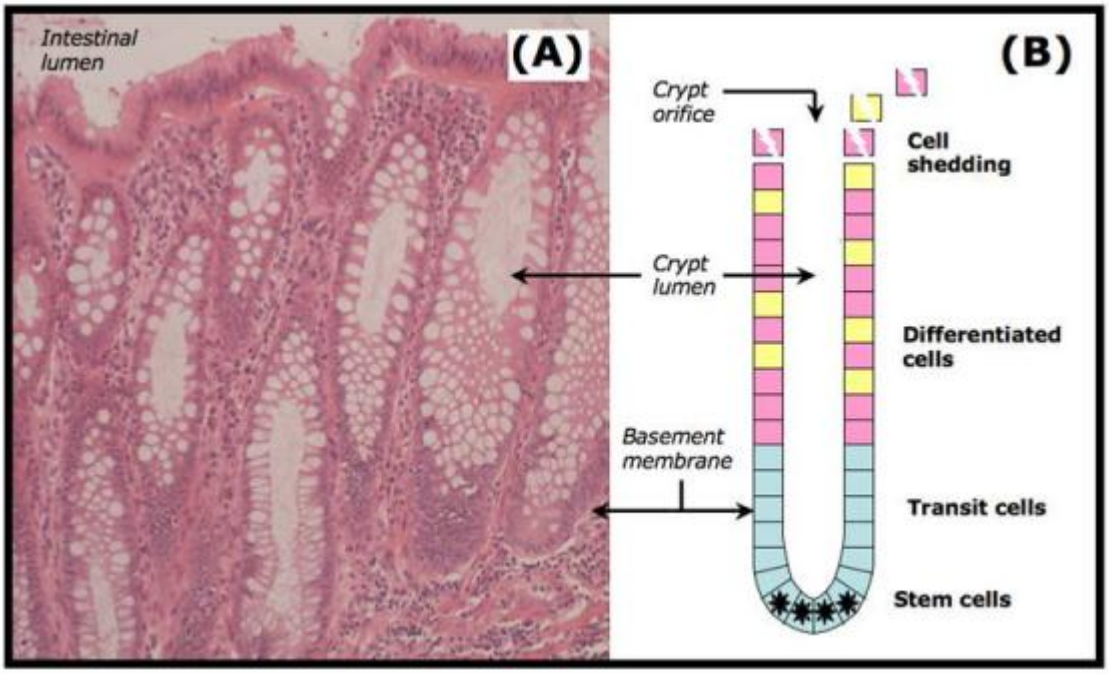

Figure 1.1. Normal colonic mucosa (9). *A*, Microdissection image courtesy of the Department of Pathology, Ninewells Hospital, Dundee, UK. The mucosa is characterized by the presence of numerous crypts of Lieberkuhn. Murine and human intestinal crypts contain about 250 and 2000 epithelial cells, respectively. *B*, Schematic of a normal colonic crypt. These glands have the geometry of a 'three-dimensional test-tube' and are embedded in soft tissue; their orifices open to the intestinal lumen. The epithelial cells lining the crypt are tightly attached to their neighbours and to the underlying basement membrane, to maintain the integrity of the epithelial sheet and, thereby, it functions as a protective barrier and nutrient absorption engine (9).

Colon Cancer Pathogenesis and Progression

Colonic carcinogenesis. Colon cancer is a type of cancer for which the stages and progression are fairly well understood. Most colon cancers progress through a series of morphological stages (Figure 1.2). The colonic polyp is widely recognized as the

predominant form of preneoplastic lesion in the colon. These pre-malignant growths are often non-cancerous, but have the potential to progress to a malignant state. The process by which these benign masses become cancerous is often referred to as the adenoma-carcinoma sequence (10). This phenomenon is marked by multiple mutations to genes resulting in the deregulation of the processes of proliferation, growth, migration and apoptosis. During normal cell growth, there is a tightly maintained balance between proliferative and apoptotic signals.

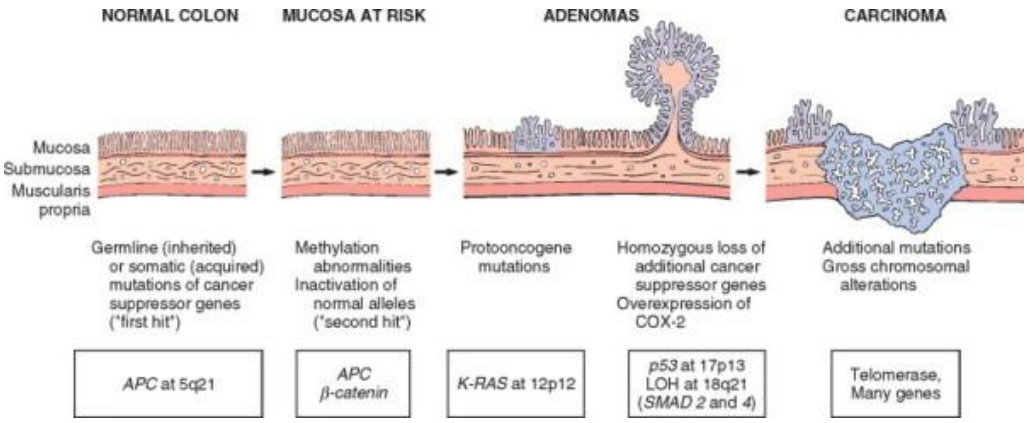

Figure 1.2. Adenoma-carcinoma sequence (11). Morphologic and molecular changes in the adenoma-carcinoma sequence..

Mutations to genes that promote unregulated cell growth/proliferation (oncogenes) or that regulate these processes (tumor suppressors), prime tissues for malignant transformation (12). Accumulation of these mutations leads to three distinct stages of carcinogenesis: initiation, promotion, and progression/malignant transformation (13).

Initiation is the first stage and is marked by the first mutation(s) that result in a change to the genetic profile of the cell. These changes may be spontaneous or due to

exposure to a carcinogen. Changes found during initiation may involve DNA base pair modifications resulting in mismatch errors during gene expression. If not corrected, these may lead to mutation when the DNA replicates. This damage rarely results in cancer because there are multiple DNA repair mechanisms in place within the cell (14). If repair does not take place, and the DNA damage affects a gene involved in growth, proliferation, DNA repair, or the regulation of these processes; the cell becomes more prone to cancerous transformation. The majority of mutations seen during the initiation stage enhance proliferation and resistance to apoptotic signals allowing a cell to enter the promotion stage (15).

Promotion is characterized by increased cellular growth and division, where a single mutant cell becomes a distinct population of cells. It is at this stage where the first microscopic evidence of abnormal cellular growth is observable. This can be seen as altered crypt morphology and aberrant crypt formation. Aberrant crypt foci (ACF) are the congregation of abnormal crypts, and result in regions within the colon that are more prone to cancerous transformation (16). This is often due to clonal expansion of the original mutated cell until it makes up the majority of a crypt, and is eventually the progenitor of multiple crypts (5). These ACFs have been identified as precursors to cancerous lesions within the colon often leading to the progression stage (17).

During progression, tumor cell growth is increased relative to normal cells. The DNA in tumor cells is more prone to mutation due to loss of DNA repair function and/or cell growth checkpoint functions caused by earlier mutations. Many of the resulting mutations accumulate in pathways that result in further dysregulation of cell growth and proliferation. Malignant transformation is characterized by the ability of the tumor to

invade the submucosal layer of the colon, resulting in change of tumor categorization from adenoma to carcinoma. This leads to increased angiogenesis, sustained proliferation and further resistance to apoptosis; all signs of malignancy. It has been demonstrated that mutations to genes within at least four or five key pathways are required for formation of a malignant tumor (18-20).

A combination of the activation of oncogenes and the inactivation of tumor suppressor genes are necessary for colorectal tumors to develop. Numerous studies have identified the tumor suppressor gene, adenomatous polyposis coli (APC), to be a gene often involved at an early stage of this process (Figure 1.2) (21). Mutations within this gene are found in ~60% of patients with early stage colon tumors, linking its mutation to an early stage in tumor development.

Ras is an oncogene that is also commonly mutated in colorectal cancer, and has been identified as activated in ~50% of colon carcinomas (18). Ras mutation is generally considered to take place during progression (Fig. 1.2). Though mutations leading to malignant transformation will occur sequentially, the sequence may vary and it is the sum result of the changes associated that result in a tumor's cancerous phenotype.

Risk Factors for Colon Cancer

The mutations leading to colon cancer are often spontaneous in humans and can be influenced by a variety of different factors. The most influential of these is age, with approximately 90% of cases occurring in people over the age of 50 (22, 23).

Race and ethnicity are also significant factors. In the US, Native Americans have the lowest incidence rate, followed by Asians, Hispanics, and Caucasians, with African

Americans having the highest incidence. While the difference in incidence rates between Caucasian and African American is about 10%, the increase in mortality rate is nearly 50% in African Americans (23, 24).

Family history of colon cancer is another risk factor, with about 30% of people having a first degree relative with history of this disease (23). Inflammatory bowel disease and diabetes are also medical factors that increase risk. Other factors that increase risk include obesity, red meat consumption, smoking, and alcohol consumption. However, physical activity, calcium consumption, and milk consumption are factors that have been linked to decreased risk (2).

Incidence rates of colon cancer also vary by sex (25, 26). Risk of colon cancer significantly increases in women as they become post-menopausal (27). Levels of estradiol (E_2) and other female hormones such as progesterone fluctuate throughout the cycle of a reproductive age woman. These levels drop dramatically in post-menopausal women (28-30). This suggests the possibility that hormonal differences between the sexes may alter susceptibility to this disease state.

Hormone/estrogen replacement therapy. There is a general decrease in risk of colon cancer associated with use of hormone replacement therapy (HRT) in post-menopausal women. Data from the Women's Health Initiative (WHI) study showed more than 30% reduction in colon cancer risk in women using HRT (31). Additional clinical studies have also observed a reduction in susceptibility to this disease associated with HRT in post-menopausal women (32-34). In one study, a 30-40% decrease in risk has been associated with those currently using HRT. The greatest effect was seen in current users of HRT who had been undergoing this therapy for five or more years and

the effect was lost after HRT use was stopped (35). Estrogen replacement therapy (ERT) has also been shown to decrease risk of colon cancer in some studies (36), 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. The WHI and other studies showed that HRT and ERT led to increased risk of breast cancer (31).

Estrogens and Estrogen Receptor Function

Estrogens. Estrogens are the primary sex hormones in females and are named based on their importance in the estrous cycle. There are three natural estrogens present in most animals: estriol, estrone and estradiol. These estrogens are steroid hormones and are synthesized from androgens through aromatase enzyme activity (37).

Estrogens play important roles in development, pregnancy, and other physiological processes. They are the primary trigger for the development of female secondary sex characteristics, as well as regulating the menstrual cycle (38). Estrogens are also important in males, and have been identified as playing a role in the male reproductive system (39).

Estrogen receptors. Estrogen receptors are nuclear receptor transcription factor superfamily members that are activated by ligand binding of estradiol or other estrogens. There are two primary forms of estrogen receptor identified ERα and ERβ. They primarily function as transcription factors either enhancing or inhibiting production of mRNAs of the target gene when activated.

These proteins contain five structural characteristics common to all members of this superfamily: regions A-F (40). There are three major functional domains: an N-terminal activation function domain-AF1 (A/B), a DNA binding domain-DBD (C) and a C-terminal ligand binding domain-LBD/activation function domain-AF2 (E/F). The AF1 domain has weak ligand independent activity and is responsible for constitutive transcriptional activity (41, 42). The DBD is a highly conserved region and is the portion of the protein that recognizes the estrogen response elements found in the promoter regions of genes whose transcription is modified by ER activity (43). The LBD is the portion of the protein that binds E_2, and when activated by ligand binding the AF2 domain becomes a site for cofactor binding and dimerization which significantly increases transcription factor activity (43, 44). Homology between the AF2 domain in ERα and ERβ is only 53% and is likely the primary source for differential functional effects between the two receptors.

ER signaling. Upon ligand activation, ERs can regulate biological processes by two primary mechanisms (45). Classical signaling occurs through direct binding of ER dimers to estrogen-responsive elements (ERE) found within the regulatory regions of genes whose activity is modified by ER activity (46). This is followed by the recruitment of co-regulators to the transcription start site. The consensus ERE is made up of two 5 base pair palindromes separated by a 3 base pair spacer: GGTCAnnnTGACC (47). There are however, many natural EREs that deviate from this sequence (48, 49). A second mechanism involves the interaction of activated ER with another transcription factor such as activating protein-1 (AP-1) or stimulating protein-1 (Sp-1) (50, 51). This is referred to as transcription factor cross-talk. There have also been non-genomic

functions of ERα identified which involve the binding of the receptor to activate protein kinases and modulate their signaling pathways (52).

ERβ is the predominant form of the receptor found in the colon (53). This form of ER was first identified and characterized in 1996 (54, 55). It has been suggested that ERα is the primary receptor involved in pro-proliferative estrogen signaling, while ERβ is the primary receptor involved in anti-proliferative signaling (56, 57). Additional studies have demonstrated that ERβ activity is often antagonistic to that of ERα (58, 59).

ERβ activity has been linked to both cell cycle arrest in several *in vitro* models (60, 61). Other studies have demonstrated increased apoptotic activity associated with ERβ activation *in vitro* (56, 62). However, data is less conclusive in colon cancer cell lines. A few studies have shown an E_2 response in colon cancer cell lines (63, 64), but the majority of studies have found colon cancer cell lines to be non-responsive to E_2 treatment (65-67). Insertion of functional ERβ into colon cancer cell lines can reactivate E_2 induced anti-proliferative activity (68). These data suggest that ERβ activity and E_2-induced protection against colon cancer are likely early stage events and lose effect in more advanced stages of cancer development.

Estrogen therapy in animal models of colon cancer and the role of the estrogen receptor. Animal models have long been used to study colon cancer as a means to study the disease in a controlled environment. E_2 treatment in ovariectomized (OVX) rats exhibited reduced tumor development in a dimethylhydrazine induced model of colon cancer (69). Orally administered estrone in OVX, ERα +/+ and -/- mice was observed to reduce tumor numbers in an azoxymethane (AOM) induced model of carcinogenesis (70). Schleipen et al. demonstrated that treatment with an ERβ specific agonist induced

apoptosis in the colon of OVX rats (71). These studies demonstrate that ERα is not the mediator of the estrogenic protection observed.

Apoptosis and p53

Apoptosis. The cellular process apoptosis mentioned previously as an anti-proliferative outcome, is one of the cell's most effective methods of limiting the spread of harmful mutations. Apoptosis is the process of programmed cell death and was first described by Carl Vogt in 1842 (72). It differs from necrosis, an uncontrolled form of cellular death reaction, in that it is a very organized destruction of a cell and is governed by a strict set of signals (73).

Apoptosis is primarily triggered through two pathways: extrinsic and intrinsic (Fig. 1.3). In both cases, pathways lead to the activation of proteases (known as caspases) that are held in an inactive state (procaspases), until an apoptotic signal reaches it (74, 75). The process often starts by uninhibiting initiator caspases (8/9), which then target effector caspases (3/6/7) for activation and lead to signals ranging from permeabilization of the mitochondrial membrane, to destruction of the cytoskeleton, and cleavage of genomic DNA. Apoptotic cells also display cell surface markers signaling to surrounding cells that the cell is apoptotic. This allows the surrounding cells to internalize pieces of the dying cell, to use for their own function (74).

Extrinsic apoptosis begins with the binding of a ligand to a cell surface death receptor, such as Fas receptor (FasR) binding of Fas ligand, tumor necrosis factor α receptor 1 (TNFαR1) binding of TNFα related apoptosis-inducing ligand (TRAIL) (76).

After ligand binding, effector proteins such as Fas-associated death domain (FADD), are recruited by the receptor to activate caspase 8 and begin a caspase cascade (73, 76).

The intrinsic pathway is often initiated as a response to DNA damage or other severe types of cellular stress. It involves the release of intracellular pro-apoptotic proteins that create pores in the mitochondrial membrane releasing cytochrome c. Cytochrome c then coordinates with apoptotic proease-activating factor 1 (Apaf-1) to activate caspase 9 initiating a caspase cascade (Fig. 1.3) (76, 77).

The tumor suppressor protein p53 can be involved with initiation of both of these apoptotic pathways. The extrinsic pathway can be initiated by p53 induced expression of Fas, TRAIL receptor 2, TRAIL death ligand (TNFSF10) and Fas death ligand (FasL) (78-80). Other genes in this pathway influenced by p53 include Apaf-1 and caspases 6&8 (78). Bcl-2 associated X protein (Bax) is involved in the intrinsic pathway where it inserts itself into the mitochondrial membrane and causes pore formation (Fig. 1.3) (81-83). p53 can induce the expression of Bax, as well as other pro-apoptotic genes like p53-upregulated mediator of apoptosis (PUMA), while repressing anti-apoptotic genes like Bcl-2 (83).

p53. p53 was first reported in several studies published in 1979. This protein was originally thought to be oncogenic, but would lead to significant leaps in the understanding of cell cycle regulation, cellular senescence, apoptosis and cancer (84-87). Experiments inducing tumorigenic conditions in hamsters by viral infection with Simian

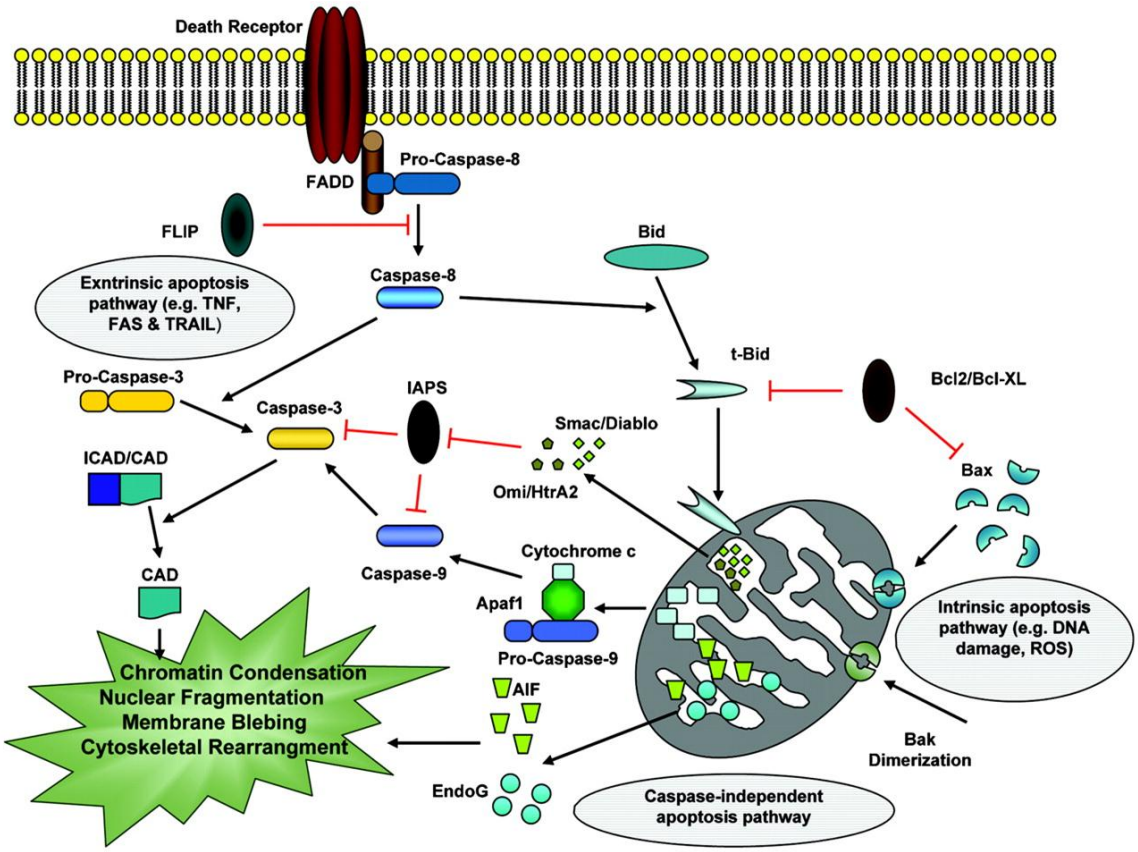

Figure 1.3. The molecular mechanisms of apoptosis (88). Apoptosis pathways can be initiated via different stimuli—that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway). Stimulation of death receptors results in receptor aggregation and recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) and caspase-8. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. Mitochondria are engaged via the intrinsic pathway, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumour suppressor genes (that is, p53), viral virulence factors, and most chemotherapeutic agents. Mitochondrial membrane permeabilisation is regulated by balance of opposing actions of proapoptotic and antiapoptotic Bcl2 family members (Bax, Bak, Bcl2 and Bcl-XL, Mcl-1). Following mitochondrial permeabilisation, mitochondrial pro-apoptotic proteins like cytochrome c, Smac/Diablo, Omi/HtrA2 (caspase dependent), AIF, and Endo G (non-caspase-dependent) release via transmembrane channels across the mitochondrial outer membrane (see main text for more details). CAD, caspase activated DNase; FAS, fibroblast associated antigen; ICAD, inhibitor of CAD; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF related apoptosis inducing ligand. (88)

virus 40 (SV40) led to the discovery of a 53 kDa protein that was pulled down by the SV40 large T antigen. This 53 kDa protein is now recognized as a tumor suppressor and is often referred to as “guardian of the genome”, but was originally believed to be an oncoprotein (87). Early experiments supported this hypothesis, but its true role and importance in the cell has been elucidated over the last thirty years.

p53 is found to be mutated in more than 50% of all human cancers (89). p53 mutation is generally believed to occur later in the process (Fig. 1.2). Some of these mutants are thought to be gain of function, promoting tumorigenesis over and above the normal loss of tumor suppressor function (90). It is claimed that nearly 11 million people have tumors harboring mutations in *TP53*, the gene coding for p53, and another 11 million have tumors with mutations involving other proteins in p53 pathways (91). One example of this is oncogenic transformation of Mdm2 which aggressively degrades p53 (92, 93). These data suggest that loss of p53 function is a primary risk factor in cancer development.

During normal cellular growth and development p53 activity is non-essential, as demonstrated by normal development and maturation of p53 knock-out mice (94). However these same mice are much more prone to spontaneous tumor development as they age. In normal, healthy cells p53 is capable of initiating cell cycle arrest, senescence or apoptosis in response to cellular stress. One of the most common reasons is DNA damage. This can be caused by irradiation, alkylation, depurination, telomere shortening, substitution or mismatch errors during replication (95). Each of these problems has proteins and pathways specifically designed to detect and report them, but they all operate by signalling p53. DNA damage is not the only source of stress that can result in

activation of p53 pathways; glucose starvation, hypoxia, and oxidative stress, among other causes can trigger a p53 response (95, 96). Finally, p53 can also respond to oncogenic activation of proteins, such as Ras and E2F (97-99).

Wild type p53 is a transcription factor that activates downstream target genes in response to DNA damage (100, 101). These target genes can include cyclin dependent kinase 1 (p21), BAX, PUMA and other effector genes (83, 102-104). p53 is also capable of directly and indirectly contributing to DNA repair (105, 106). Deletion or mutation of p53 enhances cell proliferation in cells with DNA damage, due to the loss of its ability to arrest the cell cycle and initiate apoptosis.

The role of p53 in E_2 induced apoptosis. E_2 treatment in cells containing ERβ has demonstrated the ability to increase apoptosis. Furthermore, ERβ has been shown to have a role in p53 expression and function in many cell types. The levels of p53 and its downstream target cyclin dependent kinase inhibitor 1 (otherwise known as p21 a cell cycle regulator) are increased in estrogen dependent breast cancer (MCF-7) cells transfected with a vector expressing ERβ and treated with E_2 (107). E_2 treatment in osteoblasts also increases the levels of ERβ, p53 and its downstream targets murine double minute 2 (Mdm2) and p21 (108). HHUA endometrial cancer cells have been shown to have an increased ratio of ERβ:ERα, and E_2 treatment decreased oncogene Bcl-2 (B-cell lymphoma, a negative modifier of apoptosis) levels with no significant change to p53 levels (109). In normal breast epithelial (HC11) cells transfected to express ERβ, treatment with an ERα specific agonist led to increased proliferation, while treatment with an ERβ specific agonist led to increased expression Bax and associated apoptosis (56). These data are evidence of a role for E_2, modulated through ERβ, to

increase p53 activity/expression and thereby increase apoptosis. This identifies p53 signalling as a potentially critical pathway in E_2 chemoprevention of spontaneous colon cancer.

Summary

Colon cancer is a major cause of mortality in most developed countries and is becoming a more prominent cause of death in many developing countries. Epidemiological and experimental evidence suggest that presence of the female hormone estrogen at premenopausal levels, decreases risk of colon cancer in post-menopausal women and ovariectomized animals. Further experimental data suggests that this effect is not ERα mediated. However, the molecular mechanisms and cellular pathways involved have not been studied significantly. Therefore, we determined the physiological response to E_2 treatment in non-malignant colonocytes and the role that ERβ and p53 play in E_2 protection against colon carcinogenesis.

Hypotheses and specific aims

Hypothesis 1. E_2 treatment will reduce non-malignant colonocyte growth.

Aim 1. Determine the effect of E_2 on YAMC cell growth.

Aim 2. Determine the physiological basis for decreased cell growth in YAMCs treated with E_2.

Hypothesis 2. Mice lacking ERβ will lose protection against colon cancer development provided by E_2 exposure.

Aim 1. Determine the effect of E_2 on ACF formation in WT and ERβKO mice.

Hypothesis 3. E_2 treatment will increase p53 expression or increase its stability.

Aim 1. Determine p53 activity and expression in YAMC bleo/neo cells with and without E_2 treatment.

Aim 2. Determine the differential effects of E_2 treatment in YAMC mp53/neo cells.

Hypothesis 4. E_2 treatment increases the expression of downstream targets of p53, such as Bax, Bcl-2, and p21, involved in the induction of apoptosis.

Aim 1. Determine the expression levels of p53 downstream targets (Bax, Bcl-2, NOXA, PUMA, p21 and p27) in YAMC bleo/neo and mp53/neo cells with and without E_2 treatment.

Hypothesis 5. E_2 treatment enhances the cells ability to respond to DNA damage based stress.

Aim 1. Determine the effect E_2 treatment has on the formation of γH2A.X foci formation after irradiation.

Hypothesis 6. E_2 treatment will decrease ACF formation in wild type mice, but this effect will be lost in p53 KO mice.

Aim 1. Determine the effect of E_2 on ACF formation in WT and Heterozygous p53KO mice.

Aim 2. Determine if E_2 treatment alters apoptosis and/or proliferation in colonic crypts of WT and Heterozygous p53KO mice

CHAPTER II

ESTRADIOL ALTERS CELL GROWTH IN NON-MALIGNANT COLONOCYTES AND REDUCES THE FORMATION OF PRE-NEOPLASTIC LESIONS IN THE COLON[1]

Colon cancer incidence is higher in men (62.9 in 100,000) than in women (45.8 in 100,000) (110). This suggests a possible protection in women due to hormonal differences. Data from the Women's Health Initiative study showed a significant decrease in the incidence of colorectal cancer in postmenopausal women receiving hormone replacement therapy (HRT) compared with placebo (31). In fact, the majority of clinical studies have shown that either HRT or estrogen replacement therapy (ERT) can significantly reduce the risk of colon cancer in postmenopausal women (33-36, 111). Hoffmeister and colleagues (112) found that HRT and ERT both significantly reduce colorectal cancer incidence, but there was no significant difference in effect between the two therapies. The level of protection seen from ERT correlates with the degree of use, seen as a 29% reduction in colon cancer risk, and if ERT had ever been used, this increases to 45% for those currently undergoing ERT (36).

Animal study data support the theory of a significant protective effect against colon cancer in animals treated with estradiol (E_2). E_2 treatment in ovariectomized rats reduced dimethylhydrazine-induced tumor numbers in the colon by 71% (69). Orally administered estrone in ovariectomized wild-type (WT) and estrogen receptor (ER) α

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knockout mice inhibited formation of azoxymethane (AOM)–induced tumors (70). Data from this study are important because the fact that estrone suppressed tumor formation in both WT and ERα knockout mice shows that ERα is not the primary mediator for estrogenic protection in the colon. However, several studies have shown that ERβ expression is inversely associated with colon tumor incidence (53, 113). Most of these studies have correlated reduced ERβ expression with increased risk of colonic malignancy. However, no studies have definitively shown the necessity of ERβ expression in colonocytes to observe this protection.

In an effort to identify the mechanism by which E_2 affects cell growth, numerous different colon cancer cell lines have been used as in vitro models; however, a majority of studies have shown that E_2 treatment does not influence cell growth in these cell lines (65-67, 114, 115). Based on these data, we hypothesized that E_2 would alter the physiology of nonmalignantly transformed colonocytes and that this response would result in reduced formation of premalignant lesions in mice exposed to carcinogen. Furthermore, we hypothesized that this effect would be modulated through ERβ. To test our hypotheses, we characterized the effects of E_2 in young adult mouse colonocytes (YAMC) and in WT and ERβ knockout mice (ERβKO). YAMCs are a well-characterized nonmalignant cell line derived from the Immorto mouse (116). The data presented here show that E_2, through an ERβ-mediated response, affects cell growth in nontransformed colonocytes, resulting in reduced incidence of premalignant lesions in the colon.

Materials and Methods

Cells. YAMC and YAMC-Ras cells were graciously provided by Dr. Robert Chapkin (Department of Nutrition and Food Science, Texas A&M University, College Station, TX; 117). For general maintenance, cells were cultured in RPMI 1640 (Sigma) with 5% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); 1% penicillin/ streptomycin; and 1% Glutamax-1 (Invitrogen). Cells were maintained under permissive conditions, 33°C with 10 units IFNγ/mL (Roche) medium.

Cell growth assays. Forty-eight hours before plating, YAMC or YAMC-Ras cells were transferred to medium containing 5% charcoal-dextran–stripped FBS, 1% Glutamax, 1% penicillin/streptomycin, and 0.1% ITS. Cells were seeded at 30,000 per well on six-well plates. Twenty-four hours after plating, cells were exposed to individual treatments of 0, 100, 1,000, or 10,000 pmol/L E_2 or in combination with 1 μmol/L ICI 182,780 (ICI; fulvestrant, Sigma-Aldrich), and 48 h after the first treatment, the medium was changed and a second dose of the given treatments was delivered. All treatments were diluted in DMSO as 1,000× stocks and delivered as 1 μL/mL medium to achieve the final dose listed. At the end of the 96-h treatment period, cells were trypsinized and prepared for counting. The experiments at nonpermissive conditions were carried out as above but at 39°C and without IFNγ. Cell concentration was determined using a Beckman Coulter particle counter. Twenty microliters of sample were diluted in 10 mL Isotone II diluent (Beckman Coulter), and each sample was counted thrice. All experiments were performed at the permissive temperature unless otherwise specified.

Three wells per treatment per experiment were used and either three or four replicate experiments were conducted.

SV40 protein levels. YAMC cells (250,000) were seeded in 25-mm flasks and grown at 33°C in stripped serum medium. Cells were treated with 1 nmol/L E_2 or vehicle for 96 h with medium and treatment was changed after 48 h. Protein was extracted by adding 1.5 mL lysis buffer to the flask for 30 min at room temperature. After incubation, solution was mixed gently with a pipette and the contents were transferred to microcentrifuge tubes. Protein was quantified by UV spectrometry. Protein concentration was determined by Western blot using the Immobilon Western Chemilunescent Horseradish Peroxidase (HRP) Substrate kit (Millipore); the methodology was previously described (118). Antibodies used were SV40 Large T antigen antibody (Calbiochem) and goat anti-mouse IgG-HRP (Assay Designs).

Reverse transcription-PCR. YAMC and YAMC-Ras cells were grown in T-25 flasks under permissive conditions. Cells were trypsinized and centrifuged. The colon from a WT untreated mouse was removed and used for RNA isolation using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen). The Invitrogen protocol was followed for both cell and tissue extractions. Reverse transcription-PCR (RT-PCR) samples contained 10 μL TaqMan One Step RT-PCR Master Mix, 1 μL primers (Mm00599821 ERβ, Mm00433149_m1 ERα, or Hs99999901_s1 18s; Applied Biosystems), 8 μL RNase-free water, and 1 μL RNA (20 ng/μL). RT-PCR was run on a Bio-Rad iQ5 thermocycler for 40 cycles.

Cellular apoptosis. Cells were grown in stripped serum medium for 48 h before plating. Cells (30,000) were seeded on six-well plates and grown in stripped serum

medium under permissive conditions. Cells were treated for 96 h, with 0, 100, 1,000, or 10,000 pmol/L E_2 treatments changed every 48 h. At the end of the treatment period, cells were trypsinized and collected. After collection, cells were centrifuged and the medium was replaced with lysis buffer from the EnzChek Caspase-3 Assay kit no. 2 (Invitrogen). The Invitrogen protocol was followed for this procedure. Apoptosis was measured as increased fluorescence measured on a TECAN infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

Mice. Heterozygous ERβKO (+/−) c57BL6/J mice were obtained from The Jackson Laboratory. Mice were bred and housed at the Laboratory Animal Resources and Research facility at Texas A&M University. ERβKO and WT mice were produced from the original breeding pairs and genotyped from genomic tail DNA (Appendix B-14). All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Carcinogen treatment and aberrant crypt foci analysis. Female mice between the ages of 2 and 8 mo were ovariectomized, and either 20 mg cholesterol (Sigma-Aldrich) or 18 mg cholesterol/2 mg E_2 pellet was implanted s.c. on the back at the base of the neck at the time of ovariectomy. Pellets were prepared as described in ref. (119). Pellets were replaced 8 wk later. Mice were transferred to a phytoestrogen-free diet at the time of surgery and allowed food and water ad libitum. Two weeks after surgery, mice received the first of six weekly injections of AOM (Sigma-Aldrich) at 10 mg/kg body weight. Eight weeks post-AOM, animals were killed. Blood was collected through cardiac puncture. The colon was resected and 1-cm sections from the distal end were

cassetted and fixed in 4% paraformaldehyde (Mallinckrodt Baker, Inc.). The remainder of the colon was flattened between sheets of filter paper and fixed in 70% ethanol. Ethanol-fixed colons were stained with 0.5% methylene blue (Sigma-Aldrich) and aberrant crypt foci (ACF) were counted as previously described (120. 121).

Plasma E_2. Plasma E_2 concentrations were determined using an Estradiol EIA kit (Cayman). Fifty microliters of plasma/sample were added to sample wells, and samples from E_2-treated animals were diluted 1:10. Fifty microliters of Tracer and antiserum were added per sample well and the plate was incubated for 1 h. The wells were washed five times with wash buffer. Ellman's Reagent (200 μL) was added per well and incubated in the dark for 60 to 90 min while shaking. Wells were read on a plate reader at 415 nm.

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

Statistical analysis. Statistics were performed using a Student's t test, one-way ANOVA with Tukey's correction, or two-way ANOVA, depending on the outcome analyzed. t test analyses were performed in Excel, and ANOVA data were run in Minitab 15. A 99% confidence interval for the mean count difference (estrogen minus

control) and a one-sided test of whether mean estrogen counts were less than control counts were calculated for ACF data. Also, for total ACFs and high-multiplicity ACFs, we specifically tested for a nonzero interaction in the effect of estrogen by mouse type (knockout or WT). All values listed are group mean and error bars are presented as SEM.

Results

E_2 inhibits nonmalignant cell growth. First, we measured the effect of E_2 treatment on cell growth in nonmalignant colonocytes. YAMC cells treated with 0 to 10,000 pmol/L E_2 exhibited reduced cell growth when treated for 96 h under permissive conditions (Fig. 2.1A). As the E_2 dose increased, there was a corresponding decrease in cell growth ($P < 0.0001$). These data show a growth inhibitory effect in nonmalignant colonocytes exposed to physiologic levels of E_2.

We then treated these cells with the same doses of E_2 under nonpermissive conditions (39°C) to see if similar responses will be observed when cells were in a more primordial state. Again, E_2 suppressed cell growth under these conditions in a dose-dependent manner ($P < 0.0001$; Fig. 2.1B).

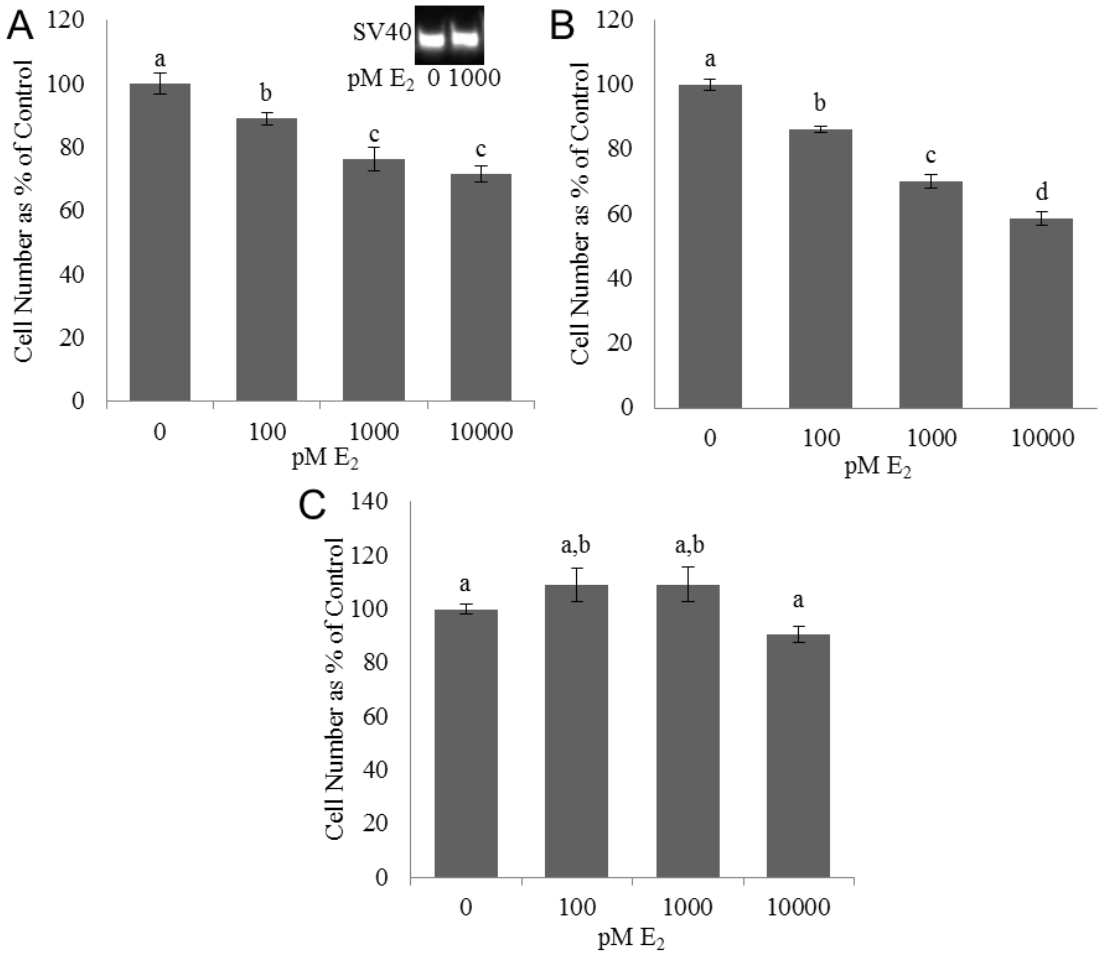

Figure 2.1. E_2 inhibits growth of YAMC cells but not YAMC-Ras cells. *A*, YAMC cells were grown at 33°C in the presence of IFNγ. Cells were transferred to a charcoal dextran–stripped medium 48 h before plating. Twenty-four hours after plating, cells were treated with 0 to 10 nmol/L E_2, and the medium and treatments were replaced 48 h later. *B*, the above experiment was repeated, changing from the permissive temperature of 33°C to the nonpermissive temperature of 39°C and removing IFNγ. *C*, YAMC-Ras cells were grown under the same conditions as in A. There was no significant difference between the control and any of the E_2 treatments. Data are expressed as percentage of growth of the DMSO control group. Columns, mean (n = 12) from four replicate experiments; bars, SEM. Bars without a common letter differ; $P<0.01$. Inset, bands from a Western blot showing SV40 protein levels do not change in response to E_2 treatment.

Because the conditional immortalization of these cells operates through the expression of the temperature-sensitive SV40 large T antigen, we wanted to rule out a possible interaction between SV40 and E_2 as the mechanism of effect. We chose to

measure the levels of this protein under permissive conditions with and without a 1 nmol/L E_2 treatment. E_2 treatment did not alter SV40 protein levels when compared with control at the permissive condition (Fig. 2.1A, inset).

Effect of E_2 on YAMC-Ras cell growth. Next, we determined if E_2 treatment would result in a similar effect on cell growth in an isogenic cell line with a single malignant transformation. YAMC-Ras cells contain an activated v-Ha-Ras oncogene. These cells, when treated with E_2, show no reduction in cell number (Fig. 2.1C). This finding was in stark contrast to what was seen in nonmalignant cells and suggests that a single malignant transformation could alter the ability of colonocytes to respond to the presence of E_2.

ERβ expression in YAMC, YAMC-Ras, and WT mouse colonic tissues. To see if the change in E_2 responsiveness was a result of altered ERβ expression, RT-PCR was performed to evaluate differences in ERβ expression between YAMC and YAMC-Ras cells. Relative expression of ERβ was not significantly different between the YAMC and YAMC-Ras cell lines and was similar to that seen in colonic tissues collected from a female WT mouse (Fig. 2.2A), and no detectable ERα expression was observed at the mRNA level (supplemental data A-1). When YAMC cells were treated with E_2, relative expression of ERβ was enhanced compared with vehicle controls (Supplementary Fig. S1). Collectively, these studies show that ERβ is expressed in YAMC cells and its

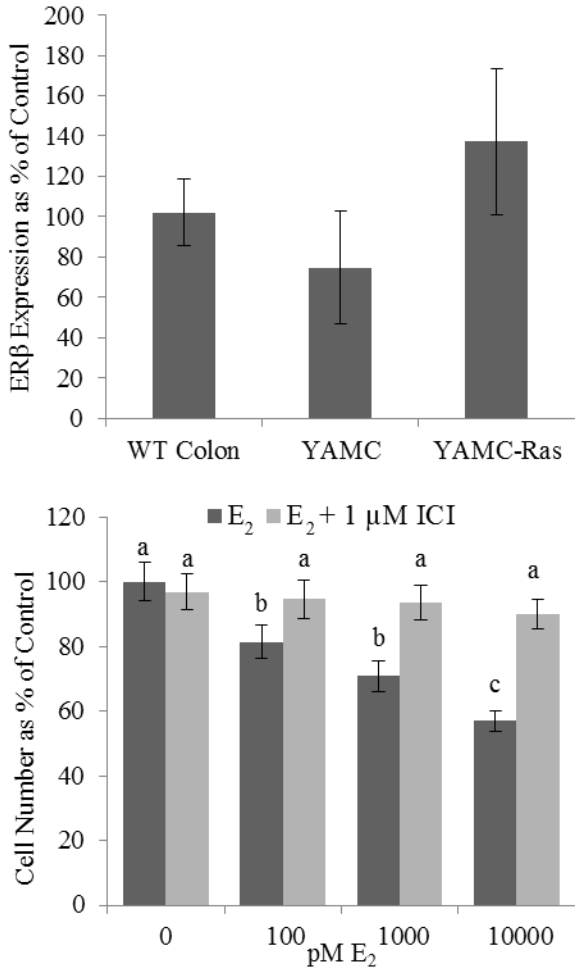

Figure 2.2. ERß expression in YAMC and YAMC-Ras cells and ER function in cell growth inhibition. *A*, RNA levels were measured in colonic tissue from a WT mouse, YAMC, and YAMC-*Ras* cells. *Columns*, mean (n = 12); *bars*, SEM. Bars without a common letter differ; $P<0.05$. *B*, cotreatment with ICI inhibits the effects of E 2 in YAMCs. YAMC cells were transferred to a charcoal dextran–stripped medium 48 h before plating. Cells were seeded at 30,000 per well on six-well plates and treated every 48 h for a total of 96 h. ICI treatment inhibited the effects of E 2 on cell growth. Data are expressed as percentage of growth of the DMSO control group. *Columns,* mean (n = 9) from three replicate experiments; *bars,* SEM. Bars without a common letter differ; $P<0.01$.

expression changes in the presence of E_2. In addition, these data suggest that the loss of E_2 responsiveness in the YAMC-Ras cells is not the result of suppressed ERβ expression.

Cotreatment of ICI with E_2 prevents cell growth inhibition. Having shown ERβ to be present in both cell lines, we next wanted to determine if the growth-inhibitory effect of E_2 treatment was mediated through the ER. YAMC cells were treated with 0 to 10,000 pmol/L E_2 concentrations either in the presence or absence of 1 μmol/L ICI, a well-described ER antagonist that inhibits both receptor subtypes (Fig. 2.2B; refs. 122, 123). With each E_2 dose, cotreatment with ICI fully inhibited the growth suppression induced by E_2. In fact, cotreating with ICI resulted in cell numbers that were not significantly different than the untreated control group. ICI antagonism of E_2 growth inhibition strongly supports the theory that this is an ER-mediated response.

Apoptosis in YAMC and YAMC-Ras cells treated with E_2. Next, we wanted to identify the physiologic response involved with estrogenic growth inhibition. Caspase-3 activation was used to determine the relative numbers of apoptotic cells when YAMC and YAMC-Ras cells were treated with 0 to 10,000 pmol/L E_2 for 96 hours. YAMC cells exhibited an increase in caspase-3 activity in a dose-responsive manner ($P < 0.001$; Fig. 2.3A). In contrast, YAMC-Ras cells showed no significant increase in caspase-3 activity at any concentration of E_2 (Fig. 3B). These data point to apoptosis being a mechanism involved in the E_2-induced growth inhibition of nonmalignant colonocytes.

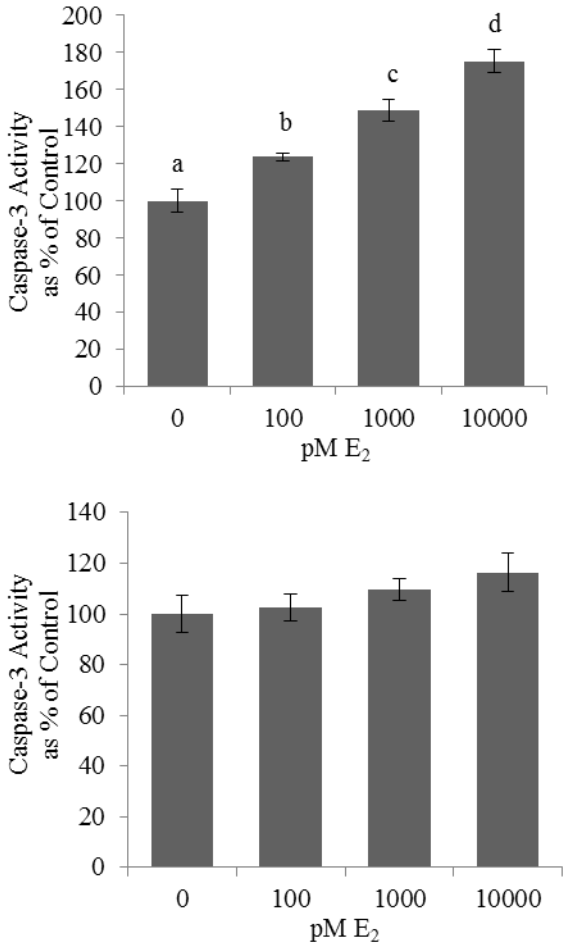

Figure 2.3. Apoptosis in YAMC and YAMC-Ras cells treated with E_2. *A*, YAMC cells were grown under permissive conditions and transferred to a charcoal dextran-stripped medium 48 h before plating. After 96 h of E_2 treatment, cells were collected and the EnzChek caspase-3 assay was performed. *B*, the above experiment was repeated with YAMC-*Ras* cells. Data are expressed as increased fluorescence measured when compared with the blank. *Columns*, mean (n=9) from three replicate experiments; *bars*, SEM. Bars without a common letter differ; $P<0.001$.

***Formation of ACF*.** We next wanted to investigate the role of E_2 treatment in the colon at a premalignant stage of disease. We began by exploring the ability of E_2 to suppress the formation of ACFs, premalignant lesions that are predictive of tumor formation. ACFs were only observed in the distal two thirds of the colon, with the majority found in the most distal region. WT animals treated with the vehicle control had

the highest number of ACFs averaging 29 ACFs per animal, whereas WT animals treated with E_2 showed a significant reduction (14.9 ACFs per animal; $P < 0.0001$; Fig. 2.4A). More importantly, when analysis is narrowed to high-multiplicity ACFs (foci involving at least four crypts), the difference between groups becomes more pronounced (Fig. 2.4B). WT animals receiving E_2 averaged 3.2 high-multiplicity ACFs per animal, whereas WT control mice averaged over 10.6 high-multiplicity ACFs per animal ($P < 0.0001$). These data show a pronounced reduction in preneoplastic lesions associated with E_2 treatment.

ERβKO animals had similar numbers of ACFs in both control (21.1 ACFs per animal) and E_2-treated (20.2 ACFs per animal, $P = 0.2$) mice (Fig. 2.4A). There was a slight but significant difference ($P = 0.01$) in high-multiplicity ACFs in the ERβKO mice between treatments, with E_2-treated animals averaging 8.4 and control animals averaging 9.4 ACFs per animal (Fig. 2.4B). Together, these data distinctly show a relationship between ERβ and E_2 in the colon.

Plasma E_2 concentrations. We measured plasma E_2 levels in vehicle control and E_2-treated animals. Mice receiving cholesterol-only pellets showed plasma E_2 levels averaging 22.4 pmol/L in WT and 33.9 pmol/L in ERβKO animals (Fig. 2.5). Those receiving E_2-containing pellets showed average E_2 levels of 2.9 nmol/L in WT and 3.0 nmol/L in ERβKO animals ($P < 0.0001$).

Apoptosis within the colonic crypt. Having found a distinct phenotype associated with E_2 treatment in WT animals, we analyzed apoptosis to see if there was an association with E_2 treatment. The induction of colonocyte apoptosis was determined in

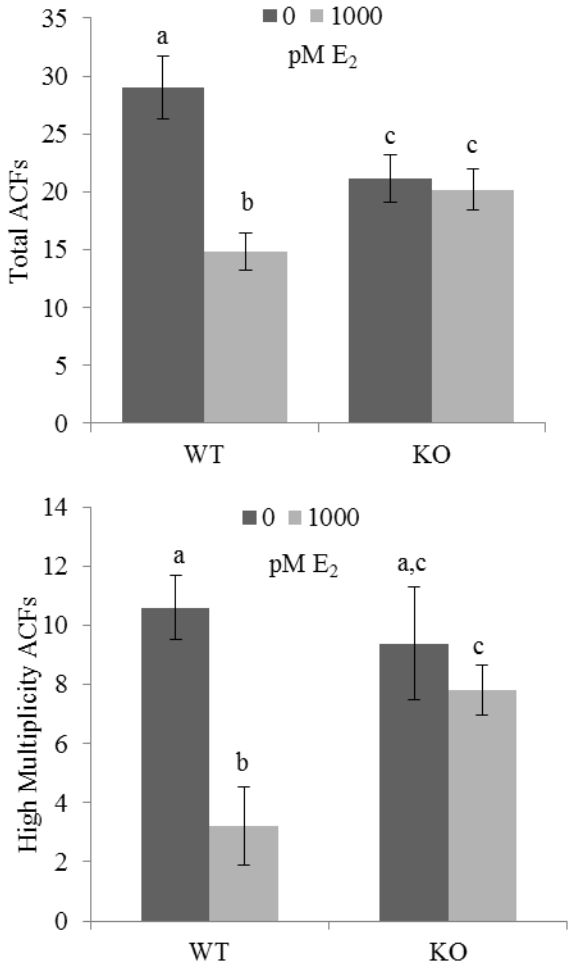

Figure 2.4. ACFs in the distal colon. *A*, total ACFs identified per animal. *B*, high-multiplicity ACFs identified per animal. *Columns*, mean (n=5-13 animals analyzed per treatment group); *bars*, SEM. Bars without a common letter differ; P<0.001.

tissues collected from each experimental group. Within the distal colon, crypts analyzed from WT mice receiving E_2 exhibited a significantly higher degree of apoptotic cells compared with those from the control. ERβKO mice showed a slight but significant difference in apoptosis with E_2 treatment with more apoptotic cells per crypt than the ERβKO control mice (Fig. 2.6A). As can be seen in both images, it is common for cells

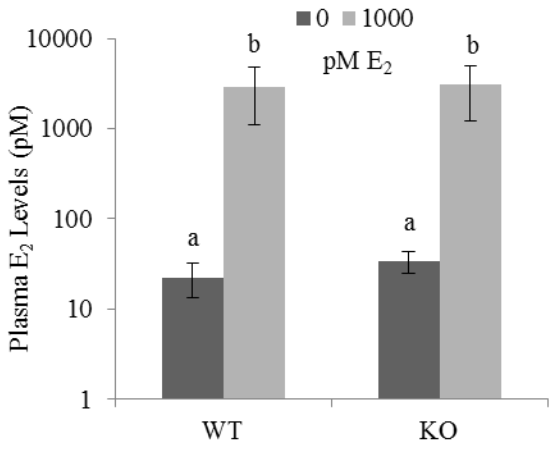

Figure 2.5. Plasma E_2 levels in carcinogen-treated animals. *Columns*, mean (n = 5–13 animals analyzed per treatment group); *bars*, SEM. Bars without a common letter differ; $P<0.0001$.

on the luminal surface outside of the crypt structure to become apoptotic in association with these cells being sloughed off. Therefore, only cells within the crypt were analyzed. Interestingly, the increase in apoptosis in E_2–treated WT mice was associated with an increase in apoptotic cells observed in the lower regions of the crypt in WT animals treated with E_2 (Fig. 2.6B and C).

Discussion

Limited data have shown that E_2 treatment reduces cell growth in three colon cancer cell lines, DLD-1, HCT116, and LoVo (63, 64, 124). However, the majority of cell culture studies have shown no reduction or an increase in growth in response to E_2 in colon cancer cells that are fully transformed (65-67, 114, 115, 125). Collectively, these data highlight the need to study the effects of E_2 in other cell models and specifically in nonmalignantly transformed colonocytes. YAMC cells are

morphologically primitive epithelial cells, with no evidence of differentiation (116). For the first time, we show that E_2 can alter the growth of nonmalignant colonocytes by showing a significant reduction in cell number in YAMC cells treated with E_2. This response was also observed in YAMC cells maintained at nonpermissive conditions, suggesting that E_2 has similar actions in colon cells that are more primordial in nature. YAMC-Ras are isogenic cells that were developed from YAMCs and contain an activated v-Ha-Ras oncogene. These cells are fully transformed and will continue to grow under nonpermissive conditions and form colonies in soft agar (126). The Ki-Ras oncogene is present in ~50% of colonic adenomas and carcinomas and was found to be common in tumors from all stages, including villous adenomas and villoglandular polyps that are nonmalignant but often progress to malignancy (127, 128). Data from YAMC-Ras cells show that overexpression of an activated v-Ha-Ras oncogene negates the growth inhibition due to E_2 exposure.

Having identified an effect of E_2 on cell growth, we wanted to determine if this response was ER mediated. ERβ is the predominant form of ER found in the colon and is expressed in some colon cancer cell lines (63, 67). Cotreatment of E_2 with ICI, an ER antagonist, resulted in a loss of the growth inhibition induced by E_2. Because ERβ expression levels were not significantly different between YAMC and YAMC-Ras cell lines, the question of mechanism of activity arises. It is possible that a nonfunctional protein is being produced in the YAMC-Ras cells or that malignant transformation affects the pathway at a point after receptor activation. Future studies will address these important questions.

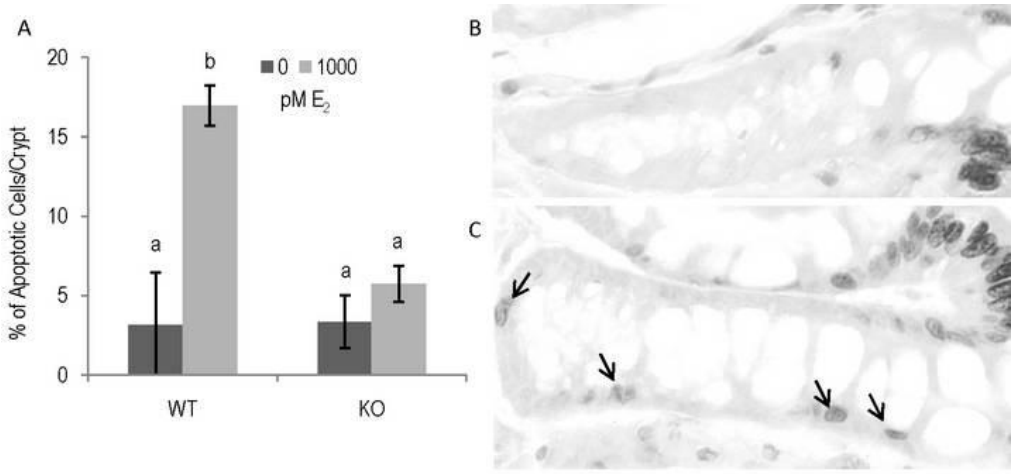

Figure 2.6. Apoptosis within the colonic crypts of the distal colon. *A*, a TUNEL assay was performed on sectioned tissue from the distal colon. Data are expressed as percentage of apoptotic cells found compared with total number of cells found in the crypt. These data are representative of at least 20 well-oriented crypts per animal and 5 to 13 animals per treatment group. *B*, representative picture of TUNEL-stained crypts from a WT cholesterol-treated animal. Some nonspecific staining can be seen in cells at the luminal surface, but very few within the crypt. *C*, representative picture of TUNEL-stained crypts from a WT E_2 -treated animal at ×400. Arrows, apoptotic cells in different regions of the crypt and other positively stained cells can be seen throughout the crypt.

We next measured apoptosis to begin identifying the cellular responses to E_2 that result in growth inhibition. As with cell growth, some studies have shown apoptosis to be increased with E_2 treatment in colon cancer cell lines (125, 129), but others show no response. We observed a significant induction of caspase-3 activity in YAMC cells and no significant response in YAMC-Ras cells when the cell lines were treated with E_2. We also conducted experiments to determine if E_2 treatment altered the progression of cells through the cell cycle, but no significant differences were seen between treatments (supplemental data A-2). These data suggest that E_2 reduces cell growth in noncancerous

colonocytes by induction of apoptosis; however, this response is lost following malignant transformation.

To investigate how E_2 affects the development of preneoplastic lesions, we first analyzed colonic tissue from WT animals receiving chemical carcinogen to better define the effects of E_2 at a premalignant stage of colon carcinogenesis. Ovariectomized WT mice exhibited a significant reduction, both in total number of ACFs and high-multiplicity ACFs, when treated with E_2 compared with those treated with vehicle control. The profound reduction of high-multiplicity ACFs in E_2-treated mice is of significant interest because these lesions are most predictive of eventual tumor formation (130). These data support other findings showing E_2 to be protective against tumor formation (131-133). However, our studies were focused on examining premalignant tissue and show a chemoprotective role for E_2 therapy before tumor formation.

We then compared the ACF data from WT animals with that from ERβKO mice. Clinical studies have shown reduced ERβ expression in colonocytes within tumors when compared with adjacent uninvolved tissue (134-136). A functional ERβ was previously determined to be involved with E_2 protection in APC Min mice (113). We have studied premalignant carcinogenesis and have shown that E_2 has a profound effect at this stage. In the presented studies, the reduction in the number of ACFs in WT animals treated with E_2 was pronounced and almost completely lost in ERβKO mice. These data clearly define the necessity for functional ERβ expression for E_2 treatment to exhibit a protective effect against cancer development in the colon.

Plasma E_2 concentrations in E_2-treated mice were marginally higher than those seen in a non-pregnant cycling woman, but well below those seen during pregnancy (29,

30). Mice receiving control pellets were below the threshold associated with menopause in women (137). Collectively, our in vitro and in vivo data show the influence of E_2 on cell growth in noncancerous colonocytes, but additional studies are needed to determine the minimal dose required to observe those effects.

We then sought to determine if apoptosis is induced in the colonocytes of animals treated with E_2. There was a significant induction of apoptosis in colonocytes in sections taken from WT animals treated with E_2 when compared with tissues from control mice. These cells are likely to have chemically induced DNA damage, but are not yet to the point of malignant transformation. Collectively, the YAMC and tissue data show that E_2 treatment induces apoptotic activity in nonmalignant colonocytes. Furthermore, these findings suggest that the YAMC cell line is a suitable model for studying the role of E_2 on nonmalignant colonocytes. One interesting observation from analysis of tissues collected from the mice was that apoptotic cells in the WT E_2-treated animals were found throughout the depth of the crypt, whereas the vast majority of apoptotic cells in the WT control–treated group and both treatment groups in the ERβKO mice were confined to the luminal third of the crypt. Generally, the majority of apoptosis seen within a colonic crypt occurs near the luminal surface, before cells are sloughed off. This increased range of apoptotic activity suggests a specific response to carcinogen-induced damage in WT animals treated with E_2. Preliminary data do not show this increased apoptotic activity in the lower portion of the crypt in non–AOM exposed WT animals treated with E_2 (data not shown). This phenomenon has been referred to as directed apoptosis and may be similar to the short-term apoptotic induction seen in rats after AOM exposure (138).

In conclusion, E_2 treatment showed a distinct phenotype in YAMC cells and colonocytes within WT mice. Treatment with E_2 reduced cell growth and induced apoptosis in nonmalignantly transformed cells in culture; however, expression of activated Ras in an isogenic cell line nullified this effect. In addition, E_2 treatment in ovariectomized WT mice exhibited a significant protection against preneoplastic lesions. This protection was lost in the absence of ERβ and definitively shows that ERβ is the primary mediator of this protective effect. For the first time, these data present evidence that E_2 treatment protects colonocytes from malignant transformation by increased apoptotic activity and begin to define the role of E_2 in nontransformed colonocytes. These findings are first steps toward identifying ERβ as an important target for potential chemopreventative agents in reducing the risk of tumor formation in the colon.

CHAPTER III

p53 MEDIATES ESTRADIOL INDUCED ACTIVATION OF APOPTOSIS AND DNA REPAIR IN NON-MALIGNANT COLONOCYTES[2]

In general, women have a reduced risk of developing colon cancer (23). Data from The Women's Health Initiative study suggests that post-menopausal women receiving hormone replacement therapy (HRT) have a lower incidence of the disease compared to the placebo group (31). Several other clinical studies have generated data supporting both HRT and estrogen replacement therapy (ERT) as viable interventions for the reduction of colon cancer risk in post-menopausal women (27, 35, 36, 112). Animal studies offer further evidence supporting the theory that estrogens may play a protective role against the development of colon cancer. Estradiol (E_2) treatment in OVX rats suppressed tumor development in a dimethylhydrazine-induced model of colon cancer (69), and orally administered estrone reduced colon tumor number in azoxymethane (AOM) treated mice (70). Additionally, our laboratory has demonstrated E_2 treatment in mice reduced the formation of pre-neoplastic lesions (139), suggesting that estrogenic protection occurs at an early stage of tumor development. This effect is lost in estrogen receptor β (ERβ) knockout mice indicating this is likely a receptor mediated event. Our previously reported data point towards increased apoptosis within the colonic crypts of animals exposed to carcinogen as a major mechanism of E_2 mediated protection against colon carcinogenesis (139).

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While in vivo studies have produced promising data supporting estrogen as a chemoprotective agent against the development of colon cancer, the majority of in vitro studies have not shown similar results (65-67, 114, 115). This is likely due to the use of colon cancer cell lines as the most common models for in vitro experiments. As a result, our laboratory has begun to characterize how non-malignant colonocyte cellular physiology is altered by E_2. The Young Adult Mouse Colon (YAMC) cell line has been successfully used as an in vitro model to investigate the physiological responses of non-malignant colonocytes to stimuli, such as poly-unsaturated fatty acids and carbon monoxide (140, 141). Our data have shown that YAMCs do in fact respond to E_2 treatment in a manner that is predictive of what occurs in non-malignant colonocytes *in vivo* (139). Furthermore, the ability of these cells to exhibit physiological changes due to E_2 treatment is lost with malignant transformation. Together, these data suggest a chemo-protective role for E_2 in the non-malignant colon, and warrant further study into the estrogenic mechanism of action in colonocytes.

The process of cancer progression often involves loss of heterozygosity of wild type p53 or dominant negative mutations in this gene. Point mutations in p53 occur in approximately 50% of colorectal carcinomas, and these mutations are often associated with disease severity (142-146). The p53 protein is a tumor suppressor and triggers a wide array of physiological functions within the cell in response to DNA damage, aberrant growth signals, and oxidative stress (147). These include initiation of cellular senescence, induction of apoptosis, and initiation of DNA repair mechanisms in response to varying forms of cellular stress, such as DNA damage (148). E_2 treatment has been shown to alter p53 protein levels and expression of its downstream targets when ER. is

present in MCF7 breast cancer cells, HC11 murine breast epithelial cells, HHUA endometrial carcinoma cells and osteoblasts (56, 107-109). The relationships between E_2 treatment and p53 activity seen in these and other cell types, along with previously observed apoptosis in YAMCs, suggest that E_2 triggered p53 activity can play an important role in the cellular response to DNA damage. As such, the E_2 induced up-regulation of p53 activity is likely to have an important function in the normal biological activity of non-malignant colonocytes in vitro, and the data reported herein begin to elucidate the role p53 plays in estrogenic chemo-protection within the colon.

Materials and methods

Cells. YAMC bleo/neo and mp53/neo cells were provided by Dr. Hartmut Land (University of Rochester Medical Center; 149). For general maintenance, cells were cultured in RPMI 1640 (Sigma Aldrich) with 10% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); and 1% gentamicin (GIBCO). Cells were maintained under permissive conditions, 33°C with 5 units γ-interferon (IFNγ)/mL medium (Roche) on plates coated with rat tail collagen type I (BD Biosciences). Forty-eight hours before plating for all experiments, YAMC bleo/neo and mp53/neo cells were transferred to medium containing 10% charcoal-dextran stripped FBS, 1% gentamicin, and 0.1% ITS. β-Estradiol and Fulvestrant, ICI 182,780, (ICI, Sigma Aldrich) were DMSO as 1000× stocks and delivered as 1 μL/mL medium to achieve the final dose listed.

Cell number assay. YAMC bleo/neo and mp53/neo cells were seeded at 15,000 cells per well on six-well plates and grown under non-permissive conditions, 37°C and

absence of IFNγ. Cells were exposed to individual treatments of vehicle or 1 nM E_2 alone or in combination with 1 μM ICI for 96 h, and 48 h 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, cells were trypsinized and prepared for counting. Cell concentration was determined using a Beckman Coulter particle counter. Twenty micro-liters of sample were diluted in 10 mL Isotone II diluent (Beckman Coulter), and each sample was counted thrice. Three wells per treatment per experiment were used and three replicate experiments were conducted.

Apoptosis. YAMC bleo/neo and mp53/neo cells (15,000/well) were seeded on six-well plates and grown in stripped serum medium under non-permissive conditions. Cells were treated for 96 h, with vehicle or 1 nM E_2 treatments changed every 48 h. At the end of the treatment period, cells were trypsinized and collected. After collection, cells were centrifuged and the medium was replaced with lysis buffer from the EnzChek Caspase-3 Assay kit no. 2 (Invitrogen). The Invitrogen protocol was followed for this procedure. Apoptosis was measured as increased fluorescence measured on a TECAN infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

p53 transcriptional reporter assay. The PG13-Luc transcriptional reporter contains 13 copies of the p53 response element (5‘-CCAGGCAAGTCCAGGCAGG-3‘) driving expression of the luciferase gene (102). YAMC bleo/neo cells (15,000/well) were seeded in 12-well plates and maintained at 33°C in stripped serum medium. Cells were grown for 72 h under non-permissive conditions. After 48 h, cells were transiently transfected with plasmids containing β-galactosidase and the pG13 p53-luciferase

reporter construct. Transfection was performed using the Effectene transfection reagent (Qiagen) using 0.5 μg β-gal and 4 μg pG13 per plate. Cells were treated with 1 nM E_2 or vehicle for the final 0, 4, 8, or 24 h vehicle or 1 nM E_2 alone or in combination with 1 μM ICI for the final 18 h. Media was then removed from cells, and the cells washed with PBS and lysed using 200 μL Reporter Lysis Buffer (Promega). Cell lysates were combined with Luciferase reagent or β-galactosidase reagent (Promega) and activity measured on a TECAN infinite M200 plate reader.

Protein measurements. YAMC bleo/neo cells (25,000/well) were seeded in 6-well plates and maintained at 33°C in stripped serum medium. Cells were grown for 72 h under non-permissive conditions and treated with 1 nM E 2 or vehicle for the final 0, 4, 8, or 24 h. Protein was extracted by adding 100 μL lysis buffer to the flask for 30 min at room temperature. After incubation, solution was mixed gently with a pipette and the contents were transferred to microcentrifuge tubes. Total protein was quantified by UV spectrometry. Protein concentration was determined by Western blot using the Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate kit (Millipore); the methodology was previously described (118). Antibodies used were p53 polyclonal antibody (905-510), Mdm2 polyclonal antibody (905-462), goat anti-rabbit IgG (Assay Designs), and ERβ antibody (ab3577, Abcam).

RT-PCR. YAMC bleo/neo and mp53/neo cells (25,000/well) were seeded in 6-well plates and maintained under non-permissive conditions in stripped serum media. Cells were grown for 72 h and treated with 1 nM E 2 or vehicle for the final 24 h. Cells were trypsinized and centrifuged, and RNA isolation conducted using the RNAqueous-4PCR kit (Ambion). 1 μg total RNA was used for cDNA synthesis using the

Transcriptor First Strand cDNA Synthesis kit (Roche). RT-PCR samples contained 9.5 μL FastStart Universal SYBR Green Master Mix (Roche), 1.25 μL forward and reverse primers (18s F-TCA AGA ACG AAA GTC GGA GGT T, 18s R-GGA CAT CTA AGG GCA TCA CAG, p53 F-AAA GAA AAA ACC ACT TGA TGG AGA GT, p53 R-CGG AAC ATC TCG AAG CGT TTA, Mdm2 F-TGA ATC CTC CCC TTC CAT CA, Mdm2 R-TCG TCT GGA AGC CAG TTC TCA, Bax F-CAC CAG CTC TGA GCA GAT G, Bax R-GCG AGG CGG TGA GCA CTC C, Bcl-2 F-ATC TTC TCC TTC CAG CCT GA, Bcl-2 R-TCA GTC ATC CAC AGG GCG AT, NOXA F-GAA ATG CCT GGT ATT GGA TGG A, NOXA R-GAA CTC AT CCT ATC TCC TTC ATC AT, p21 F-TTC CGC ACA GGA GCA AAG T, p21 R-CGG CGC AAC TGC TCA CT, p27 F-GGC CAA CAG AAC AGA AGA AAA TGT, p27 R-GGG CGT CTG CTC CAC AGT or PUMA F-GCG GCG GAG ACA AGA AGA, PUMA R-GGA GTC CCA TGA AGA GAT TGT ACA; Sigma–Aldrich), 11 μL RNase-free water, and 2 μL cDNA. RT-PCR was run on a Bio-Rad iQ5 thermocycler for 45 cycles.

DNA damage assay. YAMC bleo/neo and mp53/neo cells (15,000/well) were seeded on 22 mm × 22 mm cover slips in six-well plates and grown in stripped serum medium under non-permissive conditions. Cells were exposed to vehicle or 1 nM E_2 alone or in combination with 1 μmol/L ICI for 72 h. At the end of the treatment period, cells were irradiated with 2 Gy followed by a 1 h incubation period at 37°C. Cells on cover slips were then fixed in 4% paraformaldehyde for 10 min. Cells were then incubated overnight at 4°C with phosphohistone H2A.X (Ser139) primary antibody (Cell Signaling). Alexa Fluor 488 goat anti-rabbit IgG and Prolong Gold + DAPI (Invitrogen) were used as secondary antibody and nuclear counterstain, respectively. Visualization of

the stain was conducted using a Zeiss Axiovert 200 microscope with Axiocam MRc. The number of foci/cell was counted in 30 cells per slide.

Statistical analysis. All data are presented as mean ±SEM. Experiments were conducted in triplicate and repeated three times. Data for cell growth experiments were transformed to percentage of control to eliminate false error that might be introduced based on differences in average cell number between replicates. Analysis for all data was determined using a student t-test, or one-way ANOVA with Tukey's post hoc test. Differences were considered significant if $p < 0.05$.

Results

Wild type p53 protein is required for E_2 regulation of cell number. The YAMC cell line was originally derived in the Whitehead laboratory from normal colonic epithelial cells isolated from the H-2Kb-tsA58 transgenic mouse (116). These cells are conditionally immortalized by SV40 inactivation of p53, but while under non-permissive conditions (described in Section 2.1) p53 concentrations and its activity return to normal. Our previous data indicates that E_2 does not influence SV40 expression in these cells (139). The presence of ERα and ERβ in bleo/neo and mp53/neo cells was verified by RT-PCR and ERβ in Western blot (A-2). The YAMC bleo/neo and mp53/neo cell lines were created by inserting vectors containing bleomycin alone or bleomycin in conjunction with the $p53^{175H}$ mutant gene into YAMC cells as described by Xia and Land (149). These cell lines were characterized by Xia and Land, notably exhibiting no p53 phosphorylation at Ser15 or acetylation at Lys382 in mp53/neo cells conferring loss of wild type (WT) p53 function in these cells. To explore the role p53 plays in the

physiological response of non-malignant colonocytes to E_2 exposure, YAMC bleo/neo and mp53/neo cells were treated with 0, 0.1, 1, or 10 nM E_2 with or without 1 μM ICI, an estrogen receptor antagonist. In cells with WT p53, E_2 treatment reduces cell number in a dose dependent manner (Fig. 3.1A), with an average of a 40% decrease in cell number at the highest dose ($P < 0.01$). The addition of ICI completely abrogates this reduction in cell number. However, in cells containing the p53 mutant, E_2 treatment had no effect on cell number (Fig. 3.1B).

E_2 induced apoptosis requires WT p53. We further investigated p53 activity by studying the role it plays in the induction of apoptosis by E_2. YAMC bleo/neo and mp53/neo cells were treated with 1 nM E_2 or vehicle for 96 h prior to cell lysis. At the end of the treatment period, cells were lysed and a Caspase-3 assay was conducted. The E_2 treated YAMC bleo/neo cells exhibited double the Caspase-3 activity compared to the vehicle treated cells, indicating an increase in apoptosis ($P < 0.001$; Fig. 3.2A). Conversely, there was no significant difference in Caspase-3 activity between treatments in the YAMC mp53/neo cells (Fig. 3.2B). Furthermore, the overall degree of apoptotic activity in cells with mutant p53 was approximately one third that observed in the vehicle treated YAMC bleo/neo cells.

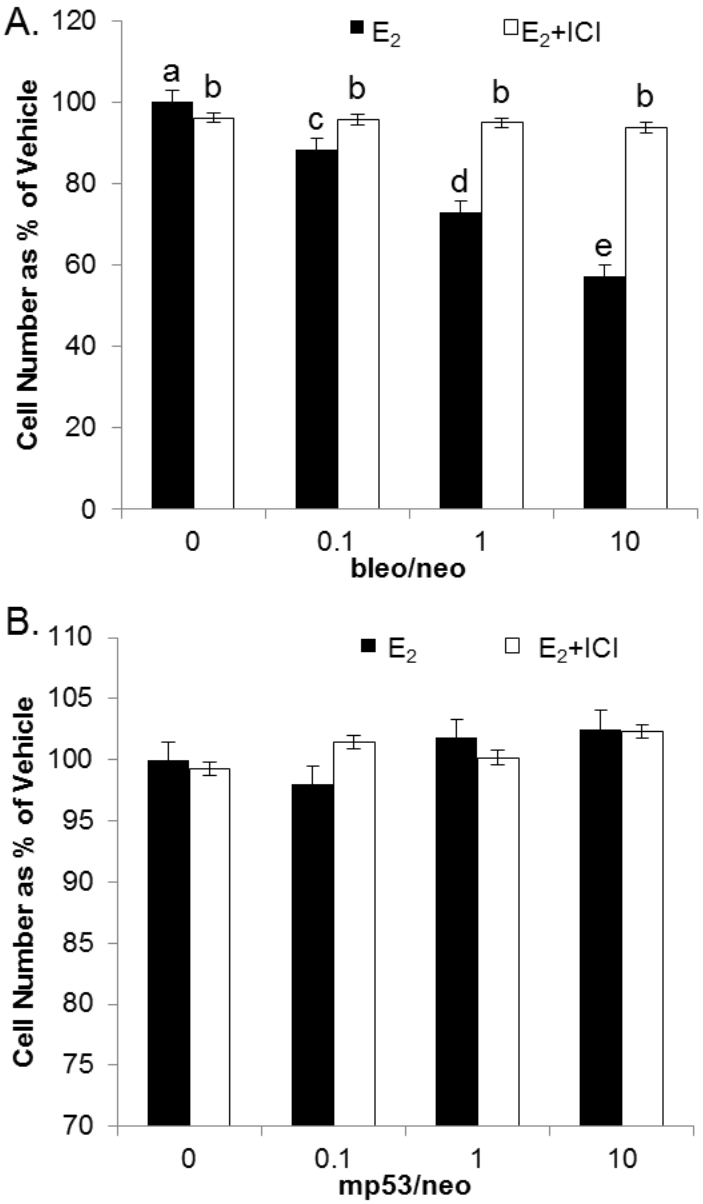

Figure 3.1. E_2 inhibits growth of YAMC cells with a functional p53. *A*,YAMC bleo/neo cells and *B*,YAMC mp53/neo cells were grown at 37°C and in the absence of IFNγ. Cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. 8 h after plating, cells were treated with the listed treatments, media and treatments were replaced 48 h later. After 96 h total treatment time, cells were collected and counted. Data are presented as percentage of vehicle control. Values are means ± SEM, n = 9. Bars without a common letter differ, $P<0.01$.

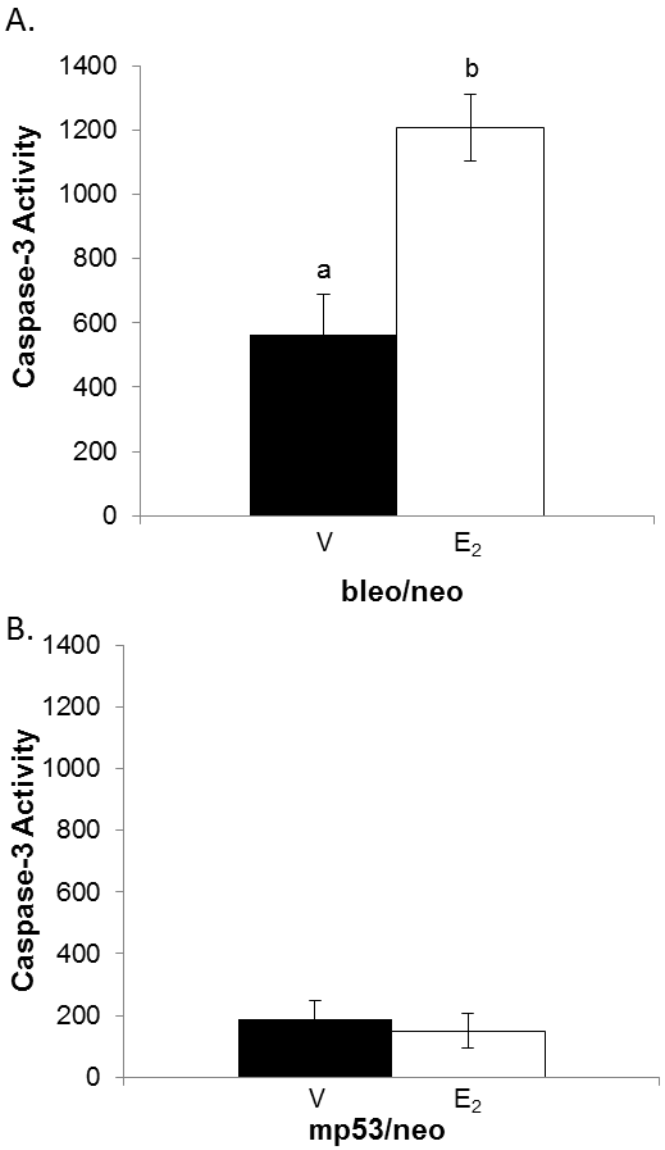

Figure 3.2. Functional p53 is necessary for E_2 stimulated apoptosis. *A*, YAMC bleo/neo and *B*, mp53/neo cells were grown under permissive conditions (33°C and presence of IFNγ) and transferred to charcoal dextran stripped serum media 48 h prior to plating. After 96 h of 1 nM E_2 treatment, cells were collected and the EnzChek Caspase-3 assay was performed. Data are expressed as relative fluorescence. Values are means ± SEM, n=9. Bars without a common letter differ, P <0.001.

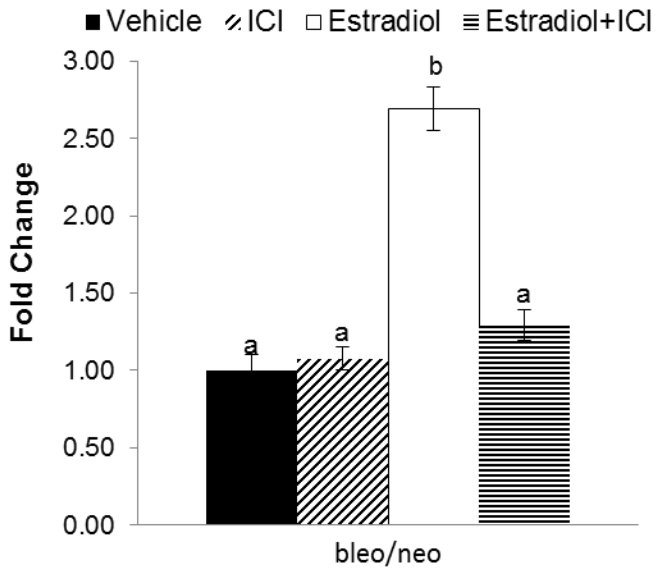

Figure 3.3. E_2 enhances p53 transcriptional activity in YAMC cells. YAMC bleo/neo cells were grown at 37°C and in the absence of IFNγ. Cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. Cells were grown under nonpermissive conditions (37°C and in the absence of IFNγ) for 72 h. After 48 h, cells were transiently transfected with pG13 p53 reporter construct and β-gal construct and treated with vehicle or 1 nM E_2 for the final 24 h. Cell lysates were collected and used to run luciferase and β-galactosidase assays, results were measured on a plate reader. Data are presented as percentage of vehicle control. Values are means ± SEM, n = 9. Bars without a common letter differ, $P<0.01$.

E_2 treatment enhances p53 transcriptional activation. To determine if E_2 treatment alters p53 transcriptional activation, p53 response in the form of a p53-luciferase reporter construct was analyzed. YAMC bleo/neo cells transiently transfected with the PG13-Luc p53 reporter construct and treated with 1 nM E_2 for 24 h exhibited more than a 2.5 fold increase in p53 transcriptional activation compared to cells treated with vehicle alone ($P < 0.01$; Fig. 3.3). As with cell growth, co-treatment with 1 μM ICI completely inhibited E_2 induced increases in p53 transcriptional activation.

E_2 treatment increases p53 protein while decreasing Mdm2. Having observed increased p53 transcriptional activation, p53 protein levels were analyzed. The amount

of p53 protein was measured in cell lysates collected from YAMC bleo/neo cells treated with or without 1 nM E_2 for 0, 4, 8, or 24 h. Cells showed a progressive increase in p53 protein across both treatments, with an overall increase in E_2 treated cells compared to vehicle treatment as time increased, beginning at 4 h, becoming significant at 8 h, with a maximal increase of ~1.5 fold at 24 h ($P < 0.01$; Fig. 3.4C). However, levels of p53 transcript were not significantly different after 24 h exposure ($P < 0.001$; Fig. 3.4A). Murine double minute 2 (Mdm2) was also analyzed because of its regulatory relationship with p53, in which Mdm2 binds to inactive p53 and initiates its export from the nucleus and degradation. The transcript levels for Mdm2 were significantly reduced with E_2 treatment (~55% reduction, Fig. 3.4A) while Mdm2 protein levels were decreased to a lesser degree (~20% reduction, Fig. 3.4B). These combined data suggest post-transcriptional mechanisms are likely involved in elevating p53 protein concentrations.

E_2 treatment selectively alters pro-apoptotic p53 target gene expression. Further investigation of p53 function in E_2 treated, nonmalignant colonocytes was conducted by analyzing changes in gene expression of p53 down-stream targets. RNA from YAMC bleo/neo cells and mp53/neo cells were collected after treatment with or without 1 nM E_2 for 24 h. Levels of the pro-apoptotic genes Bax, phorbol-12-myristate-13-acetate-induced protein (NOXA) and p53-upregulated modulator of apoptosis (PUMA) were significantly increased following E_2 treatment in YAMC bleo/neo cells ($P <0.02$; Fig. 3.5A). The increase in gene expression of Bax was much more pronounced than the changes in other targets. Conversely, the anti-apoptotic gene Bcl-2 transcript was downregulated ($P < 0.001$). There was no significant change in gene expression for the

cell cycle arrest signaling proteins p21 and cyclin dependent kinase inhibitor 1B (p27). When gene expression of these targets were measured under the same treatment conditions in YAMC mp53/neo cells, no change in transcript levels was measured between treatments for Bax, Bcl-2, p21 and p27 ($P < 0.001$; Fig. 5B). However, the gene expression of both NOXA and PUMA were significantly decreased in the E_2 treated cells.

E_2 treatment reduces radiation induced DNA damage. DNA repair was used as a functional measurement of p53 activity in response to E_2 treatment. Repair of irradiation induced double stranded DNA breaks was analyzed by means of γ-H2AX foci quantification. YAMC bleo/neo and mp53/neo cells were treated for 72 h with 1 nM E_2 or vehicle followed by irradiation. Cells were then immunohistochemically stained for γ-H2AX foci and the foci number counted (Fig. 3.6A–D). Irradiated cells had more than 4 times the number of foci (~56 vs. ~13 in vehicle treatments and ~27 vs. ~5 in E_2 treatments) as those seen in non-irradiated controls ($P < 0.05$; Fig. 3.6E). Irradiated cells treated with E_2 had a ~50% reduction in the number of observed foci. Similarly, in non-irradiated cells E_2 significantly decreased the number of foci compared to control ($P < 0.001$). In cells without functional p53, E_2 treatment provided no protection against the

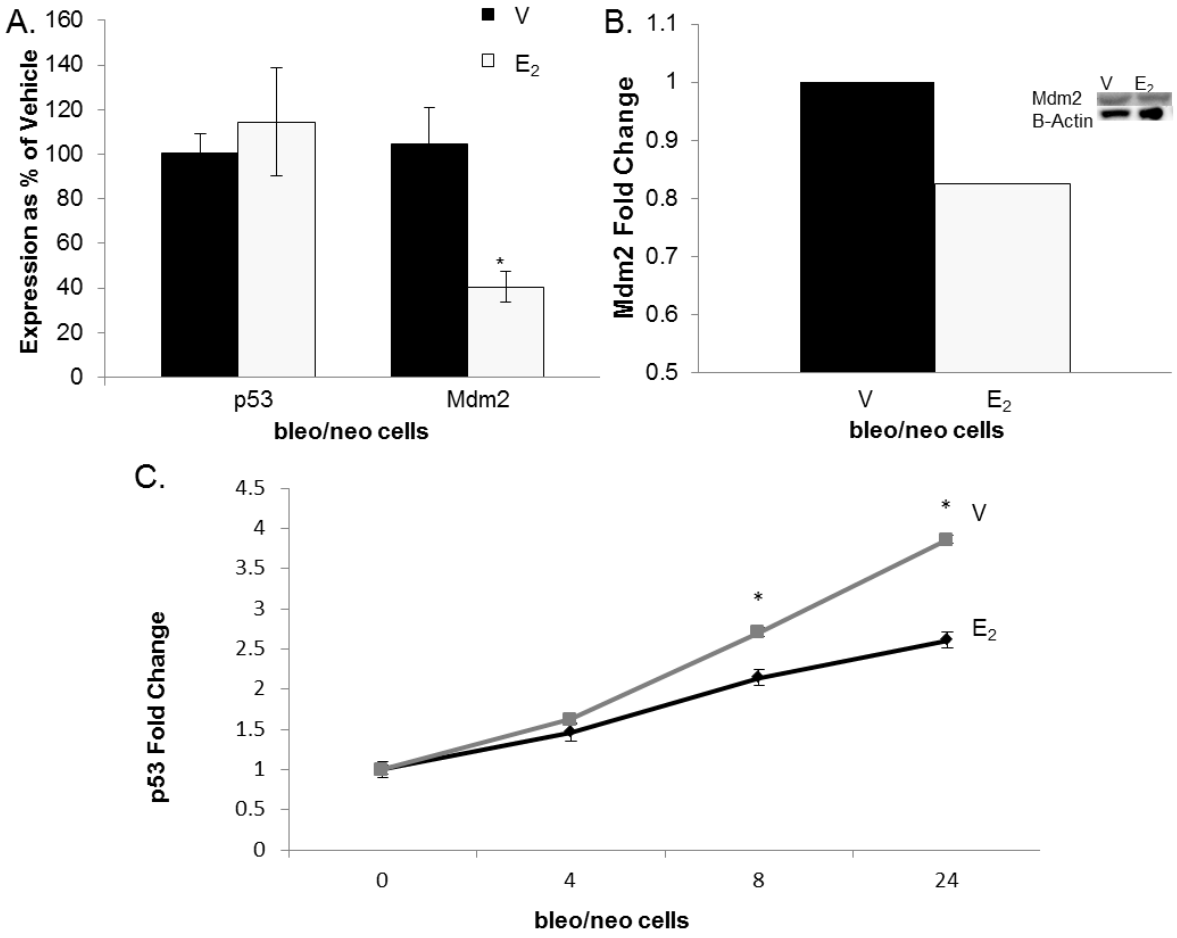

Figure 3.4. E_2 increases p53 protein levels, but not transcript levels. YAMC bleo/neo cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. Cells were grown for 72 h under non-permissive conditions (37°C and in the absence of IFNγ). *A,* Cells were treated with vehicle or 1 nM E_2 for the final 24 h, followed by cell lysis and collection of RNA. Two step RT-PCR was conducted using 1 µg RNA for cDNA synthesis and 2 µL cDNA in a 25 µL SYBR green RT-PCR reaction. Data are presented as percentage expression compared to vehicle control. Values are means ± SEM, n = 9. Asterisk indicates significant difference, $P<0.05$. *B,* Cells were treated with vehicle or 1 nM E_2 for 24 h, followed by cell lysis and collection of proteins. Western blot using total Mdm2 and β-actin antibodies was then conducted. Data are presented as fold change compared to vehicle treated bleo/neo. Values are means ± SEM, n=9. Asterisk indicates significant difference, $P<0.05$. *C,* Cells were treated with vehicle or 1 nM E_2 for the final 0, 4, 8, or 24 h. At the end of the treatment period cells were lysed and proteins collected. Western blot using a total p53 and β-actin antibodies was then conducted. Data are presented as fold change compared to 0 h vehicle control. Values are means ± SEM, n=9. Asterisk indicates significant difference between treatments at that time point, $P<0.015$.

irradiation damage (data not shown), suggesting wild type p53 is necessary to the pathway involved in E_2 mediated DNA repair in this model.

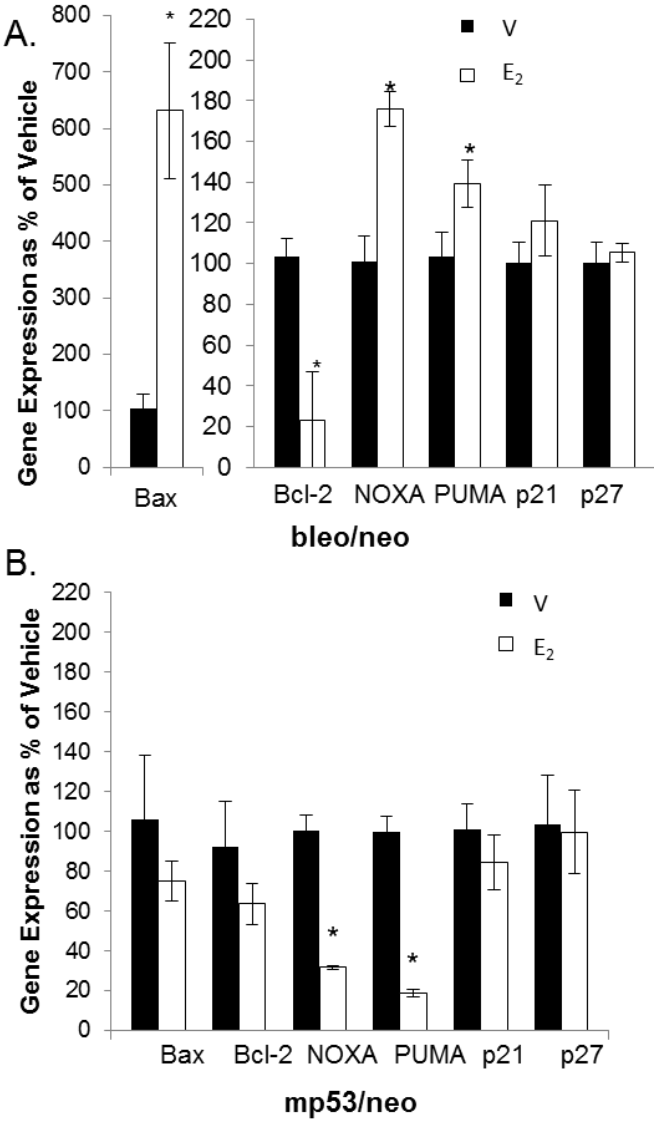

Figure 3.5. E_2 alters expression of p53 downstream targets in a pro-apoptotic manner in YAMC cells with a functional p53. *A*, YAMC bleo/neo and *B*, YAMC mp53/neo cells transferred to charcoal-dextran stripped serum media 48 h prior to plating. Cells were grown under non-permissive conditions (37°C and in the absence of IFNγ) for 72 h, with vehicle or 1 nM E_2 treatment for the final 24 h. At the end of the treatment time, cells were lysed and RNA collected, and 1 µg total RNA was used to make cDNA. SYBR green based RT-PCR was then conducted using 2 µL cDNA for the listed targets. Values are presented as percentage expression compared to vehicle control treatment ± SEM, n=9. Bars with asterisks differ from vehicle treatment, $P<0.02$.

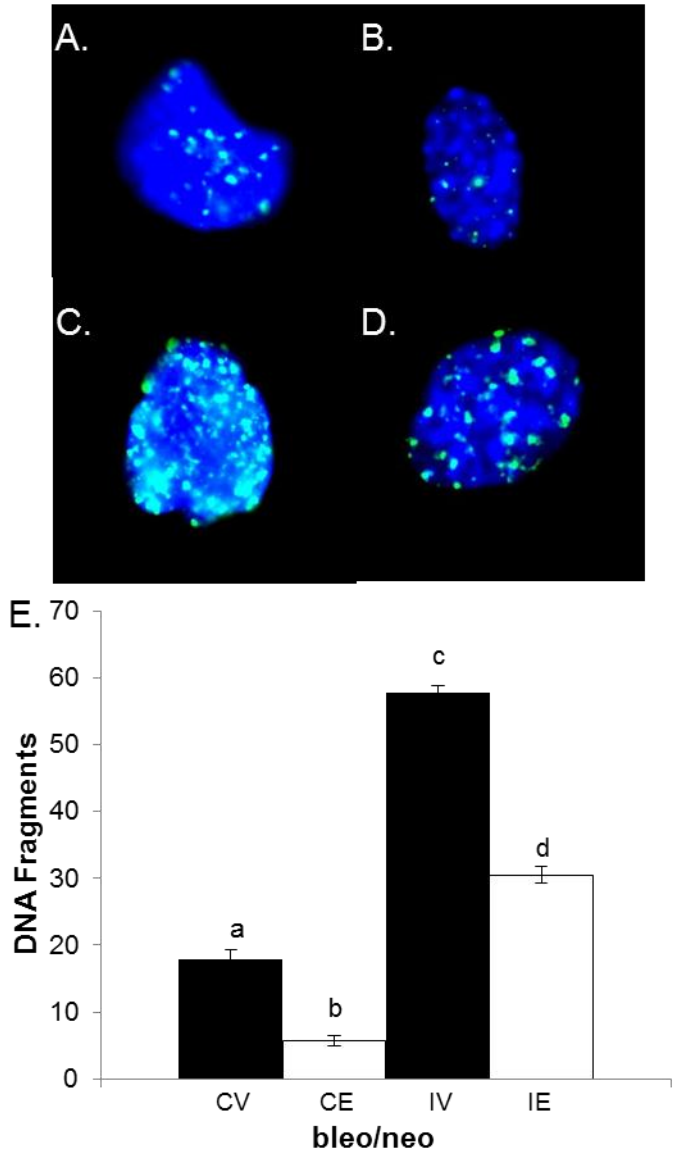

Figure 3.6. E_2 enhances repair of radiation induced DNA damage in YAMC cells with a functional p53. YAMC bleo/neo cells were transferred to charcoal-dextran stripped serum media 48 h prior to plating. Cells were grown on 22 mm×22 mm cover slips in 6-well plates under non-permissive conditions (37°C and in the absence of IFNγ) for 72 h with vehicle or 1 nM E_2 treatment. At the end of the treatment time, cells were irradiated with 2 Gy followed by incubation at 33°C for 1 h. Cells were fixed in 4% PFA and cell membrane perforated with 0.3% Triton X-100 in 10% BSA for 1 h. Cells were then incubated overnight with polyclonal anti-phospho γ-H2AX antibody in 10% BSA at 4°C, followed by incubation with AlexaFluor 488 antibody in 10% BSA for 45 min at room temperature. Cover slips were then treated with ProLong GOLD-DAPI anti-fade solution and mounted on microscope slides. Slides were stored at 4°C and visualized the next day. Representative pictures for each treatment are shown *A*, non-irradiated vehicle (CV), *B*, non-irradiated E_2 (CE), *C*, irradiated vehicle (IV), and *D*, irradiated E_2 (IE). *E*, Values are presented as average number of foci observed in 30 cells per slide ± SEM, n=9. Bars without a common letter differ, $P<0.001$.

Discussion

The p53 protein is a well-studied marker of cancer risk and tumor progression. The loss or mutation of p53 is found in a large percentage of tumors and is often associated with a more severe disease state and poorer prognosis for the patient (150-153).

Many of the p53 point mutations associated with increased tumor risk are believed to be gain of function in nature, thus offering growth advantages in excess of simple loss of normal p53 function (154). The $p53^{175H}$ mutant found in the mp53/neo cells is one of the more common point mutations of p53 found in colon cancer (142). The $p53^{175H}$ mutant is not degraded by Mdm2 like WT p53 allowing it to be the dominant form of the protein in cells carrying this mutant (155). The p53 protein's role in the initiation of apoptotic pathways in response to DNA damage, aberrant growth signals, or oxidative stress are likely responsible for the decreases in cell growth observed in E_2 treated non-malignant colonocytes. Analyses of markers of apoptotic activity shows that YAMC cells require WT p53 activity in order to exhibit E_2 induced apoptosis. We have previously shown that apoptosis is increased in YAMC cells treated with E_2 The introduction of the dominant negative mutant $p53^{175H}$ not only prevents induction of apoptosis by E_2, but significantly down-regulates baseline levels of apoptosis in these cells, highlighting the role of p53 in the regulation of E_2-induced apoptosis. The overall decrease in apoptosis is similar to that found in Hep3B hepatocellular carcinoma cells with the $p53^{175H}$ mutant (156). This suggests that WT p53 is requisite for E_2 induction of apoptosis in colonic epithelia, and further demonstrates that the $p53^{175H}$ mutation alters the ability of p53 to induce apoptosis within these cells.

Likewise, our data suggests that the ER is important in the influence of E_2 on p53 activity. The primary ER in the colon is ERβ, and we have shown that YAMC cells have upwards of 200 fold higher expression of ERβ than ERα (A-3). Our previous data demonstrates that ERβ expression mediates E_2 induced apoptosis in non-malignant colonocytes. Similarly, it was recently shown that over-expression of ERβ in DLD-1 colon adenoma derived cells results in suppression of proliferation when treated with phytoestrogens (157). It is clear that ER subtypes influence p53 in varying ways. ERα activation leads to export of p53 from the nucleus in MCF-7 cells, however, when ERβ is inserted into these cells p53 activation is up-regulated rather than down-regulated (107). The ICI abrogation of the E_2 response in bleo/neo cells, which appears to be p53 mediated, suggests that this is an ER dependent event. More compelling is that ICI significantly suppressed E_2 induction of p53 transcriptional activity. Collectively, these data begin to point to one possible mechanism behind the differences in tumorogenicity seen with E_2 exposure between an ERα dominated system, like the breast, compared to an ERβ dominant system, like the colon. Further study using ER subtype specific agonists and antagonists are ongoing to fully determine the role that ERβ plays in this system.

The p53 protein primarily functions by modifying transcriptional regulation of its target genes, such as those promoting apoptosis. There are p53 response elements within the promoter regions of p53 target genes that the DNA-binding region of the p53 protein recognizes and binds to, in order to regulate transcriptional activation (102). Thus, a p53 response element construct was used in these studies to demonstrate E_2 treatment significantly stimulates the ability of p53 transcriptional activation.

Increased levels of p53 production or increased stabilization of the p53 protein could contribute to this increased p53 transcriptional activation. The lentiviral insertion of ERβ into SW480 colon cancer cells showed increased p53 protein levels with E_2 treatment (68). Our data demonstrates that p53 mRNA levels are not altered inresponse to E_2 treatment, indicating that increased p53 production is unlikely. However, increases in p53 protein levels in E_2 treated cells compared to vehicle treated cells suggest that regulation of p53 is taking place at a post-transcriptional level. The basal level of increase in p53 across time points is likely due to the fact that these cells slowly lose viability in cell culture under non-permissive conditions. The amount of p53 protein present in the cells at any time is primarily controlled by the rate of protein degradation (158). This takes place, in part, through degradation initiated by the ubiquitination of p53. Mdm2 is one of the proteins responsible for this ubiquitination (159, 160). Mdm2 and p53 interact in a regulatory feedback loop in which p53 activates transcription of the Mdm2 gene followed by Mdm2 protein binding p53 and signaling its export from the nucleus and degradation (161). Thus, p53 in the absence of an activating stimulus will fluctuate slightly, but maintain a relatively stable protein level. Our data demonstrates that E_2 treatment in these cells decreases both Mdm2 transcript and protein levels, further indicating the possibility of increased p53 protein stability as a possible cause of increased p53 protein levels. These data underscore the mechanism of interaction between E_2 treatment and Mdm2 transcriptional regulation as methods of p53 protein regulation and warrant further study.

The integral role of p53 on the ability of non-malignant colonocytes to exhibit a physiological response to E_2 treatment is further supported by gene expression changes

in downstream targets of p53. Increased levels of Bax, NOXA, and PUMA are consistent with the previously discussed increases in apoptotic activity, as is the decreased level of Bcl-2, and are indicative of p53 induced apoptosis (100). The Bcl-2 family proteins Bax, Bcl-2, NOXA, and PUMA are directly involved in the mediation of intrinsic mitochondrial signaling of apoptosis (75). Bcl-2 is an anti-apoptotic protein that competes with Bax at the mitochondrial membrane to prevent apoptosis. The balance between Bax and Bcl-2 directly affects mitochondrial membrane permeability, and the resultant release of cytochrome-c and triggering of the caspase apoptotic cascade (82, 162). NOXA and PUMA activate Bax leading to initiation of apoptosis either by direct interaction or indirectly by binding to Bcl-2. The markedly greater increase in Bax:Bcl-2 expression ratio associated with E_2 treatment points to Bax-mediated apoptosis as an important effector pathway of estrogenic chemo-protection in the colon. Decreased Bax:Bcl-2 expression ratio has been associated with tumor progression in AOM induced rat colorectal tumors demonstrating further importance of the Bax:Bcl-2 balance on cancer development (163). Finally, the transcript levels of p21 and p27, both p53 targets which are associated with cell cycle arrest and senescence, did not change suggesting that E_2-induced p53 activity enhanced apoptosis over senescence. Hartman et al. demonstrated that p21 and p27 protein levels increase in SW480 and HT-29 cells stably expressing exogenous ERβ, however, these cells contain mutant p53 and it is suggested that this is likely responsible for increases in these proteins as the mutated p53 is believed to have lost pro-apoptotic ability (68). They also demonstrate reduction of p21 protein levels in HCT-116 colon carcinoma cells expressing exogenous ERβ and treated with E_2. In the current study, the majority of the observed changes in gene expression are

lost in cells containing the p53 dominant negative mutant. However, increases in NOXA and PUMA expression are not only lost, but are down-regulated in YAMC mp53/neo cells when treated with E_2. This finding might suggest that E_2 can initiate non-p53 dependent negative regulation of these genes, or that E_2 treatment can affect the mutant p53 in a manner other than as a pro-apoptotic stimulus.

Increases in repair of DNA double stranded breaks were also observed illustrating that increased p53 activity in this model is not limited to induction of apoptosis alone. P53 plays a major role in the cellular response to DNA damage and other cellular stresses (164). Edvardsson et al. demonstrated that E_2 treatment in SW480 cells transfected with an ERβ expression vector increased expression of p53 targets and protected against cell death associated with DNA cross-linkage caused by cisplatin treatment in SW403 cells expressing exogenous ERβ (165). They also found that double-stranded DNA repair protein, Rad51-like 3 (S. cervisiae; RAD51L3), and ribonucleoside-diphosphate reductase subunit M2 B (RRM2B), a p53 target involved in DNA repair, were up-regulated. Conversely, in MCF-7 cells, E_2 activation of ERα inhibits ataxia telangiectasia and RAD3 related (ATR) phosphorylation of p53 in response to DNA damage (166). The γ-H2AX foci, also known as irradiation induce foci (IRIF), measured in the presented study, identify a specific modification to histone H2A associated with DNA double stranded breaks (167). The p53 induced gene 3 (PIG3) protein plays an important role in the formation of the γ-H2AX localized MRN repair complex (“fast repair”) containing meiotic recombination homolog 11(Mre11), DNA repair protein Rad50 (S. cerevisiae) homolog (RAD50) and Nibrin (NBS1) (168). Furthermore, p53 binding protein 1 (53bp1) recruitment to γ-H2AX foci is enhanced by

PIG3 and the absence of p53 greatly reduces PIG3 levels. As such, increased levels of p53 protein and activity, as seen with E_2 treatment in non-malignant colonocytes, may induce DNA repair in the colon. Also of importance, apoptosis itself leads to γ-H2AX foci formation. However, we did not see significant increases in γ-H2AX foci formation even though E_2 induces apoptosis in these cells. The baseline levels of apoptosis in non-malignant colonocytes are low, so the doubling in apoptotic activity we demonstrate would not lead to a large increase in observable foci. The reduction of these foci in irradiated cells treated with 1 nM E_2 suggests that E_2 can induce p53-regulated cellular responses in colonocytes other than apoptosis. Decreases in γ-H2AX foci in non-irradiated cells treated with E_2 suggest that the mechanism(s) involved in E_2 protection/repair of radiation induced DNA damage may be active under normal conditions and significantly up-regulated in response to exposure to radiation, providing the cell with additional protection against DNA damage. This demonstrates another potential role for E_2 in this system. However, these results may be specific radiation-associated DNA damage and future studies will look at other sources of insult to determine this.

In conclusion, the cellular response of non-malignant colonocytes to E_2 treatment relies on the presence of functional p53 protein. Observed phenotypes in cell number changes and induction of apoptosis are lost with the addition of the dominant negative $p53^{175H}$ point mutation. Induction of p53 activity can be observed in response to E_2 exposure along with measurable changes in gene expression levels of p53 downstream targets and stabilization of the p53 protein itself. Finally, E_2-induced repair of DNA damage indicates a potentially broader role of E_2 activity beyond the signaling of

programmed cell death due to cellular stress. These findings, when taken together, affirm a role for p53 in E_2 related chemo-protection against colon carcinogenesis.

CHAPTER IV

FUNCTIONAL P53 IS REQUIRED FOR ESTRADIOL INDUCED PROTECTION AGAINST COLON CANCER DEVELOPMENT

Colon cancer is a significant health concern worldwide. Statistics from 2008 showed that in developed areas men have an ASR (age-standardized rate per 100,000) of 37.6 for development of colon cancer by age 75 (third highest among cancer), while the women ASR in women is only 24.2 (second highest among cancer) (2). Mortality rates in colon cancer are also among the highest with a mortality ASR of 15.1 in men (second highest among cancers) and 9.7 in women (third highest among cancers) in 2008. In total this accounts for approximately 10% of all new cancer cases and nearly 10% of cancer related deaths worldwide (2).

Colon cancer incidence and mortality rates are lower in women than men, suggesting a possible gender based decreased susceptibility to this type of cancer. Data from the Women's Health Initiative further suggest that postmenopausal hormone replacement therapy (HRT) can further decrease colon cancer risk in women (31). Results from additional clinical studies using both HRT and estrogen replacement therapy (ERT) show similar protection (27, 35, 36, 112). These results have been corroborated in animal studies allowing for greater control of variables and interventions used. Estradiol (E_2) treatment in OVX rats suppressed dimethylhydrazine-induced colon tumor development (69).

A physiological protection against colon cancer development appears to be evident, however the mechanism and pathways involved in this protection have not been

thoroughly elucidated. Oral estrone administration in OVX wild-type (WT) and estrogen receptor (ER) α knockout (KO) mice inhibited the formation of azoxymethane (AOM) induced colon tumors (70). Our laboratory has demonstrated that E_2 treatment suppression of AOM-induced preneoplastic lesions is reduced in ERβKO mice compared to WT (138). These data strongly suggest that ERβ is the receptor primarily responsible for facilitating the functions of E_2 in the colon.

Increased apoptosis in colonocytes has previously been observed as an outcome resulting from E_2 treatement (125, 129, 139, 169). With induction of apoptosis as a primary outcome, it is likely that p53 plays an important role in E_2 mediated protection. The p53 protein is a well characterized tumor suppressor.

The p53 tumor suppressor protein plays a critical role in the cellular maintenance of genome integrity. Individuals with Li-Fraumeni syndrome, a hereditary disease characterized by loss of one functional copy of the *Trp53* gene, suffer from significantly increased cancer risk (170). Approximately 50% of all cancers carry a p53 mutation, while a similar number carry a mutation in one or more genes in p53 pathways (89, 91). Because p53 plays such a vital role in the maintenance of proper cell cycle and DNA replication control, it is often referred to as the "guardian of the genome" (88).

E_2 treatment has been shown to enhance p53 function in many cell types. Breast (MCF-7) and endometrial (HHUA) cancer cells have exhibited increased p53 activity when ERβ is present (107, 109). We have also demonstrated increased p53 activity resultant from E_2 treatment in non-malignant colonocytes *in vitro* (169). These are indications that p53 activity may be involved in E_2 mediated protection against colon cancer. The data presented herein further examine the role p53 plays *in vivo* in E_2

induced suppression of colon carcinogenesis at the level of premalignant lesion formation.

Materials and Methods

Mice. Heterozygous p53KO (+/−) mice on a C57BL6-Tyr$^{c\text{-}Brd}$ background mice were bred from a colony of animals originating from Baylor University. The creation and characterization of this mouse is described by Zheng et al. (171). Mice were bred and housed at the Laboratory Animal Resources and Research facility at Texas A&M University. Het p53KO and WT mice were genotyped based on coat color after verification of function by PCR of genomic tail DNA (Appendix B-14). All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Texas A&M University.

p53. Tissues were collected and fixed as above. Prepared sections were then stained for the presence of p53. Briefly, slides were deparaffinized and rehydrated by immersing in xylene twice for 12 min and immersing in a series of descending EtOH concentrations dissolved in dH_2O (100, 100, 95, 70 and 0%) for 5 min each. To block endogenous peroxidase, slides were immersed in 3% H_2O_2 in methanol for 30 min then washed with distilled water. Slides were then microwaved in a Pyrex dish in citrate buffer (pH 6) 20 min and allowed to cool to RT. Slides were then washed twice in PBS for 5 min and tissue sections were ringed with a PAP pen. 11 µL of p53 polyclonal antibody (905-510, Roche) was added to slides and incubated for O/N at 4°C in a humidified chamber. Slides were washed with dH_2O and 11 µl of goat anti-rabbit IgG (Assay Designs) was added to slides and incubated for 1h at RT. Slides were then

washed in PBS. 15 μL of freshly prepared 3,3′-diaminobenzidine (DAB) solution was added to each section and incubated for 10 min at RT. Slides were then washed in dH2O, and counterstained with 0.5% methyl green for ~45 s. The slides were then washed thrice in dH_2O and dehydrated by placing them in increasing EtOH concentrations (70%, 95%, 100%) for 5 min followed by xylene three times for 5 min each. Slides were then wet mounted with Permount and photographed by light microscope. p53 staining intensity was determined by using the program CellProfiler and the HKCellCounter pipeline (172).

Carcinogen treatment and aberrant crypt foci analysis. Female mice between the ages of were ovariectomized, and either 20 mg cholesterol (Sigma-Aldrich) or 19 mg cholesterol/1 mg E_2 pellet was implanted s.c. on the back at the base of the neck at the time of ovariectomy. Pellets were prepared as described in ref. (119). Pellets were replaced 8 wks later. Mice were transferred to a phytoestrogen-free diet at the time of surgery and allowed food and water ad libitum. Two weeks after surgery, mice received the first of six weekly injections of AOM (Sigma-Aldrich) at 10 mg/kg body weight. Eight weeks post-AOM, animals were killed. Blood was collected through cardiac puncture. The colon was resected and 1-cm sections from the distal end were cassetted and fixed in 4% paraformaldehyde (Mallinckrodt Baker, Inc.). The remainder of the colon was flattened between sheets of filter paper and fixed in 70% ethanol. Ethanol-fixed colons were stained with 0.5% methylene blue (Sigma-Aldrich) and aberrant crypt foci (ACF) were counted as previously described (120, 121).

Plasma E_2. Plasma E_2 concentrations were determined using an Estradiol EIA kit (Cayman). Fifty microliters of plasma/sample were added to sample wells, and samples

from E_2-treated animals were diluted 1:10. Fifty microliters of Tracer and antiserum were added per sample well and the plate was incubated for 1 h. The wells were washed five times with wash buffer. Ellman's Reagent (200 μL) was added per well and incubated in the dark for 60 to 90 min while shaking. Wells were read on a plate reader at 415 nm.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed using the ApopTag Plus Peroxidase In situ Apoptosis Detection kit (Millipore) according to manufacturer's instructions with slight modifications. Briefly, distal colon sections were fixed in 4% paraformaldehyde overnight, paraffin embedded, sectioned, and mounted. Tissues were deparaffinized in three washes of xylene and rehydrated. Slides were quenched with hydrogen peroxide for 8 min and counterstained in 0.5% methyl green solution for ~3 min.

BrdU. Two hours prior to killing the animals, each mouse was injected intraperitoneally with 30 mg BrdU/kg body weight. Immunohistochemical staining for BrdU protein followed the above procedure with the following alterations. Antigen retrieval was performed before rehydration of tissues. Twenty individual crypts were analyzed by counting cells on the left side of the crypt. The data were then presented as percentage of cells proliferating per crypt.

Statistical analysis. All data are presented as mean ± SEM. Experiments were conducted in triplicate and repeated three times. Data for apoptosis, proliferation, and p53 intensity stains were transformed to percentage of WT control. Analysis for all data

was determined using one-way ANOVA with Tukey's post hoc test or a student t-test. Differences were considered significant if $P<0.05$.

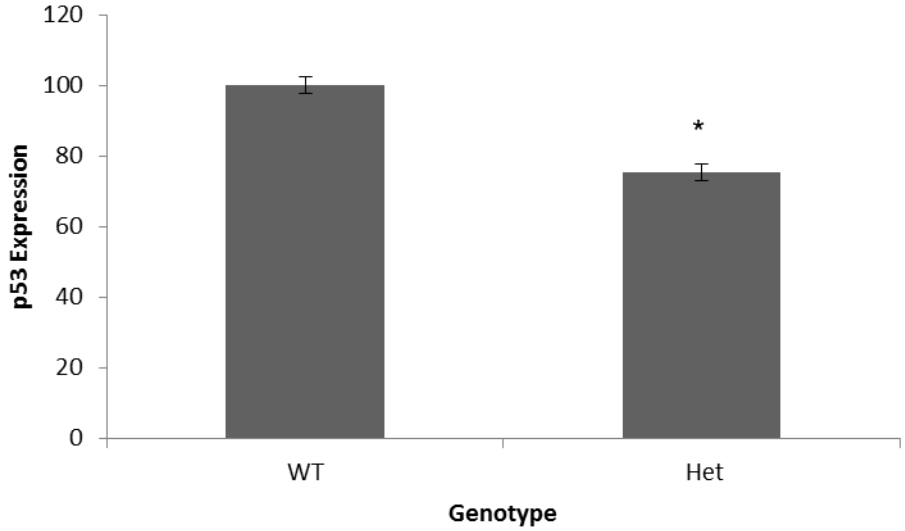

Figure 4.1. p53 expression in the colonic crypts of WT and Het p53KO animals. Columns, mean (n=10 animals analyzed per genotype); bars, SEM. Asterisks indicate significant difference from WTC, P<0.001.

Results

p53 expression within the colonic crypts. We began by verifying that the p53 expression levels differ between WT and p53KO animals. IHC measurement of p53 within the colonic crypts showed that Het p53KO animals expressed ~25% less p53 than WT animals (P<0.001; Fig. 4.1).

Formation of ACFs. Next, we examined ACF formation in WT and Het p53KO mice. ACFs were only observed in the distal two thirds of the colon, with greater frequency at the distal end. The highest frequency was observed Het p53KO animals

treated with vehicle control (34.3 ACFs per animal), while the Het p53KO animals receiving E_2 exhibited an average of 25.9 ACFs per animal ($P<0.01$; Fig. 4.2A). WT animals showed a stronger E_2 treatment effect (31.6 ACFs per animal in the vehicle control group reduced to 16.8 ACFs per animal treated with E_2; $P<0.001$).

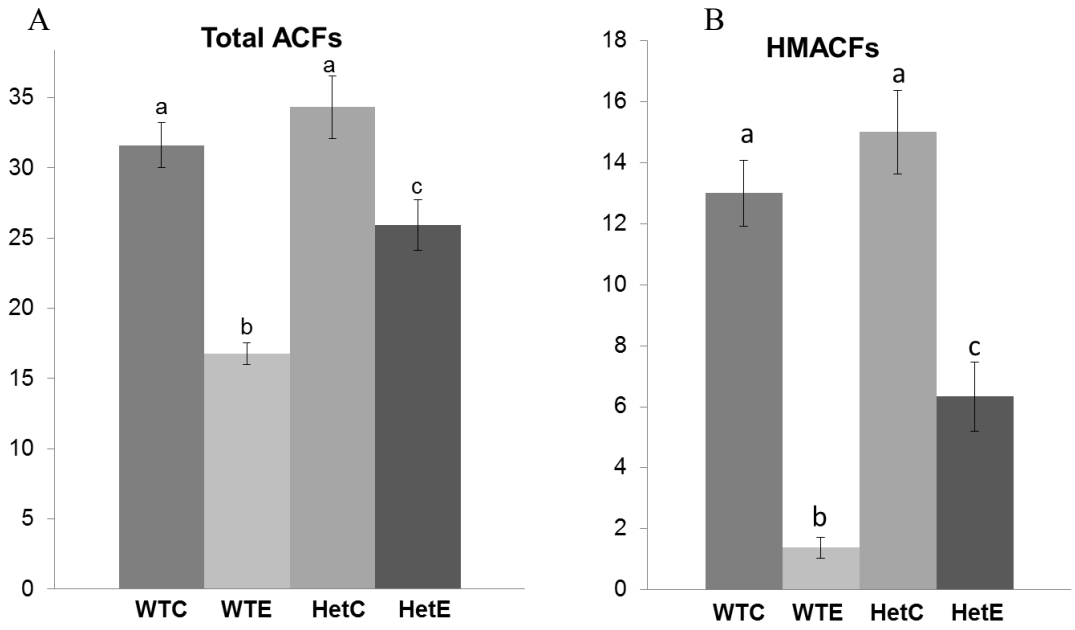

Figure 4.2. ACFs in the distal colon. A, total ACFs identified per animal. B, HM-ACFs identified per animal. Columns, mean (n=12-16 animals analyzed per treatment group); bars, SEM. Letters indicate significant difference from WTC, $P<0.01$.

Differences were more pronounced in the high multiplicity ACF (HM-ACF) subcategory. WT animals in the vehicle control treatment group averaged 13 HM-ACFs with a reduction to 1.4 HM-ACFs ($P<0.001$; Fig. 4.2B) per animal in the E_2 treatment group. The E_2 treatment effect was again diminished in the Het p53KO animals with an average of 15 HM-ACFs in the vehicle control animals dropping to 6.3 HM-ACFs ($P<0.001$) with E_2 treatment. The combinatory effect of these data suggest that partial

loss of WT p53 negatively impacts the ability of E_2 treament to reduce the risk of preneoplastic lesion formation in the AOM-induced model of carcinogenesis.

***Plasma E_2 concentrations*.** Plasma E_2 levels were measured to verify efficacy of OVX and implant function. Mice receiving E_2 implants had average plasma E_2 levels of 1.91 nM (WT) and 1.94 nM (Het), while those receiving implants with cholesterol alone had plasma E_2 levels of 0.05 nM ($P<0.001$; Fig. 4.3).

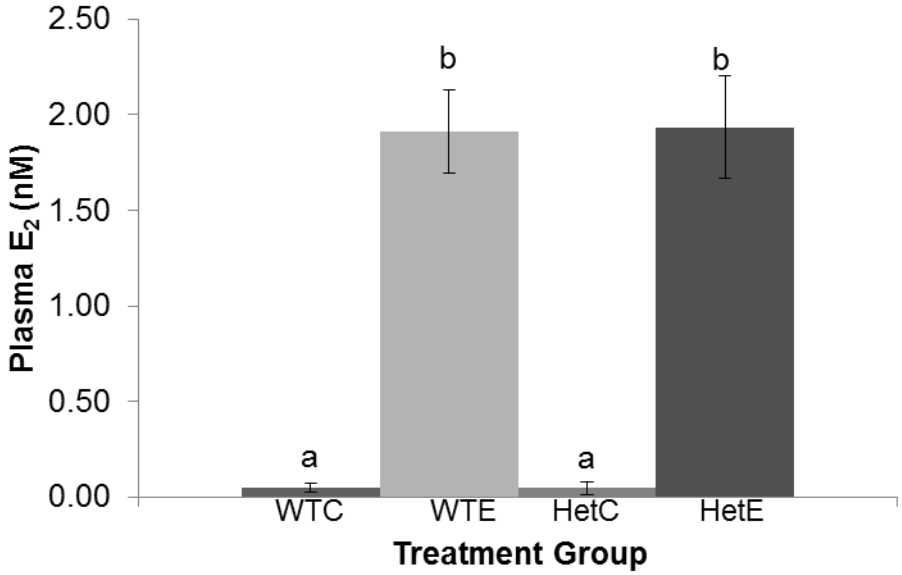

Figure 4.3. Plasma E_2 levels in carcinogen-treated animals. *Columns*, mean (n=12-16 animals analyzed per treatment group); *bars*, SEM. Letters indicate significant difference from WTC, $P<0.0001$.

***Apoptosis within the colonic crypts*.** Having previously observed increased apoptosis accompanying protection against ACF formation (139), we again analyzed apoptosis within the crypts. Apoptosis in WT animals increased by 45% in E_2 treated animals compared to vehicle control ($P<0.001$; Fig. 4.4). Apoptotic activity was reduced

in Het p53KO animals, with vehicle control treated animals exhibiting 25% less apoptosis than WTC ($P<0.001$, Fig. 4.4) and E_2 treated animals with equal apoptotic activity to WTC.

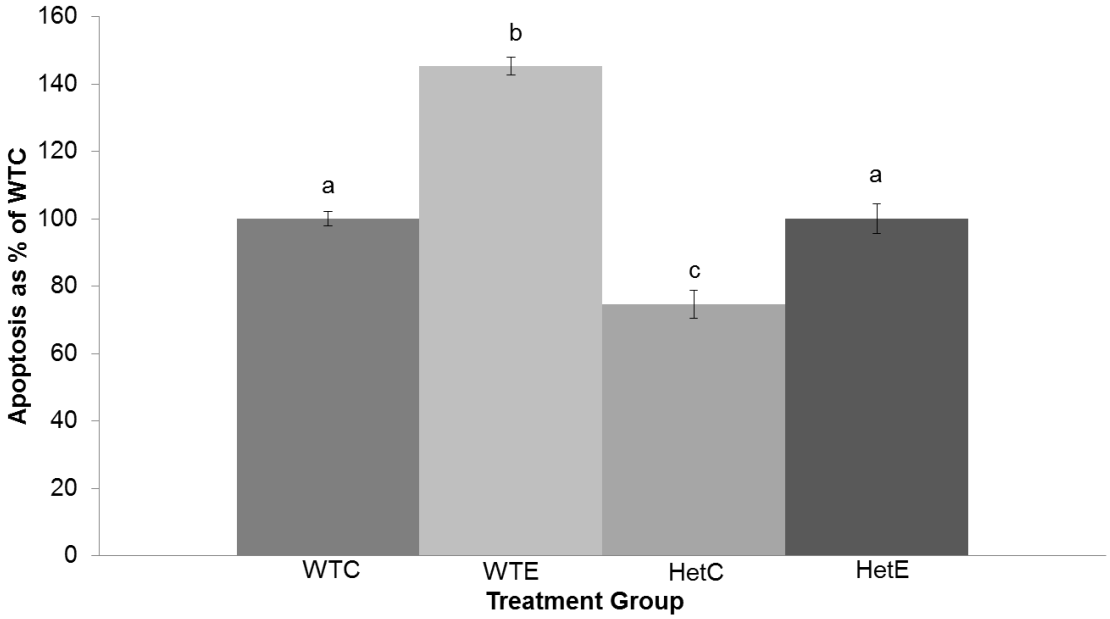

Figure 4.4. Apoptosis within the colonic crypts of the distal colon. A TUNEL assay was performed on sectioned tissue from the distal colon. Data are expressed as percentage of apoptotic cells found compared with apoptotic cells found in the WTC crypt. *Columns*, mean (n=12-16 animals analyzed per treatment group); *bars*, SEM. Letters indicate significant difference from WTC, $P<0.001$.

Proliferation within the colonic crypts. Since ACFs are premalignant lesions resulting from dysregulation of cell proliferation within the crypts, we analyzed colonocyte proliferation. A proliferation index was calculated by measuring the average percentage of proliferative cells within the crypts. WT animals in the vehicle control treatment averaged 8.4% proliferative cells per crypt. WT E_2 treated animals exhibited decreased proliferation (4.9% per crypt, $P<0.005$; Fig. 4.5). Proliferation increased in

Het p53KO animals. Animals in the vehicle control group averaged 12.6% proliferative cells per crypt, dropping to 6.7% in the E_2 treatment group ($P<0.005$).

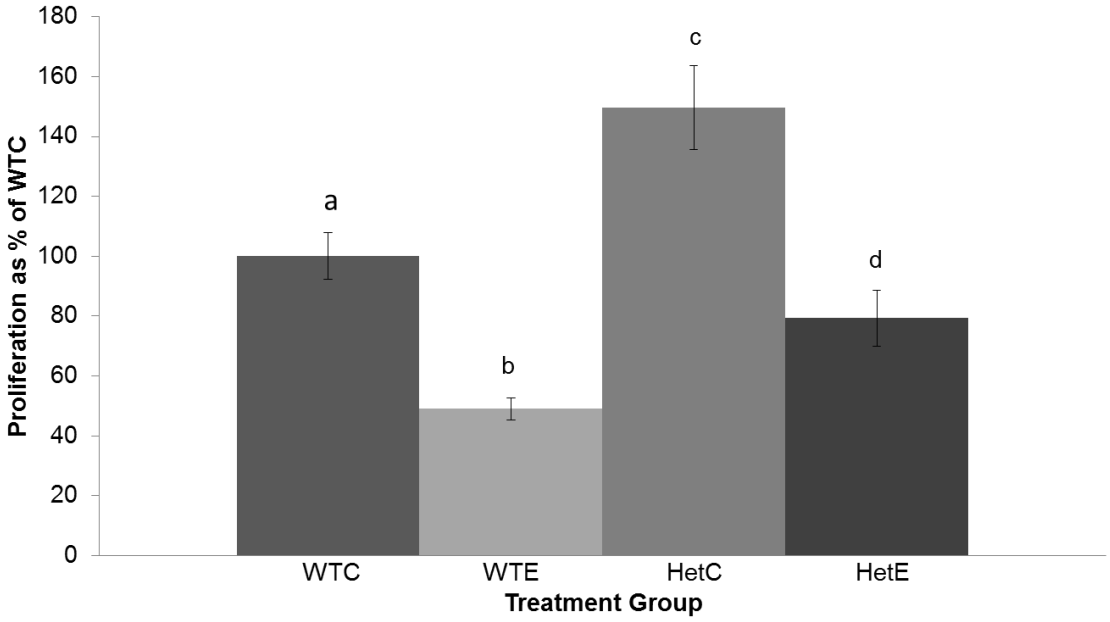

Figure 4.5. Proliferation within the colonic crypts measured by BrdU incorporation. Sectioned tissue from the distal colon was immunohistochemically stained for the presence of BrdU. Data are expressed as proportion of proliferative cells found compared with proliferative cells found in the WTC crypt. *Columns*, mean (n=12-16 animals analyzed per treatment group); *bars*, SEM. Letters indicate significant difference, $P<0.005$.

Discussion

Loss of p53 function is often associated with tumor progression and a more severe disease state (152). As p53 function is lost, the cell's ability to respond to cellular stresses like DNA damage is diminished. The decreased p53 protein levels in Het p53KO mice increase their susceptibility to carcinogenesis in a similar manner.

Having demonstrated that p53 expression levels were dependent upon genotype, we analyzed ACF formation. As previously observed (139), there were significant

reductions in total ACFs in WT E_2 treated animals compared to mice in the WT vehicle control group. Again we determined that this effect was even more pronounced when reduced to HM-ACFs, which are more a more advanced stage of these lesions and more predictive of potential tumor formation (130). Interestingly, we also observed masses in the distal colon in WT animals in the vehicle control group. Though these masses were not histologically typed, they were clearly more advanced lesions than ACFs.

After verification that the model was operating as previously observed, we compared ACF formation in Het p53KO animals to that in WT. Het p53KO animals exhibited an increased number of ACFs when compared to WT in each treatment group. While a reduction in ACFs was present in Het p53KO mice compared to vehicle control animals, the level of protection was diminished. Interestingly, E_2 treatment in Het p53KO mice exhibited a noticeably weaker protective effect than that seen in total ACFs, suggesting oncogenic p53 mutations are more prominent at this more advanced stage of carcinogenesis. Again, we observed the presence of masses in the distal colon of the Het p53KO mice, however they were present in greater numbers in the vehicle control group as well as being present in half of the E_2 treated animals. This more pronounced loss of E_2 protection in larger lesions, correlates to data suggesting that loss of normal p53 function is most strongly associated with progression in colon carcinogenesis (173).

Plasma E_2 levels in E_2 treated mice were significantly greater than those in vehicle control, with comparative levels resembling peak cycle (E_2 treatment) compared to levels similar to those seen post-menopause (vehicle control; 29, 30, 137).

Characterization of apoptosis within the colonic crypts was chosen as a functional outcome for measurement of the decreased tumor susceptibility. There was a significant induction of apoptosis within the crypts of E_2 treated WT animals compared to vehicle control animals. E_2 treatment in these cells heightens apoptosis in response to DNA damage. We have previously reported increased apoptotic activity in WT mice with E_2 treatment (139). Apoptotic activity was diminished in Het p53KO animals in both treatment groups. An E_2 induced increase in apoptosis was still present, but the magnitude of the increase was not as pronounced as that seen in WT animals. This corresponds with previously reported data where we demonstrated that functional p53 activity was requisite for E_2 induced apoptosis in non-malignant colonocytes (169). The overall reduction in apoptosis observed in Het p53KO mice could be due to changes expression of a number of p53 downstream targets. Based on data previously reported by our lab (169), combined changes in expression levels of Bax, Bcl-2, NOXA, and PUMA are likely to be involved. However, due to the relatively small difference in p53 levels observed between genotypes, these questions will be more likely to be answered successfully in a tissue specific p53KO model.

To further characterize the physiological changes taking place between treatment groups, we also measured proliferation. Proliferation rates were significantly altered with E_2 treatment in both genotypes, though proliferation was higher in Het p53KO animals compared with WT. Though proliferation rates differed between genotypes the comparative change resulting from E_2 treatment was similar in each genotype. Reduced proliferation in colon cancer cells with E_2 treatment has previously been reported (124). However, previous data collected in our laboratory, showed no such change in

proliferation rate in vitro when WT p53 function is lost (174). These data suggest that p53 is not directly involved in this aspect of the effect seen with E_2 treatment, but further analysis of this effect in a full p53KO will help determine if this is a property associated with loss of p53 in this model of carcinogenesis.

In conclusion, it is clear that p53 status affects the ability of E_2 treatment to suppress colon carcinogenesis. Increased ACF formation corresponded with apoptosis decreases in Het p53KO animals treated with E_2 over that seen in WT animals. These data further suggest that p53 is an intricate and necessary member of the pathway involved in E_2 mediated inhibition of colon carcinogenesis, further investigation of the role of p53 in these physiological responses to E_2 is warranted.

CHAPTER V

SUMMARY AND CONCLUSIONS

Estrogen receptors have long been recognized as having significant impact on health and disease. Reduced estrogen levels associated with menopause have been shown to influence risk of osteoporosis, heart disease, stroke, and numerous cancers. Estrogen replacement has been shown to reduce the risk of colon cancer in postmenopausal women, but due to harmful side effects like increased breast cancer risk this treatment is not normally recommended. Therefore, an improved understanding of the mechanisms involved with the estrogen related reduction in colon cancer risk, could lead to alternative treatments with reduced harmful side effects.

A growing body of data suggests that estrogen levels affect the process of colon cancer development, but the majority of research conducted relates to the activity and function of ERα. However, ERβ rather than ERα is the primary form of the receptor present in the colon (53). The majority of data from animal experiments demonstrate a clear reduction of colon cancer risk in mice receiving estrogen (69-71). Data also suggest that ERβ rather than ERα is responsible for mediating this protection (70). The majority of *in vitro* data do not show any reduction in proliferation or increase in anti-proliferative processes, but these studies were conducted in cancer derived sell lines (65-67). To extend our understanding of the mechanisms involved, we used a non-malignant colonocyte cell line (YAMC) to demonstrate that E_2 treatment in non-cancerous cells enhances cellular apoptosis and

reduces cell culture growth rate (Fig. 2.1 and 2.3). The presence of ERβ and lack of ERα in these cells, along with abrogation of the E_2 treatment effect with treatment of the ER inhibitor ICI, further show that this effect is ERβ mediated (Fig. 2.2B). An animal study using ERβKO mice, corroborated the findings of increased colonic apoptosis resulting from E_2 treatment (Fig, 2.6). Furthermore, this study also demonstrated that functional ERβ was requisite for E_2 induced reduction in pre-malignant lesion formation (Fig. 2.4).

The tumor suppressor p53 plays a significant role in the induction of apoptosis and response to DNA damage (78-83). To further elucidate the mechanisms involved with E_2 protection against tumor development, isogenic YAMC cell lines were used to analyze the role p53 plays in this process. Reduction in cell culture growth was shown to be lost in E_2 treated cells with mutant p53 (Fig. 3.1). Likewise, enhanced apoptosis was lost with E_2 treatment (Fig. 3.2). A p53 reporter construct was used to assess p53 transcriptional activity in YAMC cells with WT p53, showing that E2 treatment significantly enhanced transcriptional activation (Fig. 3.3). p53 protein levels were shown to increase over time with E_2 treatment, while Mdm2 protein levels were reduced after 24 hours treatment (Fig. 3.4). Expression of p53 did not change, but levels of its downstream targets: Bax, Bcl-2, Mdm2, NOXA, and PUMA were altered in a pro-apoptotic manner (Fig. 3.4A and 3.5). This effect was lost in cells with mutant p53.

To further understand how p53 status impacts the ability of E_2 to reduce colon cancer risk, an animal study using Het p53KO mice and WT littermates was conducted. ACF formation was reduced in both genotypes with E_2 treatment, but the

level of reduction was lower in Het p53KO mice (Fig. 4.2A). This reduced E_2 treatment effect in Het p53KO mice was more pronounced when ACF analysis was confined to HM-ACFs (Fig. 4.2B). Furthermore, masses were routinely observed in control treatment animals of both genotypes as well as E_2 treated Het p53KO animals, but not E_2 treated WT mice (Fig. 4.2C&D). Apoptosis within the colonic crypts was reduced with E_2 treatment in both genotypes, but again the effect was smaller in Het p53KO animals (Fig. 4.4). Surprisingly, proliferation was reduced in with E_2 treatment, an effect not previously suggested due to lack of significant change in cell cycle arrest or proliferation observed *in vitro* (Fig. 4.5). Further study involving complete p53KO animals is warranted for further analysis of the effects of E_2 treatment on p53 downstream targets.

In summary, E_2 treatment significantly enhances non-proliferative activity in non-malignant colonocytes and inhibits colon cancer development *in vivo*. We propose that these effects are resultant of a mechanism involving E_2 activation of ERβ leading to activation of p53 resulting in anti-proliferative downstream stream signaling, such as induction of apoptosis.

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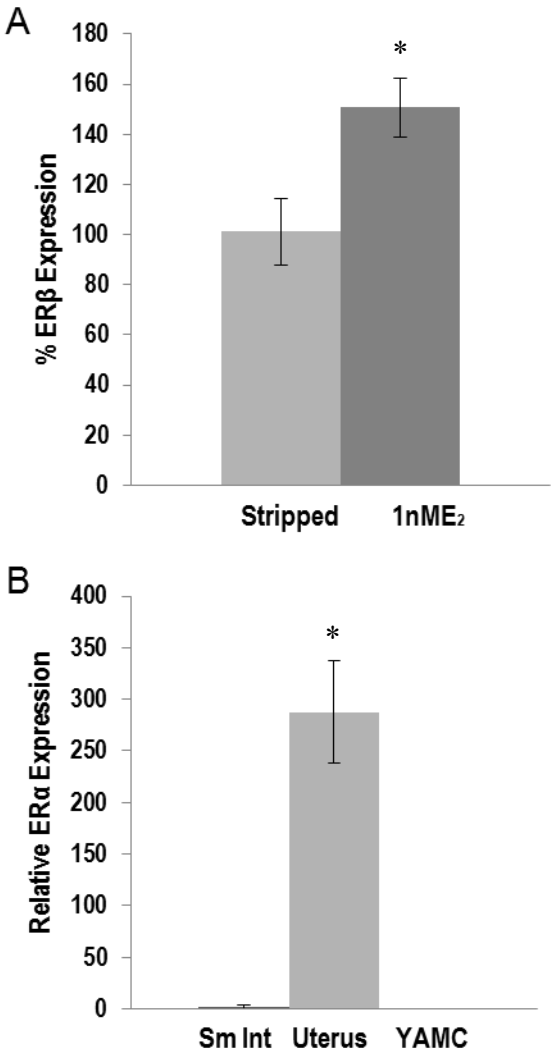
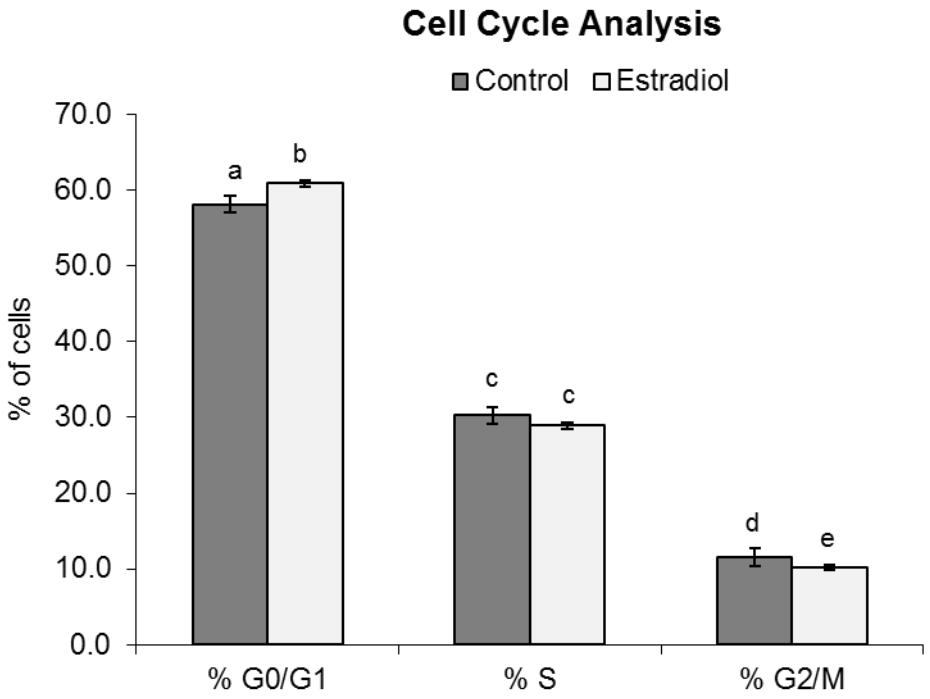
APPENDIX A

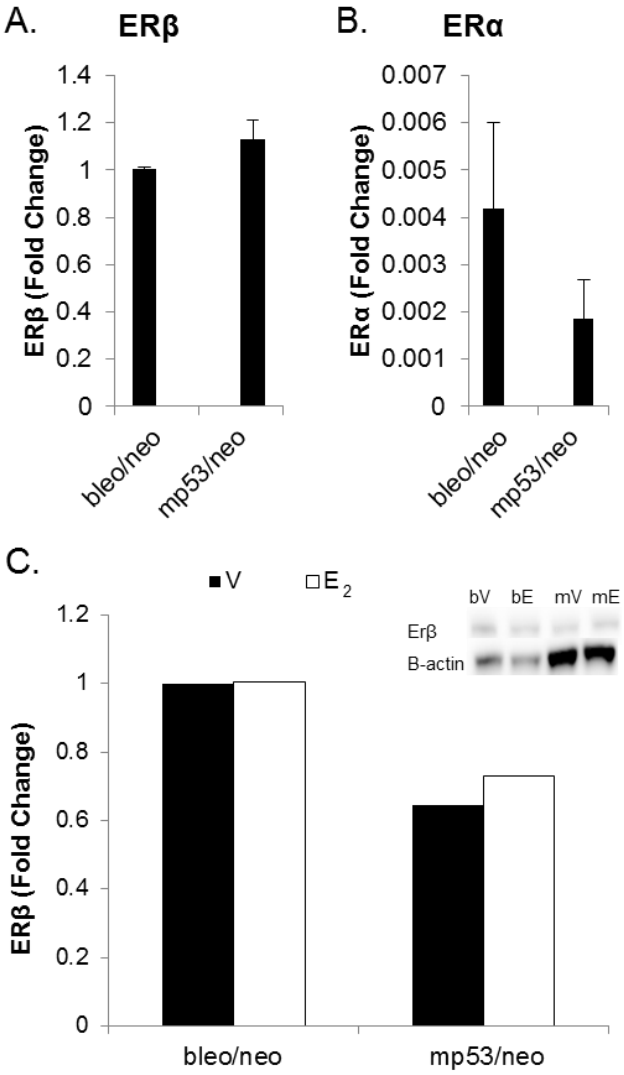
SUPPLEMENTAL DATA



A-1: **ERβ and ERα expression in YAMCs.** YAMC cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. *A&B*, Cells were grown for 96 h under non-permissive conditions. After 96 h, RNA isolation was carried out using TRIzol and chloroform. 1 μg RNA was used to make cDNA. SYBR green based RT-PCR was then conducted using 2 ng cDNA for 18s control and 20 ng cDNA for ERβ and ERα. *B*, Small Intestine and Uterine RNA were recovered from WT female organs using the Invitrogen PureLink Micro to Midi Total RNA Purification System. *Columns*, mean (*A*, n=6 *B*, n=3); *bars*, SEM. Asterisks indicate significant difference, $P<0.01$.



A-2: Cell cycle changes in YAMCs treated with E_2. YAMC were transferred to charcoal dextran stripped serum media 48 h prior to plating. Cells were grown for 96 h under non-permissive conditions. Cell cycle analysis was then conducted using flow cytometry. *Columns*, mean (n=9); *bars*, SEM. Letters indicate significant difference, $P<0.01$.



A-3: E_2 increases p53 protein levels, but not transcript levels. YAMC bleo/neo cells and mp53/neo cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. *A&B*, Cells were grown for 96 h under non-permissive conditions. After 96 h, RNA isolation was carried out using TRIzol and chloroform. 1 μg RNA was used to make cDNA. SYBR green based RT-PCR was then conducted using 2 ng cDNA for 18s control and 20 ng cDNA for ERβ and ERα. Data are presented as fold change compared to bleo/neo ERβ +/- SEM, n=6. *C*, Cells were grown for 72 h under non-permissive conditions (37°C and in the absence of IFNγ). Cells were treated with vehicle or 1nM E_2 for the final 24 h. At the end of the treatment period cells were lysed and nuclear proteins collected. Western blot using total ERβ and β-actin antibodies was then conducted. Data are presented as fold change compared to bleo/neo vehicle control.

APPENDIX B

EXPERIMENTAL PROTOCOLS

B-1. Preparation of Complete RPMI Media for YAMCs and YAMC-Ras

Materials

1) 1L Nanopure water
2) RPMI 1640 powdered media (R8755)
3) 2g $NaHCO_3$
4) 25 mL FBS
5) 532 µL ITS-(BD354351)
6) 5 mL Glutamax-1
7) 5 mL Penicillin/Streptomycin
8) IFNγ

Methods

1) Measure 800 mL H2O into graduated cylinder
2) Add RPMI 1640 powdered media
3) Add NaHCO3
4) Stir until all particles dissolved
5) Adjust pH to 7.2
6) Add H2O to 1L
7) Filter with bottle top filter
8) Store at 4°C

Working Solution

1) Transfer 500 mL to sterile container
2) Add 25 mL FBS charcoal dextran stripped FBS
3) Add 532 µL ITS-
4) Add 5 mL Glutamax-1
5) Add 5 mL Penicillin/Streptomycin
6) Filter and store at 4°C
7) Day of use add .1 µL IFNγ per mL media

B-2 RPMI Media for YAMC bleo/neo and mp53/neo

Materials

1) 1L Nanopure water
2) RPMI 1640 powdered media (R8755)
3) 2g $NaHCO_3$
4) 50 mL FBS
5) 532 µL ITS-(BD354351)
6) 125 µL Gentamicin (Gibco 15710-064)
7) 0.05 µL/mL media γ-IFN (Roche 11276905001)

Methods

1) Measure 800 mL H_2O into graduated cylinder
2) Add RPMI 1640 powdered media
3) Add $NaHCO_3$
4) Stir until all particles dissolved
5) Adjust pH to 7.2
6) Add H_2O to 1 L
7) Filter with bottle top filter
8) Store at 4°C

Working Solution

1) Transfer 500 mL to sterile container
2) Add 50 mL FBS charcoal dextran stripped FBS
3) Add 532 µL ITS-
4) Add 125 µL Gentamicin
5) Filter and store at 4°C
6) Day of use add .05 µL IFNγ per mL media

B-3 Collagen Coating of plates for YAMC bleo/neo & mp53/neo

Materials

1) 100 mm culture dishes
2) Rat Tail Collagen, Type I (BD#354236)
3) 1 N Acetic acid
4) Syringe filter
5) 1X PBS

Methods

1) Make 0.25% acetic acid
   a) Add 50 µL 1 N acetic acid to 20 mL nanopure water
   b) Filter through syringe filter and store at 4°C
2) Make 50X Collagen Stock
   a) Add 1 mL collagen (3.91 mg/mL) to 2.128 mL 0.25% acetic acid
   b) Filter through syringe filter and store at 4°C
3) Make 1X Stock
   a) Add 480 µL 50X collagen stock to 23.520 mL filtered 1X PBS (coats 8 100 mm dishes or 6-well plates)
   b) Add 3 mL 1X collagen per 100 mm dish or 0.5 mL per well in a 6-well plate
   c) Rock gently to ensure complete coverage
   d) Set with lid closed in cell hood for 90 min
   e) Remove collagen and wash with 2 mL 1X PBS
   f) Add 5 mL 1X PBS and store at 37°C

B-4 YAMC Cell Culture: Passing and Seeding

Materials

1) T-75 flask
2) RPMI 1640 complete, warmed to RT
3) Trypsin
4) IFN-γ
5) PBS

Methods

1) All procedures conducted in Cell Culture Hood unless otherwise stated
2) Passing
    a) Start with 70% confluent flask of cells
    b) Aspirate old media and replace with 5 mL PBS
    c) Add 500 µL Trypsin and incubate at 33°C for ~5 min
    d) Add 5 mL RPMI 1640 and split cells into new flask(s)
    e) Add media to 10 mL and 1 µL IFNγ
    f) Return to 33°C incubator
3) Seeding
    a) Start with 70% confluent flask of cells
    b) Aspirate old media and replace with 5 mL PBS
    c) Add 500 µL Trypsin and incubate at 33°C for ~5 min
    d) Collect cells into 15 mL tube
    e) Spin cells then aspirate off trypsin/PBS mix and add 5 mL media
    f) Load 10 µL of cell suspension onto hemacytometer
    g) Count all cells in each corner large square and center square
    h) Average counts and multiply by 25
    i) Multiply total by 10^4 to determine cells per mL
    j) Calculate volume necessary for required number of wells at seeding density
    k) Add media to reach total volume ex- 12 wells at 30000 cells/well=360,000 cells; cell density is 1,440,000 cells/mL; 500 µL of cell suspension added to 23.5 mL media; 2 mL/well

B-5 YAMC bleo/neo Cell Culture: Seeding and Passing

Materials

1) Collagen coated 100 mm culture dishes
2) RPMI 1640 complete, warmed to RT
3) Trypsin
4) IFN-γ
5) PBS

Methods

1) All procedures conducted in Cell Culture Hood unless otherwise stated
2) Passing
    a) Start with 70% confluent flask of cells
    b) Aspirate old media and replace with 5 mL PBS
    c) Add 500 µL Trypsin and incubate at 33°C for ~5 min
    d) Add 5 mL RPMI 1640 and split cells into new flask(s)
    e) Add media to 10 mL and 0.5 µL IFNγ
    f) Return to 33°C incubator
3) Seeding
    a) Start with 70% confluent flask of cells
    b) Aspirate old media and replace with 5 mL PBS
    c) Add 500 µL Trypsin and incubate at 33°C for ~5 min
    d) Collect cells into 15 mL tube
    e) Spin cells then aspirate off trypsin/PBS mix and add 5 mL media
    f) Load 10 µL of cell suspension onto hemacytometer
    g) Count all cells in each corner large square and center square
    h) Average counts and multiply by 25
    i) Multiply total by 10^4 to determine cells per mL
    j) Calculate volume necessary for required number of wells at seeding density
    k) Add media to reach total volume ex- 12 wells at 30000 cells/well=360,000 cells; cell density is 1,440,000 cells/mL; 500 µL of cell suspension added to 23.5 mL media; 2 mL/well

B-6 Cell Culture: E_2 and ICI Treatments

Materials

1) Collagen coated 100 mm culture dishes
2) RPMI 1640 complete, warmed to RT
3) Trypsin
4) IFN-γ
5) PBS
6) DMSO
7) E_2 (100 nM, 1 μM, 10 μM stocks diluted in DMSO)
8) ICI (1 mM stock in DMSO)

Methods

1) Seed wells of plates as described in B-4 or B-5 and incubate for 24 h
2) Treat wells with 2 μL of 1000x associated treatment for 6-well plates or 1 μL for 12-well plates
3) Replace media and treatment after 48 h
4) Incubate 48 h
5) Collect materials from wells using protocol B-7, B-8, B-9, B-11 or B-12

B-7 Counting cells with Coulter Counter

Materials

1) Coulter cups
2) Isotone diluent
3) PBS
4) Trypsin
5) RPMI 1640
6) 1.5 mL tubes

Methods

1) Remove media from cells and replace with 1 mL PBS
2) Add 100 µL trypsin and incubate for 5 minutes at 39°C
3) Collect cells into individual 1.5 mL tubes
4) Fill Coulter cups with 10 ml isotone diluent per vial
5) Turn power on, open door, lower stage of Coulter Counter
6) Remove vial of Coulter Clenz, replace with Isotone, raise stage
7) Readout: Setup #1 (S1). Scroll down to last line and set cell size to 20 µm
8) Select: FUNCTIONS on keypad. Scroll to Flush Asperature. Hit: START/STOP to flush. Repeat flush, then replace Isotone. Flush twice more
9) Select: OUTPUT on keypad. Scroll to Count/Concentration and select one., If "concentration"is selected, readout will be in #cells/ml. Scroll to Dilution and enter dilution factor. Leave units on um
10) Add 20 µl sample to Coulter vial (for 1:500 dilution) Place vial on stage, raise, and press START/STOP on keypad to count. Count each sample 2-3 times and average.
11) To shut down machine, replace vial of blue Coulter Clenz and flush aperature (under FUNCTIONS) 2-3 times. When flush is complete, turn off power

B-8 Caspase-3 Activity Assay

Materials

1) EnzChek Caspase-3 Assay Kit #2
2) Treated cells
3) Trypsin
4) PBS
5) Microplate
6) 1.5 mL microtubes

Methods

1) Remove media from cells and add 1 mL PBS per well of 6-well plate
2) Add 100 µL Trypsin and incubate at 39°C for 5 min
3) Collect cells, pellet and wash with PBS
4) Prepare 1X cell lysis buffer by adding 50 µL 20X cell lysis buffer to 950 µL dH_2O
5) Resuspend cells in 50 µL 1X cell lysis buffer and incubate on ice 30 minutes
6) Prepare 2X reaction buffer by adding 400 µL 5X reaction buffer, 10 µL 1M DTT, and 590 µL dH_2O
7) Centrifuge lysed cells to pellet debris, transfer 50 µL supernatant to individual wells of a microplate and add 50 µL 1X cell lysis buffer as a o enzyme control
8) Prepare 2X substrate working solution by mixing 10 µL 5 mM Z-DEVD-R110 substrater with 990 µL of 2X reaction buffer
9) Add 50 µL 2X substrate working solution to each sample and control
10) Cover the plate and incubate at RT for 30 minutes
11) Measure fluorescence (excitation/emission 496/520 nm) at 0, 15, 30 minutes

B-9 RNA Isolation

Materials

1) 2-mercaptoethanol
2) 70% EtOH
3) RNase free 1.5 mL microcentrifuge tubes
4) PBS
5) RNase free pipette tips
6) PureLink Micro to Midi Total RNA System

Methods

1) Prepare RNA Lysis Solution
   a) Use 0.6 mL RNA Lysis Solution for $5x10^6$-$5x10^7$ cells
   b) Add 1% 2-mercaptoethanol to volume of RNA Lysis Solution used
   c) Prepare RNA Lysis Solution Fresh each time
2) Lysis and homogenization
   a) Remove growth media from cells
   b) Pipet the prepared RNA Lysis Solution over monolayer
   c) Pipet up and down until cells appear lysed
   d) Transfer the lysate to a 1.5 mL tube and pass 5-10 times through a 20-gaue needle
3) Binding, washing and elution
   a) Add one volume of 70% EtOH to each volume of cell homogenate
   b) Mix thoroughly by vortexing, dispersing any visible precipitate
   c) Transfer up to 700 µL to RNA Spin Cartridge and centrifuge at 12000 g for 15s, discard flow-through
   d) Repeat c) until all of the sample has been processed
   e) Add 700 µL of Wash Buffer I to spin cartridge and spin at 12000 g for 15s, discard flow-through
   f) Place spin cartridge into new wash tube
   g) Add 500 µL Wash Buffer II to spin cartridge and spin at 12000 g for 15 s, discard flow-through
   h) Repeat g) once
   i) Centrifuge spin cartridge at 12000 g for 1 minute to dry membrane, transfer cartridge into new RNA Recovery tube
   j) Add 50 µL RNase-free water to center of spin cartridge and incubate for 1 minute at RT
   k) Centrifuge for 2 minutes at 12000 g and discard cartridge
   l) Store at -80°C

B-10 RT-PCR

Materials

1) RNA
2) Transciptor First Strand cDNA Synthesis kit
3) FastStart Universal SYBR Green Master Mix
4) Forward and Reverse primers
5) RNase-free water
6) PCR tubes

Methods

1) cDNA production
    a) Thaw samples and reagents on ice
    b) Add 1 µg total RNA, 1 µL primers, PCR-grade water to 13 µL
    c) Run in thermocycler for 10 minutes at 65°C
    d) Add 4 µL 5X Transcriptor Reverse Trascriptase Reaction Buffer, 0.5 µL Protector RNase Inhibitor, 2 µL Deoxyribonucleotide Mix, 0.5 µL Transcriptor Reverse Transcriptase
    e) Mix carefully, Incubate at 50°C for 1 h, followed by 85°C for 5 minutes
    f) Store at -20°C
2) RT-PCR
    a) Add 9.5 µL FastStart Universal SYBR Green Master mix, 1.25 µL forward primer, 1.25 µL revers primer and 11 µL PCR-grade water per sample
    b) Pipet 23 µL into each PCR tube
    c) Add 2 µL cDNA and mix by pipetting
    d) Run on Bio-Rad iQ5 thermocycler

B-11 Western Blot

Materials

1) 10X running buffer (1 L H_2O + 30.33g Tris base + 10g SDS + 144g Glycine)
2) Precast gel
3) Membrane
4) 1X transfer buffer (3.03g Tris base + 14.42g Glycine + 100 mL MetOH + dH_2O to 1L total volume)
5) Primary antibody
6) Secondary antibody
7) MetOH
8) TBS-T (Tris-buffered saline with 0.1% Tween 20)
9) Immobilon Western Chemiluminescent HRP substrate kit

Methods

1) Boil protein samples for 5 minutes, then spin for 5 minutes
2) Prepare 1X running buffer from 10X stock
3) Load precast gel in gel box and remove comb
4) Fill box to top of gel with 1X running buffer
5) Load samples, dye and size markers
6) Run, starting at 20V and increasing voltage to max by one notch every 10 minutes
7) Make 1X transfer buffer
8) Remove gel and use comb to separate plates
9) Briefly soak gel in MetOH for ~20s, then soak in transfer buffer 15-30 minutes, transfer to new dish and soak in transfer buffer 15-30 minutes
10) Saturate sponges in transfer buffer, then make sandwich (sponge, sponge, gel, membrane, sponge, sponge)
11) Place in transfer box and fill center with transfer buffer to top, fill outside with dH_2O
12) Run a 150 mA for 2h
13) Wash membrane 3 times in TBST for 10 minutes each
14) Block for 1 h
15) Primary antibody incubation overnight
16) Wash membrane 3 times in TBST for 10 minutes each
17) Secondary antibody incubation for 1 h
18) Wash 3 times in TBST for 10 minutes each
19) Add chemiluminescent substrate for 5 minutes, remove excess liquid and image
20) Store membrane in TBST in covered container at 4°C

B-12 Reporter Assay

Materials

1) PBS
2) Reporter DNA
3) Effectene transfection kit
4) Plated YAMCbleo/neo cells
5) RPMI 1640 media
6) E_2
7) ICI
8) DMSO

Methods

1) Mix 4 µg reporter DNA/0.5 µg β-gal DNA with 16 µL Enhancer and (250-DNA volume) µL Buffer EC and incubate 5 minutes
2) Add 50 µL Effectene to mixture and mix by pipetting, incubate 10 minutes
3) Wash cells with PBS and add 800 µL media per well
4) Add 4.8 mL media to transfection complex and mix by pipetting
5) Add 400 µL to each well drop-wise and gently swirl plate
6) Add treatments
7) Incubate at 39°C for 18 h
8) Mix 2 mL DI-H2O + 0.5 mL RLB)/plate
9) Remove media from cells and spin down
10) Wash cells 2 times with PBS
11) Add 200 µL RLB mixture to spun cells and then add back to original wells and rock gently to ensure complete coverage
12) Incubate at RT for 15 minutes while slowly rocking
13) Scrape all areas of well and transfer lysate to pre-labeled 1.5 mL tubes, place on ice
14) Vortex 10-15s
15) Spin at 13000 g for 2 minutes at 4°C
16) Transfer supernatant to fresh tube
17) Luciferase Assay
    a) Bring luciferase agent and cell lysates to RT
    b) Program plate reader for 2s delay:10s read
    c) Add 10 µL cell lysate/well of white 96-well plate
    d) Add 50 µL luciferase reagent/well immediately prior to reading
18) β-galactosidase Assay
    a) Thaw and mix each component
    b) Place 2X assay buffer on ice
    c) Dilute cell lysate w/RLB if necessary
    d) Add 25 µL cell lysate/well of a 96-well plate
    e) Add 20 µL 2X buffer/well

f) Incubate at 37°C for 30 minutes or until aint yellow
g) Stop reaction by adding 75 µL 1M $NaCO_3$, mix by pipetting
h) Pop any air bubbles present
i) Read absorbance at 420 nm immediately after adding $NaCO_3$

B-13 Tail DNA genotyping

Materials

1) 1.5 mL microcentrifuge tubes
2) Scissors
3) Forceps
4) Wizard SV Genomic DNA Purification System
5) Proteinase K (20mg/mL) in nuclease-free water
6) Primers (Clwt-27-5'GGAGTAGAAACAAGCAATCCAGACATC3', bNHD4-25-5'AGAATGTTGCACTGCCCCTGCTGCT3', Neo-25-5'GCAGCCTCTGTTCCACATACACTT3', p53f-5'GTGTGTGAAATGGTGGATGG3', p53r-5'AGGTGATGGCTGTGGATG3', purof-5'ATGACCGAGTACAAGCCCAC3', puror-5'GCGTGAGGAAGAGTTCTTGC3')
7) Buffer
8) MgCl2
9) dNTPs
10) TaqDNA polymerase

Methods

1) Label 1.5 mL microcentrifuge tube with animal number
2) Take ~1 cm tail snip from animal and place in labeled tube
3) Tail Digestion
    a) Prepare tissue digestion solution by adding 200 µL Nuclei Lysis Solution, 50 µL EDTA, 20 µL proteinase K and 5 µL RNase A solution per sample and store on ice
    b) Add 275 µL tissue digestion solution to each sample tube
    c) Incubate samples overnight in a 55°C water bath
    d) Add 250 µL Wizard SV Lysis buffer to each sample, vortex
    e) Transfer entire sample lysate to a Wizard V minicolumn and spin at 1300 g for 3 minutes
    f) Discard flow-through
    g) Add 650 µL Wizard SV Wash solution to each sample and spin at 13000 g for 1 minute
    h) Discard flow-through
    i) Repeat steps g) and h) 3 times
    j) Centrifuge at 13000 g for 2 minutes to dry column
    k) Transfer column to new 1.5 mL tube and add 250 µL nuclease-free water to column, incubate 2 minutes
    l) Centrifuge at 13000 g for 1 minute
    m) Add another 250 µL nucleas-free water to column and incubate 2 minutes
    n) Centrifuge at13000 g for 1 minute
    o) Store at -20 to -70°C
4) PCR
    a) Create master mix

i. WT ERβKO- 7.64 µL dH_2O + 2.5 µL Buffer + 1.08 µL $MgCl_2$ + 4.16 µL dNTPs + 5.2 µL bNHD4-25 + 5.2 µL Clwt-27 + 0.1 µL Taq DNA polymerase
ii. KO ERβKO- 12.84 µL dH_2O + 2.5 µL Buffer + 1.04 µL $MgCl_2$ + 4.16 µL dNTPs + 2.6 µL bNHD4-25 + 2.6 µL Neo-25 + 0.1 µL Taq DNA polymerase
iii. WT p53- 13.6 µL dH_2O5 µL Buffer + 2 µL dNTPs + 0.5 µL each p53f and p53r primers + 0.4 µL Taq DNA polymerase
iv. KO p53- 13.6 µL dH_2O5 µL Buffer + 2 µL dNTPs + 0.5 µL each purof and puror primers + 0.4 µL Taq DNA polymerase

b) Add 4 µL DNA to 21 µL master mix for ERβKO mice and 2 µL DNA to 23 µL p3KO mice
c) Run on thermocycler
i.

5) Run PCR product on gel

B-14 TUNEL Assay

Materials

1) Tissue sections on glass slides
2) Xylene
3) EtOH- 100%, 95%, and 70%
4) 1X PBS
5) PAP pen
6) Proteinase K dilute to 20 µg/mL in PBS immediately prior to use
7) H_2O_2
8) Methanol
9) 0.5% Methyl Green in 0.1M sodium acetate
10) Permount
11) Cover slips
12) ApopTag Plus Peroxidase *In Situ* Detection kit
    a) Equilibration buffer
    b) Working strength TdT enzyme- (Add 7.7 µL reaction buffer + 3.3 µL TdT enzyme)/ per tissue section
    c) Working strength Stop/Wash- Add 1 mL stop/wash to 34 mL dH2O
    d) Anti-Digoxigenin
    e) Working strength peroxidase substrate- Add 3 µL DAB substrate to 147 µL DAB dilution buffer

Methods

1) Daparaffinize
    a) Wash in Xylene 5 min 3X
    b) Wash in 100% EtOH 5 min 2X
    c) Wash in 95% EtOH 3 min
    d) Wash in 70% EtOH 3 min
    e) Wash in 1X PBS 5 min
2) Pretreat- Apply freshly diluted Proteinase K incubate in humidified chamber at 37°C for 5 min
3) Wash in dH2O for 2 min 2X
4) Quench Endogenous Peroxidase- Incubate in 3% H2O2 in Methanol for 5 min at RT
5) Wash in dH20 5 min 2X followed by wash in 1X PBS 5 min
6) Apply Equilibration buffer
    a) Tap off excess liquid and aspirate around tissue
    b) Apply 15 µL/cm2 equilibration buffer and incubate at least 10s
7) Apply TdT
    a) Tap off excess liquid and aspirate around tissue
    b) Apply 11 µL working strength TdT enzyme, incubate in humidified chamber 1 h at 37°C

8) Apply Stop/Wash
    a) Remove slides from humidified chamber, and agitate in 35 mL working strength Stop/Wash
    b) Incubate for 10 min
9) Wash in 1X PBS for 1 min 3X
10) Apply Anti-Digoxigenin
    a) Tap off excess liquid and aspirate around tissue
    b) Apply 11 µL Anti-Digoxigenin, incubate in humidified chamber for 30 min at RT
11) Wash in 1X PBS for 2 min 4X
12) Develop Color
    a) Tap off excess liquid and aspirate around tissue
    b) Apply 15 µL working strength peroxidase substrate, incubate for ~3 min at RT
13) Wash in dH2O for 1 min 3X followed by 1 for 5 min
14) Counterstain in 0.5% methyl green for 30s-3 min
15) Wash in dH2O 3X, dip 10X each in the first 2 washes and leave in third wash for 1 min
16) Dehydrate and mount
    a) Wash in 70% EtOH for 1 min
    b) Wash in 95% EtOH for 1 min
    c) Wash in 100% EtOH for 1 min
    d) Wash in Xylene for 3 min 3X
    e) Mount by adding 1 drop Permount before placing cover slip, let sit until dry

B-15 Immunohistochemistry

Materials

1) Xylene
2) EtOH- 100%, 95%, and 70%
3) dH_2O
4) 1X PBS
5) H_2O_2
6) Methanol
7) 0.1 M citric acid
8) 1M trisodium citrate
9) Primary Ab
    a) Anti-BrdU Ab
    b) Anti-p53 Ab
10) Secondary Ab
    a) HRP conjugated Goat-anti-mouse Ab
    b) HRP conjugated Goat anti-rabbit Ab
11) DAB
12) 0.5% Methyl green

Methods

1) Deparaffinize and rehydrate tissues
    a) Xylene 2X 12 minutes
    b) 100% EtOH 2X 5 minutes
    c) 95% EtOH 5 minutes
    d) 70% EtOH 5 minutes
    e) dH2O 5 minutes
2) Quench endogenous peroxidase activity by incubating in 3% H_2O_2 in MetOH for 30 minutes at RT
3) Wash in dH_2O 3 times for 3 minutes each
4) Antigen retrieval (Reverse steps 2 and 4 for p53)
    a) Combine 9 mL 0.1M citric acid, 41 mL trisodium citrate and 450 mL dH2O
    b) Pour over slides in Pyrex baking dish and cover, leave vented, and microwave on high for 10 minutes
    c) Microwave on half power for 10 minutes, add dH2O if needed
    d) Let cool to RT
5) Wash in PBS 2 times for 5 minutes each
6) Primary antibody incubation
    a) Prepare 1:10 for Anti-BrdU or 1:50 for Anti-p53 in 1% BSA in PBS
    b) Apply to tissues, using 1%BSA in PBS as a control
    c) Place in humidified chamber and incubate at 4°C overnight
7) Bring samples to RT

8) Wash in dH_2O 3 times for 5 minutes
9) Secondary antibody incubation
    a) Prepare a 1:200 dilution of HRP Conjugated Goat-anti-Mouse antibody(Anti-BrdU) or 1:50 (Anti-p53) in 1% BSA in PBS
    b) Incubate in humidified chamber for 1 h at RT
10) Wash in PBS 3 times for 5 minutes
11) Prepare DAB
    a) Add 15 mg DAB o 15 mL PBS, mix well then filter 10 mL into new tube
    b) Add 3.3 µL H2O2 directly before adding to tissues
    c) Add to tissues and incubate 10 minutes (Anti-BrdU) or 5 minutes (Anti-p53) at RT
    d) Deactivate DAB and all utensils used with bleach
12) Wash in dH_2O 4 times for 2 minutes and 1 time for 5 minutes
13) Counterstain in methyl green for 45 s
14) Agitate slides in dH_2O then wash 3 times for 2 minutes
15) Dehydrate tissues
    a) 70% EtOH 5 minutes
    b) 95% EtOH 5 minutes
    c) 100% EtOH 5 minutes
    d) Xylene 3 times for 5 minutes
16) Wet mount with Permount

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