INFLUENCE OF ESTRADIOL AND PHYTOESTROGENS ON COLITIS AND COLON CANCER

A Dissertation

by

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ABSTRACT

Estrogen is a female sex hormone that has a variety of biologic actions via modulation of gene expression through estrogen receptors (ERs). The protective effect of estradiol (E_2) and estrogen signaling in colon cancer has been demonstrated in epidemiological and clinical data as well as animal experiments. The overall aim of this series of studies is to determine the protective mechanism of estrogen signaling activated by E_2 and phytoestrogens on colitis and colon cancer.

First, the estrogenic effect of novel phytoestrogens, trigonelline (Trig) and 3,3-diindolylmethane (DIM) was determined in non-malignant colonocytes (YAMCs). Both molecules decreased cell growth of YAMCs, but their mechanisms of action were distinct from E_2. Trig increased apoptosis by functional ERs without direct binding to ERs while DIM altered the expression of target genes of ERs via increased ER transcriptional activity. These data suggest that phytoestrogens could activate estrogen signaling through unique mechanisms.

Second, the protective effect of estrogen signaling on colitis and colitis associated colon cancer (CAC) was demonstrated *in vitro* and *in vivo*. In the *in vitro* study, IL-6 induced cell growth was observed, and E_2 and genistein (GEN) treatment inhibited IL-6 actions via an increase of apoptosis and modulation of gene expression related to estrogen signaling. In the *in vivo* colitis experiment, chronic inflammation damaged the colon, but E_2 treatment increased the recovery of the damaged colon via an

increase of cell proliferation with modulation of cytokines. However, GEN treatment exacerbated the damage on chronic inflammation. In the CAC model, E_2 and GEN treatment suppressed the formation of aberrant crypt foci (ACF), premalignant lesions. These data suggest that E_2 protects the colon and colon epithelial cells, and the protective mechanism of estrogen signaling differs depending on the type of injury and local conditions.

Lastly, the interaction of estrogen signaling and the p53 pathway was studied using intestinal epithelial cell-specific p53 knockout mice ($Tp53^{\Delta IEC}$). The protective effect of E_2 in $Tp53^{\Delta IEC}$ mice was observed, suggesting that the suppression of ACF by estrogen signaling is partially independent of the p53 pathway.

Overall, estrogen signaling has a protective property against colitis and colon cancer but the protective mechanism of estrogen signaling could be changed from apoptosis to proliferation depending on the condition of the colon. In addition, each phytoestrogen has a distinct and unique way of influencing the colon. These data help clarify the role and the mechanism of estrogen signaling on colon cancer.

DEDICATION

“In their hearts humans plan their course, but the Lord establishes their steps.”

(Proverbs 16:9)

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NOMENCLATURE

ACF	Aberrant crypt foci
AF domain	Activation function domain
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
AMPs	Antimicrobial proteins
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ATM	Ataxia-Telangiectasia mutated
ATR	Ataxia-Telangiectasia and Rad-3-related
BAX	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
Bcl-6	B cell lymphoma 6
BrdU	5-Bromo-2'-deoxyuridine
CAC	Colitis-associated colon cancer
CCND1	Cyclin D1
CD	Crohn’s disease
COX-2	Cyclooxygenase-2
CRE	Cyclic AMP-response element
DAB	3,3’-Diaminobenzidine
DIM	3,3’-Diindolylmethane

DMSO	Dimethyl sulfoxide
DNBS	2,4-Dinitrobenzene sulfonic acid
DSS	Dextran sodium sulfate
E_1	Estrone
E_2	Estradiol
E_3	Estriol
EAE	Experimental autoimmune encephalitis
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen-responsive element
ERK	Extracellular signal-regulated kinases
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FOXP-3	Forkhead box P3
GEN	Genistein
GI tract	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130
GPR30	G-protein coupled receptor 30
H&E staining	Hematoxylin and eosin staining
HAT	Histone-acetyltransferase
IBD	Inflammatory bowel disease

ICI	ICI 182,780
IEC	Intestinal epithelial cell
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-6R	IL-6 receptor
ITS	0.1% insulin, transferrin, and selenious acid
JAK	Janus kinase
LBD	Ligand binding domain
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein 1
Mdm2	Mouse double minute 2 homolog
MIP1α	Macrophage inflammatory protein 1α
miR-146b	microRNA 146b
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide

OVX	Ovariectomization
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PI3K	Phosphotidylinositol-3 kinase
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	n-3 Polyunsaturated fatty acids
PUMA	p53 upregulated modulator of apoptosis
RAG1	Recombination activating gene 1
RORγt	Retinoid-Acid Receptor-related Orphan Receptor γ t
ROS	Reactive oxygen species
RT	Room temperature
SOCS3	Suppressor of cytokine signaling 3
SRC	Steroid receptor co-activator
STAT3	Signal transducer and activator of transcription 3
TDT	Terminal deoxynucleotidyl transferase
TGFβ	Transforming growth factor β
T_{FH}	Follicular T helper lymphocytes
T_H1	Type 1 T helper lymphocytes
T_H2	Type 2 T helper lymphocytes
T_H17	Type 17 T helper lymphocytes
TNBS	2,4,6-Trinitro benzene sulfonic acid
TNFα	Tumor-necrosis factor α

$Tp53^{Flox/Flox}$	p53 floxed mice
$Tp53^{\Delta IEC}$	Intestinal epithelial cell-specific p53 knockout mice
T_{Reg}	Regulatory T cells
Trig	Trigonelline
TUNEL assay	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay
UC	Ulcerative colitis
YAMC	Young adult mouse colonocytes

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Colon cancer statistics

Colon cancer is the third most common cancer as well as the third most common cause of death by cancers in both sexes [1]. Approximately 102,000 cases of colon cancer were newly diagnosed and over 500 deaths were estimated due to colon cancer in the U.S. in 2013. However, the diagnosis and death rate of colon cancer has continuously decreased by approximately 3% during the last 10 years [2]. The development of methods for early detection and the clarification of the pathogenetic mechanism of colon carcinogenesis are thought to be the main reason for this decline.

1.2 Colon anatomy

The colon is the final digestive organ in the gastrointestinal tract (GI tract) and its length in humans is approximately 1.1-1.9 meters [3]. The primary functions of the colon are absorption of salt and water, production of feces, and storage of feces until defecation [4]. The GI tract, including the colon, consists of 4 layers: the serosa, the muscularis externa, the submucosa and the mucosa [5]. The serosa is the outer layer of tissue covering the GI tract and protects the GI tract from friction with surrounding tissues. The muscularis externa is the smooth muscle layer of the digestive tube inside the serosa, and the submucosa is the connective tissue containing blood and lymph vessels. The mucosa is the epithelial luminal layer surrounded by the submucosa, and its

surface is highly folded, increasing the surface area for absorption of nutrients. The mucosa is divided into 3 layers: the muscular mucosa, the lamina propria and the epithelial. The epithelial layer is made up of millions of invaginations, which are called the colonic crypts, a functional unit of the colon, and a primary tissue influenced by colon carcinogenesis [6, 7].

1.3. Colon cancer progression

Cancer development is a multistep progression over many years, and colon carcinogenesis may require over 10-15 years to progress from normal epithelial tissue to invasive cancer [6, 8, 9]. The progression of colon carcinogenesis has been well established, and studies have demonstrated that it arises by accumulation of genetic and epigenetic modifications. Colon carcinogenesis is separated into 3 stages depending on their physiological features: initiation, promotion, and progression [10]. Initiation is the first stage in which DNA damage occurs in a single cell due to spontaneous mutation or carcinogen exposure. The DNA repair mechanisms existing in the cell may correct the error via repair of damaged DNA or removal of cells with DNA damage, resulting in prevention of tumors. If cells with DNA damage escape the DNA repair mechanism, they will be vulnerable to producing a malignant tumor. The second step of colon carcinogenesis is promotion in which initiated cells form polyps. Though polyps are not cancerous, 95% of colorectal cancers originate from adenomatous polyps (adenomas) [11]. In this stage, increased cell proliferation expands the initiated cell population, forming aberrant crypt foci (ACF), which is a pre-polyp structure within preneoplastic

lesions [12]. Further mutation of oncogenes or tumor suppressor genes accumulates in initiated cells and induces colon cancer progression, the last stage. During progression, aberrant cell proliferation due to accumulated genetic mutation and other factors causes malignant transformation of cells which develop from adenoma to carcinoma and ultimately become invasive to other tissues or organs during progression.

1.4 Risk factors of colon cancer

Many risk factors are identified that increase colon cancer incidence [13]. Age is the primary risk factor for colon cancer. According to colorectal cancer statistics, approximately 60% of colon cancer cases and 70% of deaths by colon cancer occur in people age 65 and older [2]. Another risk factor is genetic defect. Colorectal cancer develops with a series of genetic mutations on some oncogenes and tumor suppressor genes [9]. At least four different genetic defects play a pivotal role in the development of colorectal cancer: APC, K-RAS, p53 and SMAD4. The adenomatous polyposis coli (APC) protein is associated with Wnt/β-catenin signaling. Wnt/β-catenin signaling is essential to the development and regeneration of tissues and APC is a negative regulator of Wnt/β-catenin signaling via induction of β-catenin. Inactivation of APC causes aberrant activation of Wnt/β-catenin signaling, resulting in loss of the ability to control cell proliferation. Loss of function of APC protein is one of the primary causes in Familial Adenomatous Polyposis (FAP), an inherited condition causing numerous adenomatous polyps [9, 14]. K-RAS is an oncogene involved in EGF (Epidermal growth factor) signaling which is a growth factor inducing cell proliferation and

blocking apoptosis, and K-RAS mutation is found in 50% of colorectal cancer [15]. p53 mediates DNA repair signaling and apoptosis [16]. 50% of colorectal cancers have a p53 mutation. Loss of p53 function in mice results in a variety of cancers and high mortality at a relatively early age, due to a central role of p53 in controlling cell fates [17]. The human SMAD4 gene is located in the long arm of chromosome 18 and mutation of SMAD4 is the most common abnormality found in colon cancer [18, 19]. SMAD4 is a central mediator of transforming growth factor β (TGFβ) signaling. Down-regulation of SMAD4 is common in a variety of cancers and is associated with poor prognosis in colon cancer. A serial genetic mutation of these genes is associated with histopathological changes in the colon and promotion of colon carcinogenesis.

Various environmental factors influence the risk of colon cancer as well [13]. The environment includes all the social, economic, and cultural factors composing a lifestyle. The most critical factor from the environment is the diet. Diet and nutrients in the diet are strongly correlated with cancer incidence including colon cancer [20, 21]. Epidemiological data have demonstrated a positive correlation of high consumption of red meat and processed meat with the incidence of colorectal cancer [22]. It has been reported that a high dose of N-nitroso compounds and a high level of heme in red meat and processed meat function as an inducer of DNA damage and oxidative stress in the colon. Cooking red meat at high temperatures increases heterocyclic amines which are known as probable carcinogens involved in colon cancer [23-26]. Animal fat is also thought to be a risk factor for colorectal cancer because animal fats contain high amounts of saturated fatty acids [20, 27]. Saturated fatty acids contribute to production

of prostaglandin E_2 (PGE_2), a marker of inflammation; increased inflammatory signaling due to elevated PGE_2 is negatively correlated with survival of colon cancer patients [28, 29]. Saturated fats alter gut microbiota composition and disrupt the metabolism of bile acid, which may contribute to the possibility of colon carcinogenesis [30, 31]. Obesity, a condition characterized by accumulating excess fat in adipose tissue, is also one of the risk factors for colon cancer [20, 32, 33]. Adiposity is related to a state of mild chronic inflammation caused by an increase of pro-inflammatory mediators such as interleukin-6 (IL-6), interferon-γ (IFN-γ) and tumor-necrosis factor (TNF) [34]. Obesity also increases the level of insulin and insulin-like growth factor-1 (IGF-1), which influence colon carcinogenesis through inhibition of apoptosis and induction of epithelial proliferation [35]. In contrast, some natural dietary compounds are negatively correlated with colon cancer risk [36]. For example, fish oil, which contains n-3 polyunsaturated fatty acids (PUFA), can increase apoptosis and decrease DNA adducts in colonocytes. Likewise, curcumin, a polyphenolic compound, also prevents colon carcinogenesis via a decrease of reactive oxygen species (ROS) and inhibition of inflammation and growth factor related signaling [37-40].

1.5 Estrogen and estrogen signaling

Estrogen is a female sex hormone that plays a pivotal role in the development and function of female sex organs [41, 42]. The ovaries are the primary organ that produces estrogen, but recent studies have demonstrated that other organs such as adipose tissues can also produce estrogen. Estrogen is derived from cholesterol through a

series of enzymatic reactions. There are three naturally occurring estrogens: estrone (E_1), estradiol (E_2), and estriol (E_3) (Fig. 1.1). Similar to other steroid hormones, estrogen is hydrophobic and it can pass freely through cellular membranes and bind to estrogen receptors (ERs) in the cytosol of cells. ERs activated by estrogen can act as a transcription factor, translocate into the nucleus, and modulate the expression of target genes, which have an estrogen-responsive element (ERE) or an alternative DNA sequence such as AP-1 that interacts with ERs [43]. Estrogen can also transduce signals via binding to the transmembrane G-protein coupled receptor 30 (GPR30) [44].

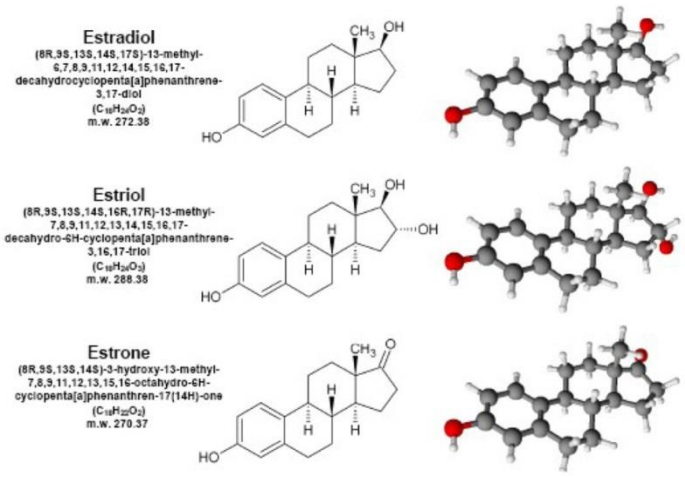

Fig. 1.1 Structures of three natural estrogens [41]

There are two primary forms of estrogen receptors: ERα and ERβ (Fig 1.2) [45, 46]. Genes for two subtypes of ERs are on chromosomes 6 and 14; their expression is tissue-specific but overlapping. ERα is predominantly expressed in the ovary, liver, and

breast, while ERβ is highly expressed in the colon and lung [47]. Though both ERs have high homology of their domains, they have different functions on target genes and biological responses. ERα is essential for ductal growth, while ERβ is associated with pro-differentiative and anti-proliferative functions in the mammary gland. In breast cancer development, several studies have shown that ERα induces carcinogenesis while ERβ protects against malignancy. This difference is partially due to the different influence of each ER on the expression of target genes. For example, cyclin D1 (CCND1) is a downstream gene of ERs via the AP-1 site, and CCND1 expression is elevated by ERα but inhibited by ERβ [48].

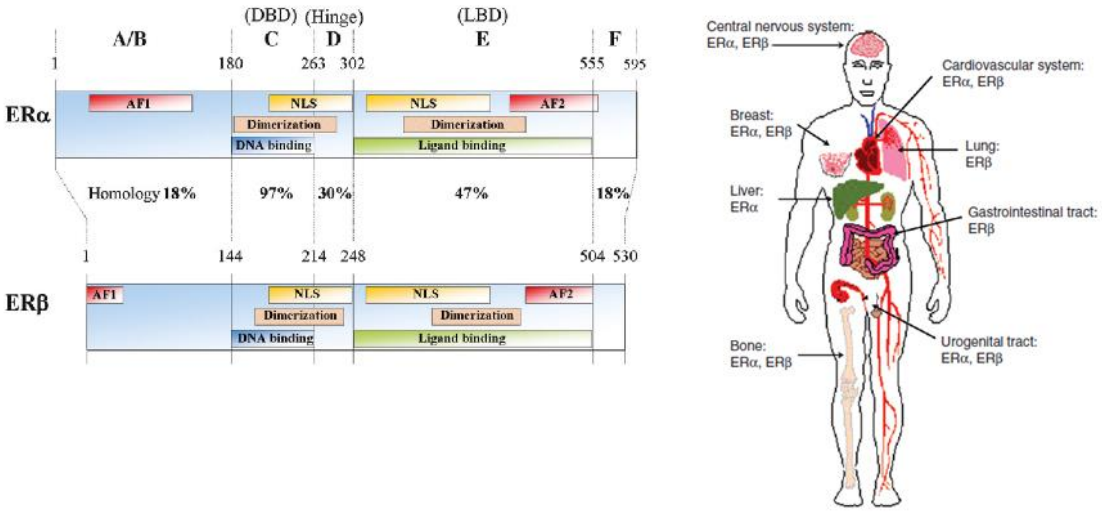

Fig. 1.2 Structure and distribution of subtypes of ERs [45, 47]

Because of low homology of the ligand binding domain (LBD) between both ERs, different binding affinity to estrogenic molecules may be anticipated (Fig. 1.2). Low homology in the activation function 2 (AF2) domain of the LBD is especially critical, because the AF2 domain is the transactivation domain, which recruits proteins to the ER transcription complex [45, 49, 50]. The AF2 domain is exposed by binding ligands, and exposed helix 12 in the AF2 domain interacts with other co-activators or co-repressors forming the ER transcription complex. Therefore, ERα and ERβ may have different co-activators due to the difference on their AF2 domains, resulting in different influences on gene profiles and cell physiology. A variety of proteins have been identified as co- activators of ERs. These co-activator proteins contain α-helical leucine-rich peptide motifs, known as LXXLL motifs, which interact with the AF2 domain of ERs and include RIP140, TRIP-1/SUG-1, TIF1, SRC-1, TIF2/GRIP-1, ACTR/SRC-3, and CBP/p300. Binding of these proteins to ERs induces the recruitment of histone-acetyltransferase (HAT), an enzyme for chromatin decondensation, making DNA more accessible to transcription factors, forming a transcription complex. Transcriptional activity of ERs thereby increases due to HAT.

1.6 Estrogen signaling on colon cancer

Clinical and epidemiological data have demonstrated the relationship between colon cancer and estrogen signaling [51-54]. Pre-menopausal women have a lower incidence of colon cancer compared to men, but the risk of the disease in women elevates to a relative risk similar to men after menopause. However, hormone

replacement therapy in post-menopausal women reduces the risk for colon cancer. Animal studies support the epidemiologic data from humans as well. Cleveland and his colleagues demonstrated that disrupted estrogen signaling increased the formation of neoplasms in $APC^{min/+}$ mice [51]. Data from our laboratory show that E_2 treatment suppresses aberrant crypt foci (ACF) formation induced by azoxymethane (AOM), a colon specific carcinogen [53]. The protective effect of E_2 treatment was reduced in ERβ knockout mice, and the expression of ERβ was decreased while ERα expression was increased during colon carcinogenesis. This indicates that ERβ, the predominant form of ER in the gastrointestinal tract, mediates the protective effect of E_2 [53, 55, 56].

1.7 Phytoestrogens

Phytoestrogens are natural compounds having estrogenic properties derived from plants (Fig. 1.3) [57]. They can be divided into three subtypes: isoflavones, lignans, and coumestans [58]. Soybeans and soy products contain abundant isoflavones, and other legumes are also good sources of dietary isoflavones. Because of their similar structure, isoflavones have high binding affinity to ERs and can induce ER-mediated transcriptional activity. Plant lignans are converted to mammalian lignans by gut microflora and the major mammalian lignans are enterolactone and enterodiol. Plant ligans are found in flax seeds, dried seaweeds, legumes and other cereals. Due to the distinct structure of lignans, they hardly bind to ERs, but they do have estrogenic properties. Lignans interrupt E_2 binding to ERs and modulate target genes of ERs [59]. Coumestans are found in a variety of plants, and some coumestans have estrogenic

properties. Coumesterol and 4'- methoxycoumestrol are found in clover. Coumesterol has weak but significant binding affinity to ERs and is capable of interacting with sex hormone binding globulin [60]. Coumesterol can inhibit the biosynthesis of estrogens via suppression of enzymes such as aromatase. In addition, recent studies have identified a new class of estrogenic molecules, which have structures distinct from natural estrogens or traditional phytoestrogens [61]. Unlike known phytoestrogens, they do not compete with E_2 for binding to ERs because of their unique structures [61, 62].

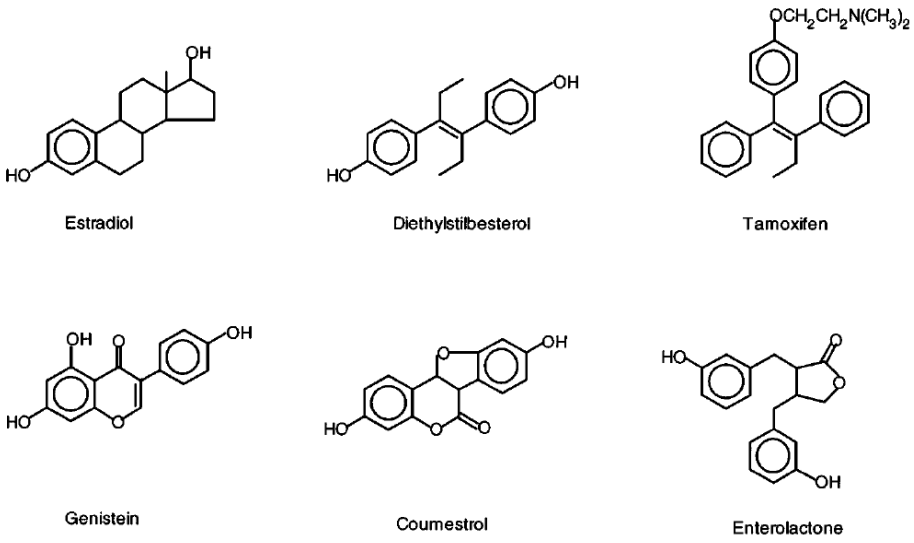

Fig. 1.3 Structures of estrogens and some phytoestrogens: E_2 (natural estrogen), diethylstilbestrol (synthetic estrogen), tamoxifen (synthetic antiestrogen), genistein (isoflavone), coumestrol (coumestans), and enterolactone (lignan) [58]

1.8 The effect of phytoestrogens on colon cancer

The effect of phytoestrogens has been well-studied as an alternative for hormone replacement therapy [63]. Genistein, coumestrol, and enterolactone have been reported to increase cell proliferation of MCF7 breast cancer cells which are estrogen-dependent [64-66]. However, phytoestrogens also have anti-estrogenic activity depending on their concentration [67]. Genistein suppressed MCF7 cell growth at 100 μM via calpain-caspase and p38 mediated apoptosis, coumestrol inhibited DNA synthesis of MCF7 and enterolactone inhibited E_2-stimulated cell proliferation of MCF7 [66-68]. In colon cancer, there is a strong epidemiological correlation between consumption of phytoestrogens and reduced incidence of colon cancer; intake of fermented soybean or tofu is correlated to a lower incidence of colorectal cancer in Asian populations [69-71]. Animal studies have demonstrated anti-tumorigenic properties of genistein or other isoflavones in colon cancer [72-75]. Genistein suppressed proliferation and induced apoptosis in the small and large intestine. Soy isoflavones reduced colon tumor formation against a high-fat diet and AOM. A previous study from our laboratory showed that genistein (GEN), a phytoestrogen from soybeans, has protective effects on young adult mouse colonocytes (YAMC) via apoptosis *in vitro* [76]. Because ERβ mediates the protective effect of estrogen signaling in colon carcinogenesis, most phytoestrogens could prevent colon cancer development via their higher binding affinity to ERβ than ERα [77]. However, the protective mechanism of estrogen signaling by E_2 and phytoestrogens against colon cancer development is still unclear.

1.9 Immune system and inflammation in the colon

The immune system is essential to protect our body and to repair the damage from an external attack. A variety of immune cells and the molecules they release are involved in immune responses. There are two general strategies of the immune system: the innate and adaptive immune responses (Fig. 1.4). The inborn ability to recognize certain infections is called the innate immune response while the adaptive immune response refers to the immune system that has been educated by an initial exposure to an infection. The innate immune response functions as the primary defense including granulocytes, mast cells, macrophages, dendritic cells, and natural killer cells [78, 79]. When components of infectious particles or infected cells are engulfed and presented by specialized phagocytes such as macrophages or dendritic cells, cells in the adaptive immune response start their role by recognition of the components presented. Adaptive immune responses are relatively slow but produce specific immune cells and antibodies specializing in the attack. T-lymphocytes and antibodies secreted from B-lymphocytes are involved in adaptive immune responses.

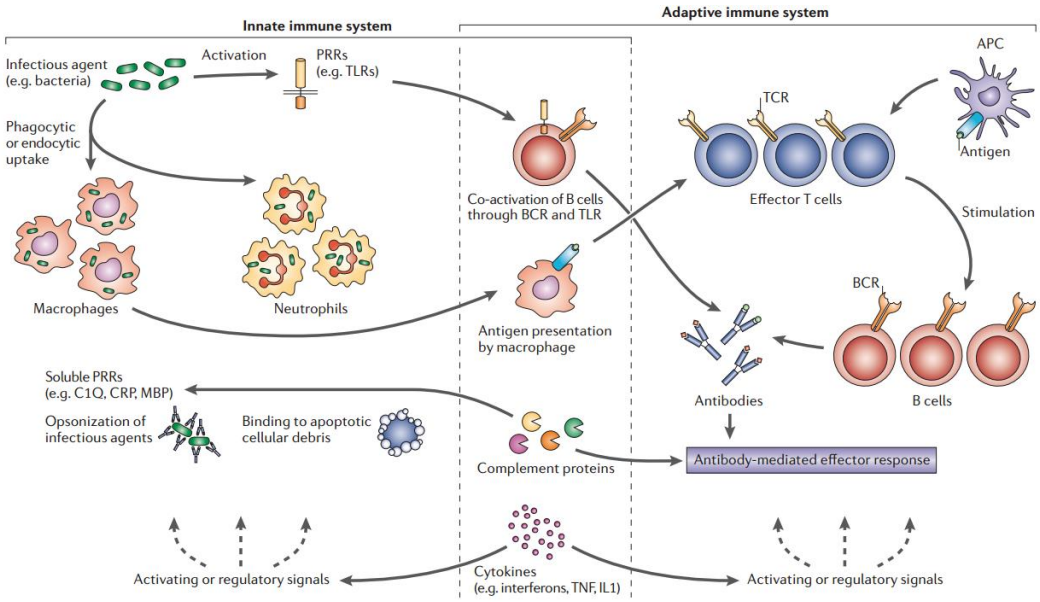

Fig. 1.4 Innate and adaptive immune system [79]

The intestinal immune system is complex because the epithelial layer of the GI tract separates the host from the outside environment (Fig. 1.5) [80]. Paneth cells and goblet cells, among intestinal epithelial cells, release mucus and antimicrobial proteins (AMPs), forming the mucus layer that acts as a primary protective barrier. If the epithelial barrier function is disrupted, the innate and adaptive immune systems are activated. In the colon, the lamina propria in the mucosa layer contains lymphoid vessels as well as a variety of innate and adaptive immune cells, and the mesenteric lymph node supports the intestinal immune system.

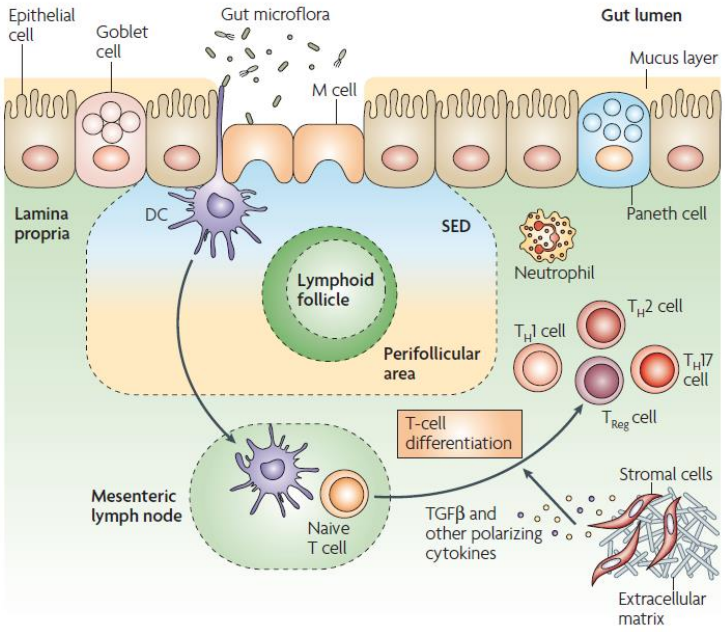

Fig. 1.5 Immune system in the colon [81]

When infected with a virus or bacteria, the body produces many molecules to fight and remove the infection using the immune response, accompanied with heat, pain and swelling [82, 83]. These symptoms and responses refer to inflammation. Inflammation includes the change of damaged cells as well as immune cells. Tissue-resident macrophages and mast cells recognize the infection or injuries that first produce inflammatory molecules such as cytokines and chemokines. These inflammatory mediators recruit adaptive immune cells into damaged tissues and induce differentiation and activation of effector cells such as $CD4^+$ T lymphocytes. After removal of pathogens, tissue recovery and remodeling occurs in the damaged area by activated mesenchymal cells. Though inflammation is essential in recovery from infection and tissue injuries,

inflammation and inflammatory signaling such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is highly correlated with cancer development [84]. Epidemiological studies have demonstrated that chronic inflammation due to chronic infection or autoimmune disease is associated with cancer, and approximately 20% of cancer cases are linked to microbial infection [85].

1.10 Inflammation on colon cancer

Inflammatory bowel disease (IBD) is a disease resulting from improper innate and adaptive immunity in the intestinal epithelial and mucosa [86, 87]. IBD is thought to originate from a combined effect of inherited genetic risk and influence of environmental factors on the immune system, resulting in aberrant inflammatory responses. Several targets have been studied to suppress aberrant inflammation in IBD patients including macrophages and T lymphocytes [88]. Clinical analysis of IBD encompasses two different types: ulcerative colitis (UC) and Crohn's disease (CD). UC primarily affects the colon and rectum, while CD arises in any part of the gastrointestinal tract from the mouth to the anus. UC is a superficial inflammation associated with type 2 T helper lymphocytes (T_H2), and CD causes a deeper ulceration featuring activated type 1 T helper lymphocytes (T_H1) and type 17 T helper lymphocytes (T_H17). To mimic IBD, animal models have been developed using chemicals [89-91]. Dextran sodium sulfate (DSS) is a toxic molecule that causes damage to the intestinal epithelial cells and disrupts the mucosal barrier. The DSS induced colitis model is regarded as a model for UC because of similar features to UC, but some research shows that DSS causes simple

chemical damage rather than chronic inflammation. 2,4,6-trinitro benzene sulfonic acid (TNBS), 2,4-dinitrobenzene sulfonic acid (DNBS), and oxazolone are haptenating agents which bind to proteins of the epithelial cell membrane and induce host immune responses. TNBS induced colitis has common features of CD, including T_H1 activation, while colitis induced by oxazolone results in T_H2 mediated immune response.

Both UC and CD are associated with increased risk of colorectal cancer, and epidemiological studies have shown that a positive correlation exists between IBD and colorectal cancer [92]. Unlike sporadic colon cancers, the mucosa is the major origin of tumor formation and p53 mutation might be required in the early stage of colitis-associated colon cancer (CAC) [93]. Therefore, CAC is an interesting topic in colon carcinogenesis. To mimic CAC, chemically induced animal models have been developed: animal models with injection of AOM and DSS are commonly used, and AOM-TNBS animal models have been reported in some studies for CAC [91, 94].

1.11 $CD4^+$ T cells

An innate immune response caused by infections induces recruitment of immune cells in the adaptive immune response, causing development of effector cells which defend against infections [95]. When an innate immune signal is given, naïve $CD4^+$ T cells, as one of the adaptive immune cells, differentiate into effector T cells including T_H1, T_H2 and T_H17 (Fig. 1.6).

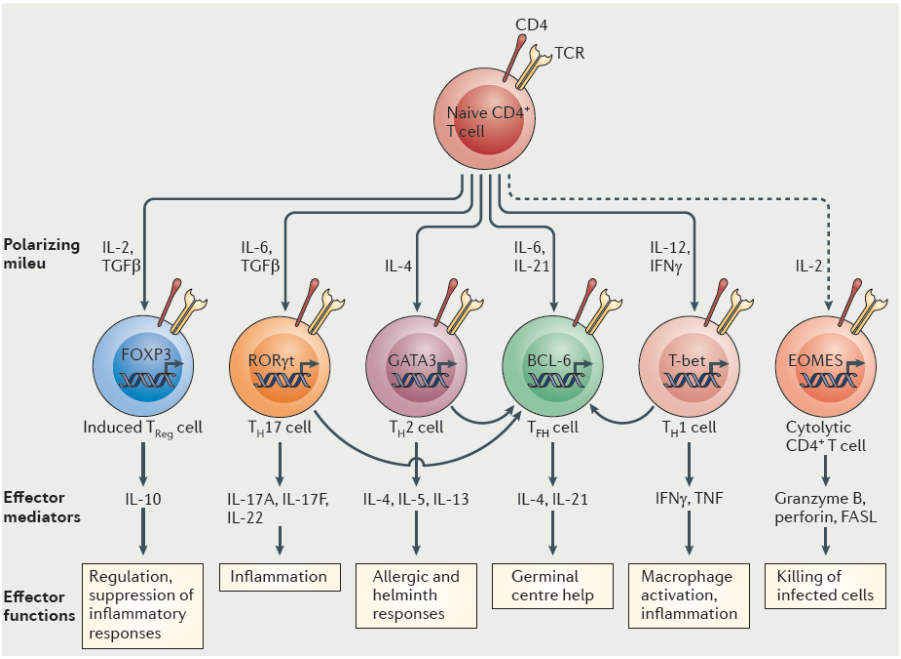

Fig. 1.6 Differentiation of effector CD4$^+$ T cells [95]

Depending on the inflammatory stimulus, cytokines produced from antigen presenting cells are variable, leading to differentiation of specific effector T cells. T_H1 cells are primarily activated when an intracellular infection such as a viral infection occurs, and produce interferon-γ (IFN-γ) and interleukin-12 (IL-12) by expressing T-bet. T_H2 cells are developed during allergic reactions or extracellular stimuli such as helminth parasites, and produce IL-4, IL-5 and IL-13 by expression of GATA3. Follicular T helper cells (T_{FH}) are a subtype of CD4$^+$ T cells that regulate antigen-specific response of B cells, and produce IL-21 by the transcription factor B cell lymphoma 6 (Bcl-6). Regulatory T cells (T_{Reg}) are thought to suppress immune responses for prevention of autoimmune disease and are characterized by production of

IL-6 and expression of FOXP-3 (Forkhead box P3) [96]. T_H17 cells were identified recently and their role is not fully understood [97, 98]. T_H17 cells have been found to play a critical role in autoimmune diseases including IBD. T_H17 cells are associated with high levels of IL-6 and featured by production of IL-17 and expression of RORγt (Retinoid-Acid Receptor-related Orphan Receptor γ t).

1.12 Role of $CD4^+$ T cells on IBD and colon cancer

Because T_H1/T_H17 are associated with CD and T_H2 is linked to UC, effector T cells are potential targets of IBD [87, 88]. Furthermore, those effector T cells and their cytokines are positively correlated to CAC [99]. RAG1 (Recombination activating gene 1) deficient mice, which have no B cells or T cells, have shown lower incidence of CAC in spite of colitis, indicating that adaptive immunity is required for tumor development [100]. The role of T_H1, T_H17 and T_H2 has been studied in CAC [94, 101]. T_H1 associated with IFN-γ has an anti-proliferative property and induces activation of cytotoxic $CD8^+$ T cells, resulting in the removal of dysplastic cells. IFN-γ deficient mice showed increased tumor formation under a chemically induced CAC model, suggesting that T_H1 has a protective effect against CAC. In contrast, IL-4 and IL-13 produced from T_H2 are inducers of cell proliferation and suppress T_H1 differentiation. IL-4 knockout mice have shown lower tumor incidence, indicating T_H2 has a promoting activity on CAC. T_H17 cells are linked to poor prognosis of patients with colon cancer and loss of their secretory cytokine, IL-17, reduced tumor formation in CAC animal models [101]. Furthermore, T_H1 and T_H17 may regulate each other, which have a similar relationship to T_H1 and T_H2

[102]. These data suggested T_H17 influences an increase of CAC risk by induction of cell proliferation and suppression of T_H1. T_H17 activation could result in a high incidence of colon cancer observed in CD patients in spite of T_H1 activation.

1.13 Interleukin-6

IL-6 is one of the cytokines that is associated with the immune response and T_H17 activation [99, 103, 104]. It is produced predominantly from monocytes and macrophages during inflammation and tissue damage as a mediator of the immune response. An increase of the IL-6 receptor or IL-6 itself is observed in colitis, psoriasis and other immune diseases, and IL-6 deficient mice are highly susceptible to infection by a variety of microorganisms. IL-6 plays a central role in animal models of IBD including T- cell transfer in immunodeficient mice or IL-10 deficient mice. The severity of colitis was ameliorated in IL-6 deficient mice, and suppression of IL-6 signaling by the antibody for IL-6 caused a decrease in epithelial cell proliferation, impairing wound healing in colitis [105-107]. These data demonstrate that IL-6, as a central mediator, regulates the immune system and increases cell proliferation to restore damaged tissues.

IL-6 binds to the membrane-bound IL-6 receptor (IL-6R), and IL-6R expression is limited to Hepatocytes and some immune cells including macrophages and some lymphocytes. The complex of IL-6 and IL-6R interacts with glycoprotein 130 (gp130), which is expressed broadly, inducing initiation of signaling. The IL-6 complex activates cell signaling molecules including Janus kinase (JAK), signal transducer and activator of transcription 3 (STAT3) and mitogen activated protein kinases (MAPK) (Fig. 1.7) [108].

JAK phosphorylated by the IL-6 complex induces phosphorylation of STAT3, causing dimerization of STAT3. A dimer of STAT3 acts as a transcription factor, translocates into the nucleus, and modulates expression of genes related to cell growth and survival. Ras is also activated by IL-6 which initiates a signaling cascade leading to activation of MAPK by phosphorylation. In addition, the phosphotidylinositol-3 kinase (PI3K) pathway is activated by phospho-JAK, causing phosphorylation of Akt, which induces dysregulated cell proliferation and survival.

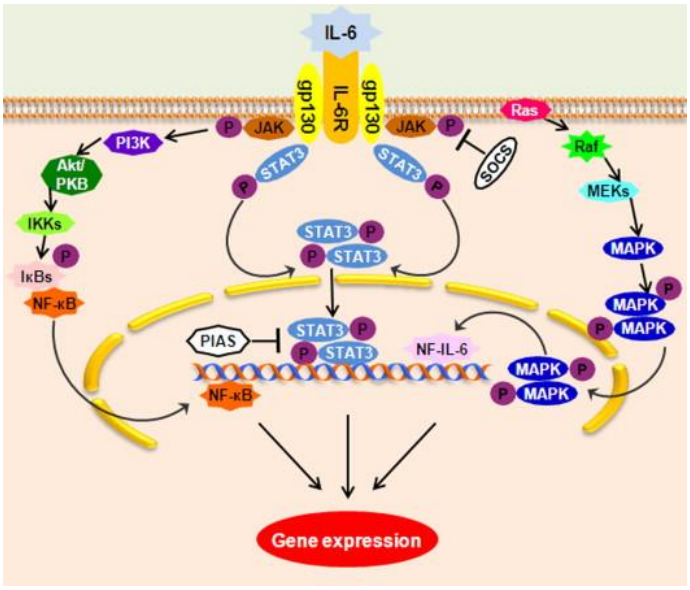

Fig. 1.7 IL-6 signaling [108]

1.14 The role of interleukin-6 on colon cancer

IL-6 is believed to be a tumor promoting factor in several human cancers including lymphoma, melanoma, and breast cancer as well as colorectal cancer [109]. IL-6 may suppress apoptosis, increase cell proliferation and induce endothelial migration which is a key step of angiogenesis. Antibodies against IL-6 ameliorate cancer related anemia and cachexia. Because IL-6 induces differentiation and activation of T_H17, T_H17 increased by IL-6 could participate in tumor formation. The tumorigenic effect of IL-6 is observed in breast epithelial cells; IL-6 expression induced by monocyte chemotactic protein 1 (MCP-1) modified cellular features and led to transformations that made the cells tumorigenic [110]. Studies have shown that the expression of IL-6 is increased in patients with colon cancer. An increased level of IL-6 is positively correlated with cancer stage, metastasis, and poor survival rate of colon cancer patients [111]. Animal studies have demonstrated that IL-6 is associated with CAC [112-114]. IL-6 signaling was required for the survival of the intestinal epithelial cells, and IL-6 and STAT-3 were increased during CAC development. In addition, IL-6 deficiency or suppression of gp130 suppressed CAC formation. These data indicate IL-6 signaling plays an important role in colon carcinogenesis.

1.15 Estrogen signaling on inflammation and CAC

Studies have shown that estrogen could modulate the immune system via disruption of immune cells and changes in cytokine production [115, 116]. ERs, as a major mediator of estrogen signaling, are expressed in most immune cells, and their

expression is slightly different between pre- and post-menopausal women [117]. ERα is highly expressed in monocytes and $CD4^+$ T cells, while ERβ is expressed more than ERα in B cells. In contrast, $CD8^+$ T cells expressed both ERs at very low levels. Furthermore, E_2 changes cytokine levels including NFκB, IL-6, IFN-L, and IL-10. The effect of E_2 is heterogeneous depending on cell type and estrogen level. For example, E_2 induces IFN-u secretion from T cells while suppressing IFN-r from macrophage and dendritic cells.

The role of estrogen signaling on IL-6 signaling remains unclear. E_2 inhibits IL-6 production during pregnancy but has no influence on IL-6 in post-menopausal women. ERs inhibit IL-6 gene expression by disruption of NFκB transactivation [118]. In mammary epithelial and breast cancer cells, growth-inhibitory effects of ERβ are repressed through activation of MAPK and PI3K, which are targets of IL-6 signaling [119]. Studies with a variety of cells demonstrated that estrogen signaling targets several IL-6 downstream genes such as STAT3, NFκB and suppressor of cytokine signaling 3 (SOCS3) [120-122]. These data indicate that there is an interaction between estrogen signaling and IL-6 signaling, and the relationship between the two signaling pathways could influence inflammation and the immune system.

It has been reported that estrogen signaling impacts autoimmune diseases and inflammation. E_2 has protective effects in animal models of arthritis though this result was not repeated in clinical studies with rheumatoid arthritis patients. E_2 shows a suppressive effect against brain inflammation in an experimental autoimmune encephalitis (EAE) animal model. This effect is mediated by ERα. In IBD patients,

epidemiological data have demonstrated that the symptoms of IBD in female patients with CD fluctuate with the estrous cycle, suggesting the level of estrogen plays a critical role in CD [123]. However, this effect of estrogen was not observed in UC patients. Animal studies with acute colitis showed the protective effect of E_2 via suppression of cytokines and decrease of macrophages [124, 125]. However, protection by E_2 on colitis is conflicting with different colitis models, DSS and DNBS. E_2 treatment exacerbated inflammation in DSS-induced colitis while ameliorating the severity of colitis by DNBS through a decrease in the expression of IFN- and IL-13. These results suggest that the protective role of E_2 could influence the specific type of colitis, CD.

Data regarding the influence of estrogen signaling is also conflicting in CAC. Saleiro and her colleagues found that loss of ERβ enhanced tumor formation by AOM and DSS, suggesting that the protective role of estrogen signaling against CAC is mediated by ERβ [126]. Previous data from our laboratory have shown that E_2 treatment suppressed ACF formation in CAC induced by AOM and DSS via suppression of ERα [55]. In contrast, Heijmans' group has observed that E_2 promotes tumor formation via an increase of cell proliferation and IL-6 in the AOM/DSS model. Furthermore, they found that loss of either ERα or ERβ reduced polyps, suggesting both ERs play a role in tumorigenesis of CAC. The differences between these experiments yielding contradictory results are ovariectomy (OVX), the time point of E_2 administration, and the dose of DSS.

Several studies have reported that phytoestrogens have anti-inflammatory properties in IBD and CAC. GEN and soy isoflavones suppressed colitis by DSS or

TNBS via modulation of cytokines and cyclooxygenase-2 (COX-2) [127, 128]. In addition, DIM inhibited PGE_2, nitric oxide (NO), and proinflammatory cytokines, resulting in a decrease of DSS-induced colitis and AOM/DSS induced CAC [129].

1.16 The role of p53 in colon cancer

p53, a tumor suppressor gene, regulates apoptosis and proliferation in cancer cells as well as normal cells. It becomes active in response to a variety of stimuli including DNA damage, metabolic stress and oncogene activation [130, 131]. Its dysfunction has a powerful impact on cancer development, and mutation or deletion of p53 is observed in 60-70% of colorectal cancers [132, 133]. 75% of somatically mutated p53 genes are found as missense mutation while nonsense or silent mutations of p53 are only found in 25% of somatic mutations in human cancers [134, 135]. Missense mutation of p53 replaces a single amino acid followed by stable expression in cancer cells not loss of expression. Most p53 mutations occur within exon 5-8, which is the DNA binding domain. Mutated p53 interferes with the activity of wild type p53 because p53 normally acts as a tetramer and mutated p53 has a dominant negative effect. However, it is hard to clarify the role of p53 in colon cancer using p53 knockout mice because the effect of p53 mutations induce a variety of cancers with a high death rate at an early age [17].

Recently a genetically modified mouse model has been developed in which p53 is knocked out in the epithelial cells of the GI tract using the Cre/loxP system [136, 137]. Cre is a site-specific DNA recombinase found in bacteriophage P1. Cre recognizes the

loxP site, which is a 34 base-pair DNA sequence, and excises DNA between two loxP sites, causing loss of a specific gene. To knockout p53 in the intestinal epithelial cells, two mouse lines are required: villin-Cre mice and $Tp53^{F/F}$ mice. In villin-Cre mice, Cre is expressed in the intestinal epithelial cells only because Cre expression is under the control of the villin promoter, which is activated in the intestinal epithelial cells only [138]. In $Tp53^{F/F}$ mice, two loxP sites flanked the intron of p53 causing deletion of exon 2-10 of p53. By mating villin-Cre mice and $Tp53^{F/F}$ mice, a mutant mouse line expressing functional p53 in all cells except for the intestinal epithelial cells has been developed, which is called $Tp53^{\Delta IEC}$. No physiological changes or infertility issues were observed in $Tp53^{\Delta IEC}$ mice, but $Tp53^{\Delta IEC}$ mice were more susceptible to colon tumor formation by AOM.

The relationship between estrogen signaling and p53 has been studied in various types of cancers but the mechanism is not fully understood. Estrogen signaling enhances the activity of p53 and regulates the expression of target genes of p53 including the p53 upregulated modulator of apoptosis (PUMA), B-cell lymphoma 2 (Bcl-2), and Bcl-2 associated X protein (BAX) [139-143]. Previous studies in our laboratory have shown that p53 is a mediator of estrogen signaling and induces apoptosis via regulation of mouse double minute 2 homolog (Mdm2) expression and downstream genes [143]. Furthermore, E_2 suppressed ACF formation was decreased in p53 heterozygous mice compared to wild type mice [144]. These data suggest that p53 plays an important role in colon carcinogenesis as a signal transducer for estrogen signaling.

CHAPTER II

THE ESTROGENIC EFFECT OF TRIGONELLINE AND 3,3-DIINDOLYMETHANE ON CELL GROWTH IN NON-MALIGNANT COLONOCYTES

2.1 Introduction

Colon cancer is the third most prevalent cancer with over 100,000 newly diagnosed cases and is estimated to be the third most common cause of cancer related deaths in the United States [1]. Studies show that pre-menopausal women have a reduced risk of colon cancer compared to age matched men while post-menopausal women have a poorer survival rate than pre-menopausal women [1, 145, 146]. Furthermore, data from 18 epidemiological studies demonstrated that hormone replacement therapy in post-menopausal women decreased the risk of colon cancer by 20 % compared to women who never used hormone replacement therapy, indicating a relationship between estrogen signaling and colon cancer [54]. The protective role of estrogenic action in colon cancer has been supported by animal experiments [51, 53, 147, 148]. The treatment of estradiol (E_2) suppressed the formation of aberrant crypt foci (ACF) in a carcinogen-induced mouse model and disruption of estrogen signaling enhances colon carcinogenesis [51, 53].

Data suggest estrogen receptor (ER) β is the primary modulator of estrogen's ability to suppress colon tumor development [53, 126]. Unique ER proteins, ERα and β, are produced from two genes and each ER is differently expressed in the body's tissues, including the gastrointestinal tract (GI tract) [149]. ERβ is the predominant form of ER

in the GI tract, and lower expression of the protein is correlated to high grade and large tumors in the colon [47, 150, 151]. A previous report from our laboratory showed that when colon tumors form, there is a novel shift in ER expression where ERβ levels decrease accompanied by a large enhancement of ERα protein expression within tumors [55]. ERβ specific agonists induced apoptosis and suppressed inflammation in the colon epithelium [75, 126]. Furthermore, ERβ suppresses micro-RNAs associated with oncogene activation in colon cancer cells [152]. Collectively, these data suggest that the activation of estrogen signaling mediated by ERβ has a protective effect against colon carcinogenesis.

While having similar chemical structures as compared to endogenous estrogens, a variety of phytoestrogens have been reported including isoflavones and flavones, and they are known to have higher binding affinity to ERβ than ERα [77, 153]. Genistein, a phytoestrogen isolated from soybeans, has anti-proliferative effects and pro-apoptotic effects on the epithelial cells of the small and large intestine in ovariectomized rats [75]. Previous data from our laboratory have demonstrated that extracts containing Sorghum phenolic compounds suppress growth of young adult mouse colonocytes (YAMCs), and this effect was the result of estrogenic action [154]. Studying the chemopreventive effects of phytoestrogens on colonocytes could give new alternatives to endogenous estrogens to control the risk of colon cancer. Interestingly, recent studies have demonstrated molecules found in plants, with distinct structures from endogenous estrogens or other commonly studied phytoestrogens, have estrogenic activity [61]. Unique to these compounds is that in classic receptor binding assays they do not displace

E_2 at any concentration, suggesting they do not need to bind in an ER binding pocket to elicit an estrogenic response [61, 62]. In this study, we chose two phytoestrogens, Trigonelline (Trig) and 3,3-Diindolylmethane (DIM) (Fig. 2.1). Trig and DIM have distinctly different chemical structures compared to other phytoestrogens or endogenous estrogens, and more importantly their mechanism to modulate estrogen signaling is not fully understood. Trig is the niacin-related molecule isolated from coffee beans and it has protective properties against diabetes and obesity [155, 156]. A previous report demonstrated Trig has an estrogenic effect in estrogen-dependent human breast cancer (MCF7) cells though it did not bind to ERs directly via the binding domain [62]. Trig can modulate the transcriptional activity of ER inducing ER-mediated target genes. However, the role of Trig in a predominant ERβ expressing tissue remains unknown and its effect on non-malignant colonocytes has never been studied. DIM is the derivative of indol-3-carbinol abundant in *Brassica* vegetables [157, 158]. Several studies have suggested DIM can suppress the growth of prostate cancer and colon cancer, and it functions as a modulator of the androgen receptor (AR) and the ER [129, 159-161]. DIM has been reported as a new class of ERβ agonist modulating ER binding partners [61]. Still, the effects of DIM on non-malignant colonocytes remain unclear, especially via estrogen signaling. In this study, we examined the effect of Trig and DIM on the cellular physiology of non-malignant colonocytes and the role of estrogen signaling on these effects.

2.2 Materials and methods

2.2.1 Reagents

17β-Estradiol (E_2), trigonelline (Trig) and 3,3-diindolylmethane (DIM) were purchased from Sigma-Aldrich. ICI 182,780 (ICI) was purchased from Tocris bioscience. Reagents were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

2.2.2 Cells

Young adult mouse colonocyte (YAMC) bleo/neo cells were provided by Dr. Hartmut Land (University of Rochester Medical Center). For regular maintenance, cells were cultured in RPMI1640 (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 in the permissive condition, 33℃, 5% CO_2 with 5 units interferon-γ (IFN-γ). For the experiments, cells were transferred to media containing 10% charcoal-dextran stripped FBS, 0.1% ITS and 1% gentamicin 48 h before the experiments. During the experiments, cells were placed at non-permissive condition, 39 ℃ with 5% CO_2.

2.2.3 Cell growth assay

Cells were seeded at a concentration of 1.5×10^4 cells/well in 6-well plates (Grenier bio-one) and grown at the permissive condition with IFN-γ for 24 h. The cells were treated with E_2, Trig, or DIM at the appropriate concentrations or 0.1% DMSO as a negative control and transferred to non-permissive condition for 96 h. 1 nM E_2 was treated as a positive control. The concentrations of Trig were 1, 10 or 100 pM, and DIM was utilized at 1, 10 or 100 nM. 48 h after the first treatment, media was replaced with

fresh media at the same concentration as treatments or 0.1% DMSO. For ICI effect, 1 µM ICI was co-treated with 1 nM E_2, 100 pM Trig or 100 nM DIM. At the end of 96 h of treatment, cells were trypsinized and collected for counting. Cell numbers were counted using a Beckman Coulter particle counter Z1. 20 µl of cells were diluted in 10 mL Isotone II diluent (Beckman Coulter) and samples were counted in triplicate. Three wells per treatment were used and three replicate experiments were conducted.

2.2.4 Flow cytometry

Cells were seeded and grown at the same conditions as the cell growth assay. Cells were collected after trypsin treatment and supernatants were removed. After washing in ice-cold 1X PBS, cells were fixed with 100% ethanol at -20℃ overnight. Fixed cells were stained with propidium iodide staining solution (50 µg/ml propidium iodide, 200 µg/ml DNase-free RNase, 4 mM sodium citrate and 0.1% Triton X-100) at room temperature (RT) for 30min. 2×10^4 live cells were analyzed using an Accuri C6 flow cytometer (BD Bioscience) for cell cycle distribution.

2.2.5 Caspase-3 activity

Cells were seeded and grown at the same conditions as the cell growth assay. Cells were trypsinized, collected and washed with PBS twice. Caspase-3 activity was measured using the manufacture's protocol for the EnzChek Caspase-3 assay kit #2, Z-DEVD-R110 substrate (Molecular Probes). Briefly, cells were lysed with 50 µl of 1X cell lysis buffer and set on ice for 30 min. Lysed cells were centrifuged at 5000 rpm for 5 min and 50 µl supernatants were transferred into a 96 well plate (BD Bioscience). 50 µl of 2X substrate was added to each well and the plates were incubated at RT for 30 min

in the dark. Fluorescence with 496 (excitation)/ 520 (emission) nm was measured at 15 min intervals.

2.2.6 Luciferase assay

Cells were seeded at a concentration of 4×10^4 cells/well in 12 well plates (Grenier Bio-One) and grown under permissive conditions with IFN-γ for 24 h. The ERE-TATA luciferase plasmid was provided by Dr. Greene (The University of Chicago). The pRL-TK vector (Promega) was utilized as an internal transfection control. Plasmids were transfected into cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Briefly, 0.4 µg of ERE-TATA plasmid and 0.2 µg of Renilla were co-transfected in each well for 4 h. Cells were treated with 1 nM E_2, 100 pM Trig, 100 nM DIM or 0.1% DMSO for 20 h. After the cells were lysed, luciferase assays were performed using a dual-luciferase assay system (Promega) by following the recommended protocol. Luminescence was determined using an Infinite M200 Tecan plate reader.

2.2.7 RNA extraction and Real-time PCR

Cells were seeded at a concentration of 1.4×10^5 cells/dish in 100 mm dishes (Corning) and grown at permissive conditions with IFN-γ for 24 h. The cells were treated with 1 nM E_2, 100 pM Trig, 100 nM DIM, or 0.1% DMSO for 24 h.

RNA was isolated using the manufacture's protocol for TRIzol Reagent (Invitrogen). In brief, 1 ml TRIzol reagent was added to trypsinized cells to lyse the cell membrane. The addition of 200 µl of chloroform separated the organic layers from lysed the cells, and the aqueous phase was transferred to a new tube. RNA was precipitated by

adding 500 µl of isopropanol. After washing with 70% ethanol, the pellet was air-dried and dissolved in RNase-free water and RNase was inactivated at 55-60℃. Isolated RNA was stored at -70℃.

cDNA synthesis was performed with the Transcriptor First Strand cDNA synthesis kit (Roche) using the manufacturer's protocol. In brief, 1 µg of RNA was incubated at 65℃ for 10 min with 1 µl oligo dT primer and 2 µl random Hexomer primer. After the addition of 4 µl reaction buffer, 0.5 µl RNase inhibitor, 2 µl dNTP and 0.5 µl reverse trancriptase, thermo cycling was performed: 25℃ for 10 min, 50℃ for 60 min and 85℃ for 5 min. cDNA was stored at -20℃.

Real-Time PCR was performed with FastStart Universal SYBR Green Master mix (Roche). The sequence of the PCR primers were as follows: Bcl-2 (Forward: ATC TTC TCC TTC CAG CCT GA, Reverse: TCA GTC ATC CAC AGG GCG AT), c-Myc (Forward: GCC CAG TGA GGA TAT CTG GA, Reverse: ATC GCA GAT GAA GCT CTG G), c-Myb (Forward: TGT CAA CAG AGA ACG AGC TGA, Reverse: CAC AGA ACC ACA CTT GCA GC), Mdm2 (Forward: TGT CTG TGT CTA CCG AGG GTG, Reverse: TCC AAC GGA CTT TAA CAA CTT CA), CCND1 (Forward: GCG TAC CCT GAC ACC AAT CTC, Reverse: ACT TGA AGT AAG ATA CGG AGG GC) and 18S rRNA (Forward: TCA AGA ACG AAA GTC GGA GGT, Reverse: GGA CAT CTA AGG GCA TCA CAG). 18S rRNA was utilized as an internal control. RT-PCR was run on a Bio-Rad iQ5 thermocycler: 95℃ 10 min, 45 cycles of 15 sec at 95℃ and 30 sec at 60℃.

2.2.8 Statistical analysis

The data are expressed throughout as the means ± SEM, which was calculated from at least three different experiments. The statistical significance among the test groups was determined by one-way ANOVA. A p-value of less than 0.05 was considered significant.

2.3 Results

2.3.1 Trig and DIM suppress the cell growth of YAMCs

The chemical structures of Trig and DIM are shown with E_2 in Fig. 2.1. Those molecules have a distinct structure from well-characterized phytoestrogens as well as estrogen. To investigate the effect of Trig and DIM, we examined the growth of YAMCs treated with 1-100 pM Trig or 1-100 μM DIM for 96 h (Fig. 2.2). 1 nM of E_2 was utilized as a positive control and 0.1% DMSO was used as a negative control. Both Trig and DIM suppressed cell growth significantly compared to control by over 20% and the effect was stronger than E_2.

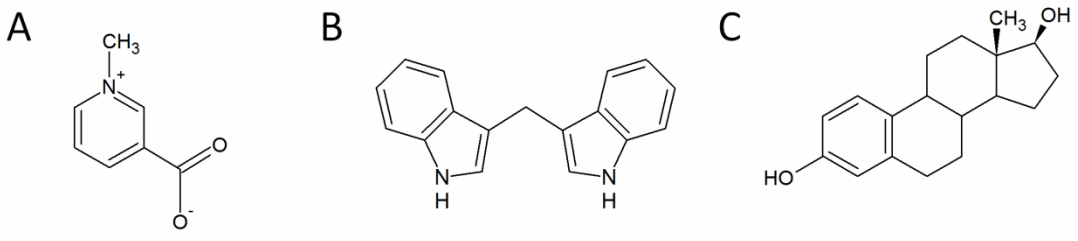

Fig. 2.1 The structure of Trigonelline (Trig) and 3,3-Diindolylmethane (DIM) A. Trig, B. DIM, and C. 17β-Estradiol (E_2) [156, 157]

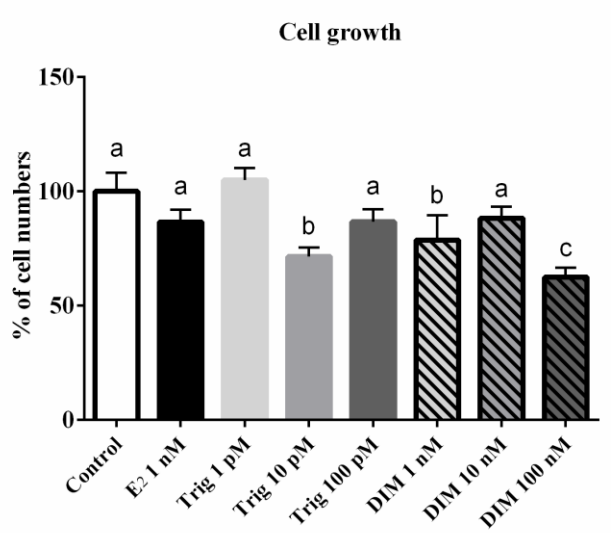

Fig. 2.2 The effect of Trig and DIM on cell growth of YAMCs. Data are expressed as percentage of cell numbers of the DMSO control group. Values are means (n = 9) ± SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

2.3.2 Trig and DIM modulate cell cycle progression and apoptosis in YAMCs

To examine the mechanism of the change in cell growth by Trig and DIM, we measured the cell cycle distribution and apoptosis (Fig. 2.3) in YAMCs after treatment of 1-100 pM Trig or 1-100 nM DIM with 1 nM E_2 as a positive control. E_2 was a weak modulator of cell cycle distribution through an increase of cells in G_2/M phase and a decrease of G_0/G_1 phase cells (Fig. 2.3A). Trig and DIM also had a significant effect on cell cycle distribution. Interestingly, Trig and DIM increased the G_0/G_1 and S phase cells not G_2/M cells.

E_2 elevated apoptosis by 40% compared to control and Trig also increased it by 30% significantly, indicating that Trig may work in part to reduce YAMC number by inducing apoptosis (Fig. 2.3B). DIM had no effect on caspase-3 activity.

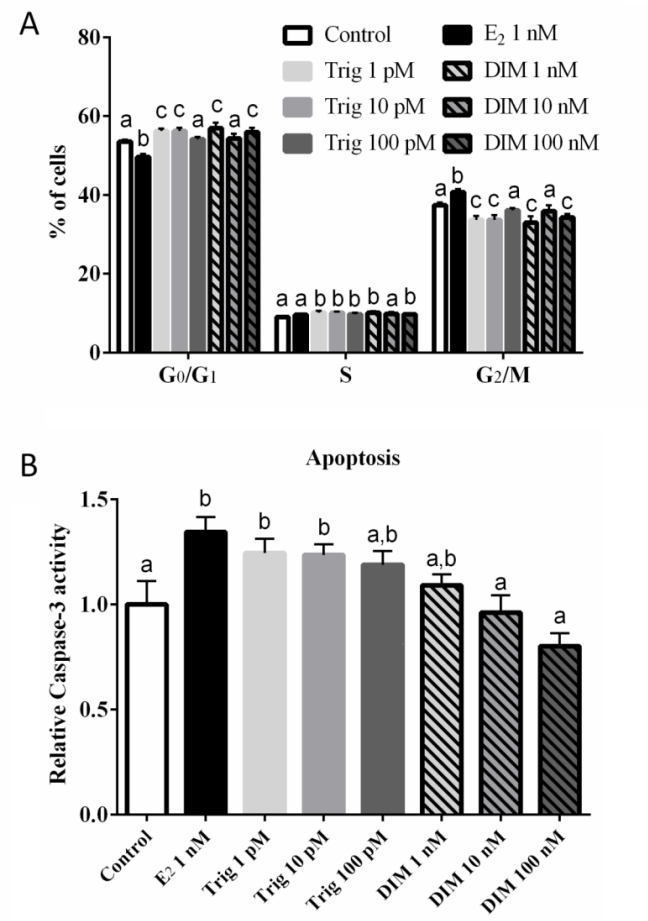

Fig. 2.3 The effect of Trig and DIM on cell cycle distribution and apoptosis. Cells were treated with 1 nM E_2, 1-100 pM Trig or 1-100 nM DIM, and treatments were replaced 48 h later. A. Cell cycle distribution was analyzed by Flow cytometry after propidium iodide staining. B. Caspase-3 activity was estimated by fluorescence. E_2 was utilized as a positive control. Data are presented as percentage of vehicle control. Values are means (n = 9) ± SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

2.3.3 The effect of Trig and DIM on suppression of YAMCs is associated with estrogen signaling

The suppression of YAMC growth by E_2 is associated with ER mediated signaling. To examine if the effects of Trig and DIM involved estrogen signaling, we co-treated cells with ICI, an estrogen receptor antagonist (Fig. 2.4A). When we co-treated, 1 μM of ICI with Trig, DIM and E_2, ICI suppressed the effect of Trig and E_2 on cell growth. However, decrease in cell number by DIM was not influenced by treatment of ICI.

To confirm the influence of Trig and DIM on ER transactivation, the cells were transfected with luciferase plasmids containing ERE-TATA (Fig. 2.4B). Renilla luciferase was used as an internal control. E_2 elevated luciferase activity significantly as we expected. Interestingly, DIM increased the reporter activity while Trig did not. It shows that DIM has effects on estrogen signaling but its action is not associated with classical ligand binding of ERs.

2.3.4 Trig and DIM regulate the gene expression related to estrogen signaling

The effects of Trig and DIM on the downstream target genes of estrogen signaling and cell growth were examined using RT-PCR (Fig. 2.5). Since Trig and DIM showed suppression of cell growth via cell cycle analysis and apoptosis, we chose genes related to these cellular processes, cell cycle and apoptosis. Bcl-2 and Mdm2 are linked to apoptosis (Fig. 2.5A and B). CCND1, c-Myc and c-Myb are regulators of cell cycle progression (Fig. 2.5C, D and E). E_2 lowered the expression of all genes but not

significantly. Trig decreased c-Myc expression significantly (Fig. 2.5C). In contrast, DIM significantly decreased the expression of all genes except for CCND1.

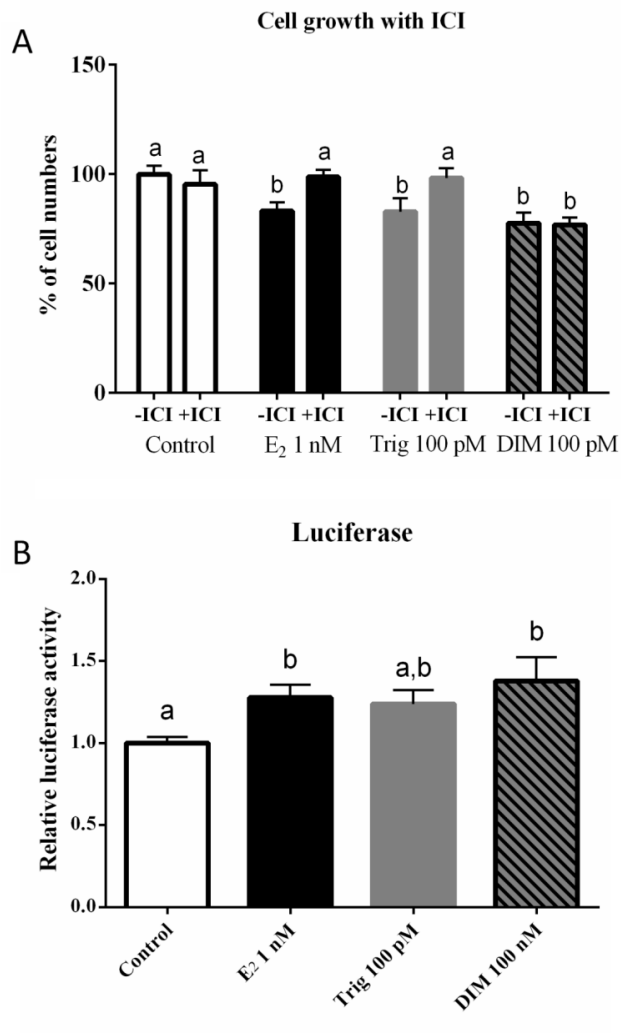

Fig. 2.4 The effect of Trig and DIM on estrogen signaling. A. 1 μM ICI 182, 780 was co-treated with Trig or DIM for 96 h to interrupt estrogen signaling on cell growth of YAMCs. B. Cells transfected by ERE-luciferase plasmids with renilla luciferase plasmids were treated with 100 pM Trig or 100 nM DIM for 20 h. Luciferase activity was estimated by luminometer. Renilla luciferase was utilized for internal normalization. Data are presented as fold change of vehicle control. Values are means (n = 9) ± SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

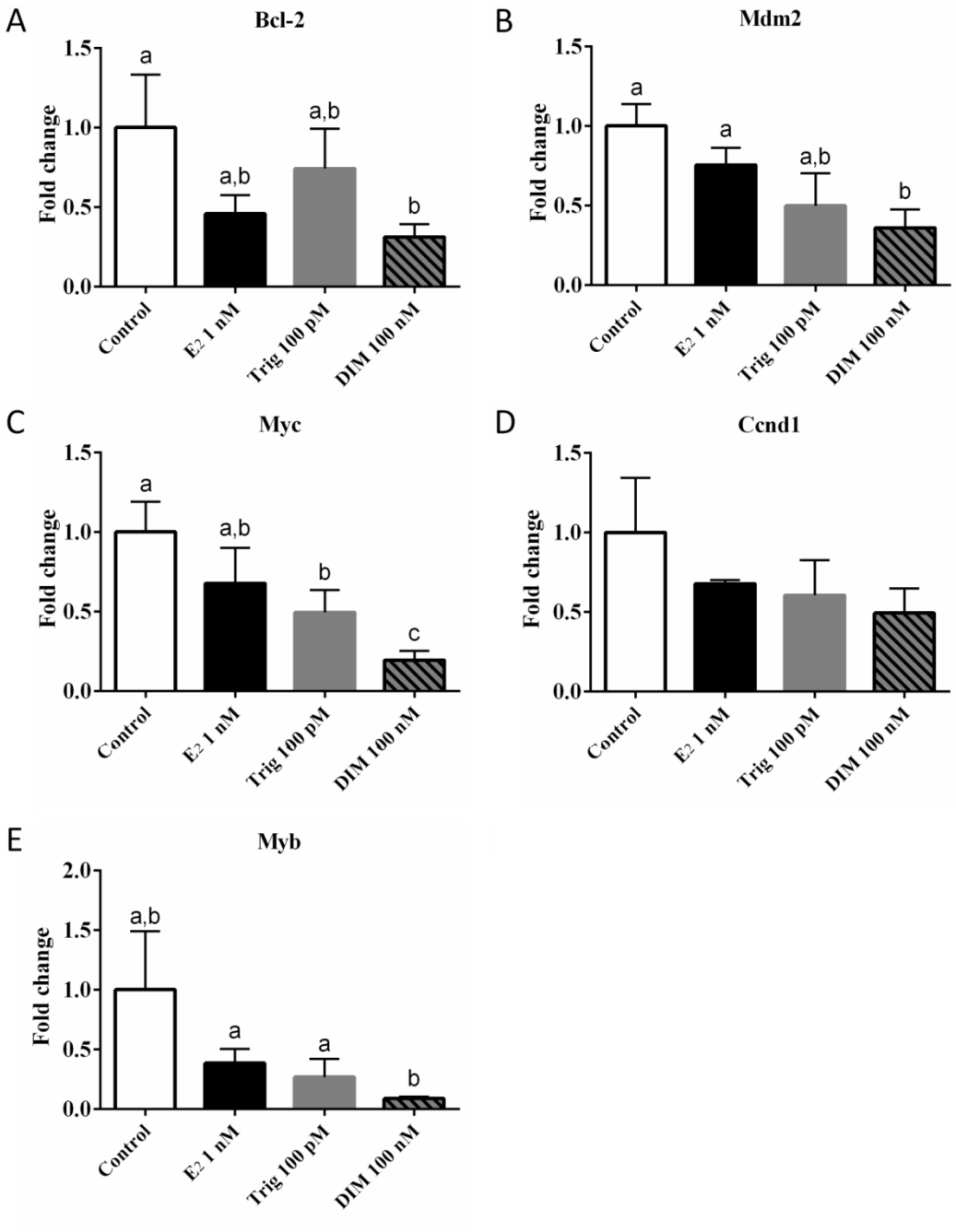

Fig. 2.5 The effect of Trig and DIM on gene expression. Cells were treated with 1 nM E_2, 100 pM Trig or 100 nM DIM for 24 h. A. Bcl-2, B. Mdm2, C. c-Myc, D. CCND1, and E. c-Myb were measured by SYBR green expression. The gene expression was normalized by 18S. Values are means (n = 9) ± SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

2.4 Discussion

Though epidemiological data have suggested a positive correlation between estrogen exposure and reduced colon cancer risk, use of traditional hormone replacement therapy has become controversial [54, 162, 163]. As such, understanding the actions of plant-derived compounds that mimic estrogenic action in the colon is an attractive alternative to suppress the risk of colon cancer [77, 164]. Though some data suggest that high concentrations of phytoestrogens suppress colon cancer growth [36], *in vitro* studies with colon cancer cell lines cannot predict the role of these compounds on non-transformed colonocytes. Therefore, the focus of the presented studies was to elicit the action of two novel phytoestrogens on non-malignant colonocytes.

Trig is a niacin-related molecule commonly found in coffee beans. Little is known about the physiological effects of Trig but it has been shown to have anti-diabetic properties [155, 156]. A previous study from our laboratory reported that Trig elicits an estrogenic response in breast cancer cells, but it does not compete with E_2 to bind to the binding domain of the ER [62]. In the present study, we evaluated the estrogenic potential of Trig in YAMCs. Treatment with the compound suppressed cell growth and this effect was inhibited by the co-treatment of ICI, suggesting that activation of the ER is necessary for Trig to modulate cell growth. ICI is a well-described antagonist of ERs [165, 166]. With a similar structure to E_2, ICI binds to the ligand binding pocket of ERs and disrupts the dimerization of proteins, resulting in the inactivation of the AF1 and AF2 domains. Our observation that ICI attenuated the suppression of cell growth by Trig, suggests the possibility that the activation of estrogen signaling by Trig involves

enhancement of the dimerization of the ERs or the activation of AF domains. Several pathways have been known to activate ERs independent of ligand binding such as phosphorylation of the proteins [167]. Furthermore, niacin, the precursor of Trig, is known as an activator of EGF receptor resulting in ERK (Extracellular signal-regulated kinases) activation [168]. Additional studies are needed to clearly elucidate how Trig modifies the activity of ERs.

Surprisingly, Trig treatment did not significantly induce ER transcriptional activity, and except for c-Myc, the expression of ER responsive genes was not significantly changed by Trig. Considering our previous data demonstrated that Trig elevated ERE activity in MCF7 cells which preferentially express ERα, these data suggest that in YAMCs that exclusively express ERβ, the classical ER transcriptional mechanism mediated through an ERE is not likely the primary mode of action for Trig [47, 62]. Furthermore, Trig induced changes in the cell cycle distribution of YAMCs differently from E_2 even though it elevated apoptosis in a similar manner as E_2. In contrast to E_2, Trig decreased the expression of c-Myc, which is one of the major modulators of the G_1/S check point in cell cycle progression and E_2 has been shown to modulate its expression [169, 170]. Our data suggests that Trig can arrest the cell cycle in G_1/S phase likely via reduced expression of c-Myc, resulting in suppression of cell growth. Furthermore, increased caspase-3 activity by Trig treatment indicates that this compound could induce apoptosis, predicting a suppressive effect of colon carcinogenesis.

ERs modulate cell physiology via genomic pathways and non-genomic pathways [45]. In genomic pathways, ERs translocate into the nucleus and alter gene expression. This regulation of gene expression is mediated by ERE in the classical pathway. Still, ERs change the expression of genes having no EREs via alternative DNA sequences including AP-1 sites, Sp1 sites and cyclic AMP-response elements (CREs). Basically, ICI induces the degradation of ERs via ubiquitination after ICI binds to the binding pocket of ERs [166]. We observed that ICI interrupted the effect of Trig on cell growth of YAMCs but found no significant change on the transcriptional activity of ERs by Trig. Present data suggest that ERs are essential for the effect of Trig and the classical pathway activated by ERs are not involved in the Trig effect because we only used traditional EREs to estimate the transcriptional activity. However, we cannot elucidate the way to clarify the transcriptional activity of ERs in a non-classical pathway because of numerous candidate DNA sequences. Instead, we measured mRNA expression of genes known as targets of ERs. DIM has been shown to be a primary metabolite of indol-3-carbinol produced by a condensation reaction when the compound is exposed to stomach acid [171]. Indol-3-carbinol is a reported chemopreventive agent in a variety of cancers including the liver and colon. Additionally, it appears to play a protective role in hormone related cancers as well, including malignancies in the breast and prostate [172]. Recent studies have suggested orally administered indol-3-carbinol is converted to DIM, which works as an active molecule to suppress tumor formation and modulate cellular signaling including via aryl hydrocarbon receptor (AhR), mammalian target of rapamycin (mTOR) and nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-κB) related pathways [157]. DIM has been reported as a new class of ERβ selective agonists which does not bind to the binding pocket of ERβ but activates ERβ via recruiting the steroid receptor co-activator (SRC)-2 [157, 159]. In our study, we found that DIM modulates transcriptional activity of ER in YAMCs. Interestingly, DIM arrested the cell cycle in the G_0/G_1 phase but did not induce caspase-3 activity. DIM decreased the expression of genes related to the cell cycle and apoptosis except for CCND1 which is a cell cycle modulator. All genes measured are known to be modulated by ER [48, 62, 143, 173-175]. Bcl-2 is an anti-apoptotic protein that has two EREs in its promoter [174]. c-Myb, an oncogene, is associated with a variety of cancers including colon cancer and ERα agonists are known to elevate c-Myb expression [175]. Mdm2 is a target gene of p53, a tumor suppressor gene as well as a main modulator of the cell cycle and apoptosis [130]. Previous studies from our laboratory have demonstrated that Mdm2 is decreased by E_2 in YAMCs [143]. Gene expression, following exposure to DIM, was similar to E_2 for these selected targets. Collectively with the ERE transcriptional activity assay, these data suggest ER-mediated signaling is similar for DIM as that of E_2 in non-malignant colonic epithelia. Since Bcl-2, Mdm2, c-Myc, and c-Myb play an important role in the decision of cell fate, down-regulation of these genes suggest that DIM suppresses cell growth by modulation of cell cycle progression. Surprisingly, CCND1 expression was not significantly changed by DIM though CCND1 is known to be modulated by ERs [48]. Unlike what was observed with Trig, ICI did not block the effect of DIM on cell growth of YAMCs. These data suggest that the primary mode of action for DIM to enhance ER co-activation is more than the classical ER

activation via ER dimerization and AF activation. Further studies will explore other mechanisms including recruitment of cofactors such as SRC-2 as previously reported [159].

In summary, we found that Trig and DIM alter cellular physiology of YAMCs and both compounds modulate cell cycle progression. Though both molecules have estrogenic effects, the way in which they modulate ER signaling seems to be different. The effect of Trig on cell growth was blocked by ICI suggesting a functional ER is critical to its actions. DIM treatment resulted in ER transcriptional activity and estrogen mediated gene expression similar to E_2. Collectively, these data suggest that compounds that demonstrate estrogenic potential are likely to result in various and unique physiological responses and changes in ER signaling in non-malignant colonocytes. We have previously shown that the YAMC model serves as a good predictor of physiological responsiveness of colonocytes *in vivo*. Cellular changes observed following treatment with Trig and DIM provide compelling evidence that Trig and DIM, though novel in structure compared to other phytoestrogens, are likely to prove to be key dietary components that may modulate colonocyte physiology and ultimately protect these cells against carcinogenesis.

CHAPTER III

ESTRADIOL AND GENISTEIN MODULATE CELL GROWTH OF NON-MALIGNANT COLONOCYTES INDUCED BY INTERLEUKIN-6.

3.1 Introduction

Inflammatory bowel disease (IBD) is an autoimmune disease associated with uncontrolled innate and adaptive immunity in the gastrointestinal tract (GI tract), resulting in chronic inflammation [176]. Though the cause of IBD is still not understood, IBD is thought to result from an aberrant immune response with increased abnormal T cell activity [86]. As IBD is related to the increase of colorectal cancer and its critical features are shared with colorectal cancer, it is an important risk factor of colitis associated colon cancer [92, 177]. Ulcerative colitis (UC) and Crohn's disease (CD) are two primary forms of IBD based on clinical features and histopathology [93, 176]. While UC is limited to the mucosa influence on the colon, CD includes whole transmural lesions in the entire GI tract. Depending on the type of IBD, the immune system and cytokines involved are different [178]. CD is an immune disease mediated by type 1 T helper cells (T_H1)/type 17 T helper cells (T_H17), which are associated with interleukin-6 (IL-6), interferon γ (IFN-γ) and tumor-necrosis factor α (TNFα). In contrast, type 2 T helper cells (T_H2) play a main role in UC with IL-13 and transforming growth factor β (TGFβ).

IL-6 is known as a pro-inflammatory cytokine and is secreted in response to tissue damage [103]. Blocking IL-6 signaling by anti-IL-6R antibody suppressed

inflammation, and T cell transfer from IL-6 deficient mice failed to induce transmural colitis. IL-6, produced from macrophages and monocytes, functions as an inducer of the activation and differentiation of T_H17, which is an IL-17 secretory T cell [179]. IL-6 is regarded as a tumor prognostic factor, because IL-6 is associated with increased cell proliferation and cell survival, and a higher level of IL-6 is positively correlated with advanced stages of colon cancer and higher mortality [104, 109]. The level of IL-6 is increased in CD and colitis-associated colon cancer (CAC), and IL-6 signaling plays a pivotal role in the survival of intestinal epithelial cells in the CAC model [111, 112]. Recently, Rokavec and his colleagues demonstrated that an increased level of IL-6 induced by monocyte chemotactic protein 1 (MCP-1) excreted from monocytes triggered an oncogenic transformation of non-malignant breast epithelial cells. This resulted in tumors in immuno-deficient mice [110]. These data suggest that IL-6 could have a tumorigenic effect on epithelial cells.

Pre-menopausal women have a lower incidence of colon cancer as compared to age-matched men but post-menopausal women have a poorer survival rate similar to men [1, 146]. Studies have shown that hormone replacement therapy (HRT) could reduce the incidence of colon cancer in post-menopausal women, suggesting the protective effect of estrogen signaling on colon cancer [52, 54]. Previous data from our laboratory showed that estradiol (E_2) suppressed the incidence of colon cancer induced by azoxymethane (AOM) and increased apoptotic molecules through the p53 pathway, demonstrating that estrogenic molecules could prevent colon carcinogenesis [53, 143]. Epidemiological and animal studies have demonstrated that estrogen ameliorated

inflammation [123, 124, 128, 180]. The symptoms of CD in pre-menopausal women worsened during menses, when E_2 concentrations are at their lowest. E_2 modulated the infiltration of immune cells and altered cytokine production in chemically induced colitis model. Recent data from our laboratory have shown that E_2 protects the colon epithelium from acute inflammation induced by 2, 4, 6-trinitro benzene sulfonic acid (TNBS) by a decrease of pro-inflammatory cytokines including IL-6 [125]. These data suggest that estrogen signaling could play a protective role in CAC as well.

The aim of our study is to examine how IL-6 influences non-malignant colonocytes, whether estrogen signaling modulates the change induced by IL-6, and what the potential mechanism of modulation by estrogen signaling is on the protection for the physiology of colonocytes. Though it has been reported that the estrogen receptors could modulate IL-6 gene expression in HeLa cells by inhibition of nuclear factor kappa B (NFκB) transactivation, the interaction between IL-6 and estrogen signaling remains unclear [118]. In this study, we examined physiological changes of young adult mouse colonocytes (YAMCs) induced by IL-6. We also estimated that the influence of estrogen signaling induced by E_2 or genistein (GEN), a phytoestrogen from soybeans, on IL-6 effect to clarify the relationship between the IL-6 pathway and estrogen signaling. GEN is a known modulator of inflammation and an enhancer of homeostasis in intestinal epithelial cells [75, 181, 182]. The present data will give insights the role of IL-6 in the development of CAC and whether estrogen signaling could suppress CAC.

3.2 Materials and methods

3.2.1 Reagents

β-Estradiol (E_2) and genistein (GEN) were purchased from Sigma Aldrich, and recombinant mouse IL-6 was obtained from Millipore. E_2 and GEN were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and IL-6 was dissolved in ultrapure water.

3.2.2 Cell culture

Young adult mouse colonocyte (YAMC) bleo/neo cells and mp53/neo cells were provided by Dr. Hartmut Land (University of Rochester Medical Center). For general maintenance, cells were cultured in the permissive condition, 33℃, in RPMI1640 media (Sigma Aldrich) with 10% fetal bovine serum (FBS, Hyclone), 1% gentamicin (GIBCO) and ITS (0.1% insulin, transferrin, and selenious acid; BD Biosciences) as well as 5 units IFN-γ. For experiments, cells were grown in RPMI1640 with 10% charcoal-dextran stripped FBS 48 h before the experiments. During the experiments, cells were grown without IFN-γ at the non-permissive condition, 39 ℃.

3.2.3 Cell growth assay

Cells were seeded at a concentration of 5×10^4 cells/well in 6-well plates (Grenier bio-one) and grown in the non-permissive condition for 24 h. The cells were treated with 1 nM E_2, 1 and 10 μM GEN with or without 15 ng/ml of IL-6 for 72 h. 0.1% DMSO was utilized as a control. After 72 h of treatment, cells were trypsinized and collected for counting. Cell numbers were counted using a Cellometer Auto 1000 (Nexcelom Bioscience) according to the manufacturer's protocol. 20 μl of cells were

loaded in the cell counting chamber, and each sample was counted twice. Three wells per treatment per experiment were used, and three replicate experiments were conducted.

3.2.4 Flow cytometry

Cells were seeded and grown with the same doses of reagents as the cell growth assay. After 72 h, cells were collected by trypsin treatment. Cells were washed with ice-cold 1X PBS and fixed with 100% ethanol in a -20℃ freezer overnight. Fixed cells were incubated with propidium iodide staining solution (50 µg/ml propidium iodide, 200 µg/ml DNase-free RNase, 4 mM sodium citrate and 0.1% of Triton X-100) at RT for 30 minutes. Cell cycle distribution was evaluated by Accuri C6 flow cytometer (BD Bioscience).

3.2.5 Caspase-3 activity

Cells were seeded and treated with the same conditions as the cell growth assay. Cells were collected with trypsin and washed with PBS twice. Cells were prepared and caspase-3 activity was estimated using the manufacture's protocol for the EnzChek Caspase-3 assay kit #2, Z-DEVD-R110 substrate (Molecular Probes). Briefly, cells were lysed with 50 µl of 1X Cell Lysis buffer and incubated on ice for 30 min. Lysed cells were centrifuged at 5000 rpm for 5 min and 50 µl supernatants were transferred into a 96 well black flat bottom plate (BD Bioscience). After adding 50 µl of 2X substrate in each well, the plates were incubated at RT for 30 min in the dark. Fluorescence with 496 (excitation)/ 520 (emission) nm was measured at 15 min intervals using a TECAN infinite M200 plate reader.

3.2.6 RNA extraction and real-time PCR

Cells were seeded at a concentration of 4.5×10^5 cells/dish in 100 mm dishes (Corning) and grown at the non-permissive condition for 24 h. The cells were treated with 1 nM E_2, 1 and 10 µM Genistein with or without 15 ng/ml IL-6 for 72 h. 0.1% DMSO was utilized as a control.

RNA extraction was performed according to the manufacture's protocol of Direct-zol™ RNA MiniPrep (Zymo Research) with TRIzol Reagent (Invitrogen). In brief, trypsinized cells were collected and 1 ml TRIzol reagent was added to lyse the cell membrane. 1 ml of 100% ethanol was added and samples were mixed by vortexing. RNA was isolated using a Zymo-Spin column and eluted in 30 µl of RNase-free water. Isolated RNA was stored in a -70℃ freezer. The concentration of RNA in each sample was determined using a Nanodrop 2000 (Thermo Fisher Scientific).

cDNA was synthesized using the manufacturer's protocol for the Transcriptor First Strand cDNA synthesis kit (Roche). In brief, 1 µg of RNA was mixed with 1 µl oligo dT primer and 2 µl random hexomer primer and incubated at 65℃ for 10 min. 4 µl reaction buffer, 0.5 µl RNase inhibitor, 2 µl dNTP and 0.5 µl reverse trancriptase were added to the RNA mixture, incubated, and thermo cycling was conducted at 25℃ for 10 min, 50℃ for 60 min and 85℃ for 5 min. Synthesized cDNA was stored in a -20℃ freezer.

Real-Time PCR was performed using FastStart Universal SYBR Green Master mix (Roche). The sequence of the PCR primers were as follows: ERα (Forward: GAC CAG ATG GTC AGT GCC TT and Reverse: ACT CGA GAA GGT GGA CCT GA),

ERβ (Forward: CAG TAA CAA GGG CAT GGA AC and Reverse: GTA CAT GTC CCA CTT CTG AC), CCND1 (Forward: GCG TAC CCT GAC ACC AAT CTC, Reverse: ACT TGA AGT AAG ATA CGG AGG GC), PCNA (Forward: TTG CAC GTA TAT GCC GAG ACC, Reverse: GGT GAA CAG GCT CAT TCA TCT CT), IL-6 (Forward: CTG CAA GAG ACT TCC ATC CAG, Reverse: AGT GGT ATA GAC AGG TCT GTT GG), and 18S as an internal control (Forward: TCA AGA ACG AAA GTC GGA GGT, Reverse: GGA CAT CTA AGG GCA TCA CAG). Real-Time PCR was run on a LightCycler® 480 II (Roche): 10 minutes at 95℃, 45 cycles of 15 seconds at 95℃ and 30 seconds at 60℃.

3.2.7 Western blot

Cells were seeded and grown in the same condition as the cell growth assay. Cells were harvested and lysed in 1X RIPA buffer with 1 mM PMSF (Cell Signaling Technology) for 15 min on ice. After two cycles of freezing and thawing, lysed cells were centrifuged at 14,000 rpm for 15 min and the supernatants were transferred into a new e-tube. The concentration of proteins was determined using the DC Protein Assay (Bio-Rad). Proteins were separated on a 10% Tris-glycine gel (Lonza) and transferred to a PVDF membrane (Immobilon-P Membrane; Millipore). Primary antibodies for STAT3 (Santa Cruz Biotechnology), phospho-STAT3 (Tyr705; Cell Signaling Technology) and β-actin (Sigma Aldrich) were utilized at a 1:1000 dilution. After incubation at 4℃ overnight with primary antibodies, the blots were incubated with goat anti-rabbit or goat anti-mouse secondary antibodies (Enzo lifescience) at a 1:2000 dilution. The blot was

visualized using the Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate Kit (Millipore).

3.2.8 Statistical analysis

The data are expressed throughout as means ± SEM, which was calculated from at least three different experiments. The statistical significance among the test groups was determined by two-way ANOVA using Graphpad. A p-value of less than 0.05 was considered significant.

3.3 Results

3.3.1 E_2 and GEN suppressed cell growth induced by IL-6

To investigate the effect of IL-6 on YAMCs, we examined the growth of YAMCs treated with IL-6 for 72 h (Fig. 3.1A). Though cells were incubated at the non-permissive condition, IL-6 increased cell growth of YAMCs by 60% ($p < 0.0001$). Co-treatment of E_2 and IL-6 elevated cell growth by 34% compared to control without IL-6 ($p < 0.0001$). The cell numbers by co-treatment of E_2 and IL-6 were 20% less than IL-6 treatment alone ($p = 0.0006$). Because E_2 suppressed cell growth by 20% compared to control without IL-6 ($p = 0.05$), the inhibition rate of cell growth by E_2 was still the same whether IL-6 was co-treated or not. Though 1 µM of GEN had no influence on cell growth, 10 µM of GEN treatment had a suppressive effect on cell growth by 20%, similar to E_2 with or without IL-6 ($p < 0.0001$). In contrast, E_2 and GEN did not change the cell growth of YAMC mp53/neo cells (Fig. 3.1B), which suggests that the apoptotic pathway is critical when estrogen signaling modulates cell growth. Furthermore, IL-6

did not influence cell growth of YAMC mp53/neo cells, indicating induction of cell growth by IL-6 could involve loss of apoptosis.

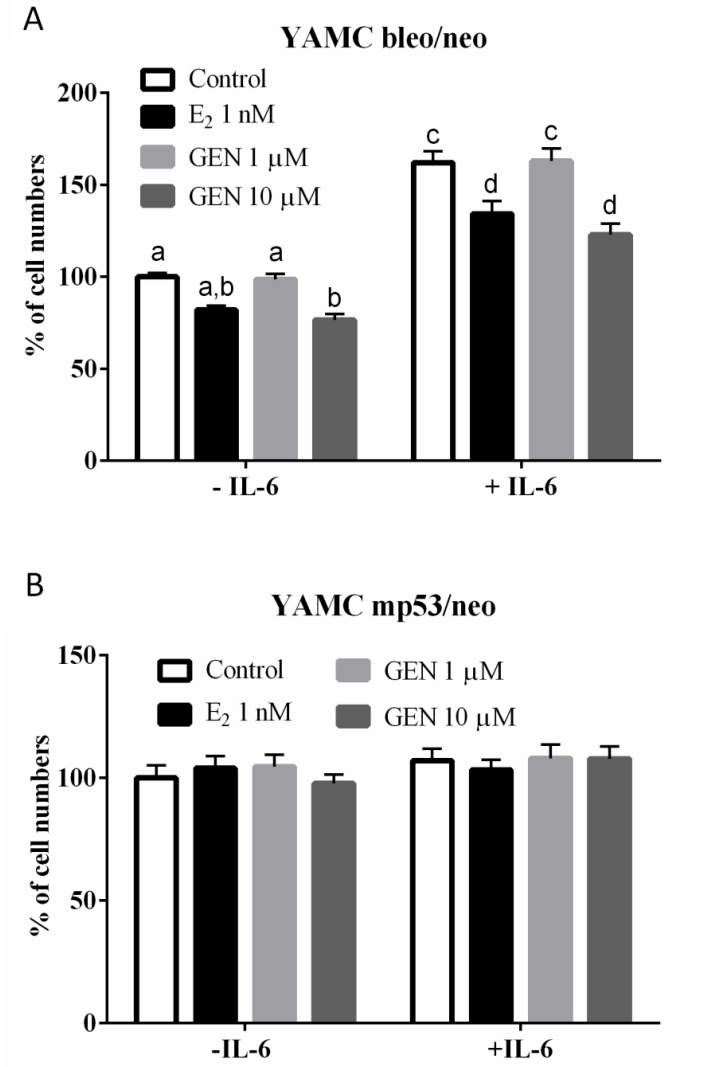

Fig. 3.1 The effect of IL-6, E_2 and GEN on the growth of YAMCs. Cells were treated with 1 nM E_2, 1 and 10 μM GEN, with or without 15 ng/ml IL-6 for 72 h at 39℃. A. Bleo/Neo. B. mp53/neo. Values are means (n = 9) ± SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

3.3.2 E_2 and GEN reversed caspase-3 activity decreased by IL-6

To examine how IL-6 induces cell growth of YAMCs, we estimated apoptosis and cell cycle distribution (Fig. 3.2). IL-6 has been reported as an anti-apoptotic molecule for T lymphocytes by activation of STAT3, and IL-6 impacts the survival of the intestinal epithelial cells in the CAC model [112, 183]. IL-6 reduced caspase-3 activity ($p < 0.0001$), suggesting a decrease of apoptosis in YAMCs (Fig. 3.2A). Similar to previous data from our laboratory, E_2 and GEN elevated apoptosis in YAMCs ($p < 0.0001$) [143]. However, the effect of E_2 was weak under IL-6 treatment while 10 μM GEN recovered apoptotic activity reduced by IL-6 ($p = 0.02$). Cell cycle distribution was analyzed by flow cytometry and IL-6, E_2 and GEN did not change cell distribution at all (Fig. 3.2B).

3.3.3 E_2 reduced PCNA and CCND1 expression elevated by IL-6 treatment

IL-6 signaling and estrogen signaling ultimately modulate transcription of genes because ERs and all IL-6 targets function as transcription factors [43, 108]. To investigate how IL-6 modulates apoptosis, we examined Real-Time PCR for analysis of gene expression (Fig. 3.3). IL-6 increased ERα expression in YAMCs (p=0.04), though there is no evidence suggesting that IL-6 signaling could change the expression of ERs (Fig. 3.3A). 1 nM E_2, 1 and 10 μM GEN treatments suppressed the increase of ERα expression by IL-6 ($p = 0.04$, $p = 0.07$ and $p = 0.02$). Though ERβ expression was slightly increased by IL-6, IL-6 suppressed the ratio of ERβ to ERα (Fig. 3.3B and C, $p < 0.0001$). Because E_2 and GEN did not reduce ERβ expression increased by IL-6, E_2 and GEN did not diminish the effect of IL-6 on the ratio of ERβ to ERα. CCND1

expression was increased by IL-6 treatment but it was not significant (Fig. 3.3D). Instead, PCNA expression was elevated by IL-6 significantly (Fig. 3.3E, $p = 0.002$). E_2 and GEN decreased neither CCND1 nor PCNA expression elevated by IL-6. These data suggest that IL-6 treatment induces cell proliferation because CCND1 is related to cell cycle progression and PCNA is a marker for cell proliferation. Interestingly, IL-6 expression was reduced by IL-6 treatment (Fig. 3.3F, $p < 0.0001$). When IL-6 was not applied, 10 μM GEN suppressed IL-6 mRNA ($p = 0.01$). Under IL-6, E_2 and GEN reduced IL-6 expression slightly more than IL-6 treatment alone but the effect was not significant.

3.3.4 E_2 and GEN have no influence on phosphorylation of STAT3 induced by IL-6 signaling

IL-6 signaling transduces its signals through signal transducer and activator of transcription 3 (STAT3), phosphotidylinositol-3 kinase (PI3K) and Ras/mitogen activated protein kinases (MAPK) [108]. To clarify the mechanism of the effect of E_2 and GEN, the change of the STAT3 protein was examined by Western blot. IL-6 treatment increased phosphorylation of STAT3 significantly (Fig 3.4). However, E_2 and GEN did not alter phospho-STAT3 elevated by IL-6. These data indicate that IL-6 signaling is activated in YAMCs, causing signal transduction via phospho-STAT3.

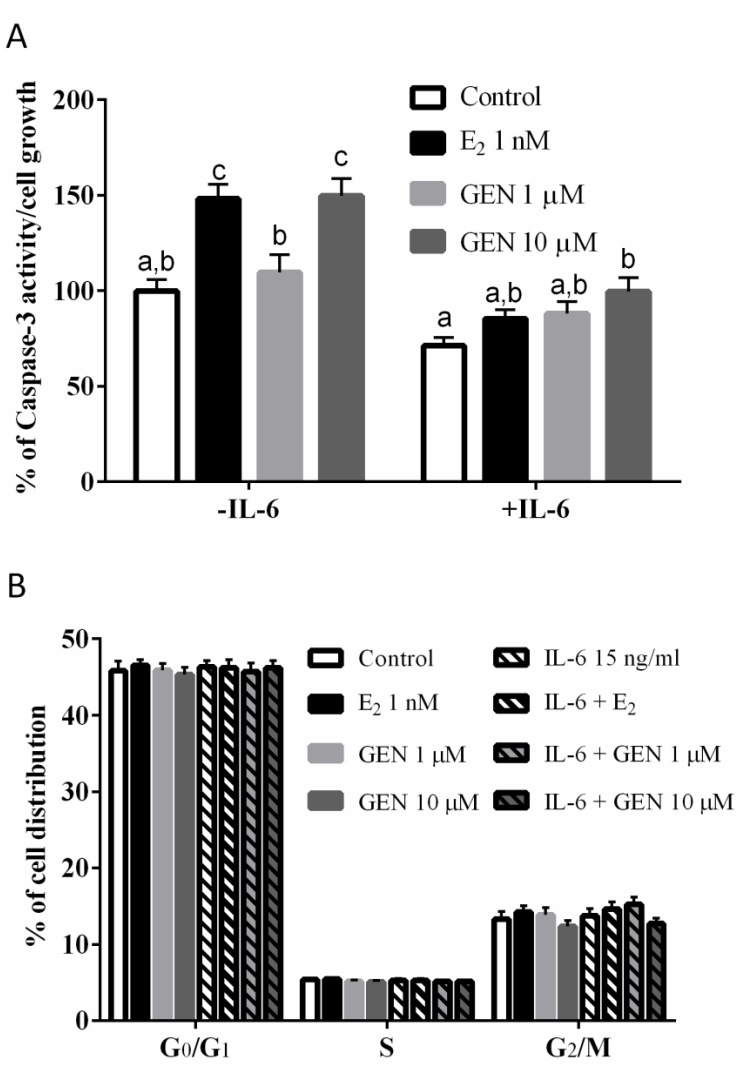

Fig. 3.2 Apoptosis and Cell cycle distribution in YAMCs treated with IL-6, E_2 and GEN. Cells were treated with 1 nM E_2, 1 and 10 μM GEN, with or without 15 ng/ml IL-6 for 72 h at 39℃. A. Caspase-3 activity. Data are expressed as increased fluorescence measured when compared with the control based on cell growth rate. B. Cell cycle distribution was analyzed by flow cytometry. Data are presented as percentage of vehicle control. Values are means (n = 9) ±SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

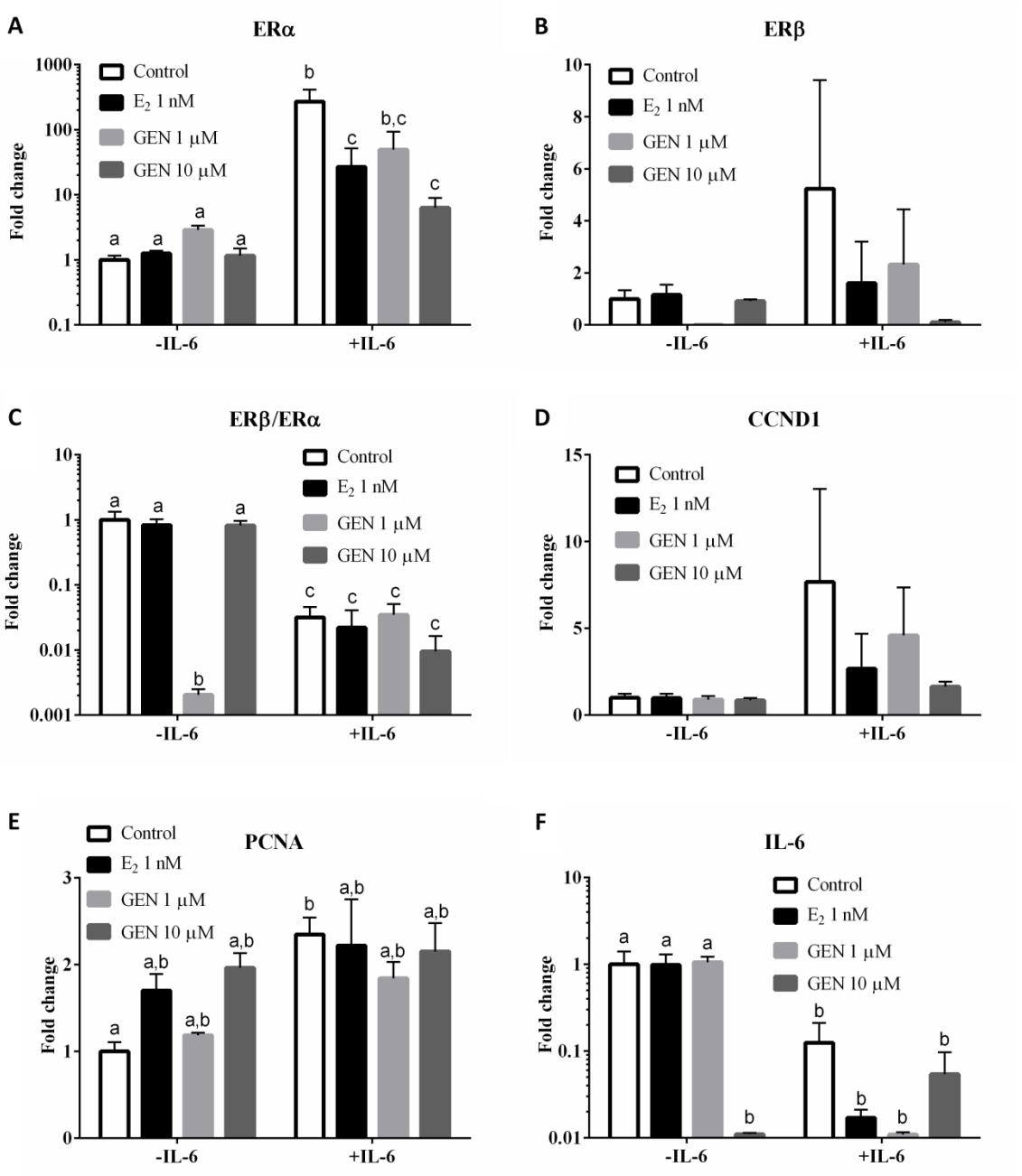

Fig. 3.3 Changes of gene expression in YAMCs treated with IL-6, E_2 and GEN. Cells were treated with 1 nM E_2, 1 and 10 μM GEN, with or without 15 ng/ml IL-6 for 72 h at 39℃. A. ERα, B. ERβ, C. Ratio of ERβ to ERα, D. CCND1, E. PCNA and F. IL-6. Values are presented as ratio of the expression compared to vehicle control. Values are means (n = 9) ±SEM from triplicate experiments. Bars without a common letter are significantly different; $p < 0.05$.

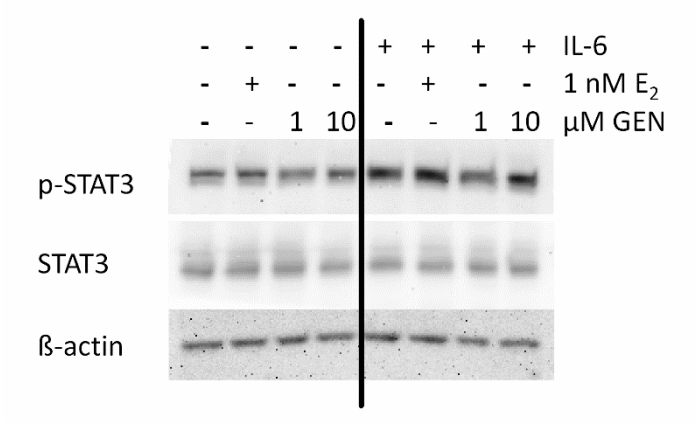

Fig. 3.4 Change of phospho-STAT3 and STAT3 in YAMC cells treated with IL-6, E_2 and GEN. Cells were treated with 1 nM E_2, 1 and 10 μM GEN, with or without 15 ng/ml IL-6 for 72 h at 39℃.

3.4 Discussion

Studies have been conducted to elucidate the role of estrogen and estrogen signaling on the physiology of cells in non-reproductive tissues. Especially in cancers, their role is changed depending on timing, tissues and the major form of ERs expressed [46, 47]. Previous articles have demonstrated that E_2 has a protective activity in colon carcinogenesis and acute inflammation through induction of the p53 pathway and this protection is mediated by ERβ, the predominant ER in the colon epithelial [53, 56, 143]. Still, to our knowledge, the role of estrogen signaling against inflammation in the colonocytes has not been fully elucidated.

Though IBD is a known risk factor for colon cancer, its mechanism for cancer progression is different from sporadic cancer [92, 93]. Because IBD is a chronic inflammation, a variety of cytokines are involved. IL-6 is one of the cytokines related to

the immune responses and IBD. IL-6 binds to the IL-6 receptor which activates downstream signaling such as STAT3, resulting in gene regulation [108]. IL-6 signaling induces cell proliferation as well as the differentiation of T_H17 cells. In the present study, we demonstrated that YAMCs, non-malignant colonocytes, responded to IL-6 treatment; IL-6 increased cell proliferation, modulated gene expression, suppressed apoptosis, as well as induced phosphorylation of STAT3. These data suggest that the coloncytes may have IL-6 receptors and IL-6 treatment induced IL-6 signaling in YAMCs. Because STAT3 is a transcription factor that controls genes related to cell survival and proliferation, increased phospho-STAT3 due to IL-6 signaling could be a mediator that changes downstream target genes such as CCND1 and PCNA. As a result, activated IL-6 signaling reduced apoptosis and increased cell proliferation in YAMCs. These data suggest that IL-6 signaling could influence the physiology of colonocytes and the inflammatory response in the colon epithelial could result in increased cell proliferation.

Interestingly, IL-6 gene expression was decreased by IL-6 treatment though IL-6 is known as a target of phospho-STAT3 [184]. The present data suggest that non-cancerous cells could have the ability to diminish IL-6 signaling, because aberrant IL-6 signaling is one of the markers for cancer. A recent study demonstrated a negative feedback loop of IL-6 in the breast mediated by microRNA 146b (miR-146b) [185]. miR-146b is highly expressed in the breast epithelial, while its expression is low in breast tumors due to methylation of its promoter. STAT3 induced miR-146b, suppressing IL-6 production via inhibition of NFκB. The present data showing that IL-6

treatment lowered the expression of the IL-6 gene could be due to the activation of a negative feedback loop for IL-6 signaling in non-malignant cells.

Recent studies demonstrated that estrogen signaling is also related to carcinogenesis. Estrogen signaling may accelerate or exacerbate cancers in tissues related to reproduction, such as the breast, because ERα is the predominant form of ER in most of these tissues. There are two primary forms of ERs, ERα and ERβ. While ERα is known to induce cell proliferation for the development of tissues such as the breast, ERβ is negatively correlated to cell growth and cancer development. Previous data from our laboratory demonstrated that E_2 has a protective effect against colon carcinogenesis because ERβ is the major form of ER in the colon, and the novel shift from ERβ to ERα occurs during the development of colon cancer. In the present study, IL-6 treatment increased ERα expression and decreased the ratio of ERβ to ERα, indicating that IL-6 signaling could be involved in the physiological changes of colonocytes progressing to malignancy.

Previous data from our laboratory showed that E_2 suppressed cell growth and increased apoptosis in YAMCs. E_2 still has the ability to modulate cell growth and apoptosis under IL-6 treatment in the present study. Furthermore, E_2 reversed increased ERα expression by IL-6. There are two possible explanations for the effect of E_2. One is that E_2 inhibited IL-6 signaling through an interaction of signaling molecules in downstream pathways of IL-6 such as STAT3. The other is that E_2 enhanced the other pathways related to tumor suppression. E_2 treatment enhanced the decrease of IL-6 expression, which could result in a decrease in IL-6 signaling. The data that phospho-

STAT3 was not changed by E_2 suggests that estrogen signaling could modulate other pathways. Still, further study is required to understand how E_2 treatment interrupts IL-6 induced cell proliferation. IL-6 induces three major signaling pathways: STAT3, PI3K and Ras [108]. Though present data suggests that E_2 has no influence on phosphorylation of STAT3, two other downstream targets of IL-6, PI3K or Ras, could be a target of estrogen signaling. The interaction between ERβ and activation of MAPK and PI3K has been reported in breast epithelial and cancer cells [119]. In addition, the data showed that E_2 decreased cell growth by 20% with or without IL-6 and the effect of IL-6 was diminished on YAMCs with mutated p53. Because increased apoptosis by E_2 is mediated by p53 and p53 suppressed IL-6 expression, these data suggest that p53 could be a mediator of estrogen signaling and the IL-6 pathway on cell growth of YAMCs [143, 186, 187].

GEN is a phytoestrogen that has a higher biding affinity to ERβ than ERα [77]. As one of the flavonoids, it has been reported that GEN has an anti-tumorigenic and anti-inflammatory capacity *in vitro* and *in vivo* [64, 68, 75, 128]. Previous data with YAMCs and mice have shown that GEN suppressed cell growth and increased apoptosis similar to E_2 [76]. In this study, 10 μM of GEN had a similar effect on cell growth and apoptosis as E_2. 10 μM of GEN also inhibited the expression of IL-6 under IL-6 was not treated. GEN had no influence on the level of phospho-STAT3. Taken together, these data suggest that the suppressive effect of GEN against IL-6 could be more than an estrogenic effect. Numerous biological properties of GEN have been reported besides the estrogenic effect: a protein kinase inhibitor, a topoisomerase II inhibitor, suppression

of phosphatidylinositol turn over, an antioxidant, an agonist for peroxisome proliferator-activated receptor γ (PPARγ), as well as an anti-inflammatory agent [144, 188, 189]. Therefore, other pathways influenced by GEN could modulate IL-6 induced cell proliferation with estrogen signaling.

In conclusion, IL-6 signaling could be activated in non-malignant colonocytes via phosphorylation of STAT3. IL-6 signaling causes an increase in cell proliferation and a decrease of apoptosis via a change of gene expression. Estrogen signaling by E_2 treatment suppressed cell proliferation against IL-6 signaling, but the effect was not mediated by STAT3. GEN has a similar inhibitory activity on increased cell proliferation by IL-6, but the influence by GEN may not include estrogen signaling, though GEN is a representative phytoestrogen. These findings together suggest that IL-6 signaling could play an important role in colon carcinogenesis, and estrogen signaling partially disrupts IL-6 signaling, resulting in the prevention of colon cancer.

CHAPTER IV

DUAL EFFECTS OF ESTRADIOL SUPPRESS COLITIS AND COLITIS ASSOCIATED COLON CANCER.

4.1 Introduction

Chronic inflammation is positively correlated with a variety of cancers including colon cancer [84]. Inflammatory bowel disease (IBD) is prevalent in the U.S., with over 1.4 million people are suffering from IBD [190]. This disease refers to a condition with chronic or recurring inflammation and/or immune response in the gastrointestinal tract (GI tract) [86, 176, 178]. The two most common forms of IBD are Crohn's disease (CD) and Ulcerative Colitis (UC). UC causes inflammation only in the colon and rectum, while CD affects the whole GI tract. In spite of their distinct features, both UC and CD are associated with an increased risk of colon cancer, called colitis associated colon cancer (CAC) [92, 177, 191].

CAC has distinct features from sporadic colon cancer [93]. Generally, CAC arises from the flat mucosa, while most sporadic cancer rises from aberrant polyps. Genetic mutations occurring during the development of CAC are different from sporadic cancer. In sporadic cancer, APC mutation occurs in the early stage, while p53 mutation is found in the relatively late stage. In contrast, p53 mutation is found in the early stage of CAC, and APC mutation occurs in the late stage. Due to these differences from sporadic cancer, transformation from IBD to colon cancer is not fully understood.

Because immune cells play a main role in IBD, research focusing on the immune systems is required in the CAC model as well.

To mimic IBD, animal colitis models were developed using inflammatory agents [91]. Dextran sodium sulfate (DSS) is a common inflammatory agent used to induce colitis in mice [91]. Because it induces superficial chemical injuries, changes of lymphocytes are not involved in DSS-induced colitis. Symptoms of DSS treatment resemble the symptoms of UC [192]. In contrast, 2, 4, 6-trinitro benzene sulfonic acid (TNBS) is a haptenating agent which induces a variety of changes in lymphocytes in the lamina propria [90]. TNBS induced colitis elevates type 1 T helper lymphocytes (T_H1) similar to CD.

Estradiol (E_2) is an active form of the female sex hormone controlling development of female sex organs such as the ovary and uterus in the human body [42, 43]. Epidemiological data have shown the suppressive property of estrogen signaling on colon carcinogenesis. The incidence of colon cancer in pre-menopausal women is lower than age-matched men, and hormone replacement therapy in post-menopausal women lowers the risk of colon cancer [52, 54]. Previous data from our laboratory have shown that E_2 inhibited formation of aberrant crypt foci (ACF), which are preneoplastic lesions, against sporadic colon carcinogenesis by azoxymethane (AOM), and the protective effect of E_2 is mediated by ERβ and p53 [53, 143].

Recent articles have demonstrated that E_2 influences the immune system [115, 116]. ERs are expressed on most immune cells, and estrogen signaling regulates the gene expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and

interleukin-6 (IL-6), which are major modulators of inflammation [117, 118, 193, 194]. Studies with experimental colitis models have demonstrated that E_2 reduced the damage of the colon via alteration of mast cell numbers and cytokine levels [124, 125, 192]. Still, the influence of E_2 against inflammation remains debatable because the effect of E_2 is conflicting; anti- and pro-inflammatory depending on the inflammatory agent. E_2 protected against acute inflammation by TNBS or 2,4-dinitrobenzene sulfonic acid (DNBS), while E_2 exacerbated the severity of damage with DSS. Epidemiological studies have shown that the severity of CD symptoms, but not UC, in pre-menopausal women depends on their menstruation cycle. This suggests that E_2 has dual effects against colonic inflammation [123]. Furthermore, most research on the relationship between inflammation and E_2 was conducted using male animals. Therefore, an experiment with female mice offers an opportunity to clarify the effect of E_2 against chronic inflammation. Studies have also examined the role of estrogen signaling on CAC but the results are debatable [55, 126, 195]. When colon carcinogenesis was induced by DSS treatment with the injection of AOM, a colon carcinogen, ACF formation was exacerbated or ameliorated by E_2 depending on the timing of E_2 treatment. Therefore, studies are required to clarify the role of E_2 on chronic inflammation in the colon and CAC.

The first aim of the present study is to clarify the influence of estrogen signaling on chronic inflammation by TNBS. Compared to DSS, the TNBS colitis model has shown similar features to CD in which E_2 plays a protective role. Though E_2 showed a protective role in an acute TNBS colitis model, chronic inflammation is a completely

different condition, more similar to IBD [125]. The secondary aim of this study is to evaluate the effect of estrogen signaling on CAC induced by the co-treatment of AOM and TNBS. Genistein (GEN) was utilized in the present study because GEN is a representative phytoestrogen isolated from soybeans and known to have anti-inflammatory activity [128]. Studies from our laboratory and other researchers suggest that ERβ mediates the protective role of E_2 in both sporadic and inflammation-associated colon cancer [55, 196]. Because GEN has a higher binding affinity to ERβ while E_2 preferentially binds to ERα, a comparison of E_2 with GEN gives more hints to understanding the role of estrogen signaling on chronic inflammation and CAC.

4.2 Materials and methods

4.2.1 Reagents

β-Estradiol (E_2), 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and azoxymethane (AOM) were purchased from Sigma Aldrich. Genistein was provided by Dr. William Helferich at the University of Illinois at Urbana-Champaign.

4.2.2 Animals

c57BL6 mice were purchased from Charles River Laboratories. Mice were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University.

4.2.3 Colitis and colitis-associated colon cancer induction

For chronic inflammation, c57BL6 mice were ovariectomized and either a 20 mg cholesterol or 19 mg cholesterol with 1 mg E_2 pellet was implanted as described previously [53]. Mice were fed a semi-purified phytoestrogen-free diet (AIN-76, Lab Supply) or genistein 1000ppm diet post surgery. Mice were allowed food and water *ad libitum*. Two weeks after surgery, mice received 1% TNBS on the skin on their back for pre-sensitization. A week later, TNBS in 50% ethanol was intrarectally injected using a flexible plastic gavage tube (Instech Solomon) once a week for 5 weeks. The concentration of TNBS continuously increased from 0.5% to 1% to maintain the inflammatory response. Two weeks after the last injection of TNBS, mice were sacrificed and tissues were collected for analysis. Colons were excised and opened longitudinally. One longitudinal half of the colon was fixed with 4% paraformaldehyde (PFA; Mallinckrodt Baker, Inc.) and processed for embedding in paraffin. The other half of the colon was frozen in liquid nitrogen for analysis of cytokines.

For inflammation associated colon carcinogenesis, c57BL6 mice were ovariectomized and either a 20 mg cholesterol or 19 mg cholesterol with 1 mg E_2 pellet was implanted as above. Mice were fed with a semi-purified phytoestrogen-free diet or genistein 1000ppm diet at the time of surgery and allowed food and water *ad libitum*. Three weeks after surgery, mice received AOM once a week for 4 weeks at 10 mg/kg body weight. 1% TNBS was treated on the skin of their back for presensitization at the first injection of AOM. Mice were intrarectally treated with 2% TNBS in 50% ethanol on day 29 and 1.75% TNBS in 50% ethanol on day 43. On day 84, mice were sacrificed,

and tissues were collected for analysis. Colons were excised and opened longitudinally. 1-cm sections from the distal end were fixed in 4% PFA. 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 [197, 198].

4.2.4 Colon cytokines

Colon tissues were snap frozen and homogenized in T-PER Tissue protein extraction reagent (Thermo Scientific). Total protein concentration was assessed by DC Protein Assay (Bio-Rad). The analysis of cytokine levels was performed using Milliplex Mouse Cytokine/Chemokine Magnetic bead panel kit (Millipore) according to the manufacturer's protocol. Briefly, 150 µg of total protein was added per well to a 96 well plate, and 25 µl of 1X magnetic bead mixture and 25 µl assay buffer were added. The magnetic beads for IL-6, IL-17, MCP-1, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP1α), IL-12, IFN-γ and TNFα were utilized. The plate was sealed, covered with foil and incubated overnight at 4℃ with gentle shaking. The plate was agitated for 1 h with 25 µl of detection antibodies and shaken for 30 minutes with 25 µl of Streptavidin-Phycoerythrin at room temperature. The beads were then resuspended in 150 µl Luminex Sheath Fluid, and the plate was run on a Luminex 200 after agitation for 5 minutes.

4.2.5 Histological scoring and immunohistochemistry

Formalin fixed tissues were embedded in paraffin and sectioned at 4 µm. Tissues were stained as described previously [55]. Hematoxylin and eosin stained (H&E) tissues

were histologically evaluated for the degree of colon damage. Injury and inflammation (Score 0-3) were graded by a board certified pathologist (Dr. Brad Weeks) on H&E stained colon sections. Immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) was performed as reported previously with minor modifications [55]. BrdU was injected at 30 mg/kg body weight 2 h before sacrifice. The activity of endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol for 30 min and antigen retrieval was achieved by microwaving in 10 mM citrate buffer for 20 min. Slides were incubated with anti-BrdU (Roche), the primary antibody, diluted 1:20 at 4℃ overnight in a humidified chamber. The primary antibody was left off of a single section in every 3 to 4 slides to serve as a negative control. Slides were washed and then incubated with the secondary antibody, goat anti-mouse-HRP (Abcam), diluted 1:250 for 2 h. Meyer's hematoxylin was used as the counter stain. Lastly, slides were dehydrated and coverslipped. Twenty well oriented crypts were analyzed per mouse and the number of positively stained cells and total cells were counted using digital images acquired by microscope.

4.2.6 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Millipore) was utilized for the TUNEL assay according to the manufacturer's protocol. Tissues were deparaffinized, rehydrated and incubated with proteinase K for 3 min at 37ºC at 10μg/ml in PBS. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide, and the slides were treated in equilibrium buffer for 1 h at room temperature

(RT). Tissues were incubated with terminal deoxynucleotidyl transferase (TDT) enzyme in Reaction buffer for 1 h at 37℃. One section per 3-4 slides was excluded when the TDT enzyme was applied as a negative control. Next, a stopwash solution was applied to the sections for 10 min and anti-digoxigenin was applied to the slides for 30 min at RT. A 0.5% 3,3'-diaminobenzidine (DAB) solution was utilized as the chromagen for 20 seconds, and hematoxylin was used as the counter-stain. Finally, the slides were dehydrated and coverslipped. Twenty well oriented crypts were analyzed per mouse and the number of positively stained cells and total cells were counted using digital images acquired by microscope.

4.2.7 RNA isolation and analysis of gene expression

For RNA analysis, the colon was washed with PBS and scraped with a glass slide to collect cells of the mucosal layer at the time of tissue collection. The mucosa was immediately frozen in liquid nitrogen and stored at -80℃. RNA was isolated using the Direct-zol™ RNA MiniPrep kit (Zymo Research). One µg cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. Real-time PCR was performed using FastStart Universal SYBR Green Master mix (Roche). The sequence of the PCR primers were as follows: ERα (Forwad: GAC CAG ATG GTC AGT GCC TT, Reverse: ACT CGA GAA GGT GGA CCT GA), ERβ (Forward: CAG TAA CAA GGG CAT GGA AC, Reverse: GTA CAT GTC CCA CTT CTG AC), CCND1 (Forward: GCG TAC CCT GAC ACC AAT CTC, Reverse: ACT TGA AGT AAG ATA CGG AGG GC), PCNA (Forward: TTG CAC GTA TAT GCC GAG ACC, Reverse: GGT GAA CAG GCT CAT TCA TCT CT),

IL-6 (Forward: CTG CAA GAG ACT TCC ATC CAG, Reverse: AGT GGT ATA GAC AGG TCT GTT GG), and 18S as an internal control (Forward: TCA AGA ACG AAA GTC GGA GGT, Reverse: GGA CAT CTA AGG GCA TCA CAG). Prepared samples were loaded into 384 well reaction plates and run for 45 cycles in a LightCycler® 480 (Roche): 10 min at 95℃, 45 cycles of 15 seconds at 95℃ and 30 seconds at 60℃. Samples were run in triplicate.

4.2.8 Statistical analysis

Data are expressed throughout as means ± SEM, which was calculated from at least 5 mice per group. The statistical significance among the test groups was determined by one-way ANOVA. To compare body weights between groups, generalized estimating equations and a Wald test were utilized with a main effect for comparison group, a natural cubic spline for the time course, and an interaction between the two, based on an assumption of an autoregressive correlation structure. For the analysis of ACF, generalized regression models assuming a Poisson distribution were carried out and ANOVA-like hypotheses were tested. A p-value of less than 0.05 was considered significant. All data were analyzed using Graphpad and a data analysis software R with a certified statistician, Dr. Alan Dabney.

4.3 Results

4.3.1 Estradiol suppressed weight gain and elongation of colon length

Female mice were chosen to examine the effect of estrogen signaling *in vivo*. Their ovaries were removed to control the level of E_2 (Fig 4.1A). There were 3 groups in

the animal study for chronic inflammation: control group (fed phytoestrogen-free diet with cholesterol pellet), E_2 group (fed phytoestrogen-free diet with E_2 pellet), and GEN group (fed GEN diet with cholesterol pellet). TNBS was injected intrarectally in mice to mimic chronic inflammation in the colon. The concentration of TNBS increased during the experiment, starting at 0.5% and reaching 1% in the end to maintain the inflammatory response. All 3 groups of mice had similar average body weight at the beginning of the study. TNBS treatment did not induce weight loss due to its low concentration, so the control group continuously gained weight (Fig. 4.1B, $p < 0.0001$). However, GEN as well as E_2 treatment inhibited weight gain during the whole experiment. When comparing body weight between groups, the treatment effect was statistically significant ($p < 0.0001$).

Because the length and weight of the colon is the primary indicator for colon developmental status, both were measured. E_2 treatment decreased the length of the colon significantly compared to control or GEN treatment ($p = 0.02$), while also elevating the weight of the colon slightly (Fig. 4.2A and 2B, $p = 0.05$). As a result, the ratio of the colon length to weight was decreased in E_2 treated mice ($p = 0.05$), as opposed to the acute TNBS study from our laboratory (Fig. 4.2C) [125]. As reported in previous data, E_2 increased the uterine weight approximately 10 times ($p < 0.0001$), and GEN increased it by 0.01 g compared to control (Fig. 4.2D).

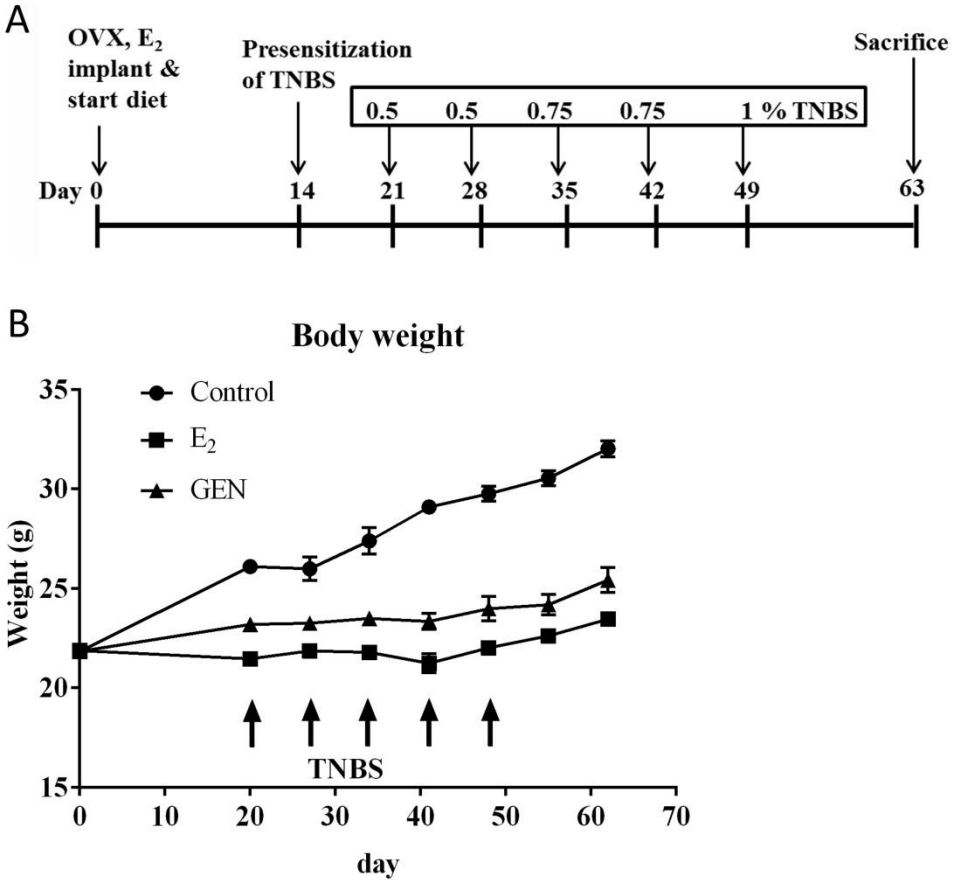

Fig. 4.1 Chronic inflammation induced by TNBS in mice A. Experimental design. B. Change of body weight. Values are means (n=17) ± SEM

Chronic inflammation by TNBS is associated with tissue damage and immune cell infiltration [199]. To examine whether E_2 protects tissues from TNBS, inflammation and injuries were scored on H&E stained tissues (Fig. 4.3). E_2 had no effect on the severity of inflammation and reduced injury only slightly. In contrast, GEN significantly exacerbated inflammation and injuries on the colon ($p = 0.007$ and $p = 0.01$).

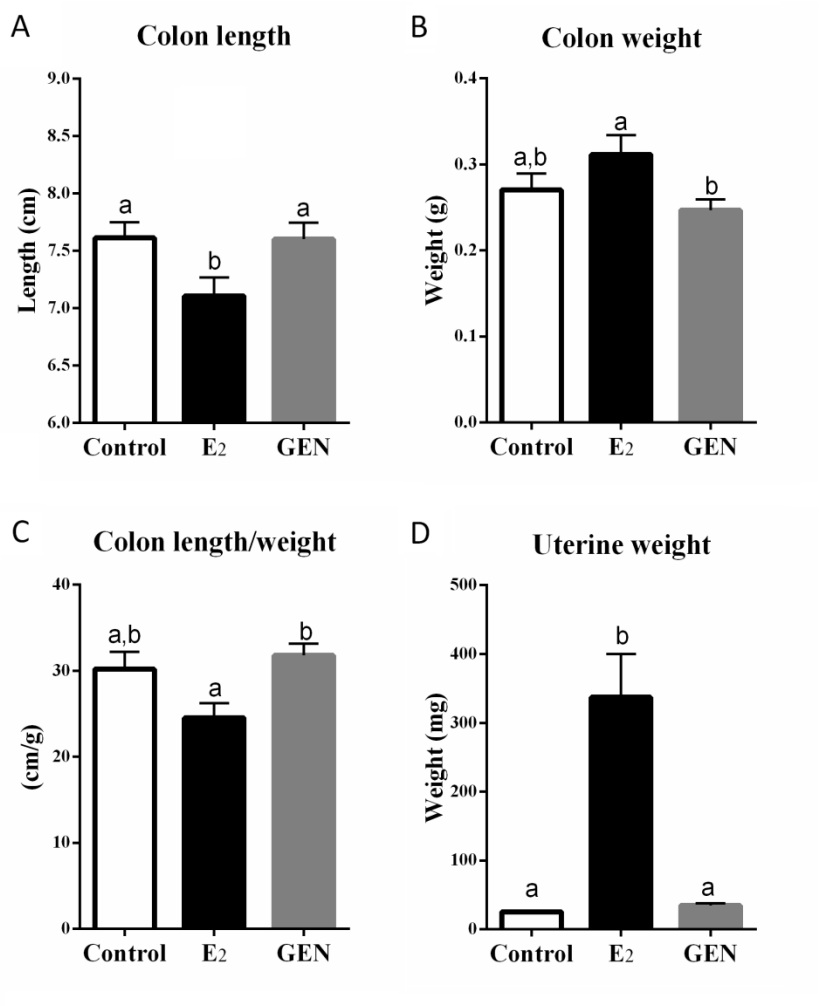

Fig. 4.2 Change of the colon and uterus A. Colon length, B. Colon weight, C. the ratio of colon length to weight, D. Uterus weight. Values are means (n =17) ± SEM, Bars without a common letter differ; $p < 0.05$.

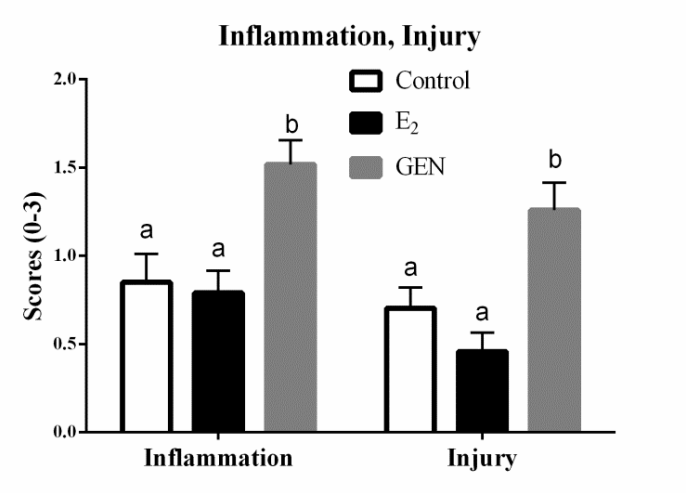

Fig. 4.3 Inflammation and injuries in the colon H&E stained tissues were examined and scored by a board certified pathologist. Values are means (n =17) ± SEM, Bars without a common letter differ; $p < 0.05$.

4.3.2 E_2 and GEN enhanced the expression of genes related to cell proliferation

Estrogen signaling modifies the expression of genes because ERs function as transcription factors [43]. To understand the influence of estrogen signaling in the colon epithelium, RNA was isolated from scraped mucosa cells inside the colon and RT-PCR was performed measuring the expression of genes related to estrogen signaling. E_2 treatment increased the expression of both ERs and GEN enhanced more ERβ expression than ERα (Fig. 4.4A-C). These data suggest that activated estrogen signaling could modulate ER expression in colonic epithelial cells. Both E_2 and GEN treatment elevated gene expression of CCND1 and PCNA (Fig. 4.4D and 4E). Though the increase of CCND1 and PCNA by E_2 and GEN were not significant, these data suggest an

increase in cell proliferation is involved in increased expression of genes associated with cell cycle progression and cell proliferation. Interestingly, IL-6 expression was decreased by E_2 and GEN treatment though IL-6 signaling is positively correlated to cell proliferation (Fig. 4.4F) [184].

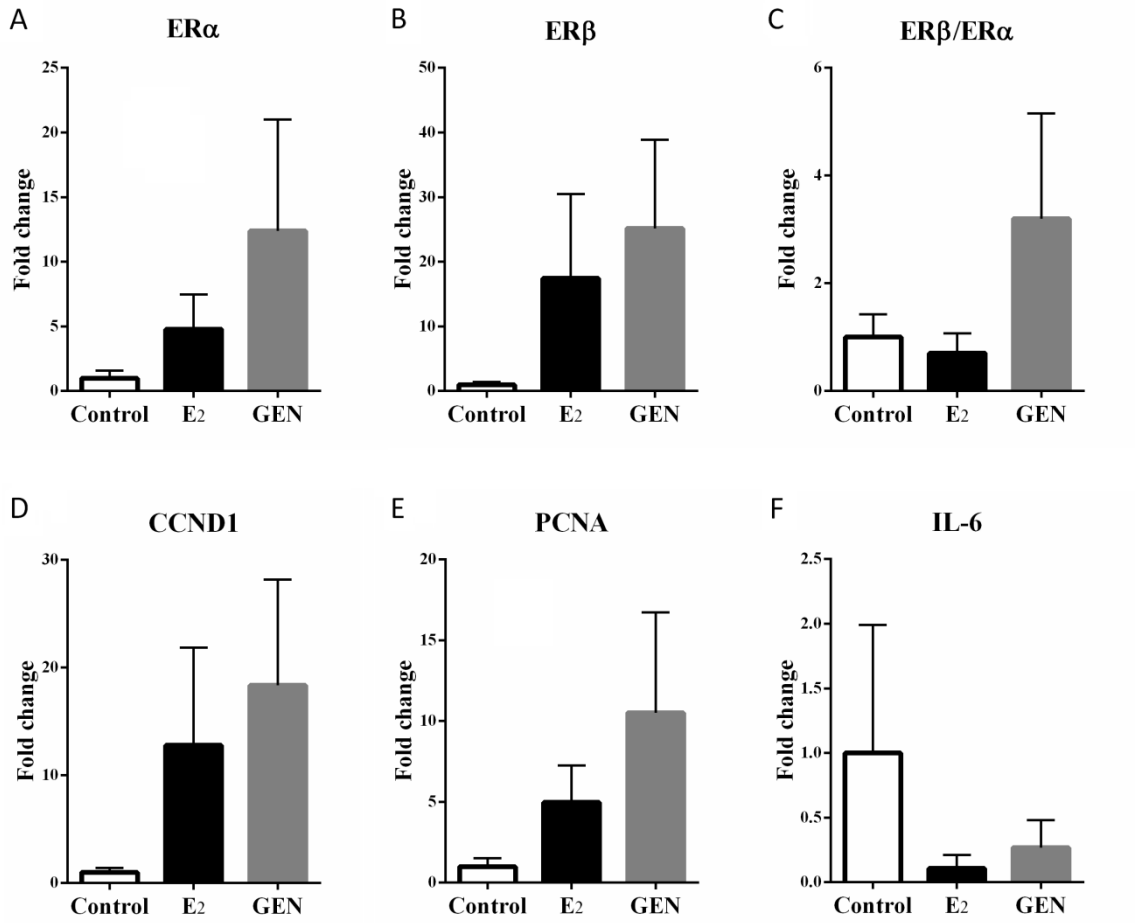

Fig. 4.4 Change of gene expression in the colon epithelial cells A. ERα, B. ERβ, C. the ratio of ERβ/ERα, D. CCND1, E. PCNA and F. IL-6 Values are means (*n* =17) ± SEM. Bars without a common letter differ; $p < 0.05$.

4.3.3 E_2 increased the level of T_H17-related cytokines and decreased the level of T_H1-related cytokines

TNBS induced colitis is mediated by effector $CD4^+$ T lymphocytes, T_H1 and T_H17, and a change of related cytokines [199]. To evaluate the change of immune responses and cytokines by E_2 and GEN, cytokine levels were examined using a Milliplex cyto/chemokine kit (Fig 4.5). IL-17 is released from T_H17 cells, while IFN-γ and TNFα are produced from T_H1 cells. IL-10 is an anti-inflammatory cytokine secreted from regulatory T cells (T_{reg}), and IL-6, MCP-1, GM-CSF, and MIP1α are released from monocytes and macrophages. IL-6, IL-17 and MCP-1 are associated with T_H17 cells while IL-12, IFN-γ and TNFα are linked to T_H1 cells. IL-6, IL-17, and MCP-1 were increased by E_2, but the changes were not significant (Fig. 4.5A, 5D, 5G). In contrast, IL-12 and IFN-γ were decreased significantly in E_2 treated mice (Fig 4.5C and 4.5F, p = 0.005 and p = 0.006). E_2 did not change the level of TNFα, IL-10, GM-CSF, or MIP1α (Fig. 4.5B, 5E, 5H and 5I). GEN treatment slightly suppressed the level of IL-12 and IFN-γ (Fig. 4.5C and 4.5F). Taken together, E_2 treatment modulated immune responses via an increase of T_H17 related cytokines and a decrease of T_H1 linked cytokines.

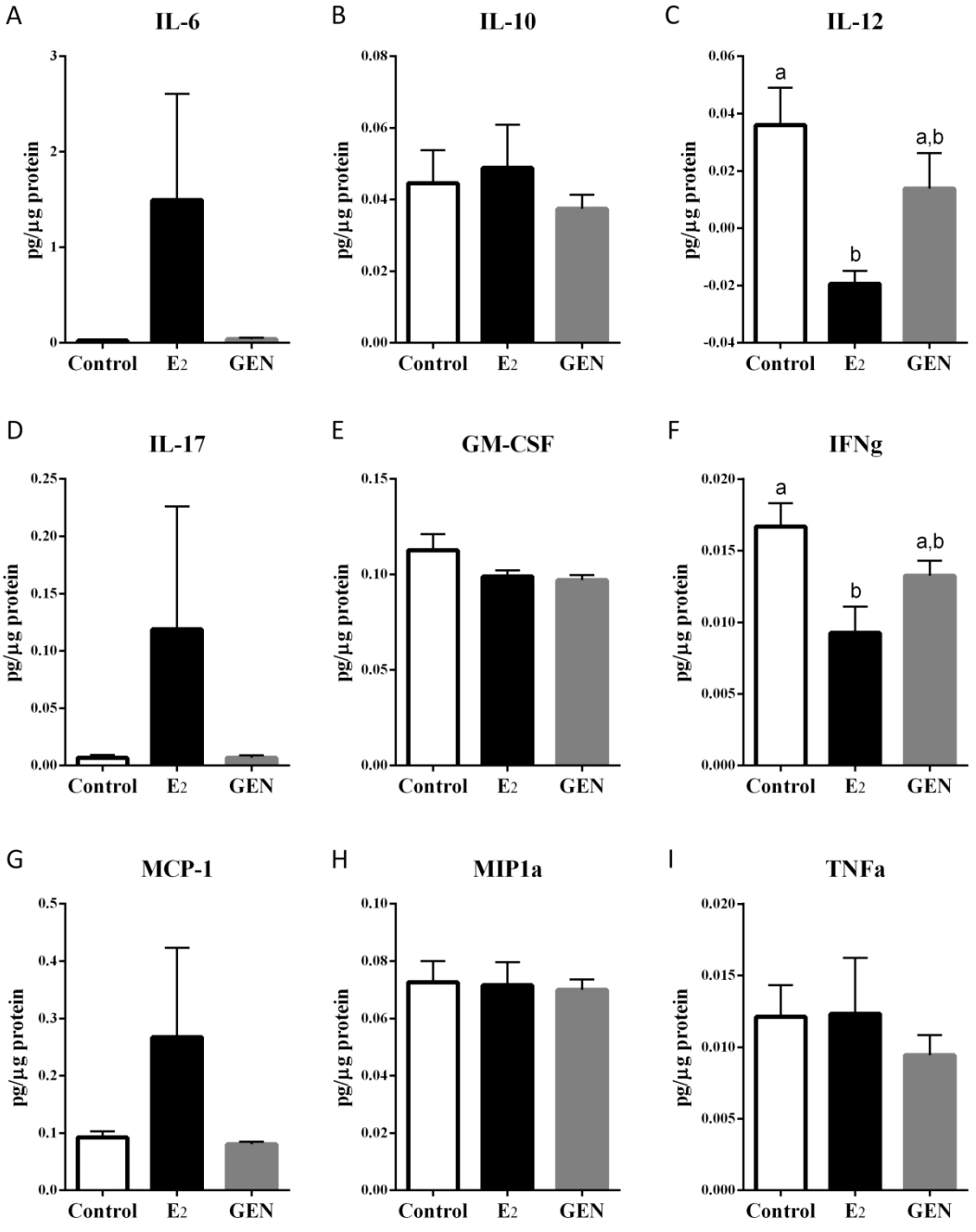

Fig. 4.5 Change of cytokines in the colon A. IL-6, B. IL-10, C. IL-12, D. IL-17, E. GM-CSF, F. IFN-γ, G. MCP-1, H. MIP1α, and I. TNFα. Values are means (n =17) ± SEM, Bars without a common letter differ; $p < 0.05$.

4.3.4 E_2 increased cell proliferation and cell numbers of colon epithelial

To estimate whether E_2 altered cell proliferation of the colon epithelial, BrdU staining was performed. Twenty complete crypts per mouse were analyzed for proliferative cells and whole cell numbers in the crypts. 1.7 cells of BrdU positive cells per crypt were found in the bottom area per crypt in the E_2 treated group, twice as many as found in the control ($p < 0.0001$). GEN had no effect on the increase of BrdU positive cells compared to control (Fig. 4.6A). The increase of BrdU positive cells by E_2 elevated the percentage of BrdU positive cells ($p = 0.0012$), demonstrating that E_2 treatment increased cell proliferation in the colonic epithelial cells (Fig. 4.6B). To estimate apoptosis of the colon epithelial cells, the TUNEL assay was performed and 20 complete crypts were analyzed per mouse. E_2 increased apoptotic cells in the top of the crypts ($p = 0.05$) while GEN had no influence on the number of apoptotic cells (Fig. 4.6C). However, the percentage of apoptotic cells was not significantly changed by E_2 treatment (Fig 4.6D). Approximately 20% more cells existed in the crypt in the E_2 treated group compared to control or GEN (Fig. 4.6E, $p = 0.0018$). Taken together, E_2 enhanced both cell proliferation and apoptosis but the effect of E_2 predominantly increased total cell numbers of the crypt.

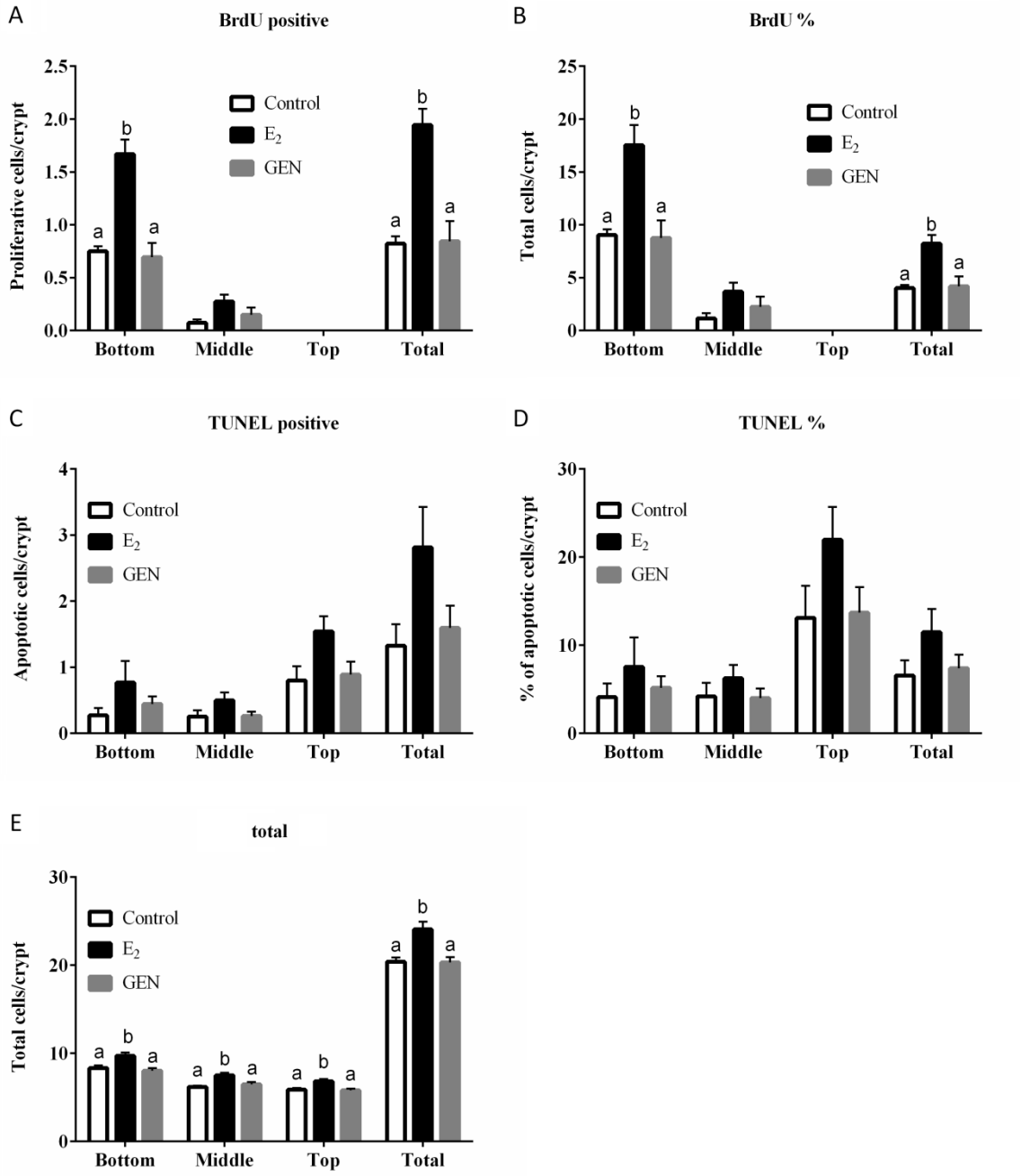

Fig. 4.6 Change of proliferative and apoptotic cells in the crypts A. Numbers of proliferative cells per crypt, B. Percentage of proliferative cells, C. Numbers of apoptotic cells per crypt, D. Percentage of apoptotic cells, and E. Total cell numbers per crypt. Values are means (n >8) ± SEM, Bars without a common letter differ; $p < 0.05$.

4.3.5 E_2 and Gen suppress ACF formation in colitis-associated carcinogenesis

To evaluate the effect of estrogen signaling on CAC development, we induced ACF formation by co-treatment of AOM, a colon specific carcinogen and TNBS in female mice (Fig. 4.7A). Three weeks after ovariectomy and implantation of pellets, AOM was injected once per week for 4 weeks to induce genetic mutation. At the 2^{nd} and 4^{th} injection of AOM, TNBS was injected intrarectally to develop CAC by a combination of genetic mutation and inflammation. E_2 treatment suppressed body weight gain compared to control and AOM with TNBS injection induced a slight weight loss (Fig. 4.7B). Change in body weight between groups was significant ($p < 0.0001$). However, GEN treatment did not influence body weight until the last injection of AOM and TNBS. Though colon length and weight were not changed significantly by any treatment, E_2 treatment slightly decreased colon length and increased colon weight, similar to the study for chronic inflammation (Fig. 4.8A and 4.8B). Uterine weight was increased by E_2 treatment as expected (Fig. 4.8C, $p < 0.0001$).

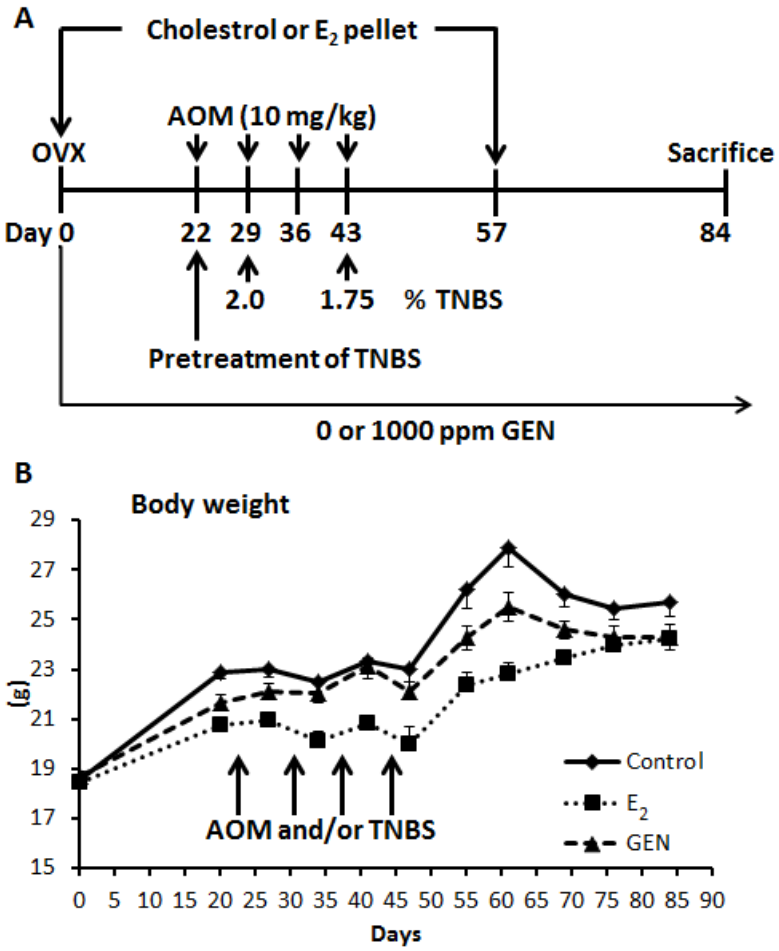

Fig. 4.7 Colitis associated colon carcinogenesis by AOM and TNBS A. Experimental design, B. Change of body weight. Values are means (n >5) ± SEM

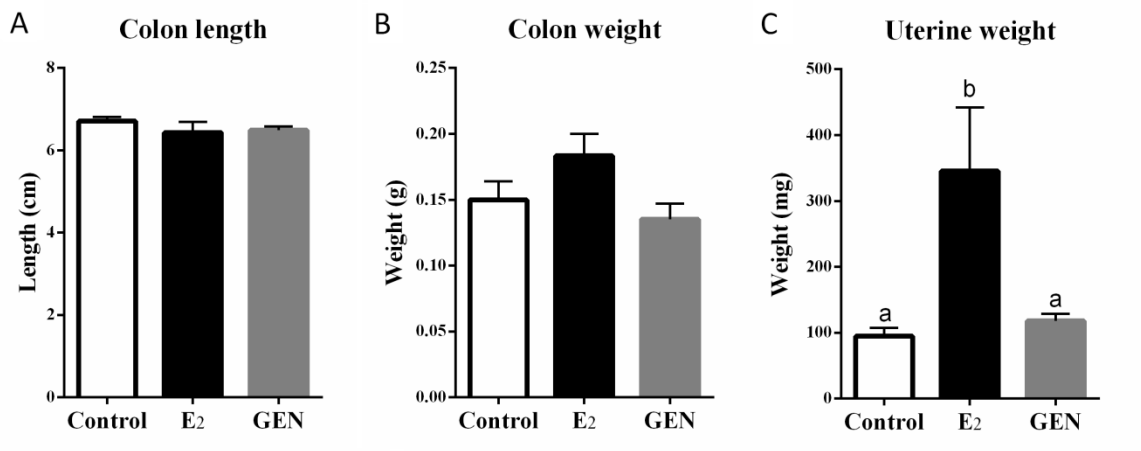

Fig. 4.8 Change of the colon and uterus on CAC A. Colon length, B. Colon weight, and C. Uterus weight. Values are means (n >5) ± SEM, Bars without a common letter differ; $p < 0.05$.

Because ACF are premalignant lesions, the number of ACF is considered a primary predictor of colon tumor formation [12]. When ACF were counted through microscopic observation after staining with 0.5% methylene blue, multiple ACF per colon were observed in all three groups (Fig. 4.9A and 9B). The ACF numbers were analyzed using Poisson model and Bonferroni adjustment for multiple tests. E_2 treatment significantly reduced high multiplicity ACF ($p = 0.02$) as well as total ACF numbers ($p = 0.0008$). GEN slightly decreased total ACF numbers ($p = 0.05$) and significantly decreased high multiplicity ACF ($p = 0.015$). To examine how ACF formation was decreased, cell proliferation and apoptosis were analyzed through BrdU immunohistochemistry and the TUNEL assay (Fig. 4.8C and 4.8D). A decrease in cell proliferation in the bottom of crypts and an increase of apoptosis in the top of crypts were observed, but these were not significantly different across treatments.

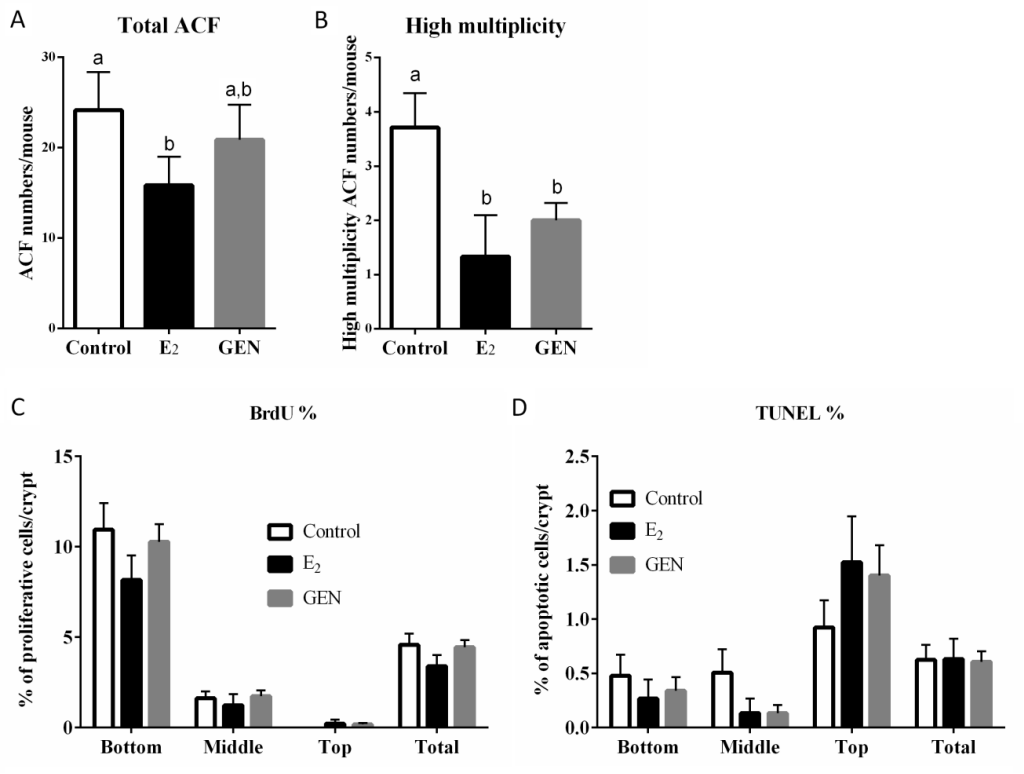

Fig. 4.9 The suppression of ACF formation by E_2 and GEN on CAC A. ACF numbers, B. High multiplicity ACF numbers, C. Percentage of proliferative cells, and D. Percentage of apoptotic cells. Values are means (n >5) ± SEM, Bars without a common letter differ; $p < 0.05$.

4.4 Discussion

It is known that chronic inflammation is associated with increased cancer incidence, and specifically, IBD is positively correlated to the risk of colon cancer [84-86]. The symptoms of Crohn's Disease (CD), one type of IBD, in women are exacerbated when the level of E_2 becomes lowest during the menstrual cycle [123]. Animal studies have suggested that E_2 has a protective effect against CAC as well as inflammation [115, 126]. To date, the role of estrogen signaling on chronic

inflammation and CAC has not yet been fully explained. In the present study, the influence of E_2 and GEN on chronic inflammation induced by TNBS and CAC induced by AOM and TNBS, was demonstrated.

Previous studies of acute TNBS treatment have shown that the colon length was shortened by TNBS compared to the untreated control [125, 200]. On the other hand, previous data from our laboratory suggest that E_2 elongated colon length, which is a part of its protective response to acute inflammation induced by TNBS. However, E_2 treatment during chronic inflammation did not increase but instead shortened the length of the colon. Though the untreated control was not involved in this study, the effect of E_2 on colon length suggested two possible mechanisms: E_2 worsened inflammation and tissue damage or affected other ways to protect tissues. Interestingly, E_2 neither increased nor decreased inflammation and injuries, suggesting E_2 treatment did not exacerbate tissue damage under chronic inflammation. Instead, E_2 increased the mRNA expression of CCND1 and PCNA, elevated proliferation and total cells per crypt, and had no significant impact on apoptosis. These data indicate that E_2 promoted the cell proliferation rate in tissues damaged by TNBS. Studies have reported that E_2 could suppress cell proliferation and increase apoptosis in the colon epithelial cells, and protect against sporadic colon carcinogenesis and CAC development [53, 126]. In contrast, a study has reported the tumorigenic effect of E_2 in CAC [195]. These conflicting data suggest that the roles of E_2 could differ depending on a variety of factors.

The major form of ER in the colon is ERβ [47]. In contrast to ERα, ERβ has a protective role in tumorigenesis via suppression of cell proliferation. Furthermore, the

expression of CCND1 was directly regulated by ERs. ERβ decreased CCND1, while ERα increased it using different partners for the transcription complex. Though the ratio of ERβ/ERα was not significantly changed, E_2 enhanced the mechanism for cell proliferation by increasing CCND1 in this study. These data suggest that E_2 could induce CCND1 expression in spite of highly expressed ERβ, resulting in cell proliferation.

The immune system plays a main role in chronic inflammation by TNBS similar to IBD [89, 90]. Communication between cytokines and roles of immune cells for immune responses are complex. Studies have reported that IBD damages colon epithelial and effector T cells are activated to fight microbial products invading the colon through impaired barrier function. In response to microbial products, myeloid cells, phagocytic cells such as monocytes, macrophages and dendritic cells, in the lamina propria release cytokines to activate effector T cells [103]. Therefore, cytokines related to effector T cells and myeloid cells were estimated in the chronic inflammation study. Myeloid cells produce a variety of cytokines including IL-6, MCP-1 and GM-CSF to recruit and activate T lymphocytes. Depending on the pathologic condition, the types of cytokines released from myeloid cells are different, leading to activation of different types of effector T cells. Here, several cytokines released from myeloid cells were examined: IL-6, MCP-1, GM-CSF, MIP1α, IL-12 and IFN-γ. Among them, IL-6 and MCP-1 were increased in E_2 treated mice. Combined with increased IL-17 level by E_2 treatment, elevation of IL-6 and MCP-1 by E_2 indicates that E_2 treatment enhanced the T_H17 mediated immune response. IL-12 and IFN-γ were decreased in E_2 treated mice, suggesting that E_2 inhibited T_H1 activation. In contrast, GM-CSF and MIP1α were not

changed by E_2, suggesting that E_2 modulates specific immune responses in myeloid cells. IL-10 is an anti-inflammatory cytokine produced from T_{Reg}, which suppresses immune responses. E_2 has no impact on IL-10, suggesting T_{Reg} activation was not changed by E_2.

It has been reported that the immune response induced by TNBS is similar to CD, which is associated with T_H1 and T_H17 [87, 89, 90]. The influence of T_H1 and T_H17 on colitis and CAC has been studied [94, 102]. Though both cells play a critical role in IBD and other immune responses, the function of T_H1 predominantly focuses on removal of damaged cells, while T_H17 enhances tissue recovery by cell proliferation [107]. T_H1 cells are positively correlated to removal of dysplastic cells because IFN-γ secreted by T_H1 cells has anti-proliferative properties in colitis and T_H1 cells induce the activation of cytotoxic $CD8^+$ T cells. Furthermore, loss of IFN-γ impaired colon tumor formation in the CAC model. In contrast, T_H17 is known as an inducer of cell proliferation due to IL-6 and IL-17. IL-6 secreted from macrophages induces activation of T_H17 cells, which produces IL-17. Increased IL-6 and IL-17 has been found in IBD and colitis-associated colon cancer patients and the level of these cytokines is positively correlated with a poor prognosis in colon cancer [98, 109]. Previous data from Chapter II has shown that IL-6 treatment increased cell growth of non-malignant colonocytes, suggesting a tumorigenic effect of IL-6 in the colonic epithelial cells. Furthermore, competition between T_H1 and T_H17 has been reported [102]. IL-17 suppresses T_H1 cell differentiation, and IFN-γ reduces T_H17 cell numbers. Therefore, suppression of T_H1 by E_2 could enhance differentiation of T_H17 cells, resulting in increased cell proliferation by alteration of the

T_H1/T_H17 ratio. These data suggest that E_2 protects the colon via enhanced recovery of the colon tissue.

IL-6 is a major cytokine in chronic inflammation, which is elevated in IBD and colon cancer [109]. Studies have reported that intestinal epithelial cells express IL-6 receptor and IL-6 signaling is activated during colon carcinogenesis via an increase of gp130, one component of the transmembrane receptor complex for IL-6 signaling [113, 201]. These data supported the hypothesis that IL-6 signaling could be activated in colonic epithelial cells during colitis and CAC. Interestingly, its expression in colon epithelial cells was suppressed slightly by E_2 in this study. It is known that estrogen signaling decreases IL-6 expression via inhibition of NFκB dimerization, not direct binding to the IL-6 promoter. Therefore, E_2 treatment tightly modulates cell proliferation via suppression of IL-6 expression in the colonic epithelial cells, though E_2 increased IL-6 secretion in the lamina propria.

In the CAC model, the change in colon length and weight was not significant between groups. Because mice were sacrificed 6 weeks after the last injection of TNBS and there is no published report that AOM injection altered colon length or weight, the effect of TNBS on the colon physiology via colitis could be weak. Still, E_2 treated mice had slightly shorter but heavier colons than control mice, indicating that E_2 shortened the colon length against TNBS as observed in the chronic inflammation study.

Though CAC development has a distinct mechanism compared to sporadic colon cancer, E_2 treatment reduced ACF formation in the present CAC model, supporting the protective effect of E_2 against CAC [93]. Surprisingly, no significant difference was

found in proliferation or apoptosis in the colon crypts in the CAC experiment. These data support the assumption that the role of estrogen signaling is complicated. Estrogen signaling enhances apoptosis not cell proliferation in healthy colonic epithelial and it is mediated by ERβ, the predominant ER in the colon [75]. E_2 treatment elevated apoptosis to protect against sporadic colon carcinogenesis by AOM [53]. However, the role of estrogen signaling was opposite in the chronic inflammation model. During chronic inflammation, E_2 treatment induced cell proliferation for recovery of damaged tissues via an increase of T_H17 and a decrease of T_H1. In the CAC model, AOM and TNBS were injected together, causing inflammation and genetic mutation at the same time. Therefore, the time at which the mice were sacrificed could be a transition time in which estrogen signaling moves its focus on apoptosis of dysplastic cells, from cell proliferation for tissue recovery because the last TNBS injection was 6 weeks before sacrifice. Changes of both cell proliferation and apoptosis were not statistically significant, possibly due to dual effects of estrogen signaling.

GEN is a phytoestrogen that has anti-inflammatory, anti-tumorigenic and pro-apoptotic properties [64, 75, 128]. GEN has more binding affinity to ERβ than ERα [77]. Because the major form of ER in the colon is ERβ, and ERβ mediates protection against colon carcinogenesis, GEN as an ERβ agonist was predicted to be a good candidate to protect the colon through ERβ against colitis and CAC in this study [47]. However, GEN treatment increased inflammation and injury in chronic inflammation and had no effect on colon length, though it suppressed body weight gain similar to E_2. Though GEN elevated CCND1 and PCNA expression, proliferative cells were not changed by GEN.

Furthermore, GEN treatment slightly suppressed activation of T_H1 without activation of T_H17, suggesting that removal of damaged cells is active but cell proliferation is limited in the colon treated with GEN. Considering the main protective effect of E_2 is due to cell proliferation to recover damaged tissues, the effect of GEN enhancing apoptosis could cause more damage to the colon. More inflammation and injuries by GEN could be due to enough T_H1 with low T_H17. GEN treatment suppressed ACF formation slightly in the CAC model. Taken together, GEN could be a good alternative to prevent CAC, but it may exacerbate the severity of colitis.

In conclusion, the present data demonstrate the dual effect of estrogen signaling on colitis and CAC. Though estrogen signaling primarily has an apoptotic effect on the colonic epithelial cells via ERβ, the effect of estrogen signaling could be pro-proliferative during colitis without a shift in the expression of ERβ to ERα. During CAC, estrogen signaling plays both roles: induction of apoptosis and cell proliferation for inhibition of ACF formation. The dual roles of estrogen signaling provide important clues to understand the potential mechanism of estrogen signaling against colitis as well as CAC, and offers new insights for the protective role of estrogen signaling on colon cancer.

CHAPTER V

ESTRADIOL SUPPRESSES COLON CARCINOGENESIS IN SPITE OF LOSS OF P53

5.1 Introduction

Cancer is a prevalent health problem worldwide and accounts for one in four deaths in the United States [1]. Though cancers are variable and distinct from one another, all cancers are derived from aberrant cell proliferation with the potential for invasion into other tissues or organs [6, 202]. Uncontrolled cell proliferation starts with genetic changes such as a loss of a chromosome or a genetic mutation. Because cell division occurs continuously to maintain human life, the chance of genetic alteration exists all the time. When a genetic change occurs in the gene critical to regulation of cell proliferation, cells lose the ability to control proliferation and are transformed into tumor cells. Still, organisms have protective mechanisms to prevent dysregulated cell proliferation. The DNA repair system fixes altered genes via Ataxia-Telangiectasia mutated (ATM) and Ataxia-Telangiectasia and Rad-3-related (ATR), reducing the chance of error during gene replication by 1 in 2×10^7 cell divisions [203, 204]. If genetic changes cannot be repaired by the DNA repair system, cells induce programmed cell death called apoptosis. Apoptosis is the process of controlled cell destruction by complex signaling cascades such as caspases [205, 206]. If cells evade these mechanisms, they transform into tumor cells and cancer develops.

p53 is a tumor suppressor gene known to be a short-lived transcription factor [16, 207]. Mutation of p53 is found in a variety of cancers, and over 20 million people have tumors in which p53 itself or p53 signaling is abrogated [131]. Most p53 mutations are found in the DNA binding domain of p53 [16, 208]. Mutated p53 has oncogenic properties and can disrupt p53 signaling. p53 is a central mediator of the protective mechanisms for controlling cell proliferation including cell cycle arrest, the DNA repair system and apoptosis in response to a variety of stresses. When any stress exists, such as DNA damage or hyperproliferative signaling, p53 is activated and active p53 modulates the expression of genes related to cell cycle progression and apoptosis. Because p53 signaling alters cell fates, it is tightly regulated by mouse double minute 2 homolog (Mdm2). Mdm2 is an E3 ubiquitin ligase that recruits p53 to degradation via ubiquitination.

p53 plays a critical role in colon carcinogenesis, and approximately half of colon cancer cases show a p53 mutation [209]. Previous data from our laboratory have demonstrated that estrogen signaling also has a protective role in sporadic colon carcinogenesis via ERβ [53]. However, studies demonstrating the relationship between p53 and estrogen signaling have mostly focused on breast cancer [210, 211]. In these studies, p53 suppressed ERα mediated estrogen signaling in breast cancer. ERβ is the major form of ER in the colon while ERα is predominant in the breast [47]. Because ERβ is linked to apoptosis, while ERα is associated with cell proliferation, the role of p53 in estrogen signaling could be different in the colon compared to the breast [211]. A previous study from our laboratory demonstrated that E_2 increased p53 expression as

well as modulated the expression of genes known as targets of p53. These findings suggest that p53 is a mediator of estrogen signaling involved in preventing colon carcinogenesis [143].

Because of the critical role of p53 in the cell cycle, DNA repair and apoptosis, p53 mutation generates a variety of tumors in a genetically modified mouse model [17]. Furthermore, p53 null mice develop tumors at a very early age and die by 10 months of age. Therefore, numerous problems accompany utilization of p53 null or knockout mice in the study of colon cancer. A previous study from our laboratory was conducted with p53 heterozygous ($p53^{+/-}$) mice to examine the role of p53 in the protective effect of estrogen signaling against colon carcinogenesis [144]. More ACF, which are premalignant lesions, were found in $p53^{+/-}$ mice treated with azoxymethane (AOM), but E_2 treatment still had a protective effect in $p53^{+/-}$ mice treated with AOM. Compared to $p53^{+/+}$ (Wild Type) mice, the protective efficacy of E_2 was decreased by approximately 50%. To conduct experiments with p53 null conditions in the colon, intestinal epithelial cell specific p53 knockout mice were developed [136]. These mice are called $Tp53^{\Delta IEC}$ and exhibit a genetic mutation that causes loss of the exons 2–10 of Tp53 in their IECs only. $Tp53^{\Delta IEC}$ mice appear healthy and are fertile, indicating that loss of p53 in IECs alone is not sufficient for colon cancer. However, when AOM was injected to induce colon cancer, these mice developed more tumors via impaired apoptosis and increased DNA damage. Therefore, $Tp53^{\Delta IEC}$ mice could be a good model to demonstrate the role of p53 in estrogen signaling during colon carcinogenesis.

The aim of this study was to demonstrate the role of p53 in the protective activity of estrogen signaling in the development of sporadic colon cancer. Though the influence of p53 on estrogen signaling is partially implied by the experiment with $p53^{+/-}$ mice, it is still not clear because $p53^{+/-}$ mice have active p53 signaling. In the present study, we utilized $Tp53^{\Delta IEC}$ mice injected with AOM, to examine whether p53 plays a pivotal role in the protective effect of estrogen signaling. These data will clarify the relationship between p53 and estrogen signaling in colon carcinogenesis.

5.2 Materials and methods

5.2.1 Animals

$Tp53^{Flox/Flox}$ mice (Background: c57BL6) were purchased from the Jackson Laboratory (Strain name: B6.129P2-*Trp53*^{tm1brn}/J). V*illin*-CRE mice (Background: c57BL6) were obtained from the National Cancer Institute/National Institutes of Health (Strain number: 01XE7). Mice were bred in the Laboratory Animal Resources and Research facility at Texas A&M University as previously reported [136]. By mating $Tp53^{Flox/Flox}$ mice and *villin*-CRE mice, offspring (F1) of genotypes $Tp53^{Flox/+}$/Cre and $Tp53^{Flox/+}$ were obtained. By mating two F1 offspring, F2 offspring were produced. Among them, $Tp53^{\Delta IEC}$ mice ($Tp53^{Flox/Flox}$/Cre) and $Tp53^{Flox/Flox}$ mice ($Tp53^{Flox/Flox}$) were identified by genotyping. These two mice were mated, and F3 offspring: $Tp53^{\Delta IEC}$ mice ($Tp53^{Flox/Flox}$/Cre) and $Tp53^{Flox/Flox}$ mice ($Tp53^{Flox/Flox}$) were produced and utilized for the present experiment. Mice were housed at the Laboratory Animal Resources and Research facility at Texas A&M University. All procedures were performed under

protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University.

5.2.2 ACF induction

$Tp53^{Flox/Flox}$ mice and $Tp53^{\Delta IEC}$ mice were ovariectomized and either a 20 mg cholesterol or a 19 mg cholesterol with 1 mg E_2 pellet was implanted as described in a previous article [53]. After 2 weeks, mice received 6 weekly injections of 10 mg/kg AOM. Pellets were replaced 8 weeks after ovariectomy. Seven weeks after the last injection of AOM, mice were injected with 5-bromo-2'-deoxyuridine (BrdU). Two h later, mice were sacrificed and tissues were collected. Blood was collected by cardiac puncture. Colons were excised and opened longitudinally. 1 cm sections from the distal end and excised tumors were 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 [198].

5.2.3 Reagents

β-Estradiol (E_2) and azoxymethane (AOM) were purchased from Sigma Aldrich.

5.2.4 Statistical analysis

The data are expressed throughout as the means ± SEM, which was calculated from at least 12 mice per group. To compare body weights between groups, generalized estimating equations and a Wald test were utilized with a main effect for comparison group, a natural cubic spline for the time course, and an interaction between the two, based on an assumption of an autoregressive correlation structure. For the analysis of

ACF and tumors, generalized regression models assuming a Poisson distribution were carried out and ANOVA-like hypotheses were tested. A p-value of less than 0.05 was considered significant. All data were analyzed using Graphpad and a data analysis software R with a certified statistician, Dr. Alan Dabney.

5.3 Results

5.3.1 E_2 suppressed ACF formation in $Tp53^{\Delta IEC}$ as well as $Tp53^{Flox/Flox}$

To investigate the effect of E_2 on ACF formation, $Tp53^{\Delta IEC}$ mice and $Tp53^{Flox/Flox}$ mice were divided into two groups: control and E_2. The average body weight in all four groups of mice began similarly and did not increase significantly during AOM treatment. After the last injection of AOM, an increase in body weight was observed (Fig. 5.1B). The difference in body weight between the control and E_2 group in $Tp53^{\Delta IEC}$ mice was not significant and only $Tp53^{Flox/Flox}$ mice treated with E_2 had a lower body weight 70 days after starting the experiment compared to the control group in $Tp53^{Flox/Flox}$ mice ($p = 0.04$). As reported in previous data, E_2 increased uterine weight approximately 10 times the weight of control (Fig. 5.1C, $p < 0.0001$).

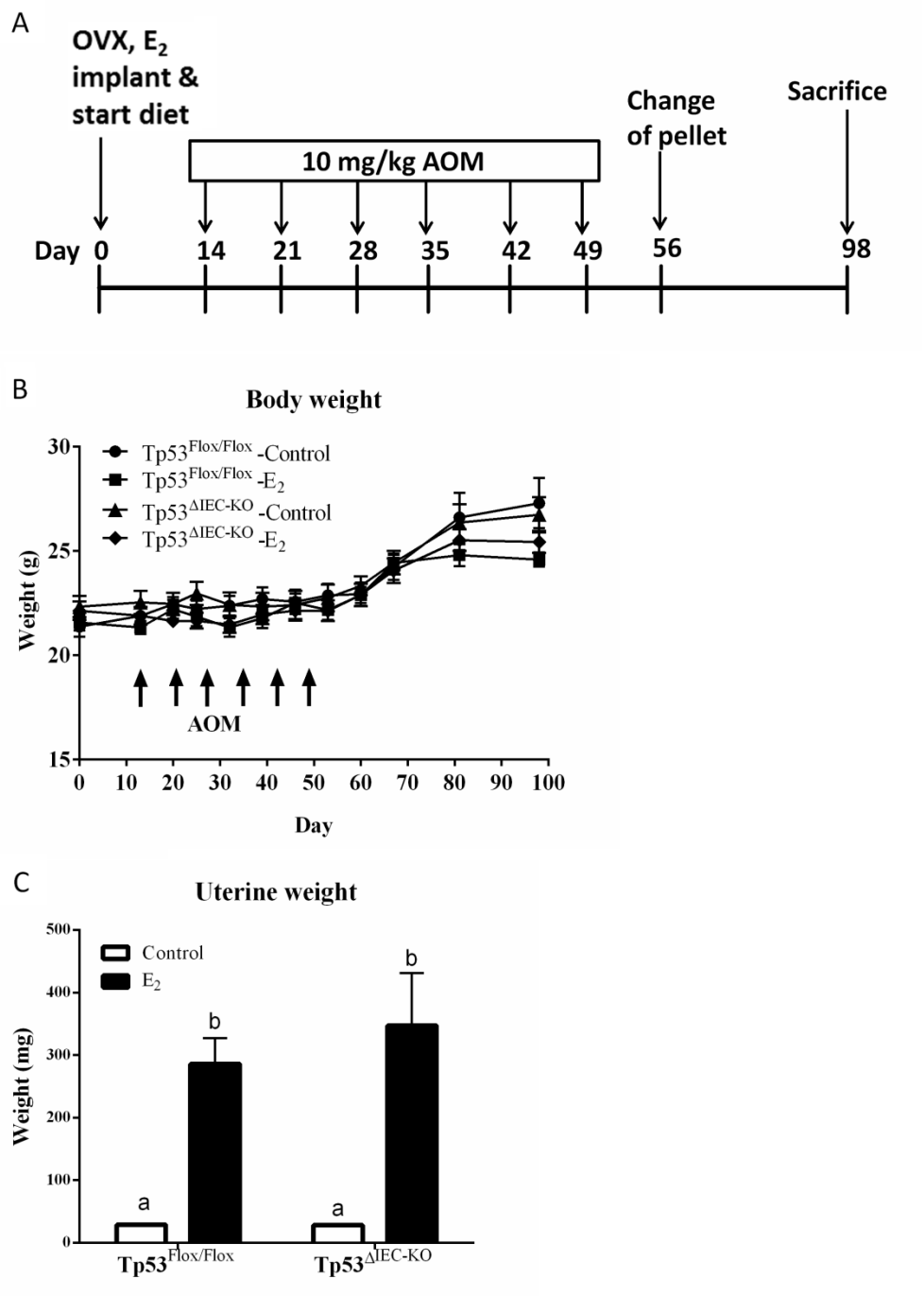

Fig. 5.1 Induction of sporadic colon cancer in Tp53$^{Flox/Flox}$ and Tp53$^{\Delta IEC}$ mice
A. The experimental design, B. Change of body weight, and C. Uterine weight. Values are means (n>11) ± SEM, Bars without a common letter differ; $p < 0.05$.

ACF and tumor numbers were counted using microscopic analysis. $Tp53^{Flox/Flox}$ mice had a lower number of ACF than $Tp53^{\Delta IEC}$ mice, but the difference of ACF numbers was not significant (Fig 5.2A). There was a slight increase in high multiplicity ACF in $Tp53^{\Delta IEC}$ mice compared to $Tp53^{Flox/Flox}$ mice (Fig. 5.2B). However, tumor incidence in $Tp53^{\Delta IEC}$ mice was high, while tumors were rarely found in $Tp53^{Flox/Flox}$ mice (Fig 5.2C, $p < 0.0001$). These data demonstrate that loss of p53 in $Tp53^{\Delta IEC}$ mice promoted ACF formation.

In $Tp53^{Flox/Flox}$ mice, mice treated with E_2 had a slightly lower number of total ACF than control mice, while also having a slightly higher number of high multiplicity ACF compared to the control, but those changes were not significant. In addition, tumor numbers were slightly decreased by E_2 treatment compared to control in $Tp53^{Flox/Flox}$ mice. E_2 treatment had a protective effect in $Tp53^{\Delta IEC}$ mice. In $Tp53^{\Delta IEC}$ mice, E_2 treatment did not decrease total ACF, but reduced high multiplicity ACF and tumor numbers compared to control (Fig. 5.2, $p = 0.13$ for high multiplicity ACF and $p < 0.0001$ for tumors).

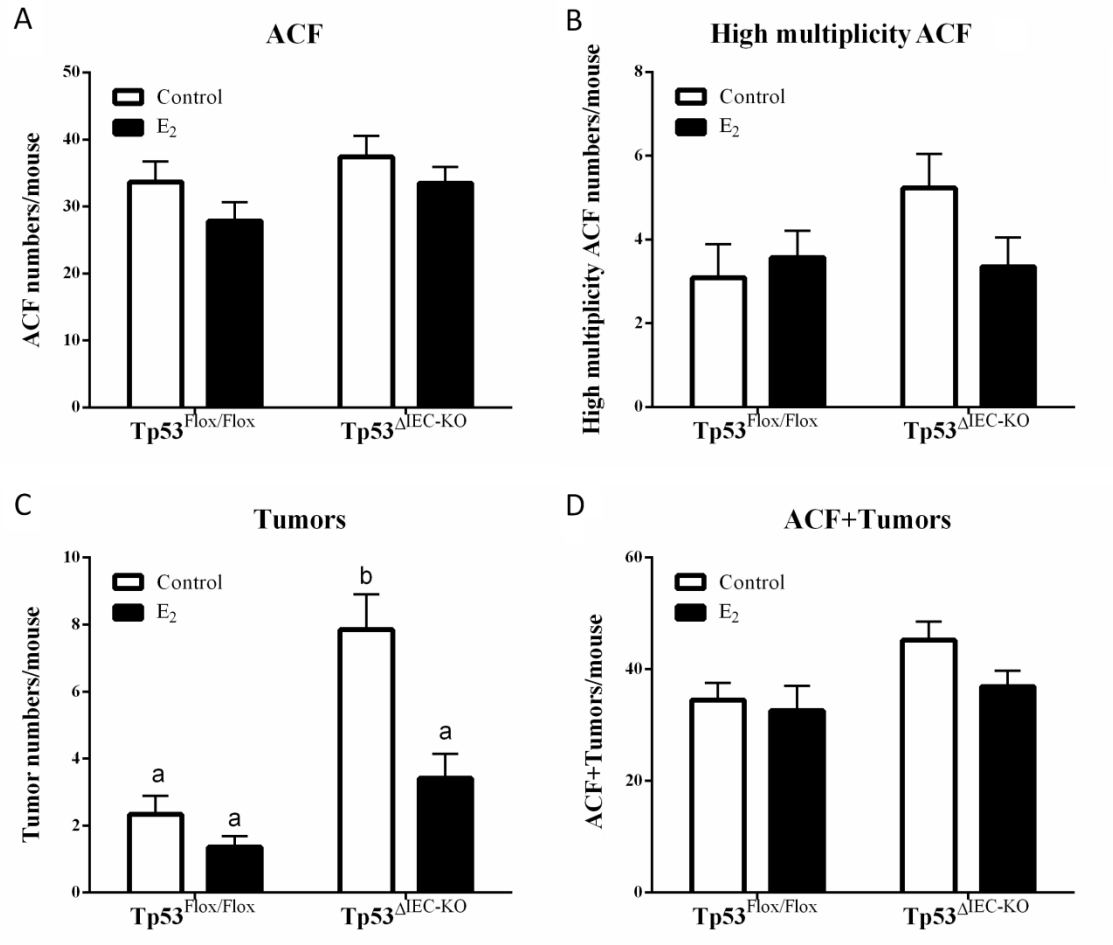

Fig. 5.2 ACF and tumor formation in $Tp53^{Flox/Flox}$ and $Tp53^{\Delta IEC}$ mice A. Numbers of ACF, B. Numbers of high multiplicity ACF, C. Numbers of tumors, and D. Numbers of ACF and tumors. Values are means (n >12) ± SEM, Bars without a common letter differ; $p < 0.05$.

5.4 Discussion

p53 is a tumor suppressor protein functioning as a key mediator that controls cell fates [16]. As a transcription factor, p53 modulates gene expression associated with cell cycle progression and apoptosis in response to a variety of stress signals. Epidemiological and clinical studies have demonstrated the suppressive role of p53 in

cancer [131, 135]. Previous studies from our laboratory have shown that the protective effect of estrogen signaling in colon cancer involves the p53 pathway in non-malignant colonocytes and $p53^{+/-}$ mice [143, 144]. Further experimentation is required to clarify the interaction between estrogen signaling and the p53 pathway in colon cancer. In the present study, IEC-specific p53 knockout mice, $Tp53^{\Delta IEC}$ mice, were utilized to induce colon cancer.

Previous data from our laboratory show that a partial loss of p53 was not sufficient for an increase in ACF in $p53^{+/-}$ mice with an AOM injection [144]. However, as previously reported, $Tp53^{\Delta IEC}$ mice were more susceptible to AOM induced ACF formation compared to $Tp53^{Flox/Flox}$ mice [136]. This was due to p53 loss, resulting in more ACF and tumors. These data suggest that the p53 null condition is critical for colon carcinogenesis.

However, E_2 treatment did not decrease ACF and tumor formation significantly in $Tp53^{Flox/Flox}$ mice. These data are different from previous data from our laboratory, in spite of the same background: c57BL6 [53]. These data could be due to the transition from ACF to tumors.

Interestingly, E_2 treatment significantly reduced tumor formation in $Tp53^{\Delta IEC}$ mice and slightly decreased high multiplicity ACF showing that E_2 still has a protective effect in colon carcinogenesis in spite of p53 loss. Though activated estrogen signaling increased p53 and p53 mediated genes in non-malignant colonocytes, these data suggest that p53 is not the only mediator for the suppression of cell proliferation by estrogen

signaling [143]. Therefore, further study is required to identify the mediator of estrogen signaling against colon carcinogenesis.

In conclusion, the present data demonstrated that estrogen signaling still has a protective effect despite loss of p53. These data suggest that estrogen signaling could protect against colon carcinogenesis independent of the p53 pathway, though the p53 pathway is activated by E_2 treatment in colonocytes. Therefore, further investigation of potential mechanisms of estrogen signaling during colon cancer progression would give more insight into colon carcinogenesis.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Colon cancer develops from complex changes in the colon epithelial layer resulting from uncontrolled cell proliferation and a series of genetic mutations [6, 9]. Numerous risk factors for colon cancer have been identified, and chronic inflammation such as IBD is one of the major predisposing conditions for colon cancer [13, 85]. Estrogen signaling has a protective effect on colon carcinogenesis and can modulate the adaptive immune system's role in inflammation [53, 115]. Therefore, studying the role of estrogen signaling on colitis and colon carcinogenesis will help clarify the potential mechanism of estrogen signaling in colon cancer. In the present studies, the protective effect of estrogen signaling has been demonstrated and the protective mechanism of estrogenic molecules was investigated *in vitro* and *in vivo* on inflammation and colon cancer models.

In the *in vitro* study, E_2 suppressed cell growth of YAMCs via alteration of gene expression associated with cell proliferation and apoptosis. However, E_2 inhibited the effect of IL-6, a pro-inflammatory cytokine, on YAMCs, suggesting that activated estrogen signaling by E_2 interrupts IL-6 action which decreases apoptosis in non-malignant colonocytes [104]. The protective effect of E_2 led to an opposite outcome in chronic inflammation. E_2 treatment increased cell proliferation of the colonic epithelial cells to promote the recovery of damaged tissues instead of increasing apoptosis. However, E_2 suppressed the formation of ACF without significant induction of apoptosis

or proliferation. Previous data from our laboratory demonstrated that E_2 protects against sporadic colon carcinogenesis via induced apoptosis [53]. In addition, E_2 decreases acute inflammation in the colon via modulation of cytokines [125]. Considering previous data, the data in the present studies suggest dual effects of E_2 on colitis and colitis-associated colon carcinogenesis in maintaining the homeostasis of colonic epithelial cells.

p53 is a major regulator of cell fates, proliferation and apoptosis, and plays an important role in colon cancer development [130, 135]. In a previous study with YAMCs, the induction of apoptosis by E_2 includes the activation of the p53 pathway [143]. In the present study, loss of p53 increased ACF formation, but E_2 treatment still showed a protective property against sporadic colon carcinogenesis. These data suggest that estrogen signaling protects the colon independent of the p53 pathway and further study is required to identify the potential mechanism of estrogen signaling that enhances apoptosis.

Phytoestrogens are natural compounds that mimic estrogen by interacting with ERs [57]. Whether directly binding to ERs or not, they induce the alteration of the expression of target genes involved in estrogen signaling. The present studies examined the effect of the phytoestrogens, Trig, DIM and GEN, in the colonocytes. Trig and DIM have a distinct structure, and they do not directly bind to ERs. Still, Trig suppressed cell growth of YAMCs via increased apoptosis, and the effect of Trig was nullified by the ER antagonist, ICI. This indicates the estrogenic effect of Trig mediates the changes in YAMCs mediated by ERs. DIM influences the expression of genes related to estrogen signaling and the effects of DIM include the change of ER transcriptional activity. GEN

has a suppressive activity on the cell growth of YAMCs similar to E_2, and the induction of apoptosis by GEN reduced ACF formation in the CAC model. However, GEN enhanced inflammation and injury under conditions of chronic inflammation because it has no impact on cell proliferation. These data from studies of phytoestrogens suggest that each phytoestrogen induces the activation of estrogen signaling but each has a unique mechanism depending on its structure and binding affinity for ERs.

Overall, the present studies demonstrated the protective role of estrogen signaling on colitis and colon cancer *in vitro* and *in vivo*. These experiments using different experimental models help clarify the dual roles of estrogen signaling in different conditions of colon damage and the partially independent mechanism of estrogen signaling on the p53 pathway. The comparison of E_2 to several phytoestrogens helps elucidate the complex mechanism of estrogen signaling. The present studies will provide new insight for the role of estrogen signaling in colon cancer.

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