THE ROLE OF P53 AND ESTRADIOL ON THE SUPPRESSION OF SPORADIC TUMOR FORMATION IN THE COLON

An Undergraduate Research Scholars Thesis

by

JENNA JEFFREY

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Clinton Allred

May 2017

Major: Nutritional Sciences

TABLE OF CONTENTS

ABSTRACT

The Role of p53 and Estradiol on the Suppression of Sporadic Tumor Formation in the Colon

Jenna Jeffrey
Department of Nutrition and Food Science
Texas A&M University

Research Advisor: Dr. Clinton Allred
Department of Nutrition and Food Science
Texas A&M University

Previous studies have shown a correlation between estradiol (E_2) levels in women and the decreased risk of colon cancer. E_2 serves a protective role in the colon by increasing apoptosis in non-malignant colonocytes that have become damaged due to different stressors within the body [1]. The p53 protein plays an important role at the cellular level by detecting DNA damage and regulating cell cycle progression. A proposed mechanism suggested by *in vitro* studies, indicates that the protective effects of E_2 on tumor formation within the colon is mediated by p53 [2]. The roles of E_2 and p53 on tumor development in the colon were analyzed through the identification of visible tumors and proliferation in the distal colons of C57/BL-6J female mice. E_2 suppressed tumor development in the presence of a functional p53 protein. Proliferation was enhanced in colonic tissue lacking a functional p53, thus suggesting the importance of p53 in suppressing tumor development. Collectively, our findings illustrate the importance of a functional p53 protein in tumor development and its role in mediating E_2 protective effects within the colon.

ACKNOWLEDGEMENTS

I would like to thank my faculty advisor, Dr. Clinton Allred, for his guidance and support throughout my time in the Undergraduate Research Scholars program, as well as throughout the course of my research experience. He has truly fostered the passion I have for research and has supported my development by encouraging me to think critically and beyond the scope of field.

I would also like to thank and acknowledge Mrs. Kimberly Allred. She was key in the development of my research skills, as she taught me the proper techniques needed to be successful in the laboratory. I am thankful for her guidance and patience, as I always seemed to bombard her with questions.

I am also incredibly grateful for all of the graduate students in Dr. Allred's lab: Jordan Hillman, Jennifer DeLuca, and Erika Garcia Villatoro. Not only have they made my time at Texas A&M University a delightful and unforgettable experience, they too were instrumental in my success at Texas A&M University by critiquing many of my works and passing down useful laboratory or life related advise.

Two other people that I would like to acknowledge are my parents, Ross and Jill Jeffrey. I am incredibly grateful for all of their love, support, and words of encouragement they have given me throughout my life. They have been my rock through some incredibly difficult times and have been my biggest advocate in pursuing my dreams.

Lastly, I would like to recognize the Department of Nutrition and Food Science through the support of the Undergraduate Research Scholarship.

CHAPTER I

INTRODUCTION

Colon Cancer and p53

Research focused on the study of colorectal cancer (CRC) represents an important topic worldwide. It has been reported that CRC is the second leading cause of cancer-related death behind lung cancer [3]. Many avenues of research are being conducted in order to better understand the physiological mechanism behind causation and potentially prevention of CRC. Development past the fifth decade of life, Inflammatory Bowel Diseases (IBD), smoking, alcohol consumption, diet, and a sedentary lifestyle are only a few of the many risk factors in the development of CRC [4]. Improvements made in health care are reducing death from CRC and decreasing incidence of the disease, through improved screening methods and removal of adenomatous polyps [5].

One of the most important defenses that the body has in fighting the development of cancer is through the tumor suppressor gene p53. A variety of cancers arise from a mutation in the p53 gene [6]. The high incidence of mutations in p53 qualifies it as an important target for cancer research. Mutation in p53 results from the inactivation of alleles apart of chromosome 17p (Ch17p) [7]. In cases of most colorectal tumors the p53 gene has been altered, attributing the loss of p53 function to the transition from an adenoma to a malignant carcinoma [4]. Lack of a functioning p53 gene prevents normal activity of apoptosis to rid the tissue of damaged cells and loses the ability to suppress cell cycle when cells become to plentiful.

Tumorigenesis arises from dysregulation of cell growth and proliferation [8]. In response to an oncogenic stress, p53 induces transcriptional and translational responses in order to prevent

abnormal cell growth by inducing cell-cycle arrest through down-regulation of cell-cycle genes [8]. The mechanism by which p53 contributes to regulating cell growth is through translational repression of key translational factors and ribosomal proteins [8]. The colon is a rapidly proliferating organ, where damages or mutations within DNA can quickly arise, thus stimulating the activation of the p53 protein [9]. A delay is made between the G_1 and S phase, where DNA repair can be carried out to fix the point mutation made [9]. These alterations initiated by p53, suppress tumor formation by not allowing the damaged cell to proceed into the next phase of the cell cycle.

There are two pathways in which CRC can develop; a prolonged duration of chronic inflammation of the gut or spontaneously [10]. A model to better illustrate the onset of inflammation-associated CRC can best be seen through those suffering from IBD. Those suffering from IBD have an increased risk of CRC due to the prolonged existence of mucosal inflammation [11]. Environmental and internal agents elicit an immune response from the damaged colonic tissue, resulting in the need for regeneration and proliferation [12]. In the event that the tissue is not repaired, the surrounding microenvironment continues efforts made in restoration and repair, leading to the continuation of unregulated proliferation and accumulation of genetic errors [12].

Sporadic CRC accounts for 70% of all detected colorectal cancers [4]. The mechanism behind the initiation of sporadic CRC is derived from point mutations in oncogenes, tumor suppressor genes, and genes responsible for DNA repair [4]. Point mutations may arise during life through exposure to mechanical and chemical agents. The progression of tumor formation begins with an adenoma and concludes in the transitional formation of a carcinoma. Most, but not all, non-malignant adenomas arise from a mutation first transcribed in the adenomatous

polyposis coli (APC), a tumor suppressor gene [4]. An APC mutation results in abnormal function of the cell due to a truncated protein product, effecting the region of protein essential for either tumor progression or suppression [13]. Mutations in KRAS and TP53 progress after a mutation in APC [4]. A mutation in KRAS increases cell proliferation as a result of constant activation of mitogen-activated protein (MAP) kinase. TP53 encodes for the tumor suppresser gene p53. A mutation in TP53 would result in unregulated entry into the cell cycle [4].

While both types of cancers occur through a multistep development with accumulations of numerous mutations, one key difference between the two cancers can best be seen in the timing of a genetic mutation in p53 [11]. A mutation in p53 arises earlier in inflammation-associated CRC when compared to the sequence of events leading to the mutation in sporadic CRC [11]. Studies have shown that in the event of mutation in the tumor suppressor gene p53, the mechanism for cell regulation becomes compromised, allowing for a tumor to progress. Not only is a functional p53 protein critical in the suppression of tumor development in the colon but recent studies and census data have suggested that E_2 also protects against CRC [14].

Colon Cancer and Estradiol

The protective role of E_2 in the suppression of CRC can best be supported by the findings from the Women's Health Initiative. A lower incidence of CRC was found among postmenopausal women receiving hormone replacement therapy (HRT), when compared to those receiving the placebo not made of estrogen and progesterone [14]. Literature also suggests the protective roles of E_2 through the decreased risk in the development of CRC seen in females with IBD compared to males with IBD [15]. *In vivo* studies have also illustrated the protective role that E_2 plays against preneoplastic lesions [1]. E_2 serves protective effects in the premalignant

carcinogenesis stage through a reduction in aberrant crypt foci (ACF), which are lesions that serve as a good indication for tumor development [1]. Collectively, these data suggest that E_2 has protective effects within the colon by suppressing the development of tumors.

Our laboratory is investigating the role that both p53 and E_2 play in congruence with one another. Through *in vitro* and *in vivo* studies, the presence of E_2 significantly increased apoptosis in young adult mouse colonocytes and colonic epithelium [1]. The induction of apoptosis indicates that E_2 triggers p53 activity. Further investigation showed that E_2 provides protective effects within the colon when p53 is expressed, and suppressed or completely lost in the absence of p53 [2]. *In vitro* studies suggest that the physiological actions of E_2 in colonocytes is, in part, mediated through p53[2].

Related to the data presented here, an *in vivo* study conducted by Yoo (2015) [16], investigated the targeted deletion of p53 in the gastrointestinal tract. Azoxymethane (AOM), a colon specific carcinogen, was used to induce sporadic tumors. Colon carcinogenesis occurs frequently in the distal part of the colon with the administration of AOM, analogous to the localization seen in sporadic CRC in humans [10]. AOM initiates tumor formation by alkylation of DNA, creating base mispairings [10]. Yoo investigated ACFs, high multiplicity ACFs, and visible tumor formation. With theses findings, our main focus for this study was the examination of proliferation with a varying presence of p53 and exposure to E_2, providing insight into the role of estrogen signaling on sporadic tumor development.

CHAPTER II

METHODS

Mouse Model

Sexually mature C57BL/6 female $Tp53^{Flox/Flox}$ mice were bred with *Villin-CRE mice* through multiple generations in order to produce an offspring containing $Tp53^{Flox/Flox}$/CRE and $Tp53^{Flox/Flox}$ that were utilized in the study. These particular mice were genetically altered to loose function of p53 in the gastrointestinal tract. At time of initiation, mice were ovariectomized (OVX) for regulation of E_2, treated with an implanted pellet, and placed on a phytoestrogen-free diet *ad libitum*. The control implant administered was a 20mg cholesterol pellet, while the treatment group was exposed to a 19 mg cholesterol and 1 mg E_2 pellet. For the induction of sporadic tumors, six weekly injections of AOM at 10mg/kg body weight were administered two weeks after ovariectomy. Replacement of cholesterol or cholesterol and E_2 pellet occurred at 56 days. Sacrifice was carried out on day 98. Two hours prior to termination, mice were injected with 5-bromo-2' –deoxyuridine (BrdU) in order to perform the proliferation assay. Tissues were excised and fixed with the use of 4% paraformaldehyde (PFA), for further analysis of proliferative cells within the colonic crypts.

Immunohistochemistry

Colonic tissue collected was stained for proliferation using the protocol set out by the laboratory. The protocol began with deparaffinizing and rehydrating the tissues. Endogenous peroxidase activity was quenched. Antigen retrieval followed after. The primary antibody was than administered through a preparation of a 1:20 dilution of AnitBrdU in PBS w/ 1% BSA. The

tissues were than placed in a humidified chamber at 4^{o}C overnight. The following day, the secondary antibody was than applied through a prepared dilution of 1:200 of HRP Conjugated Goat-anti-Mouse Antibody in PBS with 1%BSA. The tissues were placed in a humidified chamber for one hour. 1% Diaminobenzidine solution was placed on the tissues for ten minutes. Hematoxylin was than used to counterstain the tissues. The final preparations were dehydrating the tissues and mounting with permount.

Determination of Proliferation

Slides were viewed using bright-field microscopy. 20 crypts from each animal were analyzed. Each crypt was split sagittally down the center of the crypt. The right side of the crypt was used consistently throughout all animals, as to keep from any bias in occurring. The crypt was further divided into three transverse sections; the bottom, middle, and top portion of the crypt. Proliferative cells were counted within each region, and recorded as a percentage. The percentage of all proliferative cells to number of cells within the crypt was also recorded.

Statistical Analysis

All data are presented as mean ± SEM. Data from proliferation stains were transformed to percentage of cells within the crypt. Analysis for all data was determined using one-way ANOVA with Student's t post hoc test. Differences were considered significant if $P<0.05$. All statistical analyses were performed using Graphpad Prism version 6.0 for Mac OS X, Graphpad Software, La Jolia California USA, www.graphpad.com.

CHAPTER III

RESULTS

p53 and E_2 on visible tumor formation

In order to investigate the effect of E_2 and p53 on sporadic tumor formation within the colon, we first analyzed quantitative results made by p53 and E_2 in the development of visible tumor formation. Tumor number was counted using microscopic analysis. In mice with WT p53, treatment with E_2 resulted in a significantly lower number of visible tumors when compared to the respective control (Fig 1). In the absence of p53, mice treated with E_2 also had a significantly lower number in tumor formation when compared to the respective control (Fig. 1). There was no significant difference between the two genotypes when treated with the control, as well as with treatment with E_2 (Fig 1).

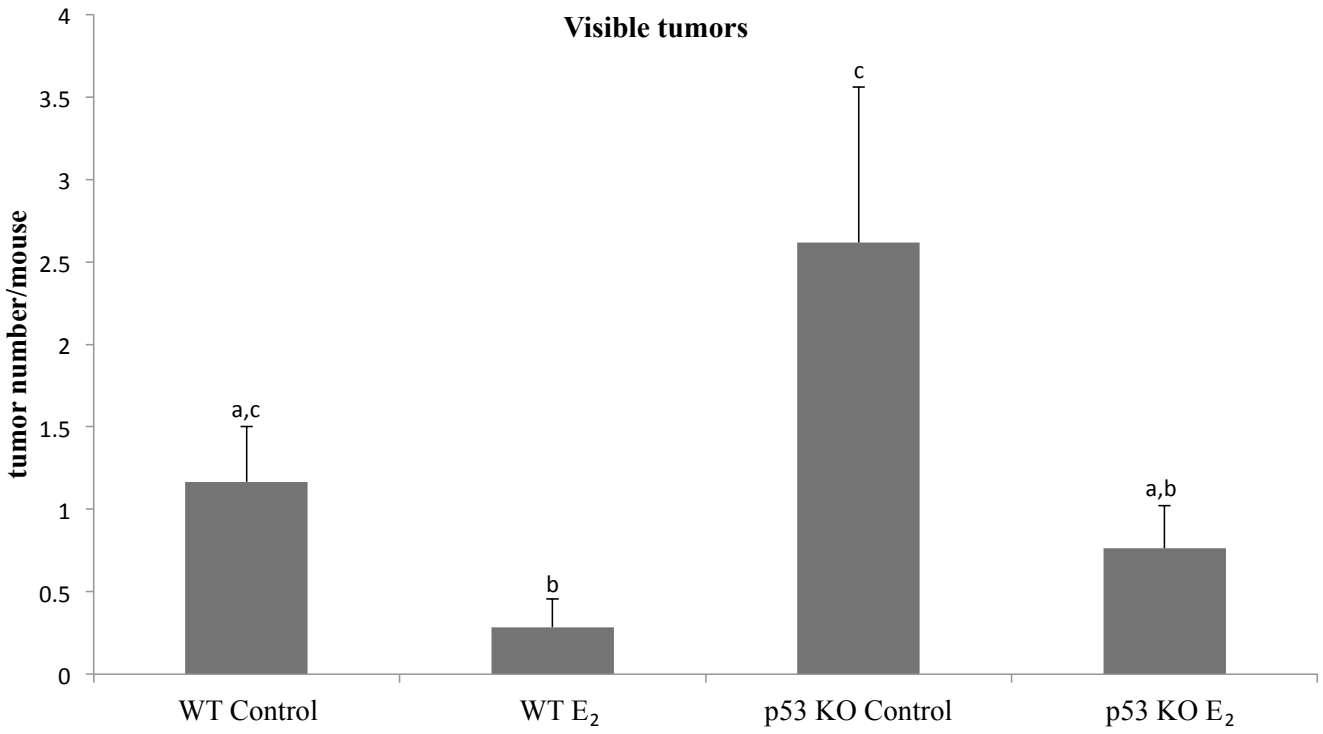

Fig. 1 *In vivo* effect of p53 and E_2 on visible tumor development during induced tumor formation with the colon. Mean (n≥12) ± SEM. Bars without a common letter differ; p<0.05.

p53 and E_2 on proliferation in the colonic crypt

In order to better understand the mechanism behind E_2 providing protective effects against the production of tumors and the role that p53 plays, amount of proliferation within the colonic crypt within each group was investigated. Loss of p53 resulted in a significant increase in proliferation in both control and E_2 treated groups (Fig. 2). A significant increase in proliferation was also found in mice with the presence of p53 and treatment of E_2 (Fig 2).

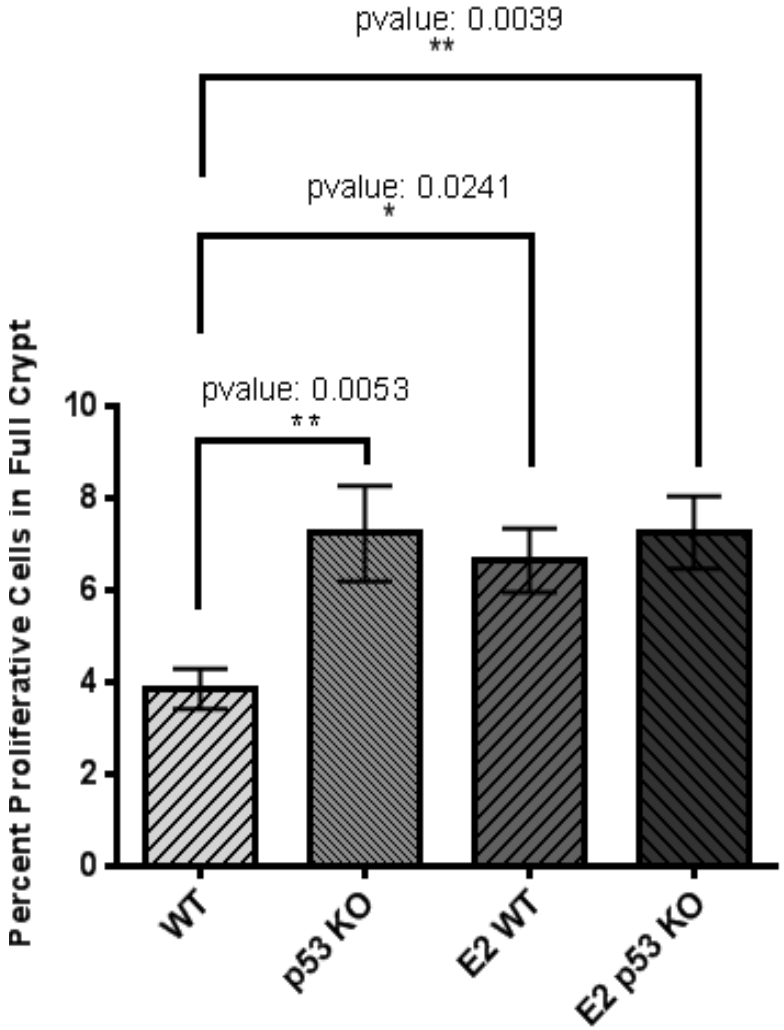

Fig. 2 *In vivo* effect of p53 and E_2 on percent proliferation in the colonic crypt during induced sporadic tumor formation. Mean ($n \geq 12$) ± SEM. Bars without a common letter differ; $p<0.05$.

CHAPTER IV

DISCUSSION

Studies have shown that E_2 provides protective effects within the colon. The Women's Health Initiative study reported a statistically significant decrease in CRC in postmenopausal women receiving HRT in the form of supplemented E_2 and progesterone [14]. The protective effects of E_2 can best be seen through the mechanism of ERβ serving as a mediator. There are two types of estrogen receptors found throughout the body: estrogen receptor α (ERα) and estrogen receptor β (ERβ). Cells of the kidney, intestinal mucosa, prostate gland, and endothelial contain ERβ, whereas the endometrium, ovarian stroma, and breast cancer cells contain ERα [17]. ERβ protein is the predominant form in colonic epithelium with no detection of ERα [18]. Although ERα and ERβ share structural similarities they differ in that they possess opposing roles. ERα is instrumental in the breast and uterine tissue by inducing proliferation for growth and development of the tissue, ERβ serves as an antagonist to ERα by counteracting the stimulation of proliferation [19].

Previous experimentation in our laboratory investigated the mediation of E_2, by ERβ, as it relates to suppression in tumor formation. A comparison of WT to ERβ knockout mice resulted in significantly fewer ACFs and increased apoptosis in WT mice treated with E_2, compared to those not treated with the control [1]. Protection of E_2 was lost entirely in ERβ knockout where significance was not detected in the presence or absence of E_2 [1]. For E_2 to impart protective effects within the colon, ERβ must be expressed.

Our laboratory further investigated this mechanism and found p53 to play a major role in E_2 protective effects within the colon, through the observation of induced apoptosis in non-

malignant colonocytes [2]. Their results were indicative of E_2 being mediated through p53. p53 is a transcription factor that is essential in the developmental process of a cell. Through regulation of gene expression in response to being exposed to different stress signals, p53 induces apoptosis, DNA repair, and/or initiation of cell cycle arrest. The tumor suppressor gene p53 is the most commonly mutated gene found in cancer [20]. A functional p53 protein is crucial in regulating tumorigenesis and stopping the development of tumors.

The reciprocal action of ERα and ERβ, and the ratio found amongst the organs, is key in understanding the susceptibility of estrogen- induced carcinogenesis [21]. Binding of estrogens to ERα induces a cancer promoting response, whereas binding to ERβ provides protective effects [21]. In the presence of E_2, colonic expression of ERα is suppressed while ERβ expression increases [21]. Previous data from our laboratory has shown in young adult mouse colonocyte (YAMC) cells, a 200-fold increase in ERβ to ERα expression [2]. With high abundance of ERβ expression found in colonic tissue, it is believed that E_2 possesses protective effects in the presence of p53, through ERβ. Findings from our study showed evidence of this concept through a significant reduction in the number of visible tumors found in the presence of p53 and treatment of E_2.

Not only did E_2 reduce the number of visible tumors in mice expressing p53, tumor formation was also significantly reduced in mice not expressing a functional p53 protein. This finding illustrates that whether p53 is expressed or not, E_2 provides protective effects within the colon. With literature providing evidence of the mediation of E_2 through p53, our data also suggests that there is another mediator channeling the protective effect of E_2. Further investigation should be conducted to explain the mechanism behind E_2 protective effects in the absence of p53.

Experimentation outside of our laboratory illustrated the role p53 plays in blocking cell proliferation and arresting cell growth through the induction of transcriptional repression and translational inhibition [8]. Due to this instrumental process, the loss of expression in p53 exposes the colon to potential tumor development. Our study has shown that in the absence of p53, a statistically significant increase in proliferation was observed in the absence and presence of E_2. p53 is instrumental in reducing tumor formation, through regulation of proliferation. In the absence of p53, the cell looses its ability to regulate growth, and uncontrolled proliferation may result.

Further investigation in this model, including analyzing apoptosis, should be carried out to further explain the mechanism by which p53 contributes to a reduction in sporadic tumor formation when in the presence of E_2. Our results in the p53 WT group treated with E_2 were not consistent with our original hypothesis. The increase in proliferation seen within this group was not consistent with our proposed mechanism. An increase in proliferation is indicative of potential tumor development. We believe that there is a timing aspect as to when E_2 provides protective or harmful effects within the colon, thus providing reasoning behind our results.

The Women's Health Initiative study, as previously stated, found that HRT resulted in a decreased risk in CRC [14]. However, when taking E_2 and progesterone at time of diagnosis, those suffering from CRC had a more advanced progression of the cancer when compared to those treated with the placebo [14]. A mechanism to better explain this effect is through the expression of ERβ. In normal colonic epithelial cells, ERβ expression is at its greatest [22]. As the tumor begins to form, expression of ERβ has been seen to decrease in expression in adenomatous tissue, and further decreases in expression as it than transitions into the formation

of a carcinoma [22]. It is plausible that the increased proliferation seen in this subgroup was due to a transition to a less expressed ERβ colonic tissue.

Overexpression in ERβ has been shown to increase p53 signaling, thus inducing apoptosis and anti-proliferation [23]. With this is mind, as an adenoma progresses into a malignant carcinoma, ERβ expression decreases through the tumor development [22]. This action inhibits stimulatory effects on p53, allowing the cell to become unregulated and grow uncontrollably. We believe that the colonic tissue had transitioned, where ERβ expression had been suppressed. The stimulatory effect that ERβ has on p53 would be inhibited, thus resulting in an increase in proliferation.

In conclusion, the loss of p53 significantly increased proliferation. E_2 has protective affects within the colon by suppressing tumor formation in the presence or absence of p53. The data suggests that there is an alternate pathway by which E_2 can protect against sporadic tumor formation in the colon. Further investigation should be carried out to provide more insight into the mechanism of E_2 signaling.

REFERENCES

1. Weige, C.C., K.F. Allred, and C.D. Allred, *Estradiol alters cell growth in nonmalignant colonocytes and reduces the formation of preneoplastic lesions in the colon.* Cancer Res, 2009. **69**(23): p. 9118-24.

2. Weige, C.C., et al., *P53 mediates estradiol induced activation of apoptosis and DNA repair in non-malignant colonocytes.* J Steroid Biochem Mol Biol, 2012. **128**(3-5): p. 113-20.

3. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2015.* CA Cancer J Clin, 2015. **65**(1): p. 5-29.

4. Marmol, I., et al., *Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer.* Int J Mol Sci, 2017. **18**(1).

5. Pignone, M., et al., *Screening for colorectal cancer in adults at average risk: a summary of the evidence for the U.S. Preventive Services Task Force.* Ann Intern Med, 2002. **137**(2): p. 132-41.

6. Greenblatt, M.S., et al., *Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis.* Cancer Res, 1994. **54**(18): p. 4855-78.

7. Goh, H.S., et al., *p53 and behaviour of colorectal cancer.* Lancet, 1994. **344**(8917): p. 233-4.

8. Loayza-Puch, F., et al., *p53 induces transcriptional and translational programs to suppress cell proliferation and growth.* Genome Biol, 2013. **14**(4): p. R32.

9. Merritt, A.J., et al., *The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice.* Cancer Res, 1994. **54**(3): p. 614-7.

10. Neufert, C., C. Becker, and M.F. Neurath, *An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression.* Nat Protoc, 2007. **2**(8): p. 1998-2004.
11. Rhodes, J.M. and B.J. Campbell, *Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared.* Trends Mol Med, 2002. **8**(1): p. 10-6.

12. Mariani, F., P. Sena, and L. Roncucci, *Inflammatory pathways in the early steps of colorectal cancer development.* World J Gastroenterol, 2014. **20**(29): p. 9716-31.

13. Jaiswal, A.S., R. Balusu, and S. Narayan, *Involvement of adenomatous polyposis coli in colorectal tumorigenesis.* Front Biosci, 2005. **10**: p. 1118-34.

14. Chlebowski, R.T., et al., *Estrogen plus progestin and colorectal cancer in postmenopausal women.* N Engl J Med, 2004. **350**(10): p. 991-1004.

15. Soderlund, S., et al., *Inflammatory bowel disease confers a lower risk of colorectal cancer to females than to males.* Gastroenterology, 2010. **138**(5): p. 1697-703.

16. Yoo, G., *Influence of Estradiol and Phytoestrogens on Colitis and Colon Cancer*. 2015, Texas A&M University.

17. Gruber, C.J., et al., *Mechanisms of disease - Production and actions of estrogens.* New England Journal of Medicine, 2002. **346**(5): p. 340-352.

18. Williams, C., et al., *Estrogen receptor beta as target for colorectal cancer prevention.* Cancer Lett, 2016. **372**(1): p. 48-56.

19. Rietjens, I.M., J. Louisse, and K. Beekmann, *The potential health effects of dietary phytoestrogens.* Br J Pharmacol, 2016.

20. Muller, P.A. and K.H. Vousden, *Mutant p53 in cancer: new functions and therapeutic opportunities.* Cancer Cell, 2014. **25**(3): p. 304-17.

21. Weyant, M.J., et al., *Reciprocal expression of ERalpha and ERbeta is associated with estrogen-mediated modulation of intestinal tumorigenesis.* Cancer Res, 2001. **61**(6): p. 2547-51.

22. Konstantinopoulos, P.A., et al., *Oestrogen receptor beta (ERbeta) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation.* Eur J Cancer, 2003. **39**(9): p. 1251-8.

23. Hsu, H.H., et al., *Apoptotic effects of over-expressed estrogen receptor-beta on LoVo colon cancer cell is mediated by p53 signalings in a ligand-dependent manner.* Chin J Physiol, 2006. **49**(2): p. 110-6.