EFFECTS OF ARYL HYDROCARBON RECEPTOR ON TUMOR PROGRESSION IN PRESENCE OF LOW AND HIGH FAT DIET

An Undergraduate Research Scholars Thesis

by

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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 2019

Major: Nutritional Science

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ABSTRACT

Effects of Aryl Hydrocarbon Receptor on Tumor Progression in Presence of Low and High Fat Diet

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The epithelium is the main barrier to the external environment in the GI tract, and it is constantly exposed to different carcinogenic factors that could cause cancer in this layer. However, there are specific signaling pathways which respond to the presence of carcinogenic molecules inside the cells and help the cells to detoxify themselves from carcinogenic elements. The ligand-activated Aryl Hydrogen Receptor (AhR) is a ligand activated transcription factor that controls expression of a diverse set of genes[1]. One role of AhR is preventing tumorigenesis and inhibiting the overgrowth of intestinal and colon cells through degradation of β-catenin protein inside the colonocytes[2]. Colonocyte proliferation as well as β-catenin intensity and nuclear localization was measured in animals of four different treatment groups, mice with and without AhR expression on either high or low-fat diet, in order to better understand AhR's role in colon tumorigenesis. The highest level of cell proliferation, β-catenin intensity and nuclear localization was expected in the mice on high fat diet which do not express AhR compared to the mice that expressed AhR and were fed a low fat diet. While β -catenin intensity and nuclear localization was increased as expected in AhR knockout and high fat diet fed animals, colonocyte proliferation was not significantly increased in these groups. These results indicate a possible mechanism by which loss of AhR can lead to increased proliferative signaling in colonocytes that could be detrimental to colon cancer prevention.

ACKNOWLEDGEMENTS

Primarily, I would like to express my gratitude to my research advisor, Dr. Clinton Allred who gave me the opportunity to do this wonderful research and whose valuable guidance and support helped me in completing it.

I also thank Jennifer AA DeLuca and Kimberly F Allred for helping me in completing the research and writing and finalizing this paper within the limited timeframe.

Secondly, I would to thank my family for their encouragement and support.

CHAPTER I

INTRODUCTION

Colon cancer is the third leading cause of cancer-related deaths in the United States in both sexes. According to American Society of Colon and Rectal Surgeons, in 2017, more than 136,000 new cases of colon cancer were diagnosed in the United States, meaning about 1 in 20 (5%) Americans are prone to developing colorectal cancer during their lifetime[3]. Although the exact cause of colon cancer is unknown, genetics, diet and age are major factors contributing to the formation and progression of cancer[4]. Increased division of epithelium cells in the colon can result in colon cancer. Humans and animals are exposed to a variety of chemicals on daily basis, some of which can be carcinogenic, leading to mutations that result in increased cell proliferation. However, specific protective mechanisms have evolved to activate in response to specific chemicals and facilitate the biotransformation and detoxification of these toxic chemicals. One example of such a mechanism is the Aryl hydrocarbon Receptor (AhR) pathway. AhR is a ligand-activated transcription factor[5]. The inactive form is found in the cytoplasm bound to its specific chaperone. When activated, AhR translocates to the nucleus and facilitates transcription of AhR target genes[5]. For more than 30 years, it was thought that AhR was only responsible for the regulation of gene expression of genes responsible for the degradation of environmental toxins. More recently, many other functions have been associated with this receptor as it is a rather promiscuous receptor capable of binding to a variety of coumponds[8]. Recent studies show that AhR plays a major role in tumor formation in the colon through various non-canonical pathways.

One such non-canonical role of AhR is to act as a ubiquitin ligase to degrade specific proteins, including β-catenin. The regeneration of the epithelial layer of colon is a coordinated process. This process begins with the proliferation of intestinal stem cells located at the bottom of each crypt[5]. Due to the importance of this process, there are specific mechanisms designated to control and facilitate epithelial regeneration and the proliferation of colonocytes[6]. The Wnt signaling pathway plays a major role in embryonic development, colonocyte proliferation, and maintenance of intestinal stem cells. This pathway is tightly regulated by E3 ubiquitin ligases which target WNT pathway signaling proteins for degradation[7]. β-catenin is an example of an important signal in the Wnt signaling pathway and is expressed in many tissues. Although its presence is vital for the cell growth, its dysregulation is associated with excessive cell proliferation and tumorigenesis[8]. Activated AhR decreases the concentration of β-catenin by marking it for ubiquitination[9]. Studies show that sustained AhR activation by its model ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), caused an overall decrease in the number of Wnt/β-catenin pathway target genes[6]. As part of its non-canonical role in proteasomal degradation, activated AhR remains in the cytosol and forms a ubiquitin ligase complex that degrades cytosolic proteins such as β-catenin[9]. Therefore, AhR indirectly affects cell proliferation and division by regulating degradation of β-catenin[8].

The gut microbiome has also been shown to impact colon cancer development and progression; one possible mechanism is through the production of metabolites that signal through the AhR. Microorganisms that live in mammalian gut system play an important role in overall health of the organism by producing byproducts that affect gastrointestinal cell metabolism such as growth[10]. Additional compounds, including those in the diet and produced by microbes can bind AhR and activate it. Activation of AhR by dietary ligands such as indole-3-acetic acid

(IAA) resulted in decreased colon tumors in mice which did not express AhR in the epithelial layer of their colon cells[6]. Microbiota metabolites have also been shown to signal through the AhR. These metabolites are often produced through metabolism of tryptophan from dietary sources[10]. It has been shown that the inefficiency of gut microbiota to produce tryptophan-based AhR ligands is involved in the pathogenesis of inflammatory bowel disease and the appearance of cancerous polyps in the colon[11]. Recent studies show that dietary tryptophan supplementation improved colitis in mice[12]. Some dietary metabolites such as the ones produced after digestion of tryptophan containing food by gut microbes can activate the AhR signaling pathway[10]. Factors such as a diet high in fat content or genetics can alter microbiota composition and as a result tryptophan metabolism[14]. Some studies even show that increased intake of dietary AhR ligands can counter-balance the negative effects of excessive AhR receptor degradation on the epithelial layer of colon[13]. This implies that diet can positively impact AhR signaling to prevent colon cancer.

In this study, we attempt to elucidate the relationship between environmental factors such as dietary fat content and intracellular signaling pathways such as AhR and Wnt which can help to better illuminate the mechanisms of colon cancer development and progression. Though loss of AhR in intestinal epithelial cells has been connected with increased colon tumorigenesis in a sporadic model, the present study will examine the impact of a diet high in fat fed during the peri-initiation period on colonocyte proliferation and β-catenin expression and nuclear localization in the resulting colon masses. We hypothesize that loss of AhR in the intestinal epithelia as well as high fat diet will result in increased cell proliferation and β-catenin expression and nuclear localization.

CHAPTER II

METHODS

Mice

C57BL/6 AhR knockout ($AhR^{f/f}$ x Villin-Cre; AhRKO) mice that lacked expression of AhR in their colon epithelial cell layer and wildtype ($AhR^{f/f}$; WT) littermates were employed to test the hypothesis. At approximately five weeks of age, animals were separated by genotype, and bedding was mixed for four weeks to homogenize the gut microbiota (**Figure 1**). After two additional weeks without mixing bedding, animals were divided into four groups (**Table 1**) and experimental diets began.

Diet

For dietary intervention, mice were randomly allocated to either a low fat diet (LFD) or a high fat diet (HFD). Provided diets contained either 10% kcal from fat (LFD; Research Diets D12450B) or 60% kcal from fat (HFD; Research Diets D12492). After three weeks on experimental diets, animals were injected with azoxymethane (AOM) once per week for 6 weeks. Animals weighing less than 40g were dosed with 10 mg/kg AOM, while animals weighing greater than 40g were dosed with 7.5 mg/kg AOM. Body weight of animals was monitored and recorded twice a week throughout the experiment. As shown in **Figure 1**, twelve weeks after the final AOM injection, HFD mice were switched to LFD.

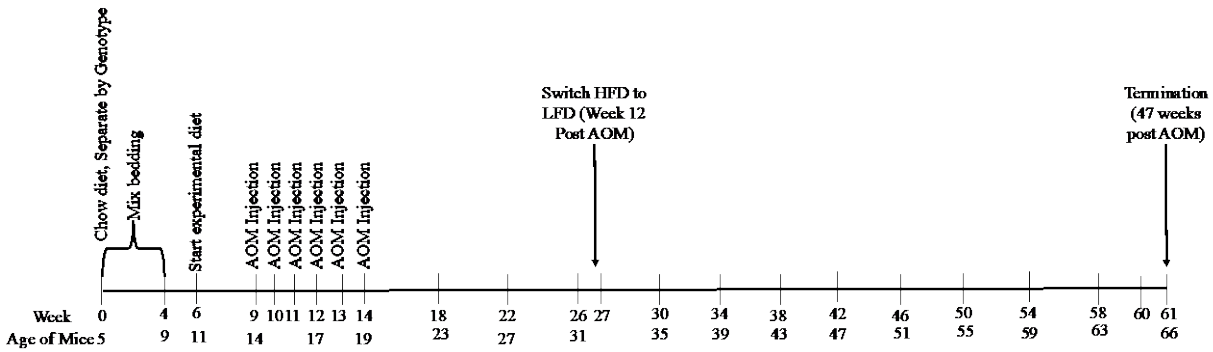

Figure 1. Experimental Timeline

Table 1. Treatment Groups

Genotype	Diet	AOM	n
AhR KO	HFD	+	14
AhR KO	LFD	+	13
WT	HFD	+	18
WT	LFD	+	17

Termination and tissue collection

Mice were terminated 47 weeks after final AOM injection. Two hours before termination, animals were injected with 5-Ethynyl-2'-deoxyuridine (EdU; Life Technologies A10044), a cell proliferation marker. Animals were terminated using a ketamine/xylazine overdose. Colons were excised, flushed with phosphate buffered saline, and opened longitudinally. Macroscopically visible colon masses were measured, mapped, and excised. After 4 hours of fixation in ice cold 4% paraformaldehyde, colon masses were washed and stored in 70% ethanol until processed, embedded, and sectioned by histology. Colon masses were diagnosed by a blinded, board-certified pathologist, and any masses containing any alteration in

mucosal proliferation were co-stained for EdU and β -catenin for further analysis. The EdU staining process followed the manufacturer's protocol (Click-iT Plus EdU Imaging Kit, Life Technologies C10637). β -catenin was subsequently co-stained as described previously[20].

Cell counting

Slides were imaged using an all-in-one fluorescent microscope (Keyence BZ-X700). 40x images of excessive mucosal proliferation were captured and overlaid to quantify proliferative cells as well as β -catenin intensity and nuclear localization in proliferative cells. Fiji ImageJ software was used for marking and counting cells for each image. During the counting procedure, 100 epithelial cell nuclei were chosen randomly on each image, and EdU positive nuclei were identified within these 100. Up to 20 proliferating cells per image were chosen for β-catenin intensity measurements. Nuclear to cytoplasmic β-catenin ratio was calculated using Microsoft Excel.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software. Outliers were removed, normal distribution was assessed, and Kruskal-Wallis was used for multi-group data which did not pass the normality test. Mann-Whitney U test was used to show whether there was a significant difference between groups. P values less than 0.05 were considered significant for this study.

CHAPTER III

RESULTS

Cell proliferation

Cell proliferation was measured using the EdU stain as a marker. At least 11 colon masses per group were analyzed in the experiment, with 500 nuclei per mass analyzed for proliferation. Overall, there was no significant difference between the two genotype groups WT vs AhRKO (P>0.05) **(Figure 2(a))**. Additionally, no significant difference was observed between high and low-fat diet treatments overall (P>0.05) **(Figure 2(b)).** HFD WT vs HFD KO were the only significantly different groups when comparing all four treatment groups (P<0.05) **(Figure 2(c))**, with fewer proliferative cells observed in the HFD KO group compared to the HFD WT group. Even though most groups in the four groups comparison test are not significantly different, there is an increasing trend in cell proliferation from LFD WT to LFD KO and HFD WT as expected.

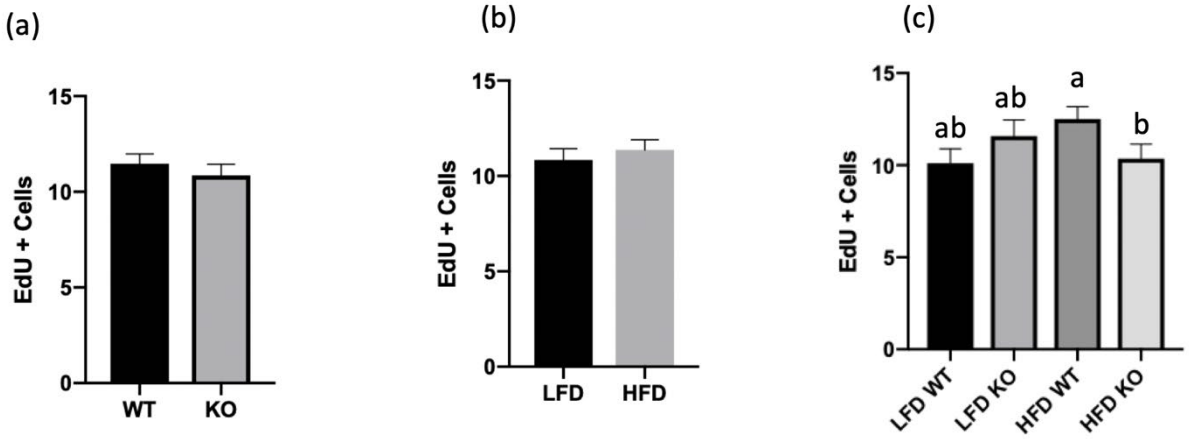

Figure 2. Cell proliferation. (a) Cell proliferation by genotype (P=0.19). (b) Cell proliferation by diet (P= 0.53). (c) Cell proliferation by four treatment groups (Kruskal-Wallis P=0.047). Mean +/- SEM. All bars without a common letter differ.

β-catenin intensity

β-catenin intensity of 100 cells per mass was measured. Overall, there was a significant increase in β-catenin intensity in the AhRKO animals compared to the WT animals (P< 0.05) **(Figure 3(a))**. A significantly different result was also observed between the diet treatments (P< 0.05) **(Figure 3(b))**, with more β-catenin intensity in the HFD group compared to the LFD group. There were significant differences among all the groups compared in the four group comparison test except the HFD WT vs HFD KO groups which were not significantly different **(Figure 3(c))**. β -catenin intensity increased significantly from LFD WT to LFD KO and HFD WT, as expected.

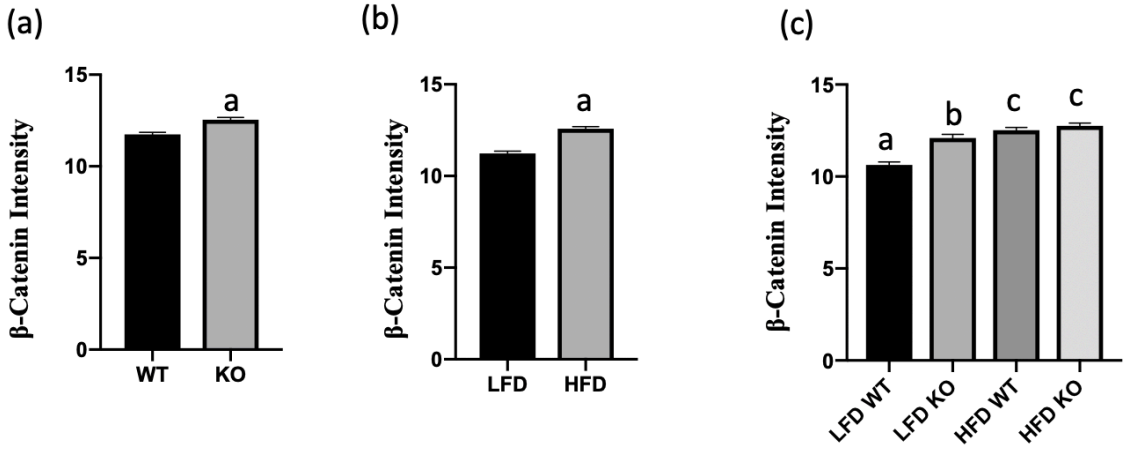

Figure 3. β-catenin intensity. (a) β-catenin intensity by genotype (P=0.001). (b) β-catenin intensity by diet (P= 0.0001). (c) β-catenin intensity by four treatment groups (Kruskal-Wallis p<0.0001). Mean +/- SEM. All bars without a common letter differ.

β-catenin nuclear to cytosolic ratio

β-catenin localization was measured using a nuclear to cytosolic ratio. This ratio was calculated by first acquiring β-catenin intensity of the whole cell and nucleus separately, subtracting the two, and then dividing the β-catenin intensity of the nucleus by the resulting intensity of the cytoplasm. The same number of cells were assessed as β-catenin intensity. Overall, there was a significant difference in β-catenin nuclear to cytosolic ratio between the two genotypes, with the KO group showing more nuclear β-catenin compared to the WT group ($P < 0.05$) **(Figure 4(a))**. A significantly different result was observed between diet treatments, with animals on HFD showing a higher nuclear to cytosolic ratio of β-catenin compared to LFD treated animals ($P < 0.05$) **(Figure 4(b)).** LFD WT vs HFD KO were the only groups of the four treatment groups which were significantly different ($P < 0.05$) **(Figure 4(c)).** While not all the groups show significantly different outcomes, there was a trend toward an increase in β-catenin nuclear to cytosolic ratio between some groups. For instance, both KO groups show higher β-catenin nuclear to cytosolic ratio compared to their wildtype counterparts fed the same diet.

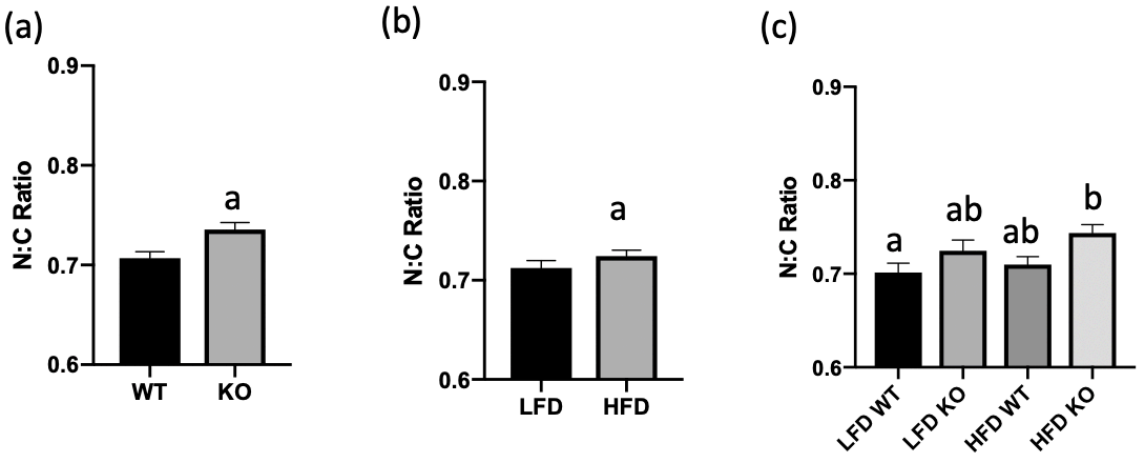

Figure 4. β-catenin nuclear to cytosolic ratio. (a) Nuclear to cytosolic ratio by genotype ($P=0.001$). (b) Nuclear to cytosolic ratio by diet ($P= 0.0414$). (c) Nuclear to cytosolic ratio by four treatment groups (Kruskal-Wallis $P<0.017$) Mean +/- SEM. All bars without a common letter differ.

CHAPTER IV

CONCLUSION

Due to the profound impact of diet and genetics on the formation and progression of tumorigenesis in the colon, we set out to define whether dietary fat content or AhR expression would affect cell proliferation in colon epithelial cells. AhR is a highly expressed ligand activated transcription factor that is found in multiple organs, and there is increasing evidence showing that this protein plays an important role in regulating cell proliferation and preventing tumor formation by regulating degradation of another protein called β-catenin[15].

Surprisingly, no significant difference in cell proliferation was observed between HFD and LFD or between WT and AhR KO mice. This unexpected result could be due to a variety of factors. For example, sample size and the number of cells counted could have affected the cell proliferation results and prevented observation of significant differences between diet or genotype. When comparing cell proliferation between the four treatment groups, while not all of the groups are significantly different from one another, they generally show the expected trend, with increased proliferation observed from LFD WT to LFD KO to HFD WT groups **(Figure 3(c))**.

β-catenin is a Wnt signaling protein expressed in multiple tissues. Although its presence is vital for cell growth and other cellular mechanisms, its dysregulation is associated with excessive cell proliferation and tumorigenesis[8]. Studies show that activated AhR decreases concentration of β-catenin by marking it for ubiquitination and degradation as part of a ubiquitin ligase complex[14]. Intracellular β-catenin intensity of colonocytes in colon masses was measured to see whether high or low-fat diet and the expression of AhR would affect β-catenin intensity. A

significant difference between AhR WT and AhRKO mice and mice on high and low-fat diet was observed. As expected, AhRKO and HFD treatments showed the highest intensity of intracellular β-catenin which was expected based on our hypothesis. When comparing all four treatment groups, all of them were significantly different except the HFD WT vs HFD KO groups. **(Figure 3(c)).** β-catenin nuclear localization was also measured, as nuclear β-catenin can act as a transcription factor to activate expression of Wnt target genes, including those involved with proliferation. Higher intensity of β-catenin in the nucleus of proliferating cells was expected in the AhRKO and HFD treated groups. As shown in **Figures 4(a)**, AhRKO mice showed significantly higher β-catenin nuclear to cytosolic ratio compared to WT mice. This result was expected because AhRKO mice did not express AhR in the epithelial layer of their colon and, as mentioned before, decreased AhR activation inside the cell is associated with an increased concentration of β-catenin[14]. In addition, mice that were on HFD also showed significantly higher β-catenin nuclear to cytosolic ratio compared to the mice that were on LFD. Even though most treatment groups did not show significant difference when compared to one another, there was an increase in β-catenin nuclear to cytosolic ratio trend when comparing WT to KO animals fed either LFD or HFD treatments.

This experiment showed that AhR expression in intestinal epithelial cells as well as dietary fat content during the peri-initiation period can play a major role in the expression of specific proteins involved in cell proliferation pathways. Further analysis of this study is needed to better elucidate the relationship between genetics and diet on colonocyte cell proliferation and β-catenin expression and nuclear localization.

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