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THE PREVENTION OF OBESITY-ASSOCIATED COLORECTAL CANCER VIA DIETARY SUPPRESSION OF INFLAMMATION- DRIVEN WNT-SIGNALING

Jinchao Li

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**THE PREVENTION OF OBESITY-ASSOCIATED COLORECTAL CANCER VIA DIETARY
SUPPRESSION OF INFLAMMATION-DRIVEN WNT-SIGNALING**

A Dissertation Presented

By

JINCHAO LI

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements of the degree of

DOCTOR OF PHILOSOPHY

May 2019

Department of Nutrition
School of Public Health and Health Sciences

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DEDICATION

To my beloved family.

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I would like to thank my advisor, Dr. Zhenhua Liu for his constant support and patience throughout the 5 years. I would never have accomplished so much without his expert guidance and encouragement. He is a great mentor in both my studies and my daily life. Thank you for believing in me and pushing me. I am thankful to Dr. Richard Wood. I admire your kind hearts, expertise, and leadership. I appreciate all your unwavering encouragement and support. Special thanks to my committee members, Dr. Richard Wood, Dr. Young-Cheul Kim, and Dr. Jing Qian for sharing their insightful comments and advice during my research.

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Thank God for blessing me with such a wonderful, Thanksgiving son.

ABSTRACT

THE PREVENTION OF OBESITY-ASSOCIATED COLORECTAL CANCER VIA DIETARY SUPPRESSION OF INFLAMMATION-DRIVEN WNT-SIGNALING

MAY 2019

JINCHAO LI, B.S., SHANDONG UNIVERSITY OF TRADITIONAL CHINESE MEDICINE

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Colorectal cancer (CRC) is the third leading cause of cancer deaths in the United States. A number of population studies have established that modifiable lifestyle factors such as obesity plays an important role in colorectal carcinogenesis. In the United States, more than one-third of adults are obese and obesity prevalence rates have no sign of decrease. Therefore, the development of effective strategy to prevent obesity-induced CRC is a public health priority. This study aimed to investigate whether genetic or dietary strategies can prevent obesity-induced CRC and determine the potential molecular mechanisms underlying the prevention effects of these strategies. We use *Apc*^{1638N} mice, germline heterozygous mutation in the *Apc* gene, and Caco-2 cell line to study intestinal tumorigenesis. Hematoxylin and eosin stain and QuickPlex SQ 120, a chemiluminescence assay, were used to measure the inflammatory status. Real time PCR, Western blot assay, and immunohistochemical analysis were used to further examine the signaling pathway status. We found that loss of Tumor necrosis factor alpha (TNF- α) decreased obesity associated intestinal tumorigenesis by decreasing the inflammation, and manipulating the β -catenin pathway and NF- κ B signaling. In addition, IKK, component of the NF- κ B signaling, was involved in the regulation of β -catenin pathway. The administration of Vitamin D (VD), at 5000 IU level, exerted an anti-inflammatory property, and led to

suppressed intestinal *Wnt*-signaling and tumorigenesis in obese mice. The molecular function of sulforaphane (SFN) on a high dose of VD supplementation, although displayed on the inhibition of HDAC and the activation of autophagy, needs further investigation. Butyrate can increase the activity of *Wnt*/ β -catenin pathway. Knocking down FFAR2 by siRNA decreased the expression of cleaved caspase 3 and the expression of phospho-GSK3 β (Ser9) and active β -catenin in Caco-2 cells, subsequently mitigated the anticancer effect of butyrate.

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CHAPTER 1

INTRODUCTION

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It is the third most common cancer in the United States, with around 140,000 new cases being diagnosed last year. The pathogenetic mechanisms underlying CRC development are complex and include hereditary and environmental factors. There is strong evidence that modifiable lifestyle factors, including obesity, play a crucial role in CRC carcinogenesis (Gunter and Leitzmann 2006).

Obesity is epidemic in most western countries and is a major preventable risk factor for CRC cancer incidence and mortality. Currently, the mechanisms underlying obesity associated CRC cancer include: chronic low-grade inflammation, altered microbiomes, and increased levels of hormones, chemokines, and adipocytokines (Berger 2014). Many studies have suggested that obesity associated chronic inflammation might mediate its complications, such as CRC. It is suggested that chronic inflammation plays a crucial role in CRC development and progression, given the fact that multiple inflammatory cells and inflammatory cytokines present in tumor microenvironments. However, the molecular mechanisms linking obesity associated inflammation to CRC cancer remain unclear.

There has been considerable interest in the use of diet for cancer prevention. A large amount of evidence has shown that diet has the potential to influence inflammation status and cancer

development. Epidemiological studies have suggested that consumption of vegetables, fruits, and whole grain is associated with a low inflammation status and reduced risk of CRC cancers. On the other hand, consumption of red and processed meat is associated with a high inflammation status and increased risk of CRC cancers. It is generally recognized that vegetables and fruits such as cruciferous vegetables (broccoli, brussels sprouts, and cabbage), fiber, whole grain, and some of the vitamins such as Vitamin D might have CRC cancer prevention effects. However, whether knockout TNF- α , supplementation butyrate, or combination of Vitamin D with SFN can prevent obesity associated CRC and the precise molecular mechanisms underlying its CRC cancer prevention effects remain unclear.

Activation of *Wnt* signaling at the bottom of the intestinal crypts is essential for intestinal homeostasis (Flanagan, Austin et al. 2018). Hyperactivation of *Wnt* pathway is present in almost all CRC. Loss of APC function is crucial in aberrant *Wnt* signaling in CRC, as previous studies have shown that different mutations of APC could result in different levels of activity of canonical *Wnt* pathway and subsequent CRC incidence (Christie, Jorissen et al. 2013). In addition, inflammatory cytokines such as TNF- α might activate *Wnt* pathway (Liu, Brooks et al. 2012; Bradford, Ryu et al. 2017).

My dissertation aims to investigate whether knockout TNF- α , combination of Vitamin D with SFN, or supplementation of butyrate, can prevent obesity associated CRC and whether inhibition of inflammation and subsequent inhibition of *Wnt* pathway are involved in the cancer prevention effects of those strategy.

CHAPTER 2

LITERATURE REVIEW

2.1 Colorectal cancer

2.1.1 Epidemiology of CRC

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States in both men and women. In 2018, it is estimated that \approx 64 640 women and 75 610 men in the United States were diagnosed with CRC and 23 240 women and 27 390 men died from CRC. The pathogenesis of CRC development is complex. Both genetic and environmental factors may play an important role in the development of CRC (De Rosa, Pace et al. 2015). Almost 75% of CRC do not have positive family history and are sporadic. Only 25% of CRC cases have positive family history.

2.1.2 Environmental factors

The environmental risk factors of CRC development include obesity, smoking, heavy drinking, high intake of red meat and processed meat. It is estimated that every unit increase of the BMI is associated with 2-3% increased risk of developing CRC. Heavy drinking and smoking is associated with 20-50% increased risk of developing CRC (Liang, Chen et al. 2009; Fedirko, Tramacere et al. 2011). Every 100 g increase of consumption of red meat and processed meat is associated with 16% increased risk of CRC (Song, Garrett et al. 2015).

On the other hand, increased intake of Vitamin D, fiber, vegetables, whole grains are associated with decreased risk of CRC. In addition, 30 minutes of moderate activity can decrease the risk of CRCs by 10%(Arem, Moore et al. 2014). Clinic studies also have shown that long term use of low-dose of aspirin can prevent the incidence of CRC (Algra and Rothwell 2012).

2.1.3 Pathogenesis of CRC

The above risk factors can cause normal colon epithelial cells into benign polyps and subsequently into malignant carcinomas by activating oncogenes and deactivating tumor suppressor genes through long term accumulation of genetic mutations and alteration of epigenetics. As shown in Figure 2.1, the top one shows the well recognized pathway: from tubular adenomas to adenocarcinomas. The bottom one shows a recently described pathway: from serrated polyps to serrated colorectal cancer.

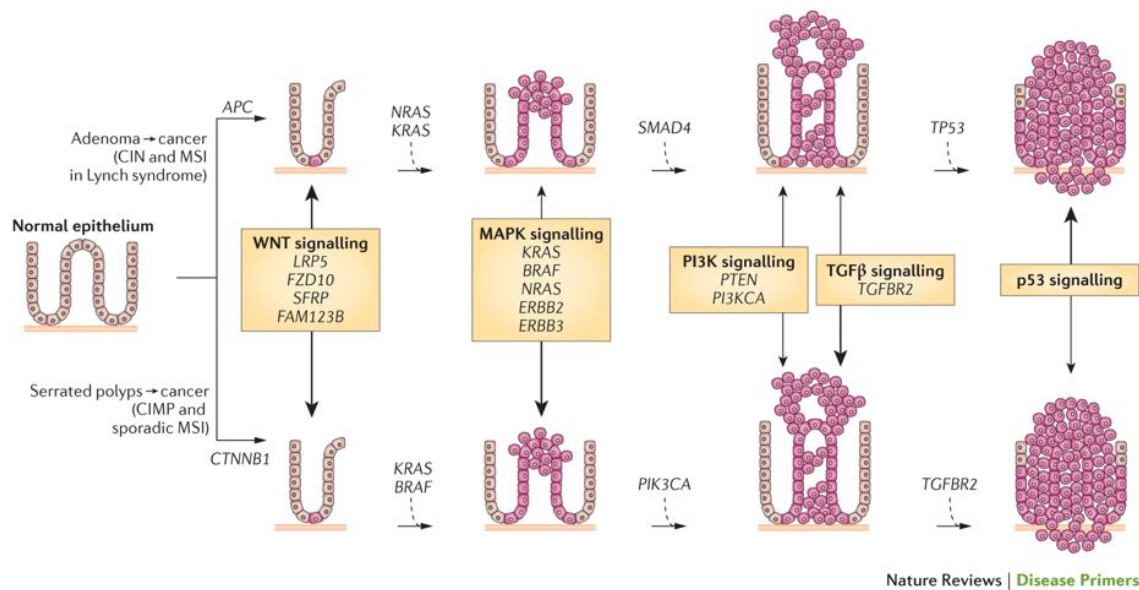


Figure 2.1 Pathogenesis of CRC. Source: Ernst J. Kuipers. Colorectal cancer. Nature Reviews Disease Primers volume 1, Article number: 15065 (2015). Available at <https://www.nature.com/articles/nrdp201565>

There are three distinct pathogenesis mechanisms by which CRC develop: the chromosomal instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP). CIN is composed of 85% of CRC. MSI is caused by loss of DNA mismatch repair activity, occurring in about 15% of all CRC (Bogaert and Preneen 2014). CIMP is characterized by a widespread CpG island methylation (Nazemalhosseini Mojarad, Kuppen et al. 2013). Loss function of APC plays a pivotal role in the development of CIN phenotype CRC. In my dissertation, I focused on the most common CRC: CIN phenotype.

2.1.4 Prevention of CRC

CRC can be prevented by altering the modifiable factors such as unhealthy diet and lifestyle factors. Since previous studies have shown that high intake of red meat, processed meat, highly refined grains and starches, and sugars are associated with increased risk of CRC. Therefore, decreasing the consumption of those foods and intake more fiber, vegetable, fruits, beans, and unsaturated fatty acids might lower the risk of CRC. There are also many studies indicating that increased intake of calcium and Vitamin D might lower risk of CRC. Long term and small dose of Aspirin and Statin usage might prevent the incidence of CRC. However the role of these interventions in the prevention of CRC remains controversial. In terms of lifestyle, researchers found that quit smoking and heavy drinking, avoiding of obesity, and regular physical activity may have the benefit of reducing the risk of CRC (Chan and Giovannucci 2010).

2.2 Obesity and obesity associated inflammation

Obesity, an epidemic, can increase many chronic diseases, such as diabetes, chronic heart diseases, and cancer, including CRC. Obesity may lead to adipose dysfunction and increased expression of proinflammatory cytokines. In the tumor microenvironments, there are many inflammatory cells and cytokines, which can stimulate cell proliferation, decrease cell apoptosis, and promote cancer metastasis. Therefore, it is believed that chronic inflammation is a central component of tumor development, progression, and metastasis. Besides inflammation, obesity associated dysregulated metabolisms, such as insulin resistance, hyperglycemia, and dyslipidemia, are also involved in obesity associated tumor incidence and growth.

2.2.1 Adipose tissue inflammation

Recently, adipose tissue is recognized as the body's largest endocrine organ. Adipose tissue can secrete many kind of cytokines and adipokines. When the energy consumed exceeds than needs, because of the needs of energy storage, adipose tissue has to remodel to accommodate this need. The adipose tissue remodeling processes include: adipose tissue expansion (increased adipocyte size and/or number); recruitment of proinflammatory immune cells; remodeling of the vasculature and the extracellular matrix. Subsequently, the above remodeling processes can lead to significant changes in the composition of the adipose tissue, and decreasing the number of the anti-inflammatory Treg and T_H2 cells, while increasing the numbers of pro-inflammatory T_H1 and CD8⁺ T cells.

2.2.2 Mechanisms of inflammatory responses in obese adipose tissue

Adipocytes play a pivotal role in adipose inflammation. Adipocytes express adiponectin and leptin. After secreted by adipocytes, leptin induces inflammatory response in obesity. However, adiponectin has anti-inflammatory effects. Circulating levels of adiponectin decrease associated

with increasing visceral obesity. Leptin plays a key role in inducing adipose proinflammatory processes (Deng, Lyon et al. 2013). Leptin stimulates the secretion of several cytokines, such as TNF- α , IL-1, IL-6, and IL-12. Leptin also increases reactive oxygen species (ROS) production and the secretion of leukotriene B₄, cyclooxygenase 2 (COX2), and nitric oxide (Carbone, La Rocca et al. 2012). Leptin also can increase T cell proliferation and T_H1 cell polarization and inhibits Treg proliferation, which is an important negative regulator of adipose inflammation. During obesity, immune cells is increased in adipose tissue, which is one of the characteristics of obesity and a major component of adipose inflammation linked to systemic complications of obesity (Xu, Barnes et al. 2003). Therefore, drugs or bioactive compounds that promote macrophages toward M2, might have the potential to inhibit adipose tissue inflammation and systemic complications of obesity (Han, Jung et al. 2013).

2.2.3 Adipose tissue and the tumor microenvironment

Tumor microenvironment (TME) plays a crucial role in the initiation and progression of CRC. The TME are composed of fibroblasts, adipocytes, cytokines, inflammatory and immune cells, blood vessels, and extracellular matrix. The TME usually has increased level of cytokines and growth factors which can increase antiinflammatory T_H2 and Tregs, and decrease proinflammatory T_H1 cells. It is believed that TME can prevent carcinogenesis in normal conditions. However, TME can also be modified and then become promoting progression and metastasis (Chen, Zhuang et al. 2015). Cancer cells, immune cells, and inflamed adipose tissue can alter TME, and subsequently promote tumor progression. For instance, inflammatory environment can induce mutations and proliferation of these cells, activate transcription factors such as STAT3, NF- κ B, and activator protein 1; increase nutrients supply by angiogenesis. Tumor-promoting cytokines are proinflammatory cytokines, such as TNF- α , IL-1 β . TNF- α , IL-1 β

can induce epithelial-mesenchymal transition (EMT), and tumor metastasis into lymphatics and remote organs.

2.2.4 Obesity and CRC

2.2.4.1 Epidemiology of Obesity associated CRC

It is estimated that around 11% of CRC cases are caused by overweight and obesity.

Epidemiological studies have shown that obesity is associated with a 30-70% increased risk of cancer. Specifically, it is suggested that visceral adiposity are more closely associated with CRC (Donohoe, O'Farrell et al. 2014). Beside the role of obesity in CRC incidence, obesity is also associated with poor prognosis of CRC. Inflammatory bowel diseases independently increase CRC risk, highlighting the role of inflammation in CRC development (Farraye, Odze et al. 2010; Khandekar, Cohen et al. 2011). Therefore, it is well accepted that obesity and chronic inflammation play as pivotal role in CRC initiation, tumor progression, and metastasis.

2.2.4.2 Obesity and cancer initiation

Epidemiological and experimental data suggest that leptin and adiponectin are associated with CRC risk (Drew 2012). Specially, increased leptin expression is associated with increased risk of CRC, whereas, decreased adiponectin is associated with CRC pathogenesis (Tutino, Notarnicola et al. 2011; Hebbard and Ranscht 2014). Hardwick et al, suggested that high fat diet (HFD) induced CRC carcinogenesis is mediated through increased serum leptin levels (Hardwick, Van Den Brink et al. 2001). The possible explanation is that, in lean adipose tissue, adiponectin suppresses secretion of adipose-derived proinflammatory cytokines such as IL-6, however, in obese adipose tissue, leptin increases production of TNF- α , IL-6, and IL-12, which

can promote CRC incidence. In consistent with this explanation, adiponectin knockout mice exhibits high level of proinflammatory cytokines (including IL-6, IL-1 β , and TNF- α) and develops larger size of CRC. Among those proinflammatory cytokines, TNF- α may activate transcription factor NF- κ B, which can increase secretion of proangiogenic factors and growth factors.

2.3 Tumor necrosis factor- α

Tumor necrosis factor- α (TNF- α), a glycoprotein, was reported that it can induce necrosis of cancer without injury to other surrounding normal tissues (Carswell, Old et al. 1975). In 1984, TNF- α gene was cloned, and recombinant human TNF- α could induce necrosis of cancer in mice, which seems have great promise in the treatment of cancer (Pennica, Nedwin et al. 1984).

2.3.1 TNF- α Signaling

TNF- α is mostly secreted by the activated inflammatory cells, such as macrophages, mast cells, and T lymphocyte, but other cell types including cancer cells also secrete TNF- α . TNF- α is first synthesized as a type II transmembrane protein (tmTNF- α) which contains an external C-terminus and cytoplasmic N-terminus (Figure 2.2). The cleavage of the extracellular domain of tmTNF- α by TNF- α -converting enzyme (TACE, a matrix metalloprotease) leads to the production of soluble TNF- α (sTNF- α).

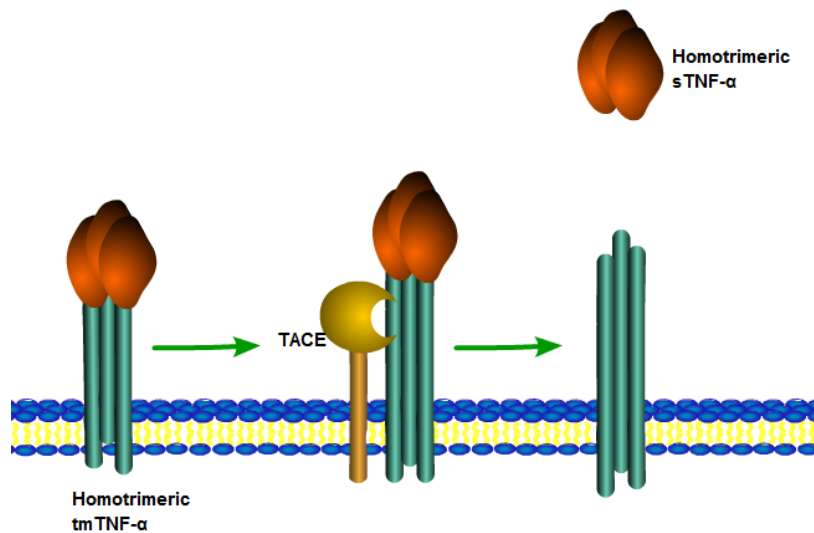


Figure 2.2. Diagram showing tmTNF- α (membrane TNF- α) cleavage by TACE into sTNF- α (soluble TNF- α).

tmTNF- α and sTNF- α are both active. TNF- α can bind with two different receptors, TNF- α receptor 1 (TNFR1) and TNF- α receptor 2 (TNFR2). Both TNFR1 and TNFR2 receptors do not have intrinsic enzyme activity and therefore they need cytosolic adaptor proteins to transduce intracellular signals. TNF- α can activate pathways that control three distinct cellular responses: cell survival and proliferation; transcription of pro-inflammatory genes; and cell death (Waters, Pober et al. 2013). TNFR1 activation can control the following signaling pathways: the nuclear factor-kappa B (NF- κ B), the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal Kinase (JNK), the p38 mitogen-activated protein kinase (p38 MAPK), the 1 acidic sphingomyelinase (A-SMase), and the neutral sphingomyelinase (N-SMase) Pathways (Olmos and Llado 2014).

After binding with TNF- α , TNFR1 will do a conformational change in its cytoplasmic portion, which will assemble the TNFR complex 1, which include TNF receptor-associated death domain (TRADD), receptor-interacting protein 1 (RIP1; also known as RIPK1), cellular inhibitor of apoptosis proteins (cIAPs), TNF receptor-associated factor 2 (TRAF2) and TRAF5 (Song, Zhou et al. 2016). Compared to the signaling pathways initiated by TNFR1, TNFR2 is the preferential receptor for tmTNF- α and is less characterized. TNFR2 can activate IKK, leading to nuclear translocation of NF- κ B through a pathway similar to TNFR1 (Sun 2011). Distinct signaling pathways upon TNFR2 binding have also been studied (Rodriguez, Cabal-Hierro et al. 2011). Previous study have shown that TNFR2 can potentiate the apoptotic response to TNF- α , suggesting that TNFR2 may function as a high-affinity trap of TNF- α that delivers the ligand signal to TNFR1 (Richter, Messerschmidt et al. 2012).

After binding with TNF- α , TNFR1 has a conformational change, leading the assembly of the TNFR complex 1. TNFR complex 1 include TNF receptor-associated death domain (TRADD), receptor-interacting protein 1 (RIP1; also known as RIPK1), cellular inhibitor of apoptosis proteins (cIAPs), TNF receptor-associated factor 2 (TRAF2) and TRAF5 (Song, Zhou et al. 2016). Unlike TNFR1, TNFR2 are more likely bind to tmTNF- α and are less studied. TNFR2 can activate IKK, resulting in nuclear translocation of NF- κ B (Sun 2011). Previous study suggested that TNFR2 can potentiate the apoptotic response to TNF- α , suggesting that TNFR2 may function as a high-affinity trap of TNF- α that delivers the ligand signal to TNFR1 (Richter, Messerschmidt et al. 2012).

2.3.2 TNF- α response in tumors

At first, TNF- α was recognized as a promising strategy for cancer treatment. However, it has also been shown that TNF- α can be derived from many tumor cells (Zins, Abraham et al. 2007; Al-Lamki, Sadler et al. 2010; Aggarwal, Gupta et al. 2012; Landskron, De la Fuente et al. 2014). As a result, the role of TNF- α in tumor survival, migration, and invasion, are more and more appreciated.

2.3.2.1 TNF- α and cancer cell death

The binding of TNF- α to TNFR1 leads to the assemble signaling complex I, which recruits Fas-associated death domain protein (FADD), forming a complex DISC (also known as complex 2) (Micheau and Tschopp 2003). Caspases-8 and caspases-10, which are initiator caspases, are activated within the DISC complex (Parrish, Freel et al. 2013). Activated caspases-8 and 10 can directly cleave and activate effector caspases, such as caspases 3 and 6. Effector caspase can then cleave nuclear lamins leading to the nuclear fragmentation. It is well known that inhibition of caspases is capable of protecting cells from apoptotic cell death. In contrast, necrosis has a feature of a gain in cell volume, swelling of organelles, and irreversible plasma membrane damage (Festjens, Vanden Berghe et al. 2006). Necroptosis can be initiated by the TNF- α DISC through RIPK-1-mediated recruitment and activation of the structurally related protein RIPK-3 (Hitomi, Christofferson et al. 2008; Bonnet, Preukschat et al. 2011; Welz, Wullaert et al. 2011). Interestingly, even absence of FADD or pro-caspase 8, TNF- α can also induce cell death. Another mechanism of how TNF- α exerts antitumor effects is through induction of autophagy, which can be a precursor to both apoptotic and necrotic cell death (Figure 2.3).

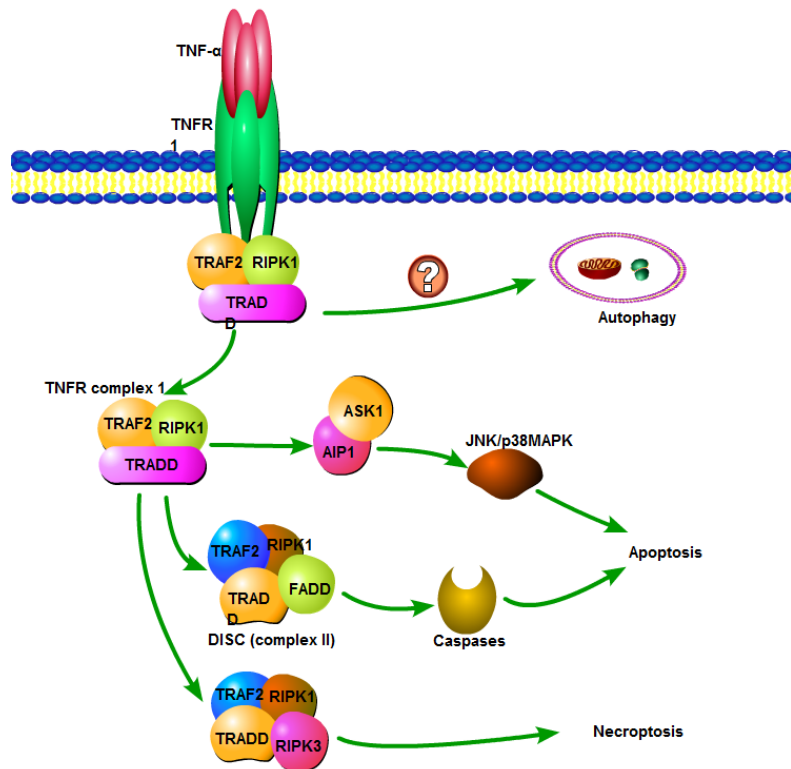


Figure 2.3. TNF- α induces tumor apoptosis and necroptosis via distinct signaling pathways.

2.3.2.2 TNF- α and cancer-related inflammation

TNF- α , being secreted in a setting such as chronic inflammation and infections, can increase the risk of cancer. For example, *Helicobacter pylori* is an important risk factor gastric cancer; inflammatory bowel disease increases the risk of colonic cancer (Wroblewski, Peek et al. 2010; Kim and Chang 2014). TNF- α might speed up cancer cell growth by modulating leukocytes, including T cells, B cells and/or tumor-associated macrophages (TAMs). TNF- α can increase TNFR1-dependent IL-17 production, subsequently, myeloid cell was recruited into the tumor microenvironment and tumor growth was accelerated (Charles, Kulbe et al. 2009). TNF- α also plays an important role in tumor angiogenesis by inducing VEGF production. B cells are important effector cells for TNF- α -mediated carcinogenesis and produce a significant amount of

TNF- α . TNF- α may modulate the activity of regulatory B cells through repressing anti-tumor immunity (Schioppa, Moore et al. 2011).

2.3.2.3 TNF- α and cell proliferation, survival, and angiogenesis

TNF- α stimulates proliferation, invasion, survival, migration, and angiogenesis via TNFR1. In addition, TNF- α binding with TNFR2 also activates the PI3K/Akt pathway leading to cell migration and proliferation (Yang, Wang et al. 2018). In vascular endothelial cells, TNF- α may activate epithelial and endothelial tyrosine kinase (Etk) and vascular endothelial growth factor receptor 2 (VEGFR2), leading to enhanced angiogenesis (Pan, An et al. 2002; Zhang, Xu et al. 2003). In renal cell carcinoma, TNF- α may increase tumor progression by acting selectively through a TNF- α /Etk/VEGFR2 pathway. (Al-Lamki, Sadler et al. 2010).

2.3.3 TNF- α as a therapy for cancer.

Although TNF- α can be used to treat cancer, it has several side effects such as fever, septic shock, and cachexia (Locksley, Killeen et al. 2001). It is estimated that the maximum tolerated dose (MTD) that can be used systemically in clinic is 10-fold lower than the dose inducing cancer cell death (Schiller, Storer et al. 1991; Skillings, Wierzbicki et al. 1992). Due to those side effects, the clinical application of TNF- α was restricted to the isolated limb perfusion (ILP) setting for soft tissue sarcoma (STS) and melanoma in-transit metastases confined to the limb. It is reported that the combination of TNF- α and melphalan may induce regression of unresectable metastases from colorectal cancer. TNF- α is believed to target the tumor vasculature, by decreasing Alpha-v-beta-3 integrin (Ruegg, Yilmaz et al. 1998; Grunhagen, de Wilt et al. 2006). It is suggested that TNF- α can increase tumor blood vessel permeability, thus leading to increased tissue concentration of chemotherapy and destroying the tumor

vasculature (Seynhaeve, Hoving et al. 2007). TNF- α may also promote tumor necrosis by inducing coagulant effect, in which TNF- α can increase fibrin deposition and thrombus formation in the cancer vasculature (Zhang, Deng et al. 1994; Zhang, Deng et al. 1996).

2.3.4 TNF- α as a target for cancer.

Komori et al first reported that TNF- α may be involved in activation of oncogene and DNA damage (Komori, Yatsunami et al. 1993). TNF- α can increase the carcinogenesis by stimulating clonal evolution (Li, Sejas et al. 2007). In addition, TNF- α can also contribute to carcinogenesis by inducing DNA damage in cancer cells, and normal lung epithelial cells (Babbar and Casero 2006; Yan, Wang et al. 2006). Furthermore, clinical trials suggested that neutralizing TNF- α might be beneficial in cancer patients. In a phase I study, the anti-TNF antibody infliximab can stabilize previously progressing advanced cancer in 7 of 41 patients (Brown, Charles et al. 2008). In a phase II study, TNF- α antagonist etanercept, a soluble TNFR2 fusion protein that binds and neutralizes TNF- α , can also stabilize diseases in 6 of 30 progressing ovarian cancer patients (Madhusudan, Foster et al. 2004), and in 14 of 30 renal cell cancer patients (Harrison, Obermueller et al. 2007). However, the molecular mechanisms of action of anti-TNF in cancer patients are still unclear. It is suggested that TNF- α antagonists inhibit cytokine and chemokine production, recruitment of inflammatory cells, angiogenesis and extracellular matrix degradation (Tracey, Klareskog et al. 2008). Some of the mouse model experiments demonstrated a role for TNF antagonists in cancer prevention. Previous studies also have shown that traditional medical herbal and the polyphenols present in tea inhibit TNF- α release (Fujiki, Suganuma et al. 2003). Recent studies suggested that tumor microenvironment levels of sTNF-R2 may represent a factor of poor prognosis and in epithelial ovarian cancer (Nomelini, Borges Junior et al. 2018). Given the fact that TNF- α plays an important role in regulating innate immunity, TNF- α antagonists might increase risk of infection. Therefore, many patients with rheumatoid arthritis

or other chronic inflammatory diseases are recruited in the clinic trials for studying cancer incidence during TNF- α antagonist treatment (Table 1).

Study Design	Diseases	Treatment	Duration	Results
Cohort study	Rheumatoid Arthritis	TNF inhibitor and rituximab	13 years	Not increase risk of cancer(Silva-Fernandez, Lunt et al. 2016)
Cohort study	Inflammatory bowel disease	TNF inhibitor	3.7 years	Not increase risk of cancer (Nyboe Andersen, Pasternak et al. 2014).
Cohort study	Rheumatoid Arthritis	TNF inhibitor	9.4 years	Not increase risk of cancer(Raaschou, Frisell et al. 2015)
Cohort study	Crohn's disease Ulcerative colitis	Vedolizumab	54 weeks	Not increase risk of cancer(Amiot, Serrero et al. 2017)
Cohort study	Rheumatoid Arthritis	TNF inhibitor	5 years	Not increase risk of cancer(Mercer, Lunt et al. 2015)
Clinical trial	Advanced cancer	Infliximab	50 weeks	No evidence of disease acceleration(Brown, Charles et al. 2008)

Cohort study	Rheumatoid Arthritis	TNF inhibitor	10 years	Not increase risk of cancer(Phillips, Zeringue et al. 2015)
Cohort study	Rheumatoid Arthritis	TNF inhibitor	14 years	Reduced risk of cancer(Wu, Chen et al. 2014)

Table 1. Effects of TNF inhibitor on risk of cancer.

2.4 Vitamin D

Vitamin D is a group of steroids with a broken ring: secosteroids. Vitamin D is grouped into two major physiologically relevant forms, ergocalciferol (vitamin D₂), which is mostly derived from plants, and cholecalciferol (vitamin D₃), which is photosynthesized in the skin of animal by the action of UVB on 7-dehydrocholesterol. The most major source of vitamin D is obtained by cutaneous production from UVB. Sincere the production of vitamin D can be interrupted by latitude, season, ageing, skin pigmentation, it is crucial to get enough vitamin D from diet to maintain a satisfied vitamin D level (Bikle 2014). In the United States, the main dietary source of vitamin D is from dietary products and some fortified foods such as breakfast cereals, soy beverages, and orange juice. Natural sources of vitamin D include fish, and egg yolks.

2.4.1 Synthesis and Metabolism of Vitamin D

Vitamin D₃ can be obtained from dairy products and fish oils or be photosynthesized in the skin by the action of sun exposure on 7-dehydrocholesterol. As shown in Figure 2.4, synthesis of vitamin D depends on the doses of UVB. Since sunscreen, skin color can block sun exposure, sunscreen and skin color decrease the production of vitamin D (Matsuoka, Wortsman et al.

1992). Vitamin D is transported to the liver in the blood vessels by binding to vitamin D-binding protein (DBP). In the liver, vitamin D is converted into 25-hydroxyvitamin D [$25(\text{OH})\text{D}_3$] by the 25-hydroxylase (CYP27A1), resulting to $25(\text{OH})\text{D}_3$. The $25(\text{OH})\text{D}_3$ is then hydroxylated in the kidney by 1-hydroxylase (CYP27B1, 1-OHase), yielding the active form of vitamin D, $1,25(\text{OH})_2\text{D}_3$. $1,25(\text{OH})_2\text{D}_3$ has different effects on various target tissues. In the kidney, 24-hydroxylase (CYP24) also can hydroxylate $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$, resulting $24,25(\text{OH})_2\text{D}_3$, $1,24,25(\text{OH})_3\text{D}_3$. Compared to active $1,25(\text{OH})_2\text{D}_3$, $24,25(\text{OH})_2\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3$ are relatively inactive (Bikle 2014). Calcitriol [$1,25(\text{OH})_2\text{D}_3$] exerts multiple cancer prevention effects on various malignant cells and animal models.

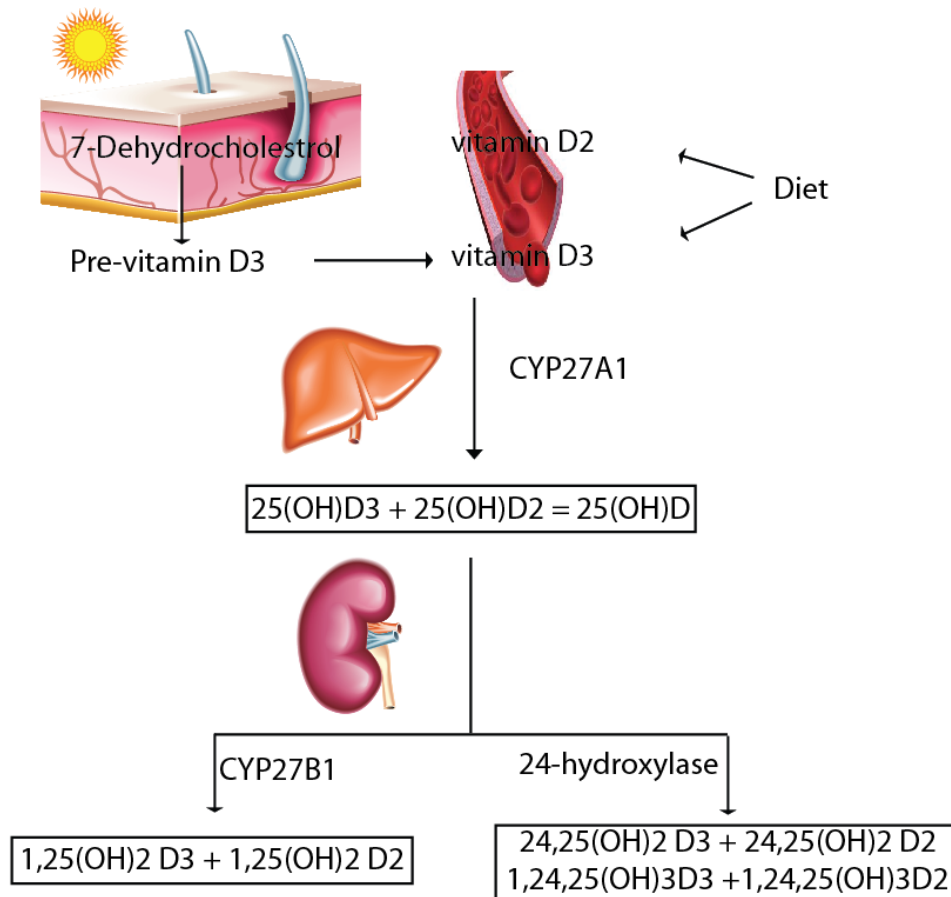


Figure 2.4. Vitamin D synthesis and metabolism

2.4.2 Mechanisms of the anti-cancer effects of calcitriol

As shown in Figure 2.5, several molecular mechanisms are involved in the cancer prevention effects of calcitriol. It is suggested that the cancer prevention effects of calcitriol is obtained by binding to nuclear vitamin D receptor (VDR), mostly via genomic actions (Deeb, Trump et al. 2007; Mikhak, Hunter et al. 2007; Ingraham, Bragdon et al. 2008; Li, Li et al. 2017).

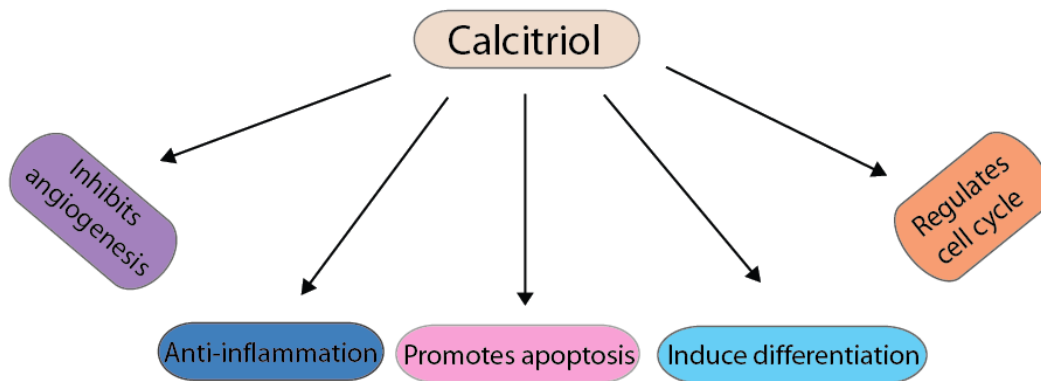


Figure 2.5. Mechanisms underlying the anticancer effects of calcitriol

2.4.2.1 Regulation of cell cycle and apoptosis

Calcitriol has anti-proliferative effect in many cancer cells by modifying cell cycle (Sarkar, Hewison et al. 2016), inducing apoptosis (Dou, Ng et al. 2016). Specifically, calcitriol decreases the proliferation of cancer cells through increasing the production of cyclin dependent kinase (CDK) inhibitors p21 and p27, and decreasing CDK activity (Flores, Wang et al. 2010). In the meanwhile, the combination of calcitriol and conventional therapies might also downregulate the expression and activity of important signaling pathways which can regulate the cell cycle. (Segovia-Mendoza, Diaz et al. 2017).

2.4.2.2 Enhancement of differentiation

Besides inhibition of proliferation, calcitriol also has the ability of inducing the differentiation of a variety of cancer cells, including CRC cells (Diaz, Diaz-Munoz et al. 2015). In addition, calcitriol can induce terminal differentiation of human myeloid leukemia cells into monocytes and macrophages (Vasylyeva, Chen et al. 2005). Previous study also has shown that calcitriol might be able to upregulate the expression of myoepithelial markers in SUM159 mammospheres (Shan, Wahler et al. 2017).

2.4.2.3 Modulation of angiogenesis

Calcitriol can decrease the angiogenesis of new blood vessel formation in cancer microenvironment. For example, in *Vdr*-null mice, there are increased expression of pro-angiogenic factors such as Hypoxia-inducible factor 1 alpha (HIF 1 α), vascular endothelial growth factor (VEGF), angiopoietin 1, platelet-derived growth factor (PDGF) in tumors (Chung, Han et al. 2009). In addition, Calcitriol can inhibit cyclooxygenase-2 (COX-2) induced production of prostaglandin E₂ (PGE₂), which can induce angiogenesis by secreting HIF 1 α in cancer cells (Fukuda, Kelly et al. 2003).

2.4.2.4 Anti-inflammation

As I discussed above, a large amount of evidence suggests that chronic inflammation plays an important role in cancer development and progression (Koul, Kumar et al. 2010; Sfanos, Hempel et al. 2014; Crusz and Balkwill 2015). Previous studies have shown that calcitriol exhibits anti-inflammatory actions in several cancers (Krishnan and Feldman 2011). Calcitriol decreases the expression of COX2, PGE2, and other inflammatory cytokines in prostate cancer cells (Moreno, Krishnan et al. 2005) and ovarian cancer cell lines (Thill, Woeste et al. 2015). This may be because calcitriol can alter several pro-carcinogenic inflammatory pathways, such

as NF- κ B(Bao, Yao et al. 2006; Cohen-Lahav, Shany et al. 2006), and p38 stress kinase pathway(Nonn, Peng et al. 2006).

2.4.3 Vitamin D in clinical trials

In WHI clinical trial, supplementation with a small amount of vitamin D₃ (400 IU per day) and calcium (1 g per day) did not show a CRC prevention effect. (Wactawski-Wende, Kotchen et al. 2006). One limitation of this study is that the low dose of vitamin D (400 IU per day) may not significantly raise the serum 25(OH)D levels (Tella, Gallagher et al. 2014). Reanalyzing the WHI clinical trial, Ding et al found that vitamin D and calcium supplementation may exert CRCs cancer prevention effects in postmenopausal women who do not use estrogen therapy (Ding, Mehta et al. 2008). Vahedpoor et al found that after 6 months of vitamin D supplementation, patients with cervical intraepithelial neoplasia grade 1 have shown cancer regression and improved metabolic status(Vahedpoor, Jamilian et al. 2017). However, in a RCT study of healthy postmenopausal women, vitamin D and calcium supplementation did not decrease the cancer risk after 4 years of supplementation(Lappe, Watson et al. 2017). One possible explanation of negative results is that the control participants have a higher baseline serum vitamin D level (32.8 ng/ml) compared with the US population. It is estimated that 75-80% the US adult population had serum vitamin D levels less than 30 ng/ml, and approximately 30% had vitamin D levels below 20 ng/ml(Yetley 2008). In patients with early recurrent prostate cancer, calcitriol therapy decreased the rate of rise of prostate specific antigen (PSA) (Gross, Stamey et al. 1998).

2.5 Sulforaphane

2.5.1 Sulforaphane Bioavailability

Sulforaphane (SFN) [1-isothiocyanato-4-(methylsulfinyl)butane], a isothiocyanate, has been reported that it has chemopreventive property. SFN contains an isothiocyanate group ($-N = C = S$) and a methylsulfonyl side chain (R-(S-O)-R). (Figure 2.6) SFN is a plant-origin isothiocyanate organosulfur compounds, which has been extensively studied. It has been suggested that SFN has many biological effects including cancer prevention (Amjad, Parikh et al. 2015), and inflammation prevention (Greaney, Maier et al. 2016).

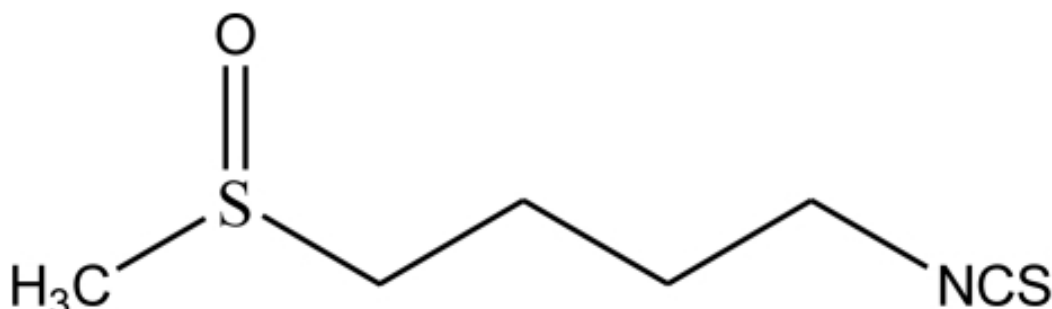


Figure 2.6. The molecular structure of sulforaphane in broccoli

Besides its well known cancer prevention effect, SFN is known to possess anti-inflammatory property (Liu and Talalay 2013). Nuclear factor kappa B (NF κ B) plays a pivotal role in the inflammatory conditions (Tak and Firestein 2001). It is well known that NF κ B activation can lead to the up-regulation of oncogenes and proinflammatory cytokines in the colon cancer and prostate cancer. It has been reported that NF κ B pathway is highly active in patients with solid cancers such as colon, prostate, breast cancers (Aggarwal and Gehlot 2009). Further studies have suggested that SFN formed adducts with cysteine residues in the extracellular domain of TLR4, resulting in the inhibition of NF κ B mediated signaling pathway.

2.5.2 Anticancer Activity of Sulforaphane

Epigenetic alterations play a pivotal role in the cancer prevention effects of SFN (Golson and Kaestner 2017). Compared with genetic alterations, epigenetic changes are reversible. There are two major types of modifications in epigenetic alterations: histone modifications, which are flexible, and DNA methylation, which are generally stable (Kouzarides 2007). Histone acetylation reduces the positive charge of the histone proteins, leading to a reduced affinity for DNA and resulting in an open chromatin structure that eases the access of transcription factors to specific gene loci. In the mean time, histone deacetylases (HDACs) can remove the acetyl group (Figure 2.7). It is well known that overexpression and / or over activity of HDAC are characteristics of cancer, which can lead to a reduced transcription of genes responsible for apoptosis and cell cycle arrest (Fraga, Ballestar et al. 2005).

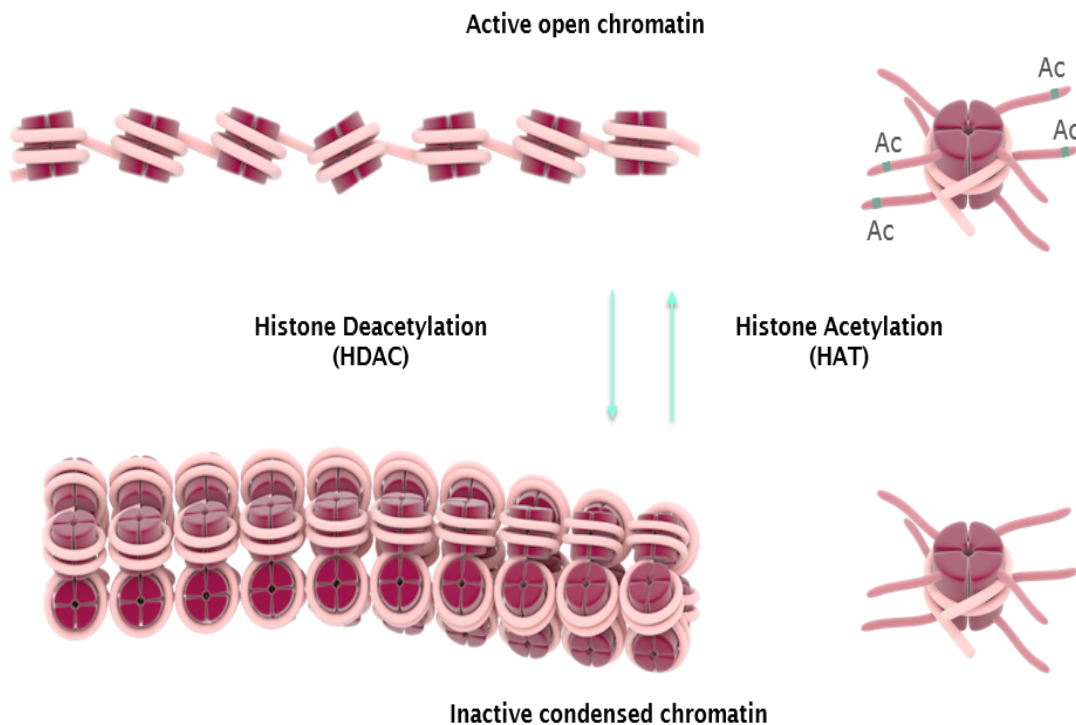


Figure 2.7. Modulation of chromatin conformation and transcriptional status by acetylation of lysine tails in histone core proteins. HDAC, histone deacetylase; HAT, histone acetyltransferase.

SFN can act as an HDAC inhibitor, resulting in cancer cell cycle arrest and apoptosis (Ho, Clarke et al. 2009). Myzak et al., first reported that SFN can increase TOPflash reporter activity without changing expression of β -catenin, indicating that SFN altered the activity of HDAC (Myzak, Karplus et al. 2004). In human embryonic kidney 293 cells, SFN dose-dependently decrease HDAC activity (Myzak, Karplus et al. 2004). It is suggested that 15 μ M SFN can significantly inhibit HDAC activity in three prostate epithelial cell lines (BPH-1, LNCap, and PC3) (Myzak, Hardin et al. 2006). Another study reported that after a single oral dose of SFN, SFN significantly inhibited HDAC activity in the colonic mucosa and suppressed tumor development in APC^{min} mice (Myzak, Dashwood et al. 2006). In HCT116 colon cancer cells, 15 μ M SFN can significantly inhibit the activity of HDAC1, HDAC2, HDAC3, and HDAC8 after 36 h of treatment (Rajendran, Delage et al. 2011).

Previous studies have shown that DNA methylation pattern is changed during cancer initiation and progression. The changes include global and site specific DNA hypomethylation as well as gene specific promoter hypermethylation (Portela and Esteller 2010; Baylin and Jones 2011). Hypomethylation of the promoter of oncogenes can increase their expression, while, DNA hypermethylation may lead to the inhibition of genes involved in cell cycle regulation and apoptosis. Regulation of DNA methylation patterns are mediated by DNA methyltransferases (DNMTs). DNMTs are overexpressed in leukemic, gastric, lung, and prostate cancer (Mizuno, Chijiwa et al. 2001; Etoh, Kanai et al. 2004; Morey Kinney, Smiraglia et al. 2008; Lin, Wu et al. 2010).

Previous studies have shown that the expression of DNMT1 and DNMT3a was inhibited by SFN treatment in human breast cancer cells and prostate cancer cell lines (Meeran, Patel et al. 2010; Hsu, Wong et al. 2011). Treatment with SFN for 24 h can decrease global methylation in LNCap cells (Kobayashi, Nakamura et al. 2009). SFN treatment can decrease methylated CpG sites in the promoter region of cyclin D2 in prostate cancer cells. In addition, SFN treatment can also decrease methylation at the binding site of the c-Myc transcription factor. However, SFN treatment failed to alter abnormal methylation patterns in critical genes involved in colon carcinogenesis in colon cancer cells (Barrera, Johnson et al. 2013).

2.5.3 Sulforaphane in Human Studies

A number of clinical trials are conducted to determine the cancer prevention effects of SFN. Kensler et al have shown that the level of SFN metabolites is inversely associated with cancer markers in resident of Qidong who have a high risk of hepatocellular carcinoma (Kensler, Chen et al. 2005). Another study shows that consumption of 400 μ M glucoraphanin may increase the excretion of airborne pollutants (Kensler, Ng et al. 2012). Kirsh et al reported that consumption of cruciferous vegetables especially broccoli is associated with a significantly decreased risk of prostate cancer (Kirsh, Peters et al. 2007). However, in another clinic study, treatment with 200 μ M per day of SFN-rich extracts did not lead to $\geq 50\%$ PSA declines in the majority of patients (Alumkal, Slottke et al. 2015).

2.6 Butyrate

2.6.1 Background

Butyrate is a four-carbon short-chain fatty acid, which can be produced by anaerobic bacteria fermentation of dietary fiber in the colon (Figure 2.8). 2 metabolic pathways are involved in the production of butyrate. In the first pathway, butyryl-CoA is phosphorylated and converted to butyrate by butyrate kinase (Louis and Flint 2017). In the other pathway, the butyryl-CoA:acetate CoAtransferase transfers the CoA moiety of butyryl-CoA to acetate, resulting to the production of butyrate and acetyl-CoA (Trachsel, Bayles et al. 2016). Butyrate is the major energy source for colonocytes (Slavin 2013; Conlon and Bird 2014).

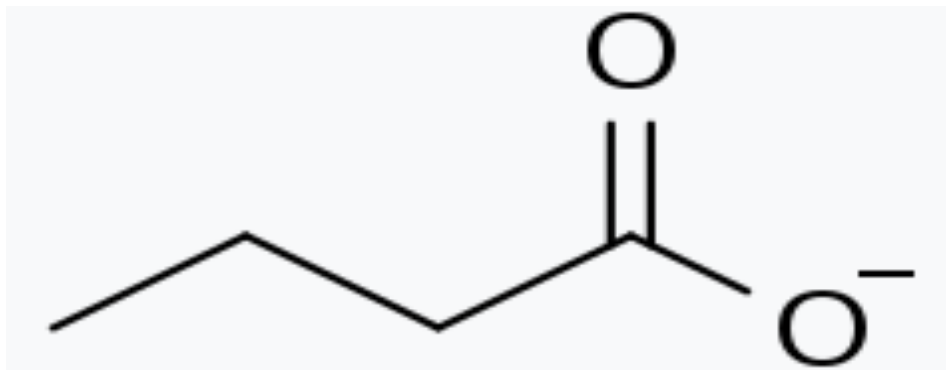


Figure 2.8. Butyrate Chemical Structure

Butyrate are absorbed across the apical membrane of the colonocytes by both diffusion and short-chain fatty acid (SCFA) transporter. There are two SCFA transporters, monocarboxylate transporter (MCT) isoform 1 (MCT1), and solute carrier (SLC) family 5 member 8 (SLC5A8) (Counillon, Bouret et al. 2016). Orphan G protein-coupled receptor 41 (GPR41) and GPR43 are the receptors for SCFA. GPR41 and GPR43 are also known as FFAR3 and FFAR2 respectively (Brown, Goldsworthy et al. 2003). Butyrate preferentially binds to GPR41 over GPR43. GPR41 is universally expressed in a variety of tissues including adipose tissue, pancreas, spleen, lymph nodes, bone marrow, and peripheral blood mononuclear cells including monocytes (Brown, Goldsworthy et al. 2003; Le Poul, Loison et al. 2003). GPR43 also expressed in colon cells, and

adipocytes, and the highest expression of GPR43 was found in immune cells such as monocytes and neutrophils (Nilsson, Kotarsky et al. 2003).

2.6.2 Butyrate and Anti-inflammation

Studies have shown that butyrate is a promising anti-inflammatory agent. Butyrate can inhibit the secretion of the proinflammatory cytokines such as IFN- γ , TNF- α , IL-1 β , IL-6, and IL-8, whereas enhance the production of the anti-inflammatory cytokines IL-10 and TGF- β . It is suggested that the molecular mechanisms underlying the inhibition of inflammation by butyrate are partially due to inhibition of NF- κ B. Further studies suggest that butyrate suppresses the NF- κ B signaling pathway activation by rescuing the redox machinery and controlling reactive oxygen species (Russo, Luciani et al. 2012). Activation of PPAR- γ by butyrate is another possible mechanism of anti-inflammatory activity of butyrate. Previous studies have suggested that PPAR- γ is a member of the nuclear receptors, and activation of PPAR- γ can exert anti-inflammatory effects (Mattace Raso, Simeoli et al. 2013). Butyrate may inhibit signaling of IFN- γ , which plays a crucial role in inflammation-associated CRC development (Schwab, Reynders et al. 2007; Zimmerman, Singh et al. 2012).

2.6.3 Butyrate and CRC

Epidemiological and animal studies have shown that dietary fiber can inhibit CRC incidence (Baena and Salinas 2015; Encarnacao, Abrantes et al. 2015). One of the explanations of CRC prevention effect of butyrate is that bacterial fermentation convert resistant starch to SCFA (Song, Garrett et al. 2015). Among SCFA, butyrate plays a pivotal role in the prevention/inhibition of colon carcinogenesis (Manning and Gibson 2004). Butyrate can induce colon cancer cell differentiation and apoptosis, and inhibit colon cancer cell

differentiation(Goncalves, Araujo et al. 2011). Similarly, several animal studies also suggested a protective effect of butyrate on CRC carcinogenesis (D'Argenio, Cosenza et al. 1996; Kameue, Tsukahara et al. 2004; Lu, Nakanishi et al. 2013). Human studies also found an inverse relationship between the levels of butyrate in the human colon and the incidence of CRC (Bingham, Day et al. 2003). The risk of developing tumors are higher in the distal colon where the concentration of butyrate is lower, implicating butyrate has CRC prevention effect (Mortensen, Holtug et al. 1988).

It seems that the underlying mechanisms of cancer prevention effect of butyrate involve regulation of gene expression, which due to its capacity of HDAC inhibition. Acting as a HDAC inhibitor, butyrate can result in hyperacetylation of histones. Beside HDAC, it is likely that butyrate has other targets, such as DNA methylation (Dehaan, Gevers et al. 1986), histone methylation (Pesavento, Yang et al. 2008), hyperacetylation of nonhistone proteins (White, Mulligan et al. 2006), inhibition of histone phosphorylation(Mathew, Ranganna et al. 2010), regulation of expression of micro-RNAs (miRNA) (Hu, Dong et al. 2011), and modulation of intracellular kinase signaling (Basson and Hong 1998). In addition, butyrate can also alter immune response in the colon and moderate gut bacteria community to maintain colonic mucosa homeostasis(Lupton 2004). The cancer prevention effect of butyrate depends on its intracellular concentration (Mariadason 2008; Ashktorab, Belgrave et al. 2009).

2.7 Wnt pathway

The *Wnts*, a group of nineteen secreted glycoproteins , which is composed of approximately 300 amino acids. The *Wnts* can activate many signaling pathways, including the canonical *Wnt*/β-catenin pathway, *Wnt*/Ca²⁺ pathway, and the *Wnt*/polarity pathway(Prakash and

Swaminathan 2015). The *Wnt* proteins are approximately 40 kDa in size. The *Wnt* signaling pathway is highly conserved and plays a central role in regulating development and stemness. Aberrant *Wnt* signaling plays an important role in the initiation of many cancers. Constitutively active *Wnt* signaling is a driver of CRCs progression.

2.7.1 Canonical and non-canonical *Wnt* signaling

The *Wnt* signaling pathway is divided into canonical (β -catenin dependent) and non-canonical (β -catenin independent) pathways. The characteristics of the canonical *Wnt* pathway are the accumulation of β -catenin in the cytoplasm, and its subsequent translocation to the nucleus. When the *Wnt* ligands is absent, cytoplasmic β -catenin is phosphorylated by a degradation complex, which is composed of the tumor suppressor adenomatous polyposis coli (APC), the scaffolding protein AXIN and two kinases CK1 (casein kinase 1) and GSK3 β (glycogen synthase kinase 3 β) (Figure 2.9). β -TrCP, a ubiquitin ligase, then recognized phosphorylated β -catenin, therefore, β -catenin is ubiquitinated and targeted for degradation by the proteasome (Tabatabai, Linhares et al. 2017). *Wnt* ligands first bind to Frizzled receptors as well as LRP5/6 co-receptors, which can induces dishevelled (DVL) phosphorylation, which subsequently recruits Axin and destruction complex to the cell membrane. Without destruction complex, β -catenin accumulated in the cytoplasmic and subsequent enter nucleus. In the nucleus, β -catenin can bind to the TCF/LEF (T-cell factor/lymphoid enhancer factor) transcription factors and recruit the transcriptional Kat3 co-activators p300 and CBP (CREB-binding protein) to initiate the transcription of *Wnt* target genes (Duchartre, Kim et al. 2016).

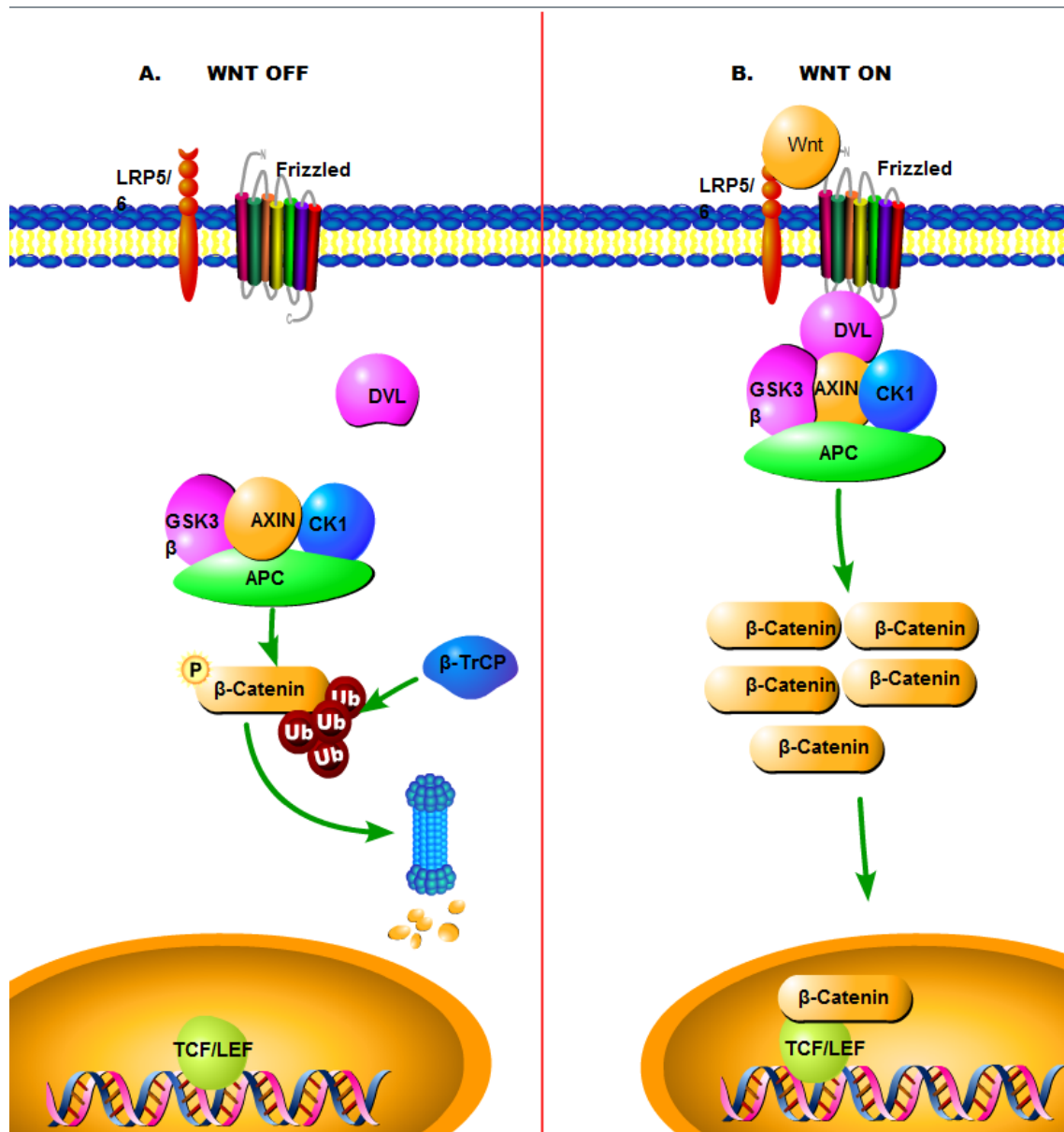


Figure 2.9. Canonical Wnt Pathway. A. *Wnt* off. When *Wnt* protein is absent, β -catenin is constantly degraded by the destruction complex which includes Axin, APC, Gsk3, and CK1. The destruction complex targets β -catenin for ubiquitylation (Ub) and subsequently degraded by proteasome. B. *Wnt* on. The binding of *Wnt* protein to the Frizzled/Lrp5/6 receptors resulting in the phosphorylation of disheveled (Dvl). Activated Dvl then recruits Axin, which can inhibit the destruction complex, leading to the accumulation of β -catenin in the cytoplasm, which then enters into the nucleus where it can bind to TCF/LEF to initiate transcription.

2.7.2 *Wnt* signaling and cancer

Wnt signaling is crucial for intestinal crypts homeostasis and maintenance of intestinal stem cells (Flanagan, Austin et al. 2018). It is well known that hyperactivation of *Wnt* pathway is present in almost all CRC. *Wnt* signaling plays a pivotal role in CRCs incidence and is the basis for CRCs tumorigenesis in patients with familial adenomatous polyposis and in *Apc* mutant mice model. It is estimated that approximately 92% of sporadic CRCs contain at least one mutation in *Wnt* signaling (2012). Loss of APC function is the main driver of CRCs carcinogenesis. Previous studies have shown that different mutations of APC result in different activity level of canonical *Wnt* pathway (Christie, Jorissen et al. 2013). Interestingly, if APC function is restored, colon adenomas could regress to normal tissue, which suggested that continuous *Wnt* signaling alteration is crucial for CRCs maintenance (Dow, O'Rourke et al. 2015). Notably, although truncated APC is present, *Wnt* secretion still can modulate the *Wnt* pathway signaling (Voloshanenko, Erdmann et al. 2013).

Besides *Apc* mutation, mutations in the R-spondin/*Lgr5*/*RNF43* are all involved in CRCs initiation (de Lau, Peng et al. 2014). Interestingly, somatic *RNF43* mutations are appeared mutually exclusive with *Apc* mutations and exhibits in 19% of total CRCs cases (Giannakis, Hodis et al. 2014). R-spondin 3 mutations and fusion proteins are occurred in 10% of CRC cases. Since *RNF43* mutant CRCs is dependent on *Wnt* secretion, *RNF43* mutant CRCs is highly susceptible to *Wnt* secretion targeted therapy (van de Wetering, Francies et al. 2015).

Chromosomal instability (CIN) is present in 65 - 70% of sporadic CRCs. CIN is involved in loss of function of *Wnt* signaling components, particularly APC (Caldwell, Green et al. 2007; Rusan

and Peifer 2008). Recent study has shown that insufficient basal *Wnt*/STOP may accelerate microtubule assembly rates and CIN in CRCs cells, while restore normal assembly rates could reverse the CIN phenotype (Ertych, Stolz et al. 2014; Stolz, Neufeld et al. 2015).

SNAI2 is a crucial transcriptional factor that plays a pivotal role in epithelial to mesenchymal transition (EMT). Canonical *Wnt* signaling pathway can phosphorylate GSK3 β and stabilize SNAI2, leading to increased EMT (Wu, Li et al. 2012). PI3K-Akt signaling inhibition can lead to accumulation of β -catenin and FOXO3a, resulting in the increased metastasis (Tenbaum, Ordonez-Moran et al. 2012). It was reported that Fzd2 expression facilitated EMT and cell migration by Fyn and Stat3. In a xenograft mouse model of colon cancer, Fzd2 inhibition leads to reduced tumor growth and metastasis (Gujral, Chan et al. 2014).

2.7.3 Targeting *Wnt* signaling in CRC

Vantictumab (OMP-18R5) can block *WNT*-FZD ligand-receptor binding at the membrane, as it is an antibody that targets the FZD receptors (Gurney, Axelrod et al. 2012). Vantictumab is not on the Phase 1b to evaluate for the effects of cancer prevention, such as breast, ovarian, and pancreatic cancers, but not CRCs. Considering the fact that the hyperactivation of *Wnt* signaling in CRCs is caused by Apc mutation, it is not surprising that blocking *WNT*-FZD ligand-receptor interaction is not a effective treatment for colon cancer. However, CRCs patients who carry RNF43 mutation are suitable for vantictumab, because ligand-receptor interaction is needed to initiate the *Wnt* response (van de Wetering, Francies et al. 2015; Madan, Ke et al. 2016).

The destruction complex degrades β -catenin, therefore, it is an promising target to alter *Wnt* pathway. It has been shown that regulation of Axin function via modulating Tankyrase activity can stabilize the destruction complex. Tankyrase enzymes (TNKS and TNKS2) can destabilize

the destruction complex by PARsylating Axin and subsequent degradation of Axin. Previous study suggested that blocking TNKS can inhibit the proliferation of APC-mutant CRC in *vitro* and in *vivo*. In addition, TNKS blockade can synergize with regular chemotherapies and drugs that inhibit RAS/MAPK and PI3K/AKT pathways (Schoumacher, Hurov et al. 2014; Schoumacher, Hurov et al. 2014; Wu, Luo et al. 2016). However, more studies need to be done to study the safety and efficacy of TNKS inhibition.

Another strategy is to hamper β -catenin binding with specific transcriptional co-activators. For example, CCT036477 is a small compound that inhibits the binding of β -catenin with CREB binding protein (CBP) (Emami, Nguyen et al. 2004; Gonsalves, Klein et al. 2011; Jarde, Evans et al. 2013). *In vitro* studies have shown CCT036477 can dramatically reduce levels of *Wnt* reporter gene activity, decrease expression of β -catenin downstream targets, and block binding to the co-activator TCF. However, little is known about the efficacy of these compounds *in vivo*.

Anti-CTLA4, anti-PD1 blocking antibodies can inhibit immune checkpoints. Interestingly, a recent study has shown that modulating of *Wnt*/ β -catenin signaling pathway may be involved in the cancer prevention effect of anti-CTLA4, anti-PD1 antibody (Spranger, Bao et al. 2015). It may be because that *Wnt*/ β -catenin signaling inhibition may decrease dendritic cells (DCs) mediated tumor tolerance (Oderup, LaJevic et al. 2013; Swafford and Manicassamy 2015). Therefore, poor response to anti-CTLA4, anti-PD1 antibodies might be due to high *Wnt*/ β -catenin signaling activity. Combination *Wnt*/ β -catenin inhibition with immunotherapy might be a promising strategy for CRCs treatment.

CHAPTER 3

PURPOSE OF THE STUDY

3.1 Research Aims and Hypotheses

3.1.1 Aim 1

The primary objective of the study 1 is to determine if inhibition of TNF- α production is an effective strategy to delay or prevent high fat diet induced colon cancer onset. To achieve this goal, we will use the *Apc*^{1638N} mice, which are heterozygous for a germline mutation in *Apc*. *Apc*^{1638N} mice have a mild tumorigenic phenotype (3 vs 100 tumors) and longer lifespan (>1 years vs 4-6 months) (Heyer, Yang et al. 1999; Taketo 2006).

Chronic inflammation is a central component of obesity associated cancer. TNF- α is a key pro-inflammatory cytokine which induces other inflammatory mediators. Therefore, many studies have done to examine the cancer prevention effects of blocking TNF- α on tumorigenesis. It has been shown that eliminating TNF- α or TNFR can inhibit tumor induction and growth (Moore, Owens et al. 1999; Suganuma, Okabe et al. 1999).

The *Wnt* pathway is crucial for maintenance of the intestinal epithelia. Hyperactive *Wnt* pathway can induce the incidence and development of colon cancer (Zhan, Rindtorff et al. 2017). It was shown that TNF- α activates *Wnt* signaling via the induction of GSK3 β phosphorylation (Oguma, Oshima et al. 2008).

We hypothesize that genetic ablation of TNF- α will be an effective strategy to attenuate the development of colon cancer in mice fed a high fat diet. Moreover, we propose that eliminating TNF- α induces a decrease of *Wnt* signaling leading to a reduced risk of colon cancer in mice fed

a high fat diet. We will evaluate the effects of genetic ablation of TNF- α on obesity associated intestinal tumorigenesis. QuickPlex SQ 120, a chemiluminescence assay, was used to measure the inflammatory cytokines. To determine the activation of *Wnt* pathway and its downstream genes, we performed Real time PCR and Western blot assay. Immunohistochemical analysis was used to further confirm the activation of *Wnt* pathway.

3.1.2 Hypothesis 1

It has been reported that inhibition of TNF- α may reduce cancer risk (Bernert, Sekikawa et al. 2003; Karabela, Kairi et al. 2011). However, it remains to be determined whether inhibition of TNF- α can decrease high fat diet induced colon cancer. The role of TNF- α in high fat diet induced colon cancer will be examined in this study using TNF- α -deficient mice crossed with *Apc*^{1638N} mice. We hypothesize that ablation of TNF- α decrease the risk of obesity associated CRCs. To test this hypothesis, *Apc*^{1638N} mice will be randomly divided into 2 dietary groups, 12 animals / group: *LF*, a low fat diet (10 cal% fat); *HF*, a high fat diet (60 kcal% fat). 12 TNF- α -deficient mice crossed with *Apc*^{1638N} mice will receive a high fat diet (60 kcal% fat). After 16 weeks on diet starting at 4-6 weeks of age, mice were euthanized with CO₂. Plasma samples were collected and stored at -80 °C for analyses of cytokines. Tissue samples were collected using the methods reported before (Liu, Brooks et al. 2012).

TNF- α is one of the central cytokines in the inflammation. Our working hypothesis is that genetic ablation of TNF- α may attenuate inflammation in the diet-induced obese mice. To test this hypothesis, the inflammatory cytokines were measured by a chemiluminescence assay using the QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD). Inflammation of the colon will be further confirmed by H&E stained histological sections.

Based on our previous studies, we have confirmed that high fat diet induced obesity is associated with a significant increase of TNF- α level in mouse colon, and activation of the *Wnt* signaling pathway. Therefore, we hypothesize that tumorigenesis effects of TNF- α is mediated by a direct activation of *Wnt* pathway, and genetic ablation of TNF- α might down-regulate *Wnt* pathway. We will analyze the effects of genetic ablation of TNF- α on key signaling proteins of *Wnt* pathway by immunoblotting analysis. We will measure the levels of phospho-GSK3 β (Ser9), the inactive form of GSK3 β , and the dephosphorylated β -catenin (Ser37 or Thr41), the active form, which are two key molecules in the *Wnt* pathway. Immunohistochemical analysis will be utilized to further determine the effect of genetic ablation of TNF- α on the activation of active β -catenin. Our expectation is that genetic ablation of TNF- α attenuates active β -catenin and its downstream targets in APC^{1638N} mice.

3.1.3 Aim 2

The study 2 aimed to test a dietary strategy, the supplementation of a high dose of vitamin D (VD) or its combination with sulforaphane (SFN), to inhibit intestinal inflammation and thereby obesity-associated tumorigenesis. VD is crucial for a variety of physiological and pathological processes in the human body. VD also can regulates numerous cellular pathways that affect cell proliferation, differentiation, and apoptosis, therefore, VD plays an important role in cancer incidence, prognosis, and mortality (Fleet 2008; Ng 2014). However, studies have shown conflicting results.

In study 2, we used a high VD (5,000 IU) with a low VD (200 IU) as a control group. The phytochemical sulforaphane (SFN) is an isothiocyanate derived in cruciferous vegetables and is

especially high in broccoli and broccoli sprouts. Epidemiologic studies suggest that higher intake of cruciferous vegetables is associated with low risk of CRC (Wu, Yang et al. 2013). It is suggested that SFN may act as a histone deacetylase (HDAC) inhibitor activity in human CRC cell lines (Ho, Clarke et al. 2009). In addition, previous studies have shown that HDAC inhibitors can sensitize cancer cells to anti-cancer drugs (Regel, Merkl et al. 2012). However, the combination effects of VD and SFN on high fat diet induced CRCs is not well studied.

3.1.4 Hypothesis 2

We anticipate that VD co-administration with SFN may further inhibit obesity associated colon cancer. To investigate the effect of VD or VD in combination with SFN in obesity-associated intestinal tumorigenesis, we evaluated the development of tumor in the APC^{1638N} mouse model with or without VD or VD+SFN supplementation.

The dietary phytochemical SFN is an isothiocyanate found in cruciferous vegetables and has a particularly high concentration in broccoli. SFN is known for its antitumor properties, but, the mechanisms by which SFN might exert anticarcinogenic effects remain unclear. SFN inhibits HDAC activity in many cancer cells (Dashwood and Ho 2008; Ho, Clarke et al. 2009; Dickinson, Rusche et al. 2015; Kim, Fujita et al. 2016). Histone deacetylase inhibitors (HDACis) can alter nuclear and cytoplasmic protein acetylation modify gene expression. Previous studies have shown that HDACis alone or in combination with other anti-cancer agents are promising treatment strategies (Thurn, Thomas et al. 2011; Arrighetti, Corno et al. 2015; Ray, Das et al. 2018). Since activity of VD receptor (VDR) can be modulated epigenetically by histone acetylation, the combination of VD and HDACi has been studied. Several studies have shown that the combination of VD and HDACi is synergistic in cancer models, especially including

those are resistant to VD alone (Rashid, Moore et al. 2001; Banwell, O'Neill et al. 2004; Khanim, Gommersall et al. 2004; Malinen, Saramaki et al. 2008). However, there are little evidence on the antitumor effect of combination of VD and SFN.

Chronic inflammation is a well-known risk factor for tumorigenesis. Previous studies have shown that high fat diet can induce inflammation, in addition, VD and SFN might have anti-inflammation property (Kim, Kim et al. 2014; Mousa, Misso et al. 2016). Therefore, we hypothesize that VD and SFN supplementation can inhibit high fat diet induced inflammation.

It is well known that aberrant *Wnt* signaling play an important role in CRCs oncogenesis. We will analyze the effects of VD and SFN on key signaling proteins of *Wnt* pathway by immunoblotting analysis. We will measure the levels of phospho-GSK3 β (Ser9), the inactive form of GSK3 β , and the dephosphorylated β -catenin (Ser37 or Thr41), the active form, which are two key molecules in the *Wnt* pathway. We hypothesis that VD and SFN alleviates obesity induced colon cancer through TNF- α /GSK3 β / β -catenin pathway.

3.1.5 Aim 3

Butyrate is an intestinal microbiota metabolite of dietary fiber. It can exhibit chemoprevention effects on CRCs development. However, the mechanistic action of butyrate remains to be determined. FFAR2 has been suggested to mitigate colonic inflammation, which plays a pivotal role in the development of colon cancer.

The current study will assess whether FFAR2 protects against colon carcinogenesis. To determine the effect of FFAR2, we will treat caco-2 cells with physiological concentrations of butyrate (1, 2, 5 μ M). To further measure the effects of FFAR2, we will perform siRNA to knockdown the expression of FFAR2. To assess the activation of *Wnt* pathway and its downstream genes, we performed Real time PCR and Western blot assay. Immunohistochemical analysis was used to further confirm the activation of *Wnt* pathway.

3.1.6 Hypothesis 3

The assumption is that treatment of Caco-2 cells with butyrate, a HDAC inhibitor, will exert antitumor effects. Caco-2 cells were cultured with the indicated concentration of butyrate for 24 h. The cell viability was determined by MTT assay and the cell apoptosis was assessed by flow cytometry and expression of cleaved caspase-3.

Based on our preliminary data, we have shown that butyrate treatment increases the protein expression of β -catenin. Therefore, it is possible that the antitumor effects of the butyrate are mediated by a direct effect of butyrate on *Wnt* signaling. To test this hypothesis, we will examine the activation of *Wnt* pathway and the expression of *Wnt* pathway downstream genes by the Real time PCR and Western blot assay and immunohistochemical analysis.

It is not known the role of FFAR2 in the antitumor effects of butyrate. FFAR2 has been suggested to regulate colonic inflammation, which is a major risk factor in colon carcinogenesis. Previous studies have shown that the expression of FFAR2 have significantly reduced in CRC(Tang, Chen et al. 2011; Bindels, Dewulf et al. 2013). Aberrant HDAC activity has been

implicated in CRC progression. Therefore, we hypothesize that Knockdown of FFAR2 might suppress the anticancer effects of butyrate. We will test our hypothesis by knockdown FFAR2 using siRNA then start the treatment of colon cancer cells with butyrate.

CHAPTER 4

TUMOR NECROSIS FACTOR-A KNOCK OUT MITIGATES INTESTINAL INFLAMMATION AND TUMORIGENESIS IN APC1638N MICE FED A HIGH-FAT DIET

4.1 Abstract

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. Previous study has shown diet-induced obesity is associated with elevated TNF- α and hyperactive of *Wnt* signaling pathway, yet the role of TNF- α has not been extensively studied in the obesity associated CRC. The present study aims to examine the role of TNF- α in the obesity associated CRC and the molecular mechanism by which obesity increases the risk of CRCs. *Apc*^{1638N} mice were given a low fat diet (LF, 10 kcal% fat), or a high fat diet (HF, 60 kcal% fat). *TNF- α* ^{-/-} *Apc*^{1638N} mice were given a high fat diet (HF, 60 kcal% fat). After 16 weeks on diet starting at 4-6 weeks of age, mice were euthanized with CO₂. TNF- α deletion decreased CRC incidence and size. Inflammation was suppressed by TNF- α deletion. TNF- α deletion also decreased the activity of *Wnt*/ β -catenin and NF- κ B pathway. In this study, we found that loss of TNF- α decreased obesity associated intestinal tumorigenesis by decreasing the inflammation, and manipulating the β -catenin pathway and NF- κ B signaling. In addition, IKK, component of the NF- κ B signaling, is involved in the regulation of β -catenin pathway.

4.2 Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It is the third most common cancer in the United States, with around 140,000 new cases being diagnosed last year. The pathogenetic mechanisms underlying CRC development are complex and include genetic and environmental factors. *Wnt*/ β -catenin pathway is crucial in maintaining colon homeostasis and colon stem cells. It is estimated that aberrant activation of the *Wnt*/ β -catenin pathway is present in over 94% of human CRCs(2012). Most of them harbor mutations in the adenomatous polyposis coli (APC) gene, which may result in the accumulation of β -catenin in the cytoplasm and subsequent translocation of β -catenin to the nucleus. In the nucleus, β -catenin binds with the TCF/LEF family members and initiates the transcription of its downstream genes.

There is strong evidence that modifiable lifestyle factors, including obesity, play a crucial role in CRC carcinogenesis (Gunter and Leitzmann 2006). Since the prevalence of obesity is nearly 40% in the US adults, the study of obesity associated CRC becomes a top priority. Although considerable efforts have been made, the mechanisms underlying the association between obesity and CRC remain elusive. It is believed that obesity associated inflammation plays a pivotal role in the CRC pathogenesis (Kolb, Sutterwala et al. 2016).

Tumor necrosis factor- α (TNF- α) is a potent pro-inflammatory cytokine which plays a key role in the immune function during inflammation. TNF- α is mainly secreted by activated immune cells, especially macrophages, mast cells, and T lymphocyte, but other cell types including cancer

cells also secrete TNF- α . TNF- α was first reported that it can induce tumor necrosis (Carswell, Old et al. 1975). Interestingly, subsequent studies suggested that TNF- α might be a potential cancer target, as it can induce cancer proliferation, migration, and angiogenesis. TNF- α induced obesity might activate *Wnt* signaling, which drives the development of CRCs (Liu, Brooks et al. 2012). However, the understanding of molecular mechanisms by which obesity increases the risk of CRCs is not complete.

As the prevalence of obesity increases alarmingly, it is urgent to find the safe and effective strategies to prevent obesity associated CRC. In this study, we found that loss of TNF- α decreased obesity associated intestinal tumorigenesis by decreasing the inflammation, and manipulating the β -catenin pathway and NF- κ B signaling. In addition, IKK, component of the NF- κ B signaling, is involved in the regulation of β -catenin pathway.

4.3 Materials and Methods

4.3.1 Animals

All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst, and animal experiments were conducted in compliance with NIH guidelines for the care and use of laboratory animals. *Apc*^{1638N} mice were used as previously described (Li, Frederick et al. 2018). *TNF- α* knockout (*TNF- α* ^{-/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). To produce *TNF- α* ^{-/-} *Apc*^{1638N} mice, *TNF- α* ^{-/-} mice were mated with *Apc*^{1638N} mice on a C57BL/6 background.

Apc^{1638N} mice were given a low fat diet (LFD, 10 kcal% fat), or a high fat diet (HFD, 60 kcal% fat) (**APPENDIX A**). *TNF- α* ^{-/-} *Apc*^{1638N} mice were given a high fat diet (*TNF- α* KO HFD, 60 kcal% fat). Fresh diet were given on a daily basis during the 16 weeks treatment. After 16 weeks on diet starting at 4-6 weeks of age, mice were euthanized with CO₂. Plasma samples were collected and stored at -80 °C for analyses of cytokines. Tissue samples were collected using the methods reported before (Liu, Brooks et al. 2012). Briefly, intestine was excised and opened longitudinally for tumor inspection, then flushed with iced phosphate buffered saline (PBS) containing a protease inhibitor cocktail and placed on a bed of crushed ice. The mucosa was gently scraped off, placed in a foil packet, frozen in -80 °C and subsequently used for all DNA, RNA and protein assays.

4.3.2 Inflammatory cytokine assays

The inflammatory cytokines were measured by a chemiluminescence assay using the QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD). Assays were performed according to the manufacturer's instructions. Briefly, on the bottom of 96-well plates, antibodies for 10 cytokines, TNF- α , IFN- γ , IL-1 β , IL-17A, IL-6, IL-2, IL-10, IL-4, IL-22, and IL-23, were coated, and then 25 μ l of calibrator standards or samples were added to each well. After washing for 3 times, 50 μ l of the detection antibody solution was added to each well. A four-parameter logistic fit curve was generated for each analyte using the standards and the levels of inflammatory cytokines in samples were calculated accordingly. Cytokines are expressed as ng of cytokine per milliliter. All standards and samples were measured in duplicate.

4.3.3 Real-time PCR for gene expression

RNA samples were extracted from the colonic scrapings with Trizol reagent (Invitrogen, Carlsbad, CA). The concentration as well as purity of RNA samples was measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA). The first-strand cDNAs were synthesized from total RNAs using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR was performed on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the SYBR green PCR reagent kit (Invitrogen, Carlsbad, CA). The copy number of each transcript was calculated with respect to the GAPDH copy number. Primer sequences and thermal cycling conditions were listed in **APPENDIX B**.

4.3.4 Western blot analyses

As reported before (Sun, Yu et al. 2011), 40 µg of protein from each treatment was separated by 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 10% instant nonfat dry milk, membranes were incubated with specific antibodies overnight at 4 °C, followed by incubation with the secondary antibody. Antibody binding was detected with the enhanced ECL detection system. Notable western blots results were quantified using Image J software after normalizing to corresponding loading controls.

4.3.5 Histopathological and immunohistochemical analysis

A section of colon, 3-5 µm, were fixed in 10% neutral buffered formalin, then processed for paraffin embedding and tissue sectioning. Slides were stained with hematoxylin and eosin (H&E) and observed under a light microscope to assess inflammation status of the colon. The histological scores were evaluated by a blinded observer and given scores according to the following measures: crypt architecture (scored 0-2 with 0 as normal and 2 as most crypt distortion); inflammatory cell infiltration (0-2 with 0 as normal and 2 as most dense inflammatory

infiltrate); goblet cell depletion (0-1 with 0 as goblet cells present and 1 goblet cell depleted) and cryptitis (0-1 with 0 as absent and 1 present). The histological score is the sum of each score. Immunohistochemical (IHC) analysis for specific proteins was performed as previously described (Liu, Brooks et al. 2012). Briefly, after the sequential processes of rehydration and antigen retrieval, tissue sections were incubated with primary antibodies overnight at 4°C, LC3B (Cell Signaling), and Active β -Catenin (Ser33/37/Thr41) (Cell Signaling). On the next day, the sections were incubated with peroxidase-labeled secondary antibody and developed using DAB (Dako, Santa Clara, CA). Positive signal was detected as a brown color under a light microscope and scored using Fiji software. Briefly, run image and color deconvolution. Chose “H DAB” as the stain. The intensity numbers of the “Color_2 image” in the results window were converted to optical density numbers and then scored relative to control.

4.3.6 Statistical analysis

Values in the text are presented as means \pm S.E.M. A one-way ANOVA (treatment) statistical analyses were performed using Graphpad Prism 5 followed by multiple comparisons (Tukey method) among all treatments (groups). To explicitly present the data in the figure, the comparisons were only shown between the HFD group and other treatment groups. Values of $p < 0.05$ were considered statistically significant among the comparisons. Fisher’s exact test was used for tumor incidence. The expression of each gene was normalized to the housekeeping gene *GAPDH* ($\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$). Statistical analyses were performed based on ΔCt and relative expression is reported as $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{Experiment}} - \Delta\text{Ct}_{\text{Control}}$.

4.4. Results

4.4.1 TNF- α deficient mice less susceptible to obesity associated tumorigenesis

Previous study has shown diet-induced obesity is associated with elevated TNF- α and hyperactive of *Wnt* signaling pathway, yet the role of TNF- α has not been extensively studied in the obesity associated CRC (Liu, Brooks et al. 2012). To determine TNF- α function in obesity associated CRC, we subjected animals from both genotypes to high fat diet. As expected, the high fat diet induced obesity mice had increased incidence of CRC (HFD, 84.6%), compared with mice fed a low fat diet (LFD, 46.2%). Loss of TNF- α decreased the incidence to 52.4% (TNF- α KO HFD) (Figure 4.1A). For the tumor size, as measured by the diameter of the tumor, the high fat diet-induced obesity significantly increased the tumor size (LFD: 0.18 cm \pm 0.03 vs HFD: 0.35 cm \pm 0.04), loss of TNF- α decreased the tumor size by 35.4% (TNF- α KO HFD: 0.23 cm \pm 0.03) (Figure 4.1B,C). These results indicate that TNF- α knockout can decrease obesity associated CRC promotion and slow CRC progression.

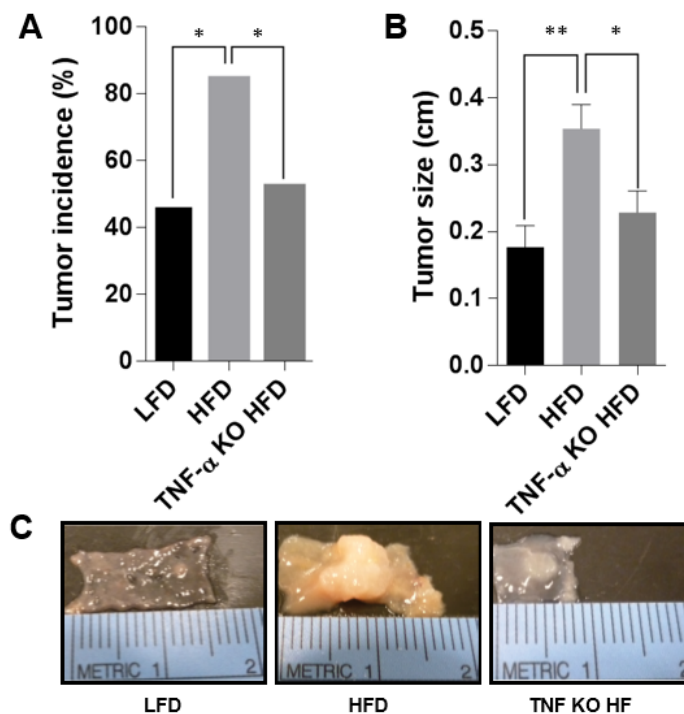


Figure 4.1. TNF- α knockout mice show less obesity associated carcinogenesis. A) Incidence of obesity associated colon cancer. B) Average tumor size. C) Representative intestinal tumors of each group. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

4.4.2 TNF- α knockout suppressed obesity associated inflammatory response

Chronic inflammation plays a pivotal role in obesity associated CRC. Obesity is recently recognized as a chronic inflammation disease. In addition, TNF- α plays a key role in systemic inflammation. We therefore determined the role of TNF- α in obesity associated inflammation. As expected, high fat diet induced obesity is associated with an increased expression of TNF- α , IL-1 β , IL-6, and IFN- γ in peripheral plasma. TNF- α deletion led to a decreased expression of those pro-inflammatory cytokines (Figure 4.2A-D). Considering the chronic local inflammation mediated by TNF- α plays a pivotal role in tumor promotion, we then measured the local inflammation status by histochemical staining. Consistently, our histochemical staining further demonstrated that high fat diet induced obesity showed an increased inflammatory cell infiltration, goblet cell loss, and cryptitis when compared to the low fat group ($p < 0.01$), TNF- α deletion mitigated the intestinal mucosal damage (Figure 4.2E-F). These observations indicated that TNF- α knockout suppressed obesity associated inflammatory response, which may involve in the CRC prevention effect of TNF- α knockout.

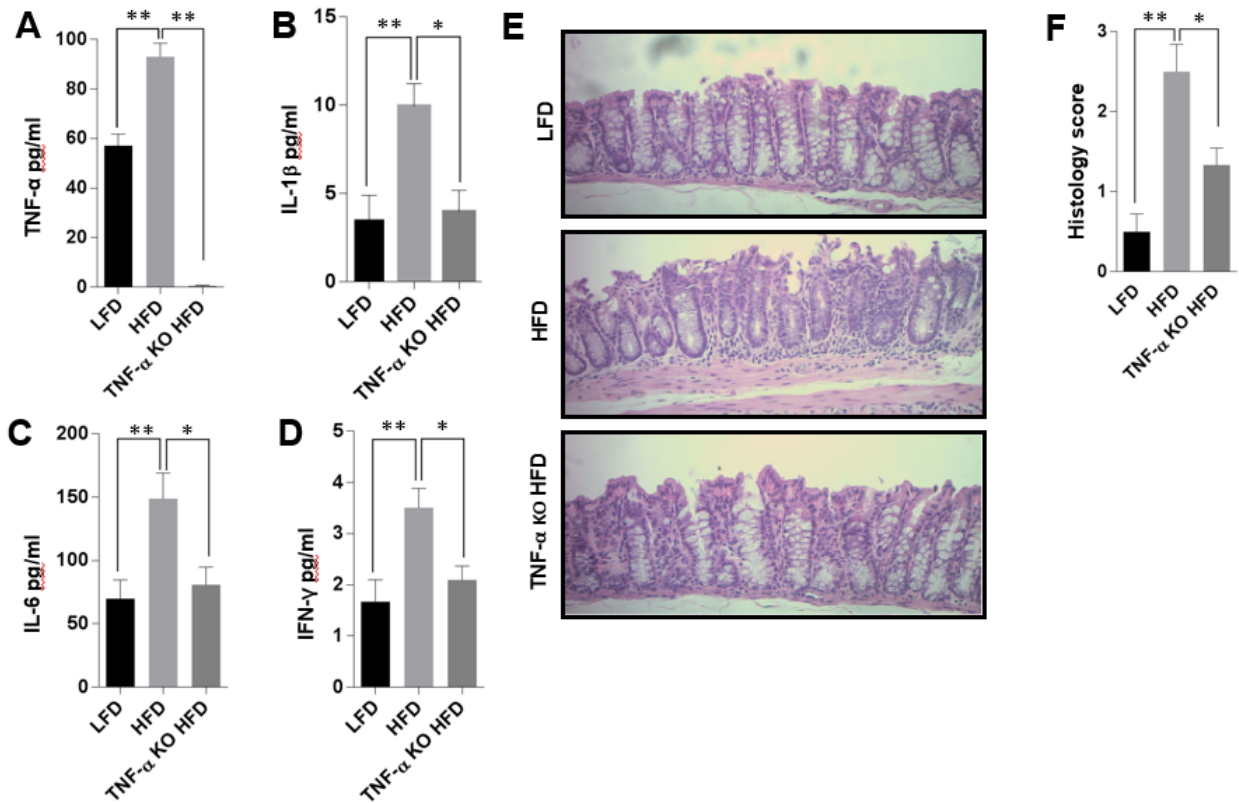


Figure 4.2. TNF- α deletion inhibits obesity associated inflammation. A-D) Analysis of inflammatory cytokines in serum. Inflammatory cytokines were analyzed by chemiluminescent assay (n = 8). E-F) Representative histopathology and inflammatory scores of the colon in each group (n = 4-6). Inflammatory cell infiltration, goblet cell loss, and cryptitis were observed in colon after feeding high-fat diet. Data are presented as the mean \pm SEM. * p < 0.05, ** p < 0.01.

4.4.3 TNF- α deletion down regulated active β -catenin and its downstream genes in APC^{1638N} mice

It is well known that aberrant *Wnt* signaling plays a crucial role in colorectal oncogenesis. We therefore examined the effects of TNF- α deletion on *Wnt* signaling in CRC. By immunoblotting analysis, we measured the levels of phospho-GSK3 β (Ser9) protein, the inactive form of GSK3 β , and the dephosphorylated β -catenin (Ser37 or Thr41) protein, the active form, which

are the two key molecules in the *Wnt* pathway. As shown in Figure 4.3A,B, high fat diet induced obesity mice had increased expression of phospho-GSK3 β (Ser9) and active β -catenin, whereas, TNF- α knockout decrease the expression of phospho-GSK3 β (Ser9) and active β -catenin. We further examined the mRNA expression of *Wnt* pathway downstream oncogenes, *Axin 2*, *c-Jun*, *c-Myc*, and *cyclin D1*. Consistent with protein data, we found that the high fat diet induced obesity mice had increased mRNA expression of these downstream genes, while TNF- α knockout decreased the mRNA expression of these downstream genes (Figure 4.3C). To further confirm these results, we performed immunohistochemistry. Immunohistochemistry also revealed less β -catenin positivity in the TNF- α knockout group compared to the HFD group (Figure 4.3D,E). Collectively, these results suggested that decreased activity of *Wnt* pathway might be responsible for the cancer prevention effect of TNF- α knockout.

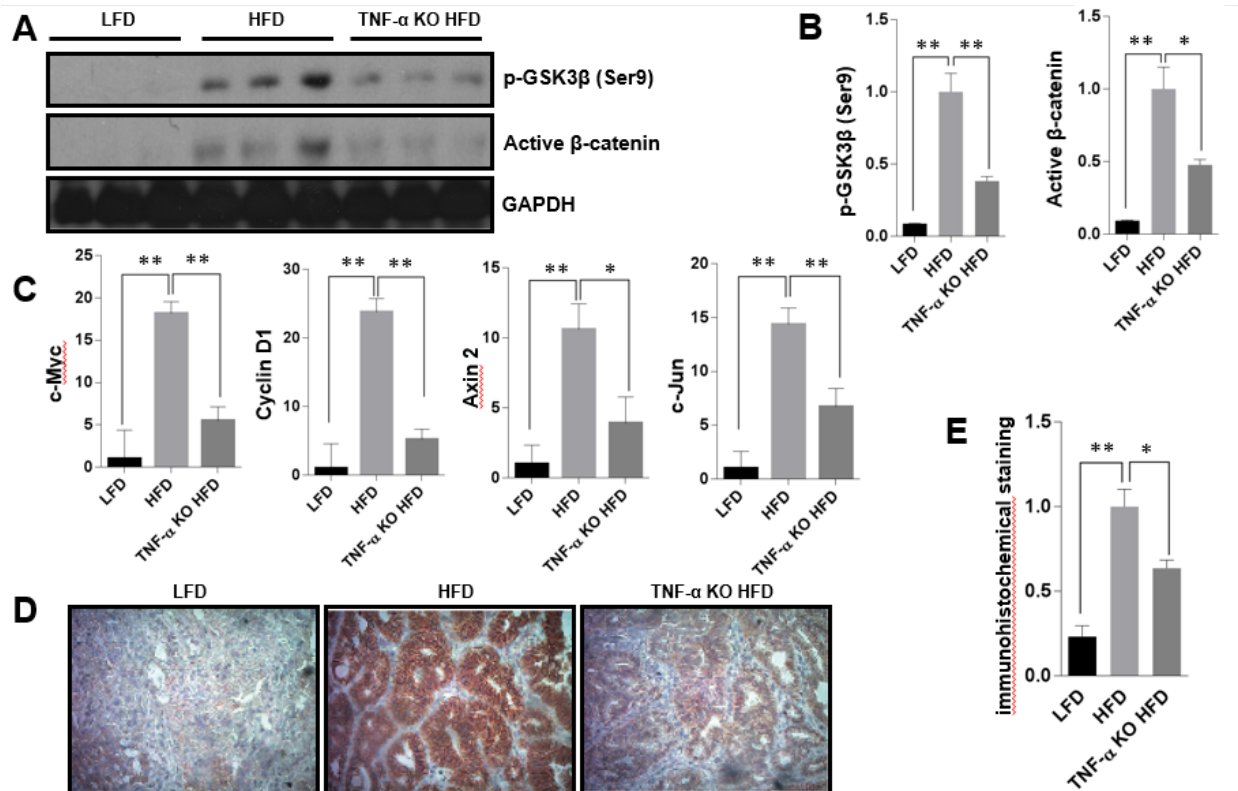


Figure 4.3 TNF- α loss affects the expression of *Wnt* signaling. A-B) Representative western blots and relative quantification of GSK-3 β Ser9 phosphorylation and active β -catenin in colonic scrapings (n = 6-8). C) mRNA expression of *Wnt* pathway downstream genes (n = 5-8). D-E) Representative β -catenin IHC in tissues from the small intestine of APC^{1638N} mice (n= 4-6) and quantification of immunohistochemical images. Data are presented as the mean \pm SEM. * p < 0.05, ** p < 0.01.

4.4.4 TNF- α deletion down regulated NF-kappa B signaling in APC^{1638N} mice

NF- κ B plays a key role in the regulation of inflammatory responses and carcinogenesis. TNF- α can activate survival pathways which is mediated by the NF- κ B (Gupta, Bi et al. 2005). We therefore, examined whether TNF- α deletion can down regulate the NF- κ B pathway. In NF- κ B pathway, cytokines such as TNF- α , IL-1 phosphorylate specific serine residues of nuclear factor

NF- κ B inhibitor kinase (IKK α and IKK β), which can then phosphorylate nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (I- κ B) and NF- κ B p65, leading to the activation of NF- κ B pathway. Consistent with previous findings (Carlsen, Haugen et al. 2009; Vykhovanets, Shankar et al. 2011), we observed that high fat diet induced obesity activated NF- κ B signaling in APC^{1638N} mice (Figure 4.4A, B). TNF- α deletion decreased the expression of p-IKK α (Ser176)/IKK β (Ser177), p-I κ B α (Ser32), and p-NF- κ B p65(Ser536), indicating the down-regulation of NF- κ B pathway.

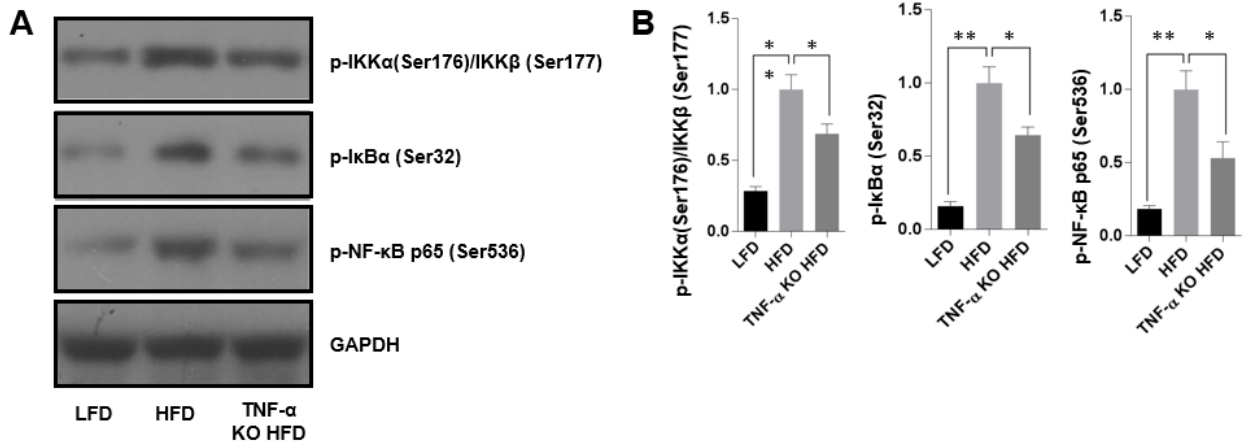


Figure 4.4 TNF- α loss down regulates NF- κ B pathway. A-B) Representative western blots and relative quantification of p-IKK α (Ser176)/IKK β (Ser177), p-I κ B α (Ser32), and p-NF- κ B p65(Ser536) in colonic scrapings (n = 6-8). Data are presented as the mean \pm SEM. * p < 0.05, ** p < 0.01.

4.4.5 IKK is involved in the obesity associated activation of β -catenin pathway

Previous studies suggested that IKK can activate *Wnt*/ β -catenin pathway (Lamberti, Lin et al. 2001; Carayol and Wang 2006). We therefore, examined whether IKK is involved in the obesity associated activation of β -catenin pathway. The effects of PS-1145, a potent IKK inhibitor, on NF- κ B pathway and β -catenin pathway were measured by western blot. Consistent with *in vivo* data, TNF- α (20ng/ml) also activated NF- κ B pathway and β -catenin pathway in Caco-2 cells. The inhibition of IKK by PS-1145 reduced the activation of NF- κ B pathway and β -catenin pathway induced by TNF- α (Figure 4.5A). These data suggested that IKK is involved in the obesity associated activation of β -catenin pathway.

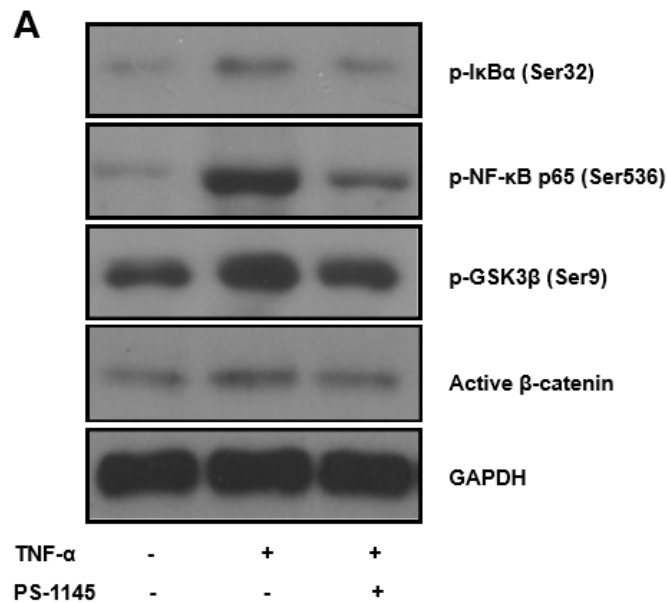


Figure 4.5. NF- κ B pathway positively regulated β -catenin pathway by IKK. A) Representative western blots p-I κ B α (Ser32), p-NF- κ B p65(Ser536), p-GSK3 β (Ser9), and active β -catenin in caco-2 cell lines. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

4.5 Discussion

Obesity, an epidemic, is a worldwide public health issues especially in developed countries. Obesity is associated with increased risk of many chronic diseases, such as cancer, diabetes, and chronic heart diseases. It is estimated that obesity attributes a 30% greater risk of CRC (Ma, Yang et al. 2013). In the United States, ~1/3 of the population is obese and there is no sign of decrease from the current prevalence of obesity. CRC is the third leading cause of cancer death in the United States in both men and women. In 2018, it is estimated that \approx 64 640 women and 75 610 men in the United States were diagnosed with CRC and 23 240 women and 27 390 men died from CRC. Therefore, it is urgent to develop safe and practical strategies to prevent obesity associated CRC.

It is believed that obesity associated inflammation is a central component of CRC development and progression. Among inflammatory cytokines, TNF- α plays a crucial role in obesity associated inflammation (Liu, Brooks et al. 2012). TNF- α was first found in 1975, recognized as a promising strategy for cancer treatment. However, previous studies have shown that TNF- α can be secreted by many tumor cells (Zins, Abraham et al. 2007; Al-Lamki, Sadler et al. 2010; Aggarwal, Gupta et al. 2012; Landskron, De la Fuente et al. 2014). As a result, the role of TNF- α in tumor survival, migration, and invasion, are more and more appreciated. In 1993, a study first suggested that TNF- α might implicate in the oncogene activation and the DNA damage (Li, Sejas et al. 2007). Subsequent clinic trails indicated that neutralizing TNF- α might be beneficial in cancer patients (Brown, Charles et al. 2008).

Popivanova et al have suggested that TNF- α plays a crucial role in the initiation of ulcerative colitis (UC) associated colon cancer (Popivanova, Kitamura et al. 2008). Blocking TNF- α can

decrease the CRC progression in mice UC model treated with azoxymethane (AOM) and dextran sulfate sodium (DSS). Liu et al reported that high fat diet induced obesity is associated with a 72% increase in the expression of TNF- α and increased expression of p-GSK3 β , β -catenin, and *Wnt* downstream genes. Previous studies have shown that TNF- α can induce GSK3 β phosphorylation and subsequently stabilize β -catenin (Oguma, Oshima et al. 2008; Liu, Brooks et al. 2012; Coskun, Olsen et al. 2014), two key components within *Wnt* pathway. Considering the fact that *Wnt* signaling is crucial for CRC incidence and progression, TNF- α deletion might decrease the *Wnt* signaling and incidence of CRC. In this study, we found that TNF- α knockout decrease the incidence of CRC from 84.6% to 52.4% and tumor size by 35.4% in *Apc*^{1638N} mice fed a high fat diet. Our observation indicated that blocking TNF- α might be a strategy to prevent obesity associated CRC. We also found that TNF- α knockout decreased obesity associated inflammation. Previous studies have suggested that TNF- α might activate *Wnt*/ β -catenin pathway through inducing GSK3 β phosphorylation (Oguma, Oshima et al. 2008; Liu, Brooks et al. 2012; Coskun, Olsen et al. 2014). In consistent with these studies, we found that TNF- α deletion decreased the expression of phospho-GSK3 β (Ser9) and active β -catenin, indicating the cancer prevention effects of TNF- α deletion might act through down-regulation of *Wnt*/ β -catenin pathway.

NF- κ B is involved in the regulation of inflammatory responses and cancer development, therefore, NF- κ B plays a pivotal role in the obesity associated CRC. IKK α and IKK β are essential for TNF- α induced NF- κ B activation (Tak and Firestein 2001). Phosphorylated IKK α and IKK β can then phosphorylate I- κ B and NF- κ B p65, leading to NF- κ B translocation to the nucleus. Consistent with previous findings (Carlsen, Haugen et al. 2009; Vykhovanets, Shankar et al. 2011), we observed that high fat diet induced obesity activated NF- κ B signaling and TNF- α deletion down regulated of NF- κ B pathway in APC^{1638N} mice. There is crosstalk between

Wnt/ β -catenin pathway and NF- κ B pathway during inflammation, for instance, IKK can activate *Wnt*/ β -catenin pathway (Lamberti, Lin et al. 2001; Carayol and Wang 2006). Our data indicated that NF- κ B signaling might positively regulate the *Wnt*/ β -catenin pathway through IKK, which can activate I κ B α and NF- κ B, at the same time activate GSK3 β and β -catenin.

4.6 Conclusions

In conclusion, we found that TNF- α deletion decreased obesity associated intestinal tumorigenesis by decreasing the production of multiple proinflammatory cytokines, and downregulated the β -catenin pathway and NF- κ B signaling. IKK, component of the NF- κ B signaling is involved in the regulation of β -catenin pathway.

Author contributions: J.L. performed all the experiments. A.M.F., Y.J., and C.G. contributed to the collection, interpretation, and analysis of data throughout the experiment; all authors contributed to the discussion. Z.L., H.X., and R.J.W. designed the research and directed the project. J.L. and Z.L. wrote the manuscript. All authors reviewed the manuscript.

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CHAPTER 5

THE PREVENTION OF A HIGH DOSE OF VITAMIN D OR ITS COMBINATION WITH SULFORAPHANE ON INTESTINAL INFLAMMATION AND TUMORIGENESIS IN APC1638N MICE FED A HIGH-FAT DIET

5.1 Abstract

Our previous study showed that obesity-promoted inflammation is responsible for the activation of the intestinal tumorigenic *Wnt*-signaling. The present study aimed to test a dietary strategy, dietary supplementation with a high dose of vitamin D (VD) or its combination with sulforaphane (SFN), to inhibit intestinal inflammation and obesity-associated tumorigenesis. *Apc*^{1638N} mice were randomly divided into 4 groups: *LF*, a low fat diet (10 kcal% fat) with 200 IU VD; *HF*, a high fat diet (60 kcal% fat) with 200 IU VD; *HFD*: a high fat diet with 5,000 IU VD; and *HFDS*, a high fat diet plus 5,000 IU VD and 0.23 g SFN per ~4,000 kcal. VD administration decreased tumor incidence and size, and the co-administration with SFN (HFDS) magnified the effects.

Inflammation and *Wnt*-signaling were suppressed by VD. The addition of SFN decreased the activity of histone deacetylase (HDAC) and increased autophagy. The administration of VD, at 5000 IU level, exerts an anti-inflammatory property, and leads to suppressed intestinal *Wnt*-signaling and tumorigenesis in obese mice. The molecular function of SFN on a high dose of VD supplementation, although displayed on the inhibition of HDAC and the activation of autophagy, needs further investigation.

5.2 Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States. In 2018, it is estimated that ~64,640 women and 75,610 men in the United States were diagnosed with CRC and 23,240 women and 27,390 men died from CRC. Epidemiological studies have shown that a number of modifiable lifestyle factors, including obesity, play important roles in colorectal carcinogenesis (Gunter and Leitzmann 2006). There is strong evidence suggesting that chronic overconsumption of a “western diet”- foods consisting high levels of fats is a major cause of obesity. In the United States, ~1/3 of the population is obese and there is no sign of decrease from the current prevalence of obesity. Therefore, developing a safe and economic strategy to prevent obesity-induced CRC is an important public health priority.

Vitamin D (VD), is a pluripotent fat-soluble steroid that is essential for a variety of physiological and pathological processes in the human body. In addition to its important role in calcium absorption and bone health (Rautiainen, Manson et al. 2016; Uday and Hogler 2017), VD regulates a number of critical cellular pathways that affect cell proliferation, differentiation, and apoptosis. Therefore, VD potentially plays an important role in modulating cancer incidence, prognosis, and mortality (Fleet 2008; Ng 2014). However, epidemiological and clinical VD supplementation studies have shown inconsistent results. For example, in a sub-study of the Women’s Health Initiative trial, dietary supplementation with 400 IU of VD did not significantly reduce the risk of CRC (Brunner, Wactawski-Wende et al. 2011), however, it should be noticed that the supplement of 400 IU of VD per day did not significantly elevate the serum 25(OH)D level, a biomarker of VD status. In another study, dietary supplementation with 2,000 IU of VD did not lower the risk of CRC (Lappe, Watson et al. 2017); However, in a phase II clinical trial, a high dose of VD (8,000 IU for two weeks, followed by 4,000 IU) suppressed the progression of colon cancer (Ng, Nimeiri et al. 2017). Therefore, in this study, we examined the protective

effect of a high dose of VD (5,000 IU per ~4,000 kcal), on the development of intestinal tumor in the *Apc*^{1638N} model.

In addition, we further examined whether sulforaphane (SFN), an isothiocyanate derived from cruciferous vegetables, can magnify the anti-cancer effects of VD supplementation.

Epidemiologic studies suggest that higher intake of cruciferous vegetables is associated with lower risk of CRC (Wu, Yang et al. 2013). Mechanisms of cancer chemoprevention by SFN include the ability to induce apoptosis of cancer cells by a mitogen-activated protein kinase (MAPK) pathway and the ability to alter carcinogen metabolism through the induction of Nrf2-regulated genes. It has also been suggested that SFN inhibits histone deacetylase (HDAC) activity in human CRC cell lines (Ho, Clarke et al. 2009), and VD may interact with SFN to affect on gene expression through epigenetic mechanisms (e.g., chromatin acetylation) (Zhang, Leung et al. 2012; Seuter, Heikkinen et al. 2013). Of note, HDAC activity was significantly inhibited in the peripheral blood mononuclear cells after consumption of 68 g (~105 mg SFN) of broccoli sprouts (Myzak, Tong et al. 2007). Therefore, SFN, even at physiologically feasible concentrations, might enhance the anti-cancer effect of VD.

5.3 Materials and Methods

5.3.1 Animals

The protocol (2013-0070) was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Amherst, and animals were maintained in accordance with NIH guidelines for the care and use of laboratory animals. *Apc*^{1638N} mice, which are heterozygous for

a germline mutation in the *Apc* gene, were utilized to study intestinal tumorigenesis. Compared to another commonly-used CRC model, the *Apc*^{Min} mice, the *Apc*^{1638N} mice have a mild tumorigenic phenotype (3 vs 100 tumors) and longer lifespan (>1 years vs 4-6 months) (Heyer, Yang et al. 1999; Taketo 2006), making it a more suitable model for dietary intervention studies since the modest degree of chemoprevention afforded by nutrients can easily be obscured in animals with more aggressive tumorigenic phenotypes. Although most genetic models, including the *Apc*^{1638N} model, produce neoplasms primarily in the small intestine rather than the colon, this does not negate their validity since mouse models with *Apc* mutations share the same genetic and phenotypic similarities to humans with familial adenomatous polyposis, and moreover, the mouse and human *APC* orthologs are approximately 90 % identical (Shoemaker, Gould et al. 1997). In many instances the modulation of small intestinal tumors recapitulates the chemopreventive effects of drugs and dietary components in human colon cancer (Yang, Edelman et al. 1998).

Apc^{1638N} mice, including both males and females, were randomly divided into 4 dietary groups, ~12 animals/group: *LF*, a low fat diet (10 kcal% fat) with 200 IU VD; *HF*, a high fat diet (60 kcal% fat) with 200 IU VD; *HFD*: a high fat diet with 5,000 IU VD; and *HFDS*, a high fat diet plus 5,000 IU VD and 0.23 g SFN on a 4,057kcal energy basis (**APPENDIX A**). Because a previous human study suggested that 68 g broccoli sprouts (~105 mg SFN) significantly inhibited HDAC activity (Myzak, Tong et al. 2007) and a typical diet contains 2,000kcal, we therefore added 0.23 g SFN on a 4,057kcal energy basis to achieve a physiologically relevant concentration. Fresh diet was given on a daily basis during the 16 weeks of dietary treatment. The 5000 IU VD, although 5 times higher than the National Research Council requirement for mice (1000 IU), is physiologically achievable without evident toxicity (Fleet, Gliniak et al. 2008). Since this study focused on evaluating the effect of a high dose of VD, and whether SFN can magnify this effect,

we did not include a group with SFN supplementation alone to demonstrate their synergistic effects. After 16 weeks on a diet starting at 4-6 weeks of age, mice were euthanized with CO₂. Plasma samples were collected and stored at -80 °C for analyses of cytokines. Tissue samples were collected using the methods reported before (Liu, Brooks et al. 2012). Briefly, intestine was excised and opened longitudinally for tumor inspection, then flushed with iced phosphate buffered saline (PBS) containing a protease inhibitor cocktail and placed on a bed of crushed ice. The mucosa was gently scraped off, placed in a foil packet, frozen in -80 °C and subsequently used for all DNA, RNA and protein assays.

5.3.2 Serum 25(OH)D Assay

Serum 25-hydroxyvitamin D (25(OH)D) was measured by radioimmunoassay using LIAISON total 25(OH)D assay (DiaSorin Inc., Stillwater, MN, USA) at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. This method has been previously validated and reported (Selting, Sharp et al. 2016).

5.3.3 Inflammatory cytokine assays

The inflammatory cytokines were measured by a chemiluminescence assay using the QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD). Assays were performed according to the manufacturer's instructions. Briefly, on the bottom of 96-well plates, antibodies for 10 cytokines, TNF- α , IFN- γ , IL-1 β , IL-17A, IL-6, IL-2, IL-10, IL-4, IL-22, and IL-23, were coated, and then 25 μ l of calibrator standards or samples were added to each well. After washing for 3 times, 50 μ l of the detection antibody solution was added to each well. A four-parameter logistic fit curve was generated for each analyte using the standards and the levels of

inflammatory cytokines in samples were calculated accordingly. Cytokines are expressed as ng of cytokine per milliliter serum. All standards and samples were measured in duplicate.

5.3.4 Real-time PCR for gene expression

RNA samples were extracted from the colonic scrapings with Trizol reagent (Invitrogen, Carlsbad, CA). The concentration as well as purity of RNA samples was measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA). The first-strand cDNAs were synthesized from total RNAs using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR was performed on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the SYBR green PCR reagent kit (Invitrogen, Carlsbad, CA). The copy number of each transcript was calculated with respect to the GAPDH copy number. Primer sequences and thermal cycling conditions were listed as supporting information in **APPENDIX B**.

5.3.5 Western blot analyses

As reported before (Sun, Yu et al. 2011), 40 µg of protein from each treatment was separated by 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 10% instant nonfat dry milk, membranes were incubated with specific antibodies overnight at 4°C, followed by incubation with the secondary antibody. Antibody binding was detected with the enhanced ECL detection system. Notable western blot results were quantified using Image J software after normalizing to a corresponding loading control.

5.3.6 Histopathological and immunohistochemical analysis

A section of colon, 3-5 μm , were fixed in 10% neutral buffered formalin, then processed for paraffin embedding and tissue sectioning. Slides were stained with hematoxylin and eosin (H&E) and observed under a light microscope to assess inflammation status of the colon. The histological scores were evaluated in a blinded manner and given scores according to the following measures: crypt architecture (scored 0-2 with 0 as normal and 2 as most crypt distortion); inflammatory cell infiltration (0-2 with 0 as normal and 2 as most dense inflammatory infiltrate); goblet cell depletion (0-1 with 0 as goblet cells present and 1 goblet cell depleted) and cryptitis (0-1 with 0 as absent and 1 present). The histological score is the sum of each score. Immunohistochemical (IHC) analysis for specific proteins was performed as previously described (Liu, Brooks et al. 2012). Briefly, after the sequential processes of rehydration and antigen retrieval, tissue sections were incubated with primary antibodies overnight at 4°C for LC3B (Cell Signaling), and active β -Catenin (Ser33/37/Thr41) (Cell Signaling). On the next day, the sections were incubated with peroxidase-labeled secondary antibody and developed using DAB (Dako, Santa Clara, CA). Positive signal was detected as a brown color under a light microscope and scored using Fiji software. Briefly, images were uploaded to Fiji, and the color was deconvoluted. Chose “H DAB” as the stain. The intensity numbers of the “Color_2 image” in the results window were converted to optical density numbers and then scored relative to control.

5.3.7 HDAC Activity Assay

Nuclear extracts, prepared as described by Dignam (Dignam, Lebovitz et al. 1983), were used for HDAC activity assay using EpiQuik HDAC Activity/Inhibition Assay Kits (Epigentek, Farmingdale, NY) according to the manufacturer’s instructions. Briefly, 10 μg of nuclear extract was incubated with specific substrate for 45 min at 37 °C, followed by incubation with capture

antibody for 60 min and then detection antibody for 25 min at room temperature. Absorbance was determined at 450 nm using a microplate spectrophotometer.

5.3.8 Statistical analysis

Values in the text are presented as means \pm S.E.M. One-way ANOVA (treatment) statistical analyses were performed using Graphpad Prism 5 followed by multiple comparisons (Tukey method) among all treatments (groups). To simplify data presentation in the figures, the comparisons were only shown between the HF groups with other treatment groups. Values of $p < 0.05$ were considered statistically significant among the comparisons. Fisher's exact test was used for tumor incidence. The expression of each gene was normalized to the housekeeping gene *GAPDH* ($\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$). Statistical analyses were performed based on ΔCt and relative expression is reported as $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{Experiment}} - \Delta\text{Ct}_{\text{Control}}$.

5.4 Results

5.4.1 Influence on serum 25(OH)D and physiology by 5000 IU vitamin D supplementation

Previous studies indicate that a concentration of 60-80 ng/ml serum 25(OH)D, the major circulating form of VD and a standard indicator of vitamin D status, may be needed to reduce cancer risk (Garland, French et al. 2011). We therefore intentionally supplemented VD with a concentration of 5000 IU for the supplemental groups vs 200 IU for the control groups, which is lower than the NRC recommended level (1000 IU) for rodents on a ~4,000 kcal energy basis. This level of vitamin D was chosen as a better mimic of human vitamin D status as described by Fleet et al (Fleet, Gliniak et al. 2008) and Kallay et al (Hummel, Thiem et al. 2012). Serum levels of 25(OH)D in LF and HF group are 23.4 ± 5.6 ng/ml, 14.3 ± 1.7 ng/ml respectively, and levels in

the two VD supplemental groups (HFD and HFDS) were 62.0 ± 2.3 ng/ml, 52.4 ± 5.4 ng/ml respectively, which were significantly higher than the levels in the LF and HF groups ($p < 0.01$) (Figure 1A). With the consideration of potential toxicity by 5,000 IU VD supplementation, we examined the physiological alterations. No significant differences were observed in liver, pancreas, spleen, and kidney between in the VD supplemental groups (HFD and HFDS) when compared to the control group (HF).

After 16 weeks treatment, the final body weights in LF, HF, HFD, and HFDS groups were 37.6 ± 1.4 g, 45.7 ± 1.8 g, 45.0 ± 1.9 g, 43.0 ± 1.4 g, respectively (Figure 5.1B, D). The final epididymal fat weights in LF, HF, HFD, and HFDS mice were 1.8 ± 0.1 g, 2.6 ± 0.2 g, 2.5 ± 0.2 g, 2.1 ± 0.3 g, respectively (Figure 5.1C). The final body weights and epididymal fat weights were significantly higher in the HF group relative to the control LF group ($p < 0.05$), but the supplementation of VD alone (HFD) or VD with SFN (HFDS) did not significantly decrease the body weight and epididymal fat weight ($p > 0.05$).

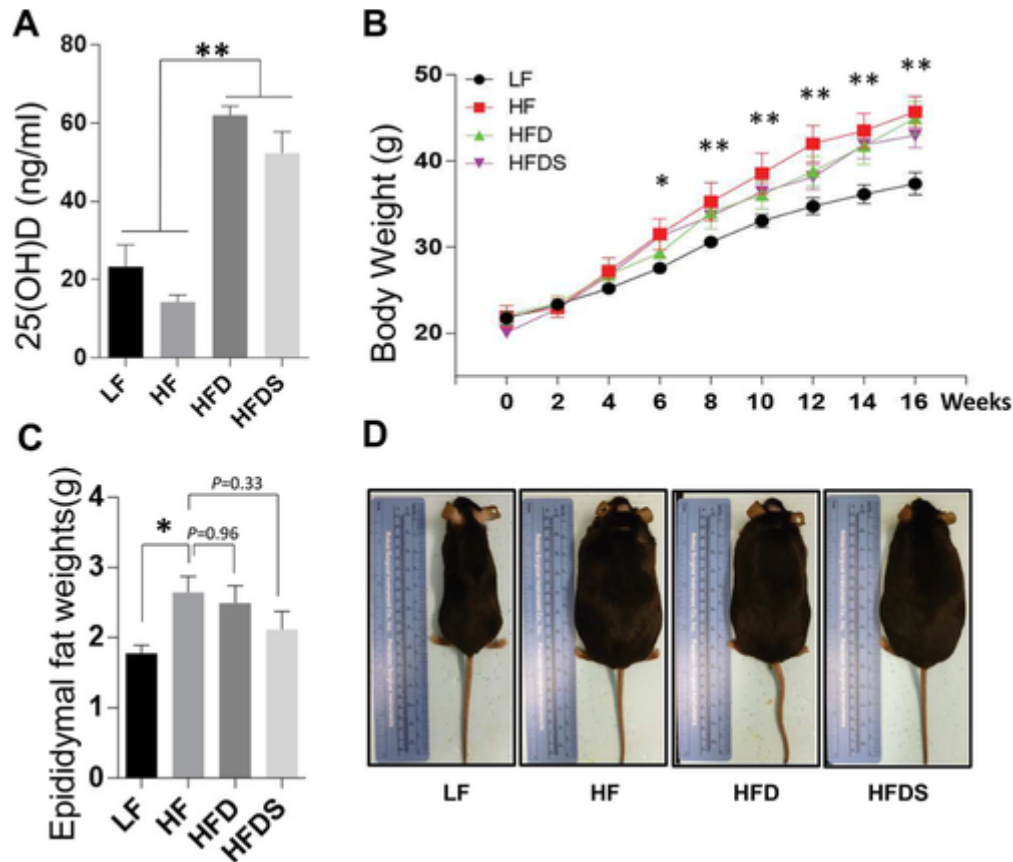


Figure 5.1. Influence on serum 25(OH)D and physiology by 5000IU kg⁻¹ vitamin D supplementation. A) Serum 25(OH)D levels among the four dietary groups. B) Growth curve: Body weights were measured every other week. C) Epididymal adipose tissue weights in each group. D) Appearance of mice at the end of diets feeding. *p < 0.05, **p < 0.01. Data are mean ± SEM.

5.4.2 A high degree of vitamin D supplementation and its co-administration with sulforaphane inhibited obesity associated-intestinal tumorigenesis in *APC*^{1638N} mice

To investigate the effect of VD or VD in combination with SFN in obesity-associated intestinal tumorigenesis, we evaluated the development of tumor in the *APC*^{1638N} mouse model with or without VD or VD+SFN supplementation. The high fat diet increased the tumor incidence from

46.1% (LF) to 84.6% (HF), whereas the supplementation of VD or VD in combination with SFN decreased the incidence to 63.6% (HFD) and 53.8% (HFDS) (Figure 5.2A). For the tumor size, as measured by the diameter of the tumor, the high fat diet significantly increased the size (LF: 0.18 cm \pm 0.03 vs HF: 0.35 cm \pm 0.04), whereas the administration of VD or its co-administration with SFN reduced the tumor size by 22.9% (0.27 cm \pm 0.02) and 37.1% (0.22 cm \pm 0.02) respectively (Figure 5.2B, C). These data indicated that the supplementation of VD or its co-administration with SFN suppressed high fat diet-induced intestinal tumorigenesis in the *APC^{1638N}* model, although only the shrinking of tumors in the co-administration group (HFDS) was reached a statistically significant degree ($p < 0.05$).

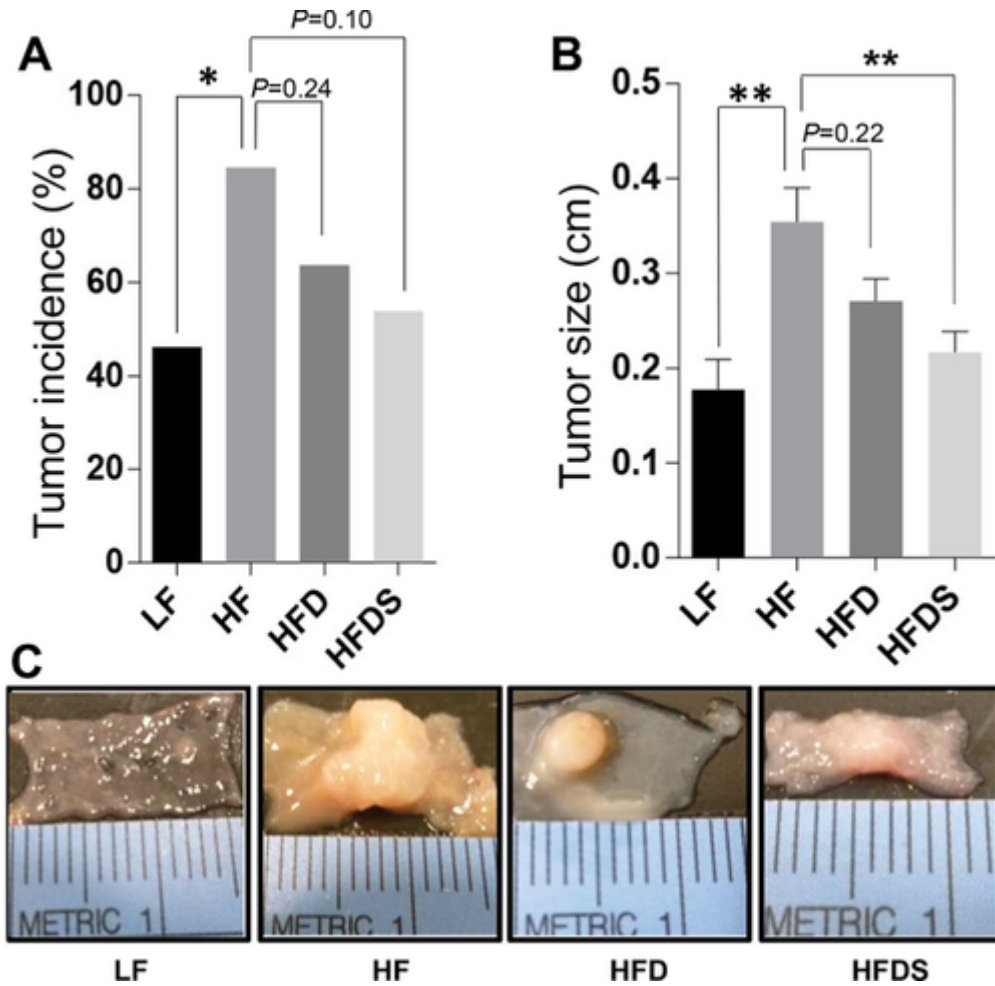


Figure 5.2. The influence of a high dose of vitamin D or its combination with sulforaphane on intestinal tumorigenesis in APC1638N mouse model. A) Incidence of high-fat-diet-induced colon cancer. B) Average tumor size. C) Representative intestinal tumors of each group. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

5.4.3 The influence vitamin D or its combination with sulforaphane supplementations suppressed high fat diet-induced inflammatory response

Chronic inflammation is a well-known risk factor for tumorigenesis. Previous studies have shown that high fat diet can induce inflammation, and VD and SFN might have anti-inflammatory properties (Kim, Kim et al. 2014; Mousa, Misso et al. 2016). Therefore, we examined to what extent VD and SFN supplementation can inhibit high fat diet-induced intestinal inflammation. We observed that VD alone significantly reduced ($p < 0.05$) the high fat diet- induced production of pro-inflammatory cytokines TNF- α , INF- γ , IL-2, and IL-17A, with a trend to decrease the production of IL-1 β , and a trend to increase the secretion of the anti-inflammatory cytokine IL-10 (Figure 5.3A). The combination of VD and SFN did not further augment the anti-inflammatory effect of VD. We did not observed any significant changes in the production of IL-6, IL-4, IL-22, and IL-23 cytokines by VD or its combination with SFN supplementations (data not shown). Our histochemical staining further demonstrated that high fat diet increased the intestinal inflammatory score when compared to the low fat group ($p < 0.01$), but VD (HFD) or VD and SFN co-supplementation (HFDS) diminished inflammatory cell infiltration, goblet cell loss and cryptitis induced by high fat diet, with a change that reached a significant degree in the HFDS group ($p < 0.05$). There were no significant differences of between HFD and HFDS group (Figure 5.3B, C).

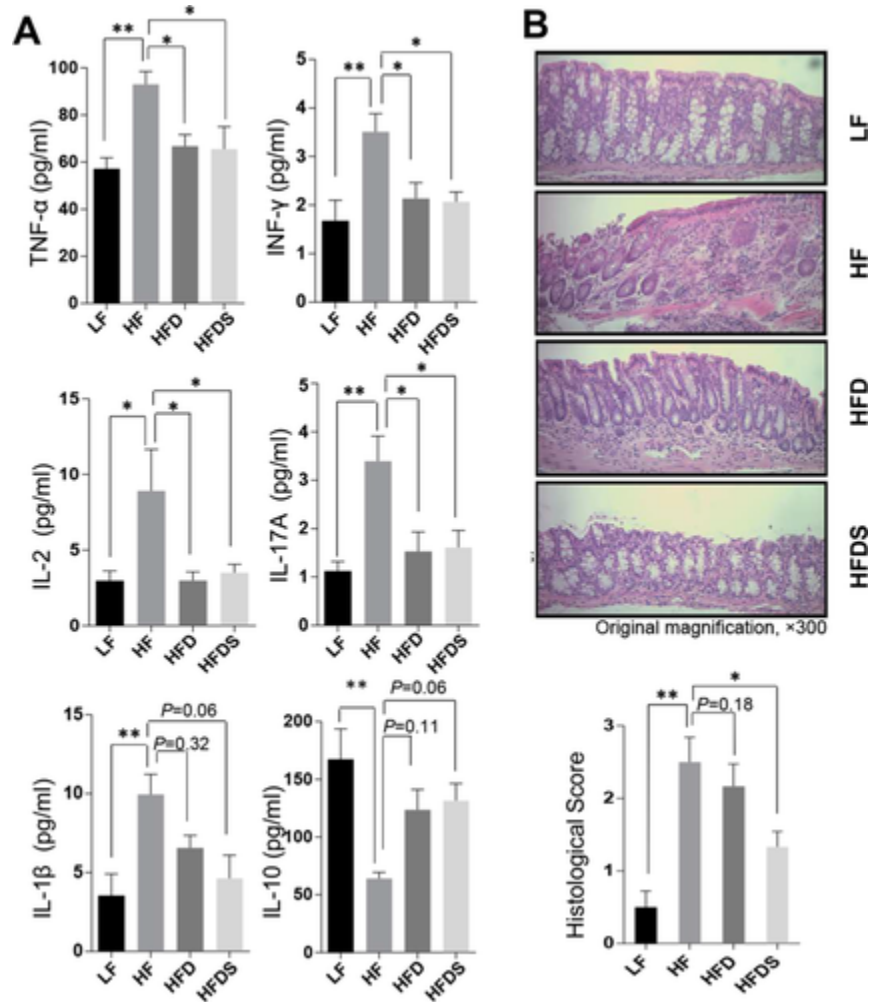


Figure 5.3. Effect of vitamin D and sulforaphane on inflammation. A) Analysis of inflammatory cytokines in serum inflammatory cytokines were analyzed by chemiluminescent assay (n = 8). B) Representative histopathology and inflammatory scores of the colon in each group (n = 6). Inflammatory cell infiltration, goblet cell loss, and cryptitis were observed in colon after feeding high-fat diet. Data are presented as the mean \pm SEM. *p < 0.05, **p < 0.01.

5.4.4 The supplementation of vitamin D and sulforaphane down-regulated active β -catenin and its downstream targets in APC^{1638N} mice

We further studied the effects of VD and SFN supplementation on signaling pathways involved in CRC. It is well known that aberrant *Wnt* signaling plays an important role in driving cell proliferation during colorectal oncogenesis. By immunoblotting analysis, we measured the levels of phospho-GSK3 β (Ser9) protein, the inactive form of GSK3 β , and the dephosphorylated β -catenin (Ser37 or Thr41) protein, the active form, which are two key molecules in the *Wnt* pathway. As shown in Figure 5.4A, the expression of phospho-GSK3 β (Ser9) and active β -catenin in the colon mucosa was increased in the HF group compared to the LF group, whereas, VD significantly decreased expression of phospho-GSK3 β and dephosphorylated β -catenin. Co-administration VD with SFN did not further decrease the expression of phospho-GSK3 β and dephosphorylated β -catenin. Immunohistochemistry also revealed less β -catenin positivity in HFD and HFDS groups compared to HF group (Figure 5.4B). We further examined the mRNA expression of *Wnt* pathway downstream oncogenes, *Axin 2*, *c-Jun*, *c-Myc*, and *cyclin D1*. We found that the mRNA expressions of these downstream genes were increased in HFD-induced obese mice, while VD alone led to decreased expressions of these genes (Figure 5.4C). However, combining VD with SFN did not further inhibit the expression of the *Wnt* pathway downstream genes.

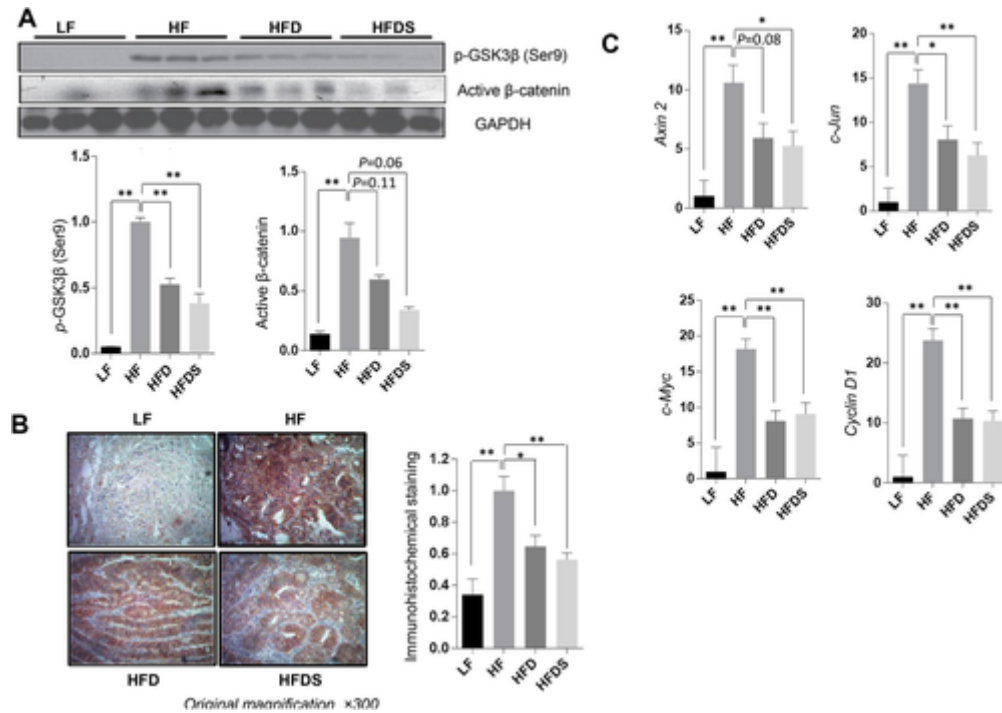


Figure 5.4. Effect of vitamin D and sulforaphane supplementation on β -catenin and its downstream genes. A) Representative Western blots and relative quantification of GSK-3 β Ser9 phosphorylation and active β -catenin in colonic scrapings (n = 6). B) Representative β -catenin IHC in tissues from the small intestine of APC^{1638N} mice (n = 5) and quantification of immunohistochemical images. C) mRNA expression of Wnt pathway downstream genes (n = 8). Data are presented as the mean \pm SEM. *p < 0.05, **p < 0.01.

5.4.5 The sulforaphane supplementation decreased the total HDAC activity.

To investigate whether the supplementation of SFN suppressed HDAC activities, the total HDAC activities were measured in nuclear extracts of mucosa. The SFN treatment group (HFDS) displayed significantly decreased level of total HDAC activities when compared to other groups (p < 0.05), and there were no differences among other groups without SFN supplementation (LF, HF, HFD) (Figure 5.5A). Western blotting results also showed that the

SFN administration decreased the HDAC6 protein level (Figure 5.5B). This provides evidence that the addition of SFN may be responsible for the enhanced antitumor effects by inhibition of HDAC activity.

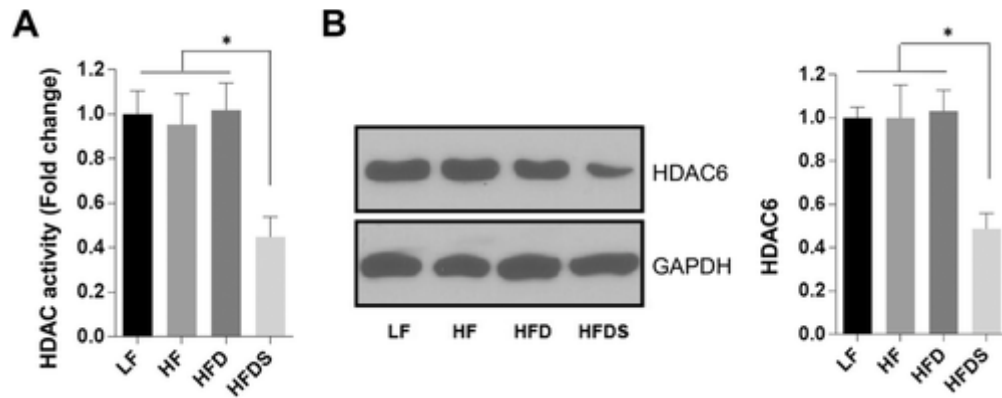


Figure 5.5. Effect of vitamin D and sulforaphane supplementation on the total histone deacetylase (HDAC) activity and the expression of vitamin D receptor. A) The total HDAC activity. B) Western blotting for HDAC6. Representative picture of Western blotting. Data are presented as the mean \pm SEM (n = 3–5). * $p < 0.05$.

5.4.6 The supplementation of vitamin D and sulforaphane enhanced autophagy in *APC^{1638N}* mice

Autophagy has been recognized as a basis for the health-promoting effects of VD (Hoyer-Hansen, Nordbrandt et al. 2010). Previous studies also suggested that SFN induces autophagy in human colon cancer (Nishikawa, Tsuno et al. 2010), prostate cancer (Herman-Antosiewicz, Johnson et al. 2006), and breast cancer (Kanematsu, Uehara et al. 2010). To examine the role of autophagy in the protective effects of VD and SFN, we analyzed the level of LC3-II, a well-known marker of autophagy. As shown in Figure 5.6A, the level of LC3-II in mice treated with VD was significantly increased (2.2 fold) when compared to mice in HF group ($p < 0.05$). The addition of SFN further significantly increased the level of LC3-II (3.8 fold) compared to mice in

HF group ($p < 0.01$). To monitor autophagic flux, we further measured the level of P62, which serves as a link between LC3 and ubiquitinated substrates and is efficiently degraded by autophagy. After treatment with VD, P62 was markedly decreased, consistent with increased autophagy activity. In addition, the addition of SFN further decreased the level of P62, indicating autophagic activation. IHC results also show that, compared to mice in HF and HFD group, a strong staining of LC3-II was detected in mice treated with the co-supplementation (Figure 5.6B). These results suggested that a high dose of VD supplementation can enhance the activity of autophagy, and the addition of SFN can magnify this effect.

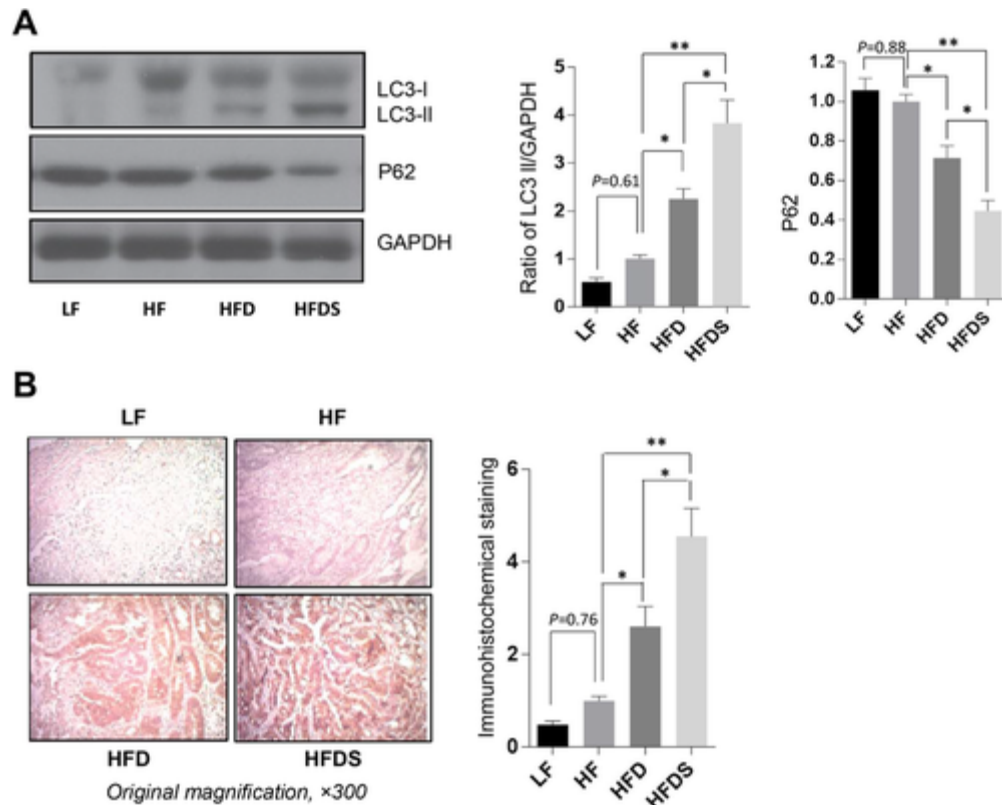


Figure 5.6. Effect of vitamin D and sulforaphane supplementation on the expression of LC3-II, a marker of autophagy. A) Representative Western blots and relative quantification of LC3-II in colonic scrapings ($n = 6$). B) Representative LC3-II IHC in tissues from the small intestine of APC1638N mice ($n = 5$) and quantification of immunohistochemical images. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

5.5 Discussion

Epidemiological studies have established obesity as a predisposing risk factors for CRC (Renehan, Tyson et al. 2008). Obesity rates have been steadily rising, and the prevalence of obesity among adults increased from 33.7% in 2007-2008 to 39.6% in 2015-2016 (Hales, Fryar et al. 2018). If this trend continues, there is no chance that the world could meet the target set by the UN as of 2025 (Di Cesare, Bentham et al. 2016). Therefore, it is necessary and urgent to understand the mechanisms by which obesity raises the risk of CRC and to design efficient and economic strategies to address this problem.

Substantial epidemiologic and experimental studies have shown that higher intake or blood levels of VD is associated with the low risk of CRC (Wactawski-Wende, Kotchen et al. 2006; Gorham, Garland et al. 2007; Jenab, Bueno-De-Mesquita et al. 2010; Lee, Li et al. 2011; Grant 2012), and a randomized, controlled clinical trial suggested that VD supplementation might decrease tumor-promoting inflammatory biomarkers such as TNF- α , IL-6, IL-1 β , IL-8 (Hopkins, Owen et al. 2011). Since the initial discovery of escalated expression of TNF- α in adipose tissue in 1993 (Hotamisligil, Shargill et al. 1993), it is now well accepted that obesity is associated with a state of chronic low-grade inflammation (Ramos, Xu et al. 2003), which is at least partially responsible for obesity-associated CRC (Coussens and Werb 2002). Therefore, VD supplementation holds promise as an effective dietary strategy for the prevention of obesity-associated CRC. However, VD clinical studies have unfortunately been disappointing (Wactawski-Wende, Kotchen et al. 2006; Baron, Barry et al. 2015; Song, Garrett et al. 2015). Major reasons that may explain the inconsistency in findings among these studies include the dosage of supplementation (Wactawski-Wende, Kotchen et al. 2006; Baron, Barry et al. 2015;

Song, Garrett et al. 2015) and the fact that nutrients in foods more likely act in concert with each other and the assumption that a single nutrient alone has a substantial individual anti-cancer effect may not be valid (Jacobs and Steffen 2003; Flood, Rastogi et al. 2008). One randomized controlled trial (RCT) study found that daily supplementation of 400 IU VD with calcium had no effect on the incidence of CRC among postmenopausal women (Wactawski-Wende, Kotchen et al. 2006). One possible explanation for this observation was that 400 IU VD daily is too low of a dose, considering the fact that the mean increment in 25(OH)D caused by an additional 1,000 IU/d is only 8 ng/ml at a starting value of 30 ng/ml (Garland, French et al. 2011). In another RCT study, supplementation with a higher VD dose (2,000 IU/d) and calcium did not result in a significantly lower risk of all-type cancers at 4 years (Lappe, Watson et al. 2017). This might be due to a relatively high mean baseline serum 25(OH)D levels (32.8 ng/ml) in these healthy postmenopausal older women. In our study, the mean baseline serum 25(OH)D levels of HF group was 14.3 ± 1.7 ng/ml. In addition, another RCT study suggested that high doses of VD (8,000 IU/d for two weeks, followed by 4,000 IU/d) suppressed the progression of colon cancer. Therefore, in the present study, we investigated the preventive effect of a high dose VD (5000 IU) and its combination with SFN on intestinal inflammation and tumorigenesis in obese *Apc^{1638N}* mice. Serum levels of 25(OH)D in the high dose VD supplementation groups (HFD and HFDS) were 62.0 ± 2.3 ng/ml, 52.4 ± 5.4 ng/ml respectively, which is consistent with a previous study (Hummel, Thiem et al. 2013).

VD, via its most active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D], plays an important role in cellular processes by binding the nuclear VD receptor transcription factor and affecting expression of key genes involved in control of cellular proliferation, differentiation, and apoptosis (Deeb, Trump et al. 2007). Possible mechanisms underlying *Wnt*/β-catenin inhibition by 1,25(OH)₂D in colon cancer is that: 1,25(OH)₂D inhibits the production of IL-1β in THP1

macrophages, and thereby inhibits the ability of macrophages to activate *Wnt* signaling in colon carcinoma cells (Kaler, Augenlicht et al. 2009). It has also been proposed that the chemopreventive properties of 1,25(OH)₂D stem from its ability to increase the binding of VDR to β -catenin, thereby hampering the formation of the β -catenin/TCF complexes, and to induce the expression of the *Wnt* inhibitor DKK1 (Aguilera, Pena et al. 2007). SFN, an isothiocyanate found in cruciferous vegetables, has the property to inhibit HDAC activity in many cancer cells (Dashwood and Ho 2008; Ho, Clarke et al. 2009; Dickinson, Rusche et al. 2015; Kim, Fujita et al. 2016). Several studies have shown that the HDAC inhibitors (HDACi) can improve the effects of VD supplementation and HDACi can cause a synergistic effect in cancer models, especially those that are resistant to VD supplementation alone (Rashid, Moore et al. 2001; Banwell, O'Neill et al. 2004; Khanim, Gommersall et al. 2004; Malinen, Saramaki et al. 2008). However, there are little evidence about the antitumor effect of the combination of VD and SFN.

In the present study, we demonstrated that a high dose of VD supplementation can significantly attenuated the obesity-induced elevated expression of TNF- α by 28.33%, IFN- γ by 39.24%, IL-2 by 66.49%, IL-17A by 54.94% by 60 kcal% high fat diet. Previous studies have shown that TNF- α can induce GSK3 β phosphorylation and subsequently stabilize β -catenin (Oguma, Oshima et al. 2008; Liu, Brooks et al. 2012; Coskun, Olsen et al. 2014), two key components within *Wnt* pathway. Our data showed that the high dose VD supplementation can decrease the level of phospho-GSK3 β and active β -catenin, in parallel with decreased mRNA expression of *Axin 2*, *c-Jun*, *c-Myc*, and *Cyclin D1*, which are reported as *Wnt* pathway downstream genes. These observations are in agree with previous studies that have shown that 1,25(OH)₂D can inhibit *Wnt*-signaling and its downstream genes, such as *c-Myc*, *cyclin D1* (Tong, Hofer et al. 1999; Larriba, Ordonez-Moran et al. 2011).

Our findings from this study demonstrated that co-administration of VD with SFN further decreased tumor incidence (HFD: 63.6% vs HFDS: 53.8%) and tumor size (HFD: 0.27cm vs HFDS: 0.22cm), but none of them reached a statistically significant degree. These observations, in agreement with previous findings (Shen, Khor et al. 2007), indicate the combination of dietary phytochemical SFN with other antitumor agents, such as VD, may be beneficial in CRC prevention. The co-administration (HFDS group) failed to further decrease tumor-promoting inflammatory cytokines and alter components of the *Wnt* pathway. Nevertheless, we observed that the addition of SFN inhibited the total HDAC activity and expression of HDAC6, which is consistent with previous studies that have shown that SFN and its metabolites SFN-Cys and SFN-NAC can inhibit HDACs (Myzak, Karplus et al. 2004; Myzak, Hardin et al. 2006). Considering HDACs are commonly overexpressed in human and mouse colon tumors (Mariadason 2008; Xu, Liu et al. 2016), SFN may be a promising adjunct dietary preventative agent.

Without observing significant influences from the addition of SFN on inflammation and *Wnt*-signaling, we examined another molecular mechanism, autophagy activity. Autophagy is an evolutionarily conserved lysosomal degradation pathway that is essential for cellular homeostasis and genomic integrity (Levine and Kroemer 2008). Its dysfunction has been linked to a wide range of diseases including cancer. Previous studies have shown that VD and its analogs can trigger autophagic death in many cancer cells (Wang, Lian et al. 2008; Tavera-Mendoza, Westerling et al. 2017). SFN can also initiate autophagy in several cell types (Jo, Kim et al. 2014; Liu, Smith et al. 2017). Our data indicates that VD can activate colonic autophagy in obese *Apc*^{638N} mice, and VD and SFN co-supplementation further activate autophagy with a statistically significant degree ($p < 0.05$). These findings suggest that the addition of SFN may magnify the influences of VD on autophagy.

5.6 Conclusions

In conclusion, we found that a high dose of VD supplementation decreased obesity-associated intestinal tumorigenesis by decreasing the production of multiple proinflammatory cytokines, and subsequently altering the tumorigenic *Wnt* pathway in the *Apc*^{1638N} model. The addition of SFN with a high dose of VD supplementation inhibited HDAC activity and further stimulated autophagy. Our findings warrant further pre-clinical and clinical studies to explore the cancer preventive effects of a high dose of VD and its combination with SFN supplementation on obesity-associated CRC.

Author contributions: J.L. performed all the experiments. A.M.F., Y.J., and C.G. contributed to the collection, interpretation, and analysis of data throughout the experiment; all authors contributed to the discussion. Z.L., H.X., and R.J.W. designed the research and directed the project. J.L. and Z.L. wrote the manuscript. All authors reviewed the manuscript.

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Conflict of Interest: No conflict of interest for all authors

CHAPTER 6

FFAR2 KNOCKDOWN MITIGATES ANTICANCER EFFECTS OF BUTYRATE BY DYSREGULATION WNT/B-CATENIN PATHWAY

6.1 Abstract

Previous studies have shown that butyrate is able to induce colon cancer cells apoptosis and differentiation. However, the detailed molecular mechanism of butyrate in suppression of colon cancer growth remains ambiguous. The present study aims to evaluate the mechanism by which butyrate modulates colon cancer growth. Caco-2 cells were treated with 0 mM, 1 mM, 2 mM, 5 mM butyrate. MTT assay showed that 2 mM and 5 mM butyrate significantly induced cell death by 17.9% and 41.2% respectively. In addition, western blot indicated that 2 mM and 5 mM butyrate can significantly increase the expression of cleaved caspase 3 by 136% and 292% respectively. We also found that butyrate can increase the activity of *Wnt*/β-catenin pathway. Knocking down FFAR2 by siRNA decreased the expression of cleaved caspase 3 and the expression of phospho-GSK3β (Ser9) and active β-catenin in Caco-2 cells, subsequently mitigated the anticancer effect of butyrate. Based on these results, we conclude that FFAR2 is required for the cytotoxicity effects of butyrate and regulation of *Wnt* pathway. Our findings warrant further studies to explore the molecular mechanisms by which butyrate induces colon cancer death.

6.2 Introduction

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It is the third most common cancer in the United States, with around 140,000 new cases being diagnosed last year. The pathogenetic mechanisms underlying CRC development are complex and include hereditary and environmental factors. There is strong evidence that dietary fiber plays a protective role in CRC carcinogenesis (Gunter and Leitzmann 2006; Sengupta, Muir et al. 2006).

The gut fermentation of dietary fiber by colonic bacteria produces short-chain fatty acids (SCFAs). Butyrate, a four-carbon fatty acid, is a major energy source for colonocytes and promotes colonocytes proliferation (Guilloteau, Martin et al. 2010). Butyrate causes reduction in cancer cell proliferation by blocking the G1/S phase of the cell cycle (Vaziri, Stice et al. 1998). Like trichostatin A (TSA), butyrate is a histone deacetylase inhibitor (HDACi), regulating pro-apoptotic protein expression by inhibiting HDACs. Cancer cells preferentially ferment glucose as an energy source even when oxygen is abundant. Therefore, butyrate is accumulated in the cancer cell cytoplasm, leading to an enhanced anticancer effects (Donohoe, Collins et al. 2012).

Free fatty acid receptor 2 (FFAR2) is a G-protein coupled receptor for SCFAs (acetate, propionate, and butyrate). FFAR2 is preferentially expressed in normal colonocytes and neutrophils. Previous studies have shown FFAR2 is crucial for the resolution of inflammation, loss of FFAR2 might promote colon cancer by epigenetic dysregulation of inflammation suppressors, such as *sfrp1*, *dkk3*, *socs1* (Pan, Oshima et al. 2018). However, the role of FFAR2 in anticancer effects of butyrate is still not fully understood.

6.3 Materials and Methods

6.3.1 Chemicals and reagents

The sodium butyrate and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT, Calbiochem®) was obtained from MilliporeSigma (Burlington, MA). Sodium butyrate was dissolved in phosphate buffered saline (1X) (PBS, Gibco™) to make 1 M, 2 M, 5 M stock solutions. MTT were dissolved in PBS to make a 5 mg/ml stock solution. Stock solutions were prepared freshly before use in the fume-hood. The stock solution were then filter-sterilized. Dulbecco's modified Eagle medium (1X) (DMEM, Gibco™), heat-inactivated fetal bovine serum (FBS, Gibco™), 0.25% trypsin-EDTA (1X) (Gibco™), penicillin-streptomycin (10,000 U/mL) (Gibco™), TRIzol® reagent (Invitrogen™), DEPC-treated water, high capacity cDNA reverse transcription kit (Applied Biosystems™) and PowerUp™ SYBR™ green master mix (Applied Biosystems™) were purchased from Thermo Fisher Scientific Co. (Waltham, MA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). phospho-GSK3 β (Ser9), active β -catenin, cleaved-caspase 3 antibodies were purchased from Cell Signaling Technology (Danvers, MA).

6.3.2 Cell culture

Human colon cancer cell line Caco-2 was obtained from American Type Culture Collection (ATCC). The Caco-2 cells were maintained in a 100 mm dish at 37°C in a 5% CO₂ incubator in Dulbecco's modified Eagle medium (1X) (DMEM, Gibco™), supplemented with a 10% heat-inactivated fetal bovine serum (FBS, Gibco™), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco™), and 1 mM sodium pyruvate. Cells were passaged at 80% confluency, and the medium was changed every 72 hours.

6.3.3 Cell-viability assay (MTT assay)

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) method, as reported previously (Li, Yue et al. 2014). Briefly, Caco-2 cells were seeded in 96-well plates with 1×10^4 cells per well. After 70% confluency, suck the medium. Cells were treated with DMEM containing 1% FBS and butyrate (1-5 mM with PBS) or control medium containing 1% FBS for 48 hours. We added medium to the blank but did not seeding it. Cells were then incubated with MTT (0.5mg/mL) for 1 hour. After dissolved in DMSO, SpectraMax microplate reader (Molecular Devices, CA) were used to measure the absorbance at 570 nm wavelength. The OD value read from treatment and control groups then subtract the average OD value read from blank group. Results are expressed as the percentage of cell viability compared to the control. Experiments were repeated at least three times to ensure consistency of results.

6.3.4 Real-time PCR for gene expression

RNA samples were extracted from the colonic scrapings with Trizol reagent (Invitrogen, Carlsbad, CA). The concentration as well as purity of RNA samples was measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA). The first-strand cDNAs were synthesized from total RNAs using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR was performed on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the SYBR green PCR reagent kit (Invitrogen, Carlsbad, CA). The copy number of each transcript was calculated with respect to the GAPDH copy number. Primer sequences and thermal cycling conditions were listed as supporting information in **APPENDIX B**.

6.3.5 Western blot analyses

As reported before (Sun, Yu et al. 2011), 40 µg of protein from each treatment was separated by 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 10% instant nonfat dry milk, membranes were incubated with specific antibodies overnight at 4°C, followed by incubation with the secondary antibody. Antibody binding was detected with the enhanced ECL detection system. Notable western blots results were quantified using Image J software after normalizing to a corresponding loading controls.

6.3.6 Small interfering RNA

Caco-2 cells were seeded in 6-well plates with 1×10^6 cells per well. Caco-2 were then transfected by combining Lipofectamine RNAiMAX with Silencer Select small interfering RNA (siRNA) targeting FFAR2 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco™) for 48 hours. After 48 hours, siRNA complexes were removed and replaced with butyrate for another 48 hours. Cells were extracted for following experiments.

6.3.7 Statistical analysis

Values in the text are presented as means \pm S.E.M. One-way ANOVA (treatment) statistical analyses were performed using Graphpad Prism 5 followed by multiple comparisons (Tukey method) among all treatments (groups). To simplify data presentation in the figures, the comparisons were only shown between the HF groups with other treatment groups. Values of $p < 0.05$ were considered statistically significant among the comparisons. Fisher's exact test was used for tumor incidence. The expression of each gene was normalized to the housekeeping gene *GAPDH* ($\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$). Statistical analyses were performed based on ΔCt and relative expression is reported as $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{Experiment}} - \Delta\text{Ct}_{\text{Control}}$.

6.4 Results

6.4.1 Cytotoxicity of human colon cancer cell line Caco-2 in response to butyrate

We first examined the cytotoxicity effects of butyrate on Caco-2 cell line by using a cell viability assay (MTT assay). MTT assay is a common colorimetric assay for assessing cell mitochondrial metabolic enzyme activity that reduces MTT. Therefore, MTT assay basically uses mitochondrial enzyme activity as an indicator of cell viability. The drugs that induce cytotoxicity and mitochondrial dysfunction will reduce the enzyme activity leading to a lower OD reader. Cytotoxicity of drugs can be indicated by the OD reader. Caco-2 cells were plated in a 96-well plate and exposed to physiologically relevant concentrations of butyrate (1 mM, 2 mM, 5 mM). After 48 hours treatment, 2 mM and 5 mM butyrate significantly induce cell death by 17.9% and 41.2%, while 1 mM butyrate has no significant cytotoxicity on Caco-2 cells (Figure 6.1).

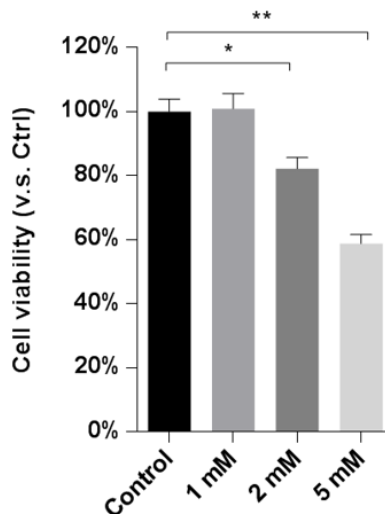


Figure 6.1. Cytotoxicity of human colon cancer cell line Caco-2 in response to butyrate. A) Caco-2 cells were treated with 0 mM, 1 mM, 2 mM, 5 mM butyrate for 48 h. Cell viability was measured by the MTT assay. * $p < 0.05$, ** $p < 0.01$. Data are mean \pm SEM.

6.4.2 Cell apoptosis of human colon cancer cell line Caco-2 in response to butyrate

Cysteine-aspartic proteases (Caspases) are a group of protease enzymes playing crucial roles in apoptosis. Caspase 3 is one of the key caspases, as it can be activated by both extrinsic and intrinsic pathway in apoptotic cells. Caspase 3 is initially synthesized as inactive proenzymes. When caspase 3 is cleaved, it can be activated. We therefore measured the protein levels of cleaved caspase 3. We found that 2 mM and 5 mM butyrate can significantly increase the expression of cleaved caspase 3 by 136% and 292% respectively, which indicating butyrate can induce Caco-2 cell apoptosis (Figure 6.2).

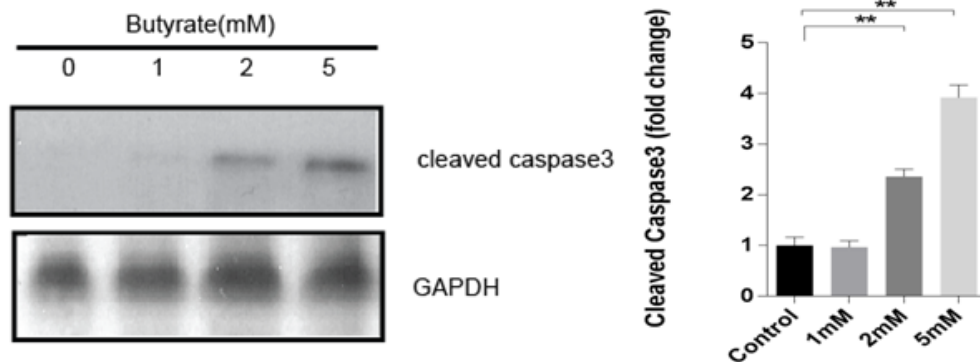


Figure 6.2. Cell apoptosis of human colon cancer cell line Caco-2 in response to butyrate. A) Representative Western blots and relative quantification of cleaved caspase 3. Data are presented as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

6.4.3 *Wnt*/ β -catenin pathway activity of human colon cancer cell line Caco-2 in response to butyrate

We further studied the effects of butyrate on signaling pathways involved in CRC. It is well known that aberrant *Wnt* signaling plays a crucial role in colorectal oncogenesis and

progression. By immunoblotting analysis, we measured the levels of phospho-GSK3 β (Ser9) protein, the inactive form of GSK3 β , and the dephosphorylated β -catenin (Ser37 or Thr41) protein, the active form, which are two key molecules in the *Wnt* pathway. As shown in Figure 6.3B, butyrate increases the expression of phospho-GSK3 β (Ser9) and active β -catenin in a dose dependent manner in Caco-2 cells. We further examined the mRNA expression of *Wnt* pathway downstream oncogenes, *c-Jun*, *c-Myc*, and *cyclin D1*. We found that the mRNA expressions of these downstream genes were increased in response to butyrate (Figure 6.3B). This observations were consistent with the previous reports (Bordonaro, Lazarova et al. 2008; Bordonaro, Lazarova et al. 2008).

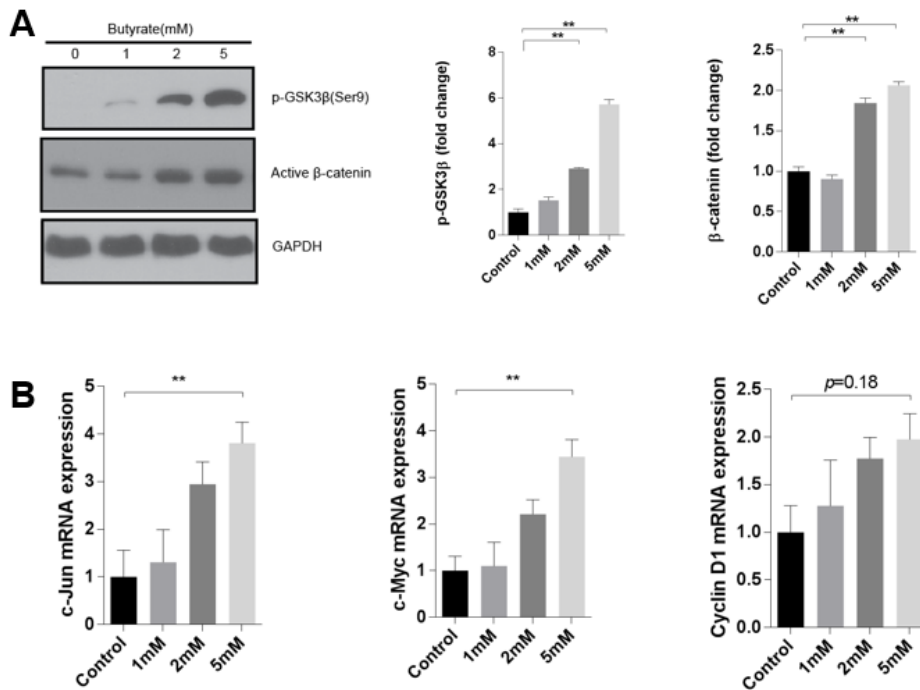


Figure 6.3. *Wnt*/ β -catenin pathway activity of human colon cancer cell line Caco-2 in response to butyrate. A) Representative Western blots and relative quantification of GSK-3 β Ser9 phosphorylation and active β -catenin in response to butyrate. B) mRNA expression of *Wnt* pathway downstream genes in response to butyrate. Data are presented as the mean \pm SEM.

* $p < 0.05$, ** $p < 0.01$.

6.4.4 FFAR2 knockdown mitigates anticancer effects of butyrate by dysregulation Wnt/ β -catenin pathway

We first measured the mRNA expression of FFAR2 (butyrate receptor) and MCT1 (butyrate transporter). As shown in Figure 6.4A, we found that butyrate dose dependently increased the mRNA expression of FFAR2 and MCT1. We then tested whether butyrate induced Caco-2 cell death and activation of Wnt/ β -catenin pathway were through the FFAR2. As shown in Figure 6.4B, knocking down FFAR2 by siRNA decreased the expression of cleaved caspase 3 and the expression of phospho-GSK3 β (Ser9) and active β -catenin in Caco-2 cells. We further examined the mRNA expression of *Wnt* pathway downstream oncogenes, *c-Jun*, *c-Myc*, and *cyclin D1*. We found that the mRNA expressions of these downstream genes were decreased in FFAR2 knock down Caco-2 cells (Figure 6.4C). As shown in Figure 6.4D, knocking down FFAR2 by siRNA mitigated the anticancer effects of butyrate. Based on these results, we conclude that FFAR2 is required for the cytotoxicity effects of butyrate and regulation of *Wnt* pathway.

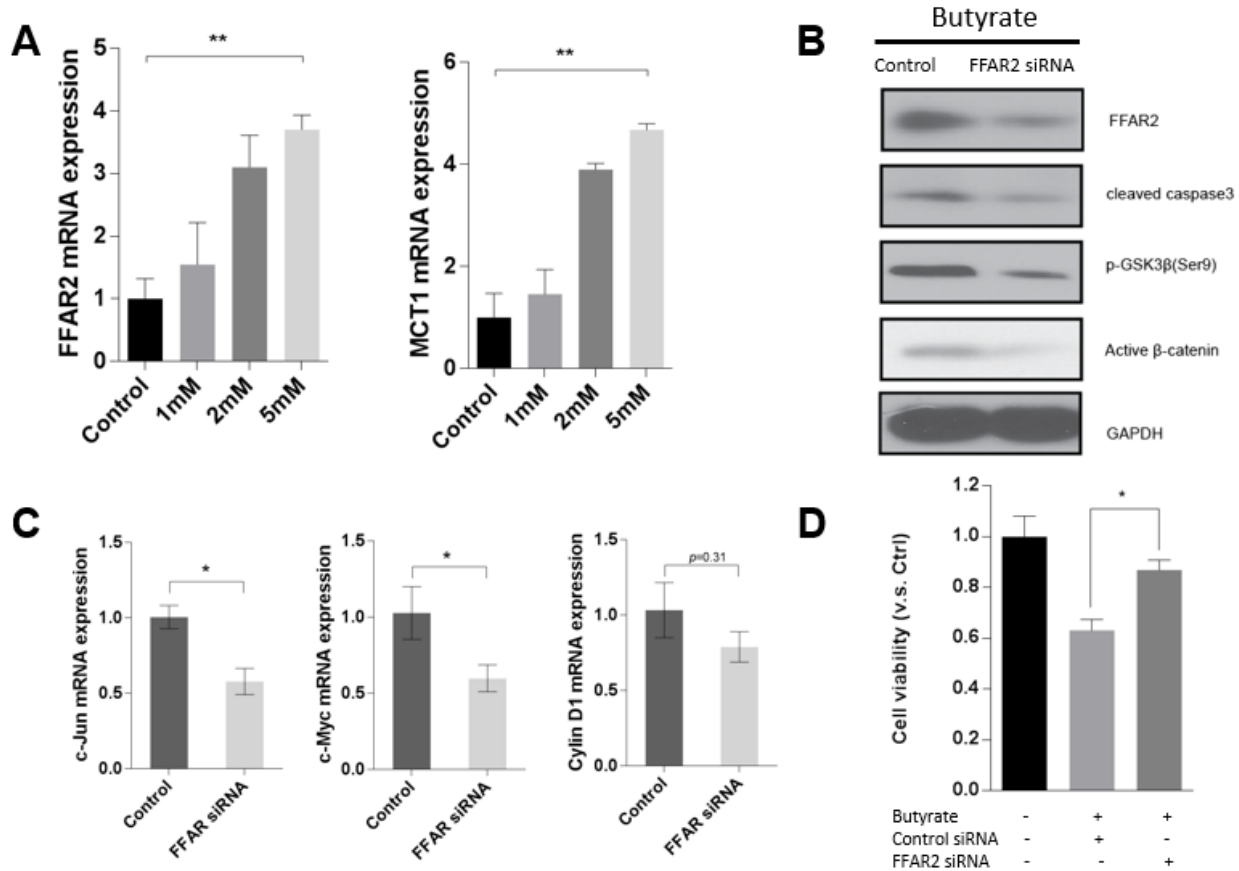


Figure 6.4. Effect of FFAR2 knock down on the Caco-2 cell apoptosis and the activation of Wnt/ β -catenin pathway. A) mRNA expression of FFAR2 and MCT1. B) Western blotting. C). mRNA expression of c-Jun, c-Myc, cyclin D1. D). Cell viability. Data are presented as the mean \pm SEM. * $p < 0.05$.

6.5 Discussion

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States in both men and women. In 2018, it is estimated that $\approx 64\,640$ women and $75\,610$ men in the United States were diagnosed with CRC and $23\,240$ women and $27\,390$ men died from CRC.

Therefore, it is necessary and urgent to design efficient and economic strategies to prevent CRC.

Substantial epidemiologic and experimental studies have shown that higher intake of dietary fiber is associated with a lower risk of CRC (Levi, Pasche et al. 2001; Aune, Chan et al. 2011; Kunzmann, Coleman et al. 2015). Colonic bacterial fermentation of dietary fiber can produce butyrate, a short chain fatty acid. One of the explanations of CRC prevention effect of butyrate is that bacterial fermentation converts resistant starch to SCFA (Song, Garrett et al. 2015). Among SCFA, butyrate plays a pivotal role in the prevention/inhibition of colon carcinogenesis (Manning and Gibson 2004). Butyrate can induce colon cancer cell differentiation and apoptosis, and inhibit colon cancer cell differentiation (Goncalves, Araujo et al. 2011). Similarly, several animal studies also suggested a protective effect of butyrate on CRC carcinogenesis (D'Argenio, Cosenza et al. 1996; Kameue, Tsukahara et al. 2004; Lu, Nakanishi et al. 2013). Human studies also found an inverse relationship between the levels of butyrate in the human colon and the incidence of CRC (Bingham, Day et al. 2003). The risk of developing tumors are higher in the distal colon where the concentration of butyrate is lower, implicating butyrate has CRC prevention effect (Mortensen, Holtug et al. 1988). In consistent with these studies, we found that butyrate (5 mM) significantly induce Caco-2 cell death by 41.2% and increased expression of cleaved caspase 3.

Butyrate can support growth of normal colonocytes, interestingly it exerts a drastic inhibitory effect on CRC (Bergman 1990). The possible explanation of this controversy might lie in the Warburg effect which is observed in various cancer cells (Bates 2012). Normal colonocytes use aerobic respiration to meet energy requirements, while colon cancer cells prefer anaerobic

glycolysis even when there is plenty of oxygen. In colon cancer cells, because of excessive anaerobic glycolysis, fatty acid oxidation is inhibited. Therefore, butyrate, a short chain fatty acid, is not used as an energy resource and subsequently accumulate in the colon cancer cells. The elevated level of butyrate can act as an HDACi and induce cancer cell apoptosis (Encarnacao, Abrantes et al. 2015; Bultman 2016).

Besides butyrate can act as an HDACi, butyrate might exert anti-proliferative and pro-apoptotic effects through manipulating several signal pathways in cancer cells. For example, sodium butyrate (2.5 mM and 5 mM) increases the mRNA and protein expression of SMAD3 and potentiates TGF- β signaling and its tumor suppressor activity in rat intestinal epithelial (RIE-1) cells (Nguyen, Cao et al. 2006). Butyrate (5 mM) pretreatment followed by TGF- β treatment increased DNA fragmentation and apoptosis in RIE-1 cells. Interestingly, without butyrate pretreatment, TGF- β treatment alone cannot induce apoptosis and DNA fragmentation (Cao, Gao et al. 2011). Butyrate (5 mM) can also arrest the RIE-1 cells in the G2/M phase of the cell cycle.

Wnt signaling is crucial for intestinal crypts homeostasis and maintenance of intestinal stem cells (Flanagan, Austin et al. 2018). It is well known that hyperactivation of *Wnt* pathway is present in almost all CRC. *Wnt* signaling plays a pivotal role in CRC incidence and is the basis for CRC tumorigenesis in patients with familial adenomatous polyposis and in *Apc* mutant mice model. Loss of APC function is the main driver of CRC carcinogenesis. However, we found that butyrate increased the expression of phospho-GSK3 β (Ser9) and active β -catenin in a dose dependent manner in Caco-2 cells. It is suggested that an optimal but not excessive activation of *Wnt*/ β -catenin pathway are favorable for cancer cell growth (Leedham, Rodenas-Cuadrado et

al. 2013). Therefore, butyrate (5 mM) might activate *Wnt*/ β -catenin pathway which surpass the optimal level in Caco-2 cells.

Free fatty acid receptor 2 (FFAR2) is a G-protein coupled receptor for SCFAs (acetate, propionate, and butyrate). Previous studies have shown that loss of FFAR2 might promote colon cancer by epigenetic dysregulation of inflammation suppressors (Pan, Oshima et al. 2018). Our finding from this study demonstrated that knocking down FFAR2 by siRNA decreased the expression of cleaved caspase 3 and the activity of *Wnt*/ β -catenin pathway. In addition, knocking down FFAR2 by siRNA mitigated the anticancer effects of butyrate. ‘

6.6 Conclusions

Based on these results, we conclude that FFAR2 is required for the cytotoxicity effects of butyrate and regulation of *Wnt* pathway. Our findings warrant further studies to explore the molecular mechanisms by which butyrate induces colon cancer death.

Author contributions: J.L. performed all the experiments. A.M.F., Y.J., and C.G. contributed to the collection, interpretation, and analysis of data throughout the experiment; all authors contributed to the discussion. Z.L., H.X., and R.J.W. designed the research and directed the project. J.L. and Z.L. wrote the manuscript. All authors reviewed the manuscript.

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Conflict of Interest: No conflict of interest for all authors

CHAPTER 7

SUMMARY AND CONCLUSIONS

CRC has a high incidence rate, and an unhealthy diet and life style is an important risk factor. The prevalence of obesity was 39.8% among US adults population and obesity can also increase the risk of CRC. Therefore, many research have conducted to study the role of obesity in the CRC risk and the mechanisms by which obesity increase the incidence of CRC. However, the detailed molecular mechanisms need to be further addressed.

In our study, we utilized genetic approach and dietary strategies to study the prevention of diet induced obesity associated CRC and determine the underlying molecular pathways that render the CRC prevention effects.

We found that loss of TNF- α decreased obesity associated intestinal tumorigenesis by decreasing the inflammation, and manipulating the β -catenin pathway and NF- κ B signaling. In addition, IKK, component of the NF- κ B signaling, was involved in the regulation of β -catenin pathway. The administration of Vitamin D (VD), at 5000 IU level, exerted an anti-inflammatory property, and led to suppressed intestinal *Wnt*-signaling and tumorigenesis in obese mice. The molecular function of sulforaphane on a high dose of VD supplementation, although displayed on the inhibition of HDAC and the activation of autophagy, needs further investigation. Butyrate can increase the activity of *Wnt*/ β -catenin pathway. Knocking down FFAR2 by siRNA decreased the expression of cleaved caspase 3 and the expression of phospho-GSK3 β (Ser9) and active β -catenin in Caco-2 cells, subsequently mitigated the anticancer effect of butyrate.

In conclusion, we found that genetic approach and dietary strategies might be an effective method to prevent CRC incidence and development.

APPENDIX A

TABLE OF COMPOSITIONS OF THE EXPERIMENTS DIETS

	LF 10%kcal fat+200IU VD		HF 60%kcal fat+200IU VD		HFD 60%kcal fat+5000IU VD		HFDS 60%kcal fat+5000IU VD+SFN	
	<i>gm%</i>	<i>kcal%</i>	<i>gm%</i>	<i>kcal%</i>	<i>gm%</i>	<i>kcal%</i>	<i>gm%</i>	<i>kcal%</i>
Protein	19	20	26	20	26	20	26	20
Carbohydrate	66	70	25	20	25	20	25	20
Fat	4	10	35	60	35	60	35	60
Total		100		100		100		100
<i>Ingredient</i>	<i>gm</i>	<i>kcal</i>	<i>gm</i>	<i>kcal</i>	<i>gm</i>	<i>kcal</i>	<i>gm</i>	<i>kcal</i>
Casein	200	800	200	800	200	800	200	800
<u>L-Cystine</u>	3	12	3	12	3	12	3	12
Corn Starch	315	1260	0	0	0	0	0	0
Maltodextrin	35	140	125	500	125	500	125	500
Sucrose	350	1400	68.8	275	68.8	275	68.8	275
Cellulose	50	0	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin D ₃ (100,000 IU/gm)	0.002	0	0.002	0	0.05	0	0.05	0
SFN	0	0	0	0	0	0	0.23	0
Total	1055.05	4057	773.85	4057	773.9	4057	774.1	4057

*The diets were formulated on an energy basis.

APPENDIX B

TABLE OF PRIMERS USED FOR REAL-TIME PCR ANALYSIS

Gene	Forward Primer	Reverse Primer
<i>Cyclin D1</i>	5'-GCGTACCCTGACACCAATCTC-3'	5'-ACTTGAAGTAAGATACGGAGGGC-3'
<i>c-Myc</i>	5'-ATGCCCTCAACGTGAACTTC-3'	5'-GTCGCAGATGAAATAGGGCTG-3'
<i>c-Jun</i>	5'-TTCCTCCAGTCCGAGAGCG-3'	5'-TGAGAAGGTCCGAGTTCTTGG-3'
<i>Axin2</i>	5'- ATGAGTAGCGCCGTGTTAGTG-3'	5'- GGGCATAGGTTTGGTGGACT-3'

*Thermal Cycling Conditions: UDG activation 50°C 2mins, Dual-Lock DNA polymerase 95°C 2mins. Denature 95°C 1s, Anneal/extend 60°C 30s, 40 Cycles.

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