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MITIGATION OF INTESTINAL TUMORIGENESIS AND CANCER CACHEXIA IN APC$^{MIN/+}$ MICE BY TABLE GRAPES (*VITIS VINIFERA*)

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ABSTRACT

Colon cancer is the 3rd most prevalent cancer in the United States and it kills over 50,000 Americans every year. Conventional chemotherapies can significantly reduce tumor growth but can have serious adverse side effects. Emerging evidence suggests that phytonutrient-rich plant foods reduce the risk of colon cancer. Grapes, one of the most widely consumed fruits in the world, contain a variety of phytonutrients such as anthocyanins, proanthocyanidins, stilbenes, flavonols, and flavanols. We have recently shown that grape compounds (resveratrol and grape seed extract) and grape powder extract can target cancer stem cells *in vitro* and *in vivo* using a carcinogen-induced rodent model of colon cancer. However, no information is available on the anti-colon cancer activity of multi-colored grapes *in vivo*. Herein, I have investigated the inhibitory efficacy of table grapes (freeze dried grape powder – FDGP) against colon cancer stem cells (CSCs) *in vitro* and intestinal tumorigenesis in the $APC^{Min/+}$ mouse, a model of genetically-induced intestinal tumorigenesis.

CSCs have been shown to be responsible for the initiation and progression of tumors in a variety of cancers. Grape powder extract (GPE) made from FDGP suppressed proliferation, sphere formation, and elevated apoptosis in colon CSCs. Furthermore, shRNA-mediated knockdown of p53, a tumor suppressor gene, in colon CSCs did not alter the efficacy of GPE, which indicates that these anti-proliferative and pro-apoptotic effects are independent of p53. These findings demonstrate the potential colon CSC inhibitory efficacy of FDGP.

Dietary supplementation of male $APC^{Min/+}$ mice with FDGP (at 3 or 6% w/w) suppressed the total number of intestinal polyps by 55%. This level of inhibition was greater than aspirin (39% at 200 ppm), which is a drug used for the prevention of colon cancer in humans. FDGP supplementation also decreased polyp growth, where the number of polyps of 1 to 2 mm in size was significantly lower than control mice. Reduced tumorigenesis was associated with downregulation of targets in the Wnt/β-catenin pathway, such as cyclin D1, c-Jun, and c-myc expression. Expression of genes linked to angiogenesis, VEGF and HIF-1α, were also lower in FDGP-treated mice.

We also looked at the effect of FDGP on cancer cachexia. Cancer-induced cachexia is a complex condition of tissue wasting which develops as a secondary disorder in cancer patients and leads to progressive functional impairment, accompanied by chronic inflammation, disrupted energy metabolism, and severe muscle wasting. It accounts for ~30% of all cancer-related deaths. In addition, the radiotherapy used to treat colon cancer can exacerbate cachexia progression. The $APC^{Min/+}$ mouse is an established model of colon cancer-induced cachexia. We found that dietary supplementation of $APC^{Min/+}$ mice with FDGP ameliorated weight loss in $APC^{Min/+}$ compared to control diet-treated mice. FDGP also countered other important markers of cancer cachexia such as endotoxemia, altered gut barrier function, and systemic inflammation in $APC^{Min/+}$ mice. FDGP supplemented mice had lower levels of circulating endotoxins/lipopolysaccharides (LPS) and LPS binding protein LBP. FDGP also suppressed the systemic and tissue levels of pro-inflammatory cytokines and upregulated the levels of anti-inflammatory cytokines. We also observed that mice treated with FDGP had greater levels of fecal

bacteria associated with reduced cachexia, and lower levels of those positively correlated with colon cancer-cachexia. These results suggest that FDGP ameliorated weight loss and other important markers of cancer induced cachexia.

In conclusion, the present study suggests the potential usefulness of table grapes for the chemoprevention of human intestinal/colorectal cancer and its associated cachexia.

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Chapter 1

Introduction

1.1 Colon cancer

1.1.1 Incidence and mortality

Cancer is a significant public health problem in the United States as well as many other parts of the world [1, 2]. Currently being the second leading cause of death in the United States, cancer is soon expected to become the first leading cause of death surpassing heart diseases. Colon cancer is the 3rd most common cancer. Estimates by the National Cancer Institute show that for the year of 2017, 95,520 new cases of colon cancer were diagnosed [2].

Incidence of colon cancer varies geographically with the countries of Australia and New Zealand, having the highest rate and western Africa having a very low rate of colon cancer [3]. Americans aged more than 50 years are at the foremost risk of developing colon cancer, and epidemiological observations show that 90% of the newly screened cases and 93% of colon cancer death occur within this age group. The median age of colon cancer diagnosis is 68 years, and the median age of death is 73 years.

Colon cancer also represents a tremendous financial burden. Approximately US $14.1 billion per year is spent on treating colon cancer in the United States alone. This is expected to exceed US $18 billion by 2020 due to the expected increase in colon cancer cases and an increasingly aging population which is more prone to colon cancer. Taking

into account costs for screening, visitations, surgeries, medication, and maintenance, a colon cancer patient spends approximately $150,000 per year for care [4].

1.1.2 Risk factors

Most cases of colon cancer are sporadic and 75% of patients have a negative family history for colon cancer. In most Western countries, the average lifetime risk for colon cancer is 3 - 5%. It doubles for people with a first-degree family member diagnosed with colon cancer [3]. It is evident that both genetic and environmental factors play a major role in the etiology of colon cancer.

With the use of a logistic regression model and taking into account the age and family history, Platz *et al*. suggested six risk factors for colon cancers which can be modified to decrease the cancer occurrence by 70% [5]. These risk factors include obesity, physical inactivity, alcohol consumption, cigarette smoking, high red meat consumption, and low intake of folic acid. Inflammatory bowel diseases are also known to be associated with colon cancer to a certain degree [6].

According to the Journal of the National Cancer Institute, about 35 % of colon cancer deaths in the United States can be attributed to dietary factors. Because of the complexity and variation in the diets, it is often difficult to determine the direct implications. The American Cancer Society suggests people for the consumption (2 ½ servings) of fruits and vegetable in a regular diet [7]. A meta-analysis of case-control studies reported that fruit and vegetable consumption, in general, was associated with a decrease in the risk of colon cancer. A meta-analysis linked regular fruit consumption to

a 13% reduction in colon cancer risk and vegetable consumption with 40% lower risk of incidence [8-10].

Countries such as the United States, which have a higher dietary fat consumption are known to have a higher incidence of colon cancer [11]. Research also links the increase in red meat consumption to increased risk of colon cancer. It is also known that the carcinogens formed during high heat cooking, grilling and charring of these meats can be a culprit [12]. Some studies showed a 12-17 % increase in risk with every 100 g of red meat consumption per day, and 49 % increase in risk for processed meats [13]. The increased colon cancer risk associated with red and processed meats may be due to the carcinogenic compounds (i.e. free heme iron, nitrosoamines, etc.) in these products, but may also result from people who consume high amounts of red meat eating fewer vegetables and less fiber, both of which have been shown to decrease the risk.

Alcohol and tobacco use have been shown to be a risk factor for colon cancer. Alcohol through its toxic effects on the colon epithelium and by reducing the bioavailability of folate has been shown to increase the risk of developing colon cancer. People consuming two to four alcoholic beverages have a 23 % higher risk than non-consumer [14]. The International Agency for Research on Cancer linked smoking tobacco to colon cancer in 2009 [15].

Inflammatory bowel disease patients have a higher risk of developing colon cancer than the general population. Long standing ulcerative colitis was shown to have a chance of developing into malignancy and colon cancer accounts for 17% of deaths in patients with ulcerative colitis [16, 17]. The duration of the disease is also known to have

an impact on the risk, with risk of developing colon cancer increasing with length of time since ulcerative colitis diagnosis.

The relationship between Crohn's disease and colon cancer risk is less clear. There are conflicting results from observational studies. In a meta-analysis of 12 articles reporting colon cancer risk, the researchers were able to calculate the risk as 2.9 % after 10 years, 5.6 % after 20 years and 8.3 % after 30 years of Crohn's disease diagnosis [18].

Family history has also been considered a significant risk factor for colon cancer. Epidemiological studies show that approximately 20% of colon cancer patients have a 5-6% chance of having a close relative who is also diagnosed with colon cancer [19]. The lifetime risk of a 50 year old person without any family history of colon cancer is only 1.8%, whereas the risk increases to 3.4% with one affected relative and 6.9% with two or more affected relatives [20, 21].

1.1.3 Screening

Because colon carcinogenesis is a long process, the disease is more suitable for population screening than many other cancer types (**Figure 1-1**). Screening provides a valuable chance to find and treat early stage adenomas, and removal and treatment of early stage cancers and adenomas has been shown to have a profound effect on colon cancer mortality. The US Polyp Study showed that the mortality rate was 50% lower in patients who went for screening and early treatment than in the control group [27]. In addition, to better disease outcomes, screening also reduces the cost of treatment. This emphasizes the importance of screening in case of colon cancer [22].

1.1.4 Diagnosis

The diagnosis of colon cancer is usually made by routine screening or after a patient presents to a primary care physician with symptoms. Symptoms associated with colon cancer include blood in the stool, sudden change in bowel habits, and the onset of abdominal pain. Uncommon symptoms include fatigue, anemia (due to blood loss) and significant weight loss [3]. These symptoms usually warrant further clinical evaluation for confirming the disease.

Colonoscopy is considered the gold standard for diagnosing colon cancer because of its high diagnostic accuracy, ease of use, and the ability to locate the tumor in the colon itself. It can also be used to acquire tissue biopsy samples for histological confirmation of the diagnosis. Colonoscopy is the only available technique which provides both diagnostic and therapeutic value. Removing adenomas using endoscopic polypectomy has been shown to significantly reduce cancer incidence and mortality [23-25]. The US National Polyp Study demonstrated the efficacy of colonoscopy in reducing the incidence of colon cancer. In recent years the image quality has been markedly improved and can now be of sufficiently high resolution to allow physicians to stage the tumors.

Capsule endoscopy is a recent advance, which uses a wireless capsule device that is swallowed by the patient undergoing screening. It allows examination of nearly the entire gastrointestinal tract without using the traditional endoscopy approach [26]. It is particularly useful in diagnosing adenomas and rectal cancer.

Computed tomography (CT) colonography is a non-invasive technique used in the diagnosis, which uses low-dose CT scanning to obtain the interior view of the colon. According to a systematic review and meta-analysis, it is shown to have a sensitivity of 96% for the detection of colon cancer [27].

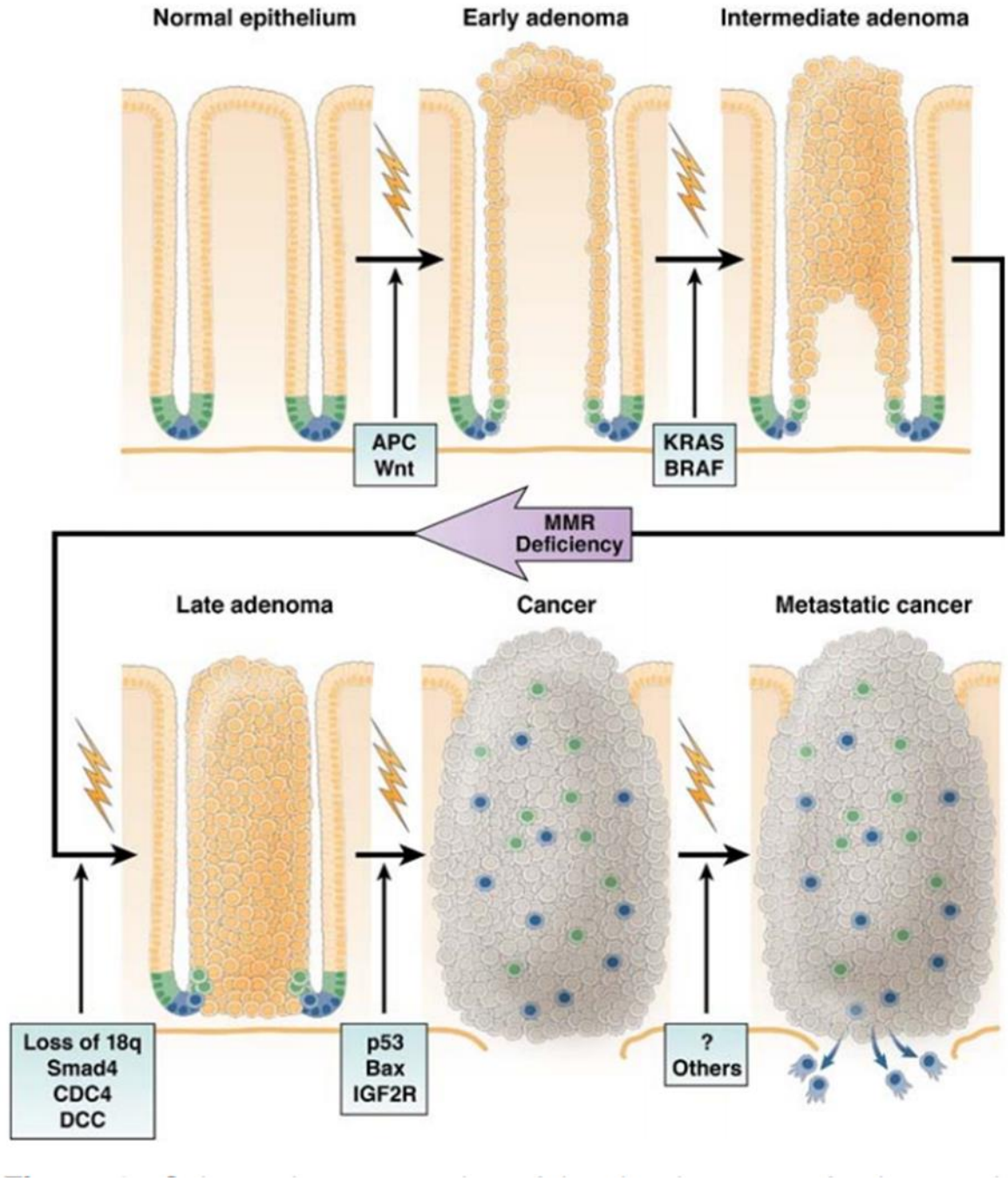

Figure 1-1: Colon cancer development. Adapted from Todaro *et al*.

1.2 Pathophysiology of Colon Carcinogenesis

1.2.1 Biology of the human intestine

In humans, the intestine is divided into two segments, the small intestine which measures around 6 meters in length and the large intestine stretching to 1.5 meters long. There are several anatomical and physiological differences between the two sections of the intestine. The small intestine contains a series of interdigitated villi and crypts of Lieberkuhn. The villi are essential for the absorption of nutrients [28]. The colon is devoid of villi and is composed of invaginated crypts on a flat surface that is folded at various intervals called rugae [29].

The colon consists of four layers – mucosa, submucosa, muscular layer, and serosa (**Figure 1-2**). The outermost colonic epithelial layer is lined by a single sheet of columnar epithelial cells which fold into finger like invaginations, supported by lamina propria to form a functional unit of the colon called crypt. The colonic epithelium is made up of several types of cells with varying levels of differentiation, which are derived from the multipotent stem cells existing at the bottom of each the crypt [30, 31] These stem cells are capable of self-renewal and divide asymmetrically to give rise to the transit-amplifying cell and a new stem cell (**Figure 1-3**). The transit-amplifying cells move upward from the bottom of the crypt, divide and differentiate into columnar, goblet, or enteroendocrine cells, which have distinct functions [31]. The colonic epithelium is renewed every 3-5 days [30]. A wide array of signaling pathways control the cellular hierarchy and homeostasis in the colonic crypts. Dysregulation of these pathways and

accumulation of genetic mutations leads to uncontrolled proliferation and finally the development of colorectal cancer [32].

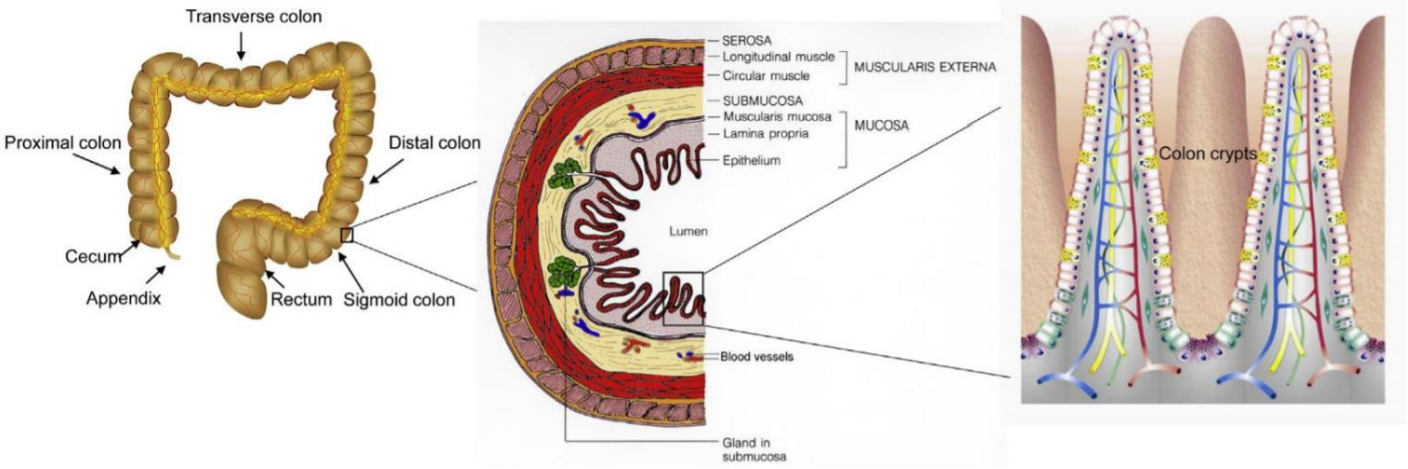

Figure 1-2: Morphology of the colon. Source: Kasdagly *et al*. (with permission)

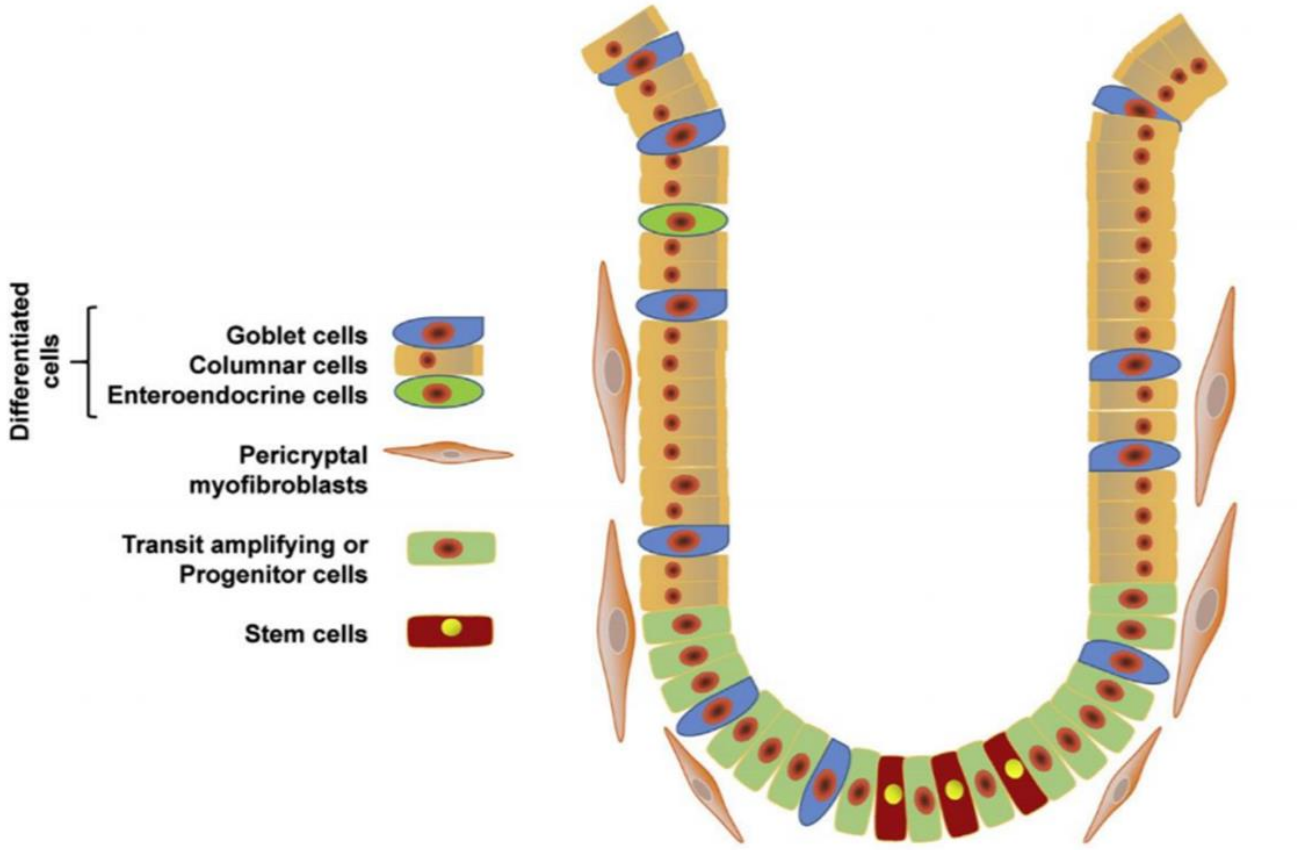

Figure 1-3: Crypt organization. Source: Kasdagly *et al* (with permission).

1.2.2 Development of human intestinal tumors

The widely accepted view of colon cancer is that it forms as a consequence of the accumulation of mutations in essential tumor suppressor genes or oncogenes, which eventually deregulate the homeostatic functions and lead to the transformation of healthy epithelial cells into cancer cells [33].

Analysis of the colon cancer genome has revealed the involvement of a small number of functionally essential gene mutations out of the hundreds of gene mutations found in colon cancer [33]. Mutations in a subset of 67 genes have been reported to be

associated with the initiation of colon cancer [34]. The environmental and genetic factors that cause colon cancer do so by promoting the hallmark phenotypes in the epithelial cells including: suppression of immune response, down regulation of growth inhibiting factors, inactivation of DNA repair mechanisms, inhibition of senescence induction, deregulation of cellular energy metabolism, promotion of inflammatory environment, induction of angiogenesis, resistance to apoptosis, and activation of invasion and metastasis pathways [34-36].

In the classic colon cancer formation model (**Figure 1-1**) the majority of cancers arise from a polyp beginning with an aberrant crypt, which then progresses into an early adenoma, less than one cm in size. Over time the adenoma grows bigger and progresses to an advanced adenoma, and it will eventually form a cancerous tumor [36]. It can take around 10 – 15 years for the polyp to develop into a malignant tumor.

This Chromosomal Instability (CIN) pathway is also known as the adenoma-carcinoma sequence is one of the most accepted sequence of events that happen in the origin of colon cancer and its progression. It follows a predictable progression of genetic and corresponding histologic changes. The genomic changes include activation of the proto-oncogene *KRAS* and inactivation of the tumor suppressor genes *APC* (chromosome region 5q21) and *P53* (chromosome region 17p13), and loss of heterozygosity for the long arm of chromosome 18 (18q LOH). Recently mutations involving other genes have been described, such as the transforming growth factor β receptor (*TGFBR*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) that are required for the adenoma-carcinoma sequence model [37, 38].

1.2.3 Colon cancer stem cells

Colon stem cells are known to be a prime suspect of cancer initiation because they have the longevity to accumulate mutations as well as the ability to self-renew [39, 40].

Colon cancer stem cells (CSCs) have characteristics similar to those of healthy colonic stem cells, with the ability to form heterogenic tumors that contain both tumorigenic and non-tumorigenic populations. CSCs are known to represent a small subpopulation of less than 1% of the overall cancer cells, but with their ability to establish and drive tumorigenesis makes them a crucial component in recurrence of tumors, resistance to therapy and metastasis [41]. CSCs may undergo a symmetrical self-renewing cell division into two identical daughter CSCs or an asymmetrical self-renewing cell division into one daughter CSC and one differentiated progenitor cell, resulting in number expansion of CSCs as well as tumor growth [42].

1.2.4 APC gene mutations and colon cancer

1.2.4.1 Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) was initially described as Gardner's syndrome. It is a condition in which germline mutations in the *APC* gene cause hundreds to thousands of adenomatous polyps in the large intestine. If left untreated the polyps which start out as benign can transform into malignant tumors. The polyp burden depends on the location of the mutation in the *APC* gene [29, 43].

1.2.4.2 Structure and transcriptional regulation of the APC gene

The human *APC* gene spans around 58kb, with a 15-exon coding region of 8529bp encoding a 2843 amino acid, 310 KD protein. The APC protein occurs in multiple forms varying in molecular weight ranging from 90-300 KD, because of the alternative splicing at the mRNA level. Post translational modifications and degradation are also known to play a role [43-45].

The APC protein consists of several functional domains. Heptad repeats mediate the APC homodimer formation at the amino-terminal end (aa 6-57). [46, 47]. Amino acids 453 to 767 share homology with the central repeat region of the *Drosophila* segment polarity protein armadillo. This particular domain interacts with APC-stimulated guanine nucleotide exchange factor (Asef) and enhances the interaction of Asef with RAC (from the Rho family of small GTPases), that govern the cellular adhesion and motility through the modulation of the actin cytoskeleton [43].

The primary function of the APC protein is the regulation of β-catenin (**Figure 1-4**). β-catenin is an essential protein involved in both the Wnt-1 signaling system and E-cadherin cell adhesion system. APC is a negative regulator of β-catenin

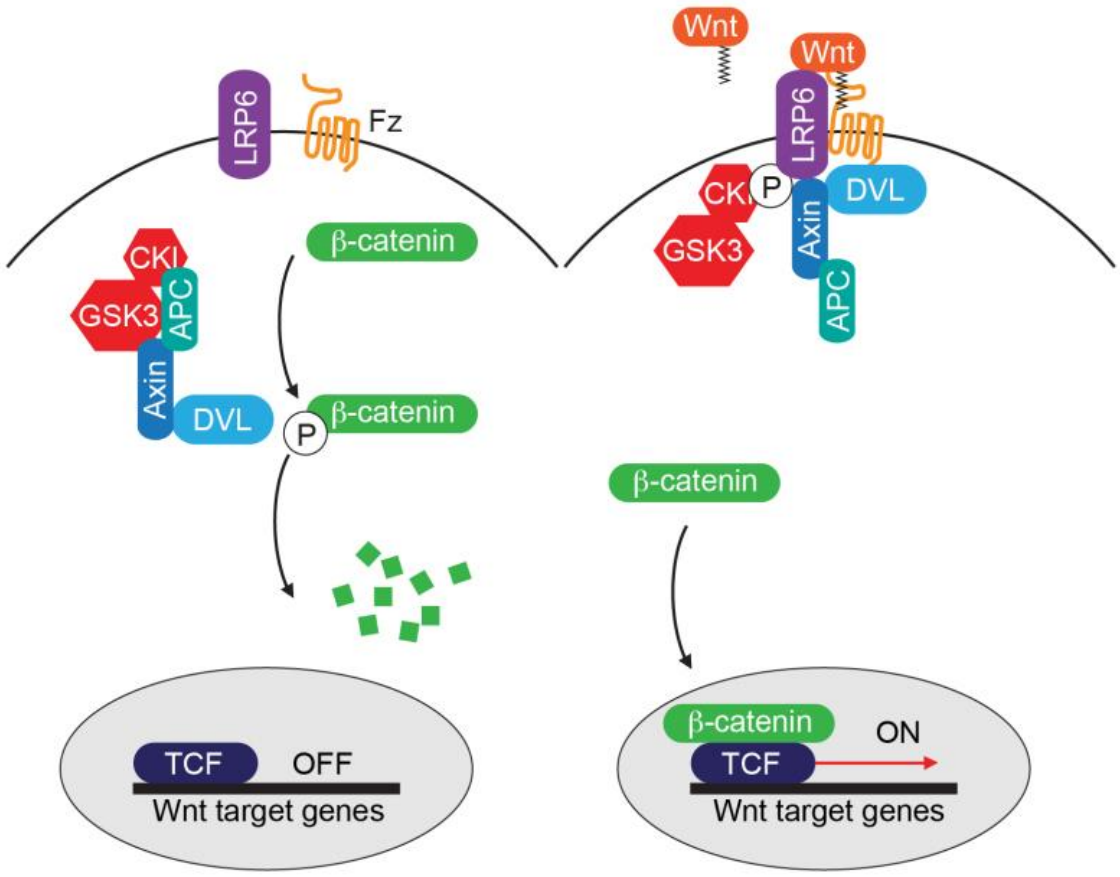

Figure 1-4: Canonical Wnt/β-catenin signaling.

levels inside the cells [48, 49]. APC forms a complex with axin, which recruits β-catenin and facilitates glycogen 3 kinase 3 (GSK3)-β phosphorylation of β-catenin at several serine-threonine residues, and targeting it for ubiquitination by β-transducin repeat-containing protein (β-TRCP). The ubiquitinated β-catenin is eventually degraded by proteosomal degradation. GSK3- β mediated phosphorylation of APC and axin increases the β-catenin binding to their complex, which in turn increases the degradation β-catenin [49-52].

When Wnt-1 binds to its transmembrane frizzled receptor, the activated disheveled protein inhibits GSK3-β. This inhibition of GSK3-β represses β-catenin degradation, and its accumulation in the cytoplasm and nucleus leads to an upregulation of gene expression. Inside the nucleus, β-catenin interacts with T-cell and lymphoid enhancer transcription factors which in turn activate the transcription of several target genes, including proto-oncogenes cyclin D1 and c-myc. Therefore by controlling the cellular accumulation of β-catenin, APC controls the upregulation of genes involved in cell cycle and progression [53-55].

1.2.4.3 Mutations in the APC gene

Most FAP patients are found to carry germline mutations in the 5'-half of the APC gene particularly two codons 1061 and 1309 are known to be mutational hotspots, and they alone account for 11% and 17% of all germline mutations in the APC gene. Bioinformatic analysis showed that 95% of the APC germline mutations are either nonsense or truncating frameshift mutations [43, 56]. Sporadic colon cancer is divided into two categories, the hypermutated (16% cases) and the non-hypermutated (84% cases). Although hypermutated cancers and non-hypermutated cancers progress through different sequences of genetic events, there is some overlap of pathways affected and APC is mutated among both groups of tumors, consistent with its role as a gatekeeper mutation in colon cancer [57].

1.2.5 APC$^{Min/+}$ min mouse model

The workhorse for preclinical colorectal cancer research over the past 30 years has been the APC $^{Min/+}$ mouse. This mouse was identified in 1990 from an ethylnitrosurea (ENU) mutagenesis screen in C57BL/6J mice. APC$^{Min/+}$ mice are considered as a genetically-relevant animal model mimicking human intestinal carcinogenesis [58]. These mice carry a germline nonsense mutation at codon 850 of the mouse homolog of human APC and spontaneously develop multiple polyps in the small and large intestines at the age of 8-12 weeks. The APC$^{Min/+}$ mouse model is unique in that tumors appear spontaneously in the gastrointestinal tract, rather than through induction by a carcinogen. Therefore, this model is particularly advantageous for testing chemopreventive agents targeted against early-stage lesions and is relevant for the design of human chemoprevention clinical trials because an adequate number of adenomas grow to a grossly detectable size within a few months. A large number of studies were conducted using This mouse model has been used to study the efficacy of several drugs including aspirin, as well as berries such as black raspberries, strawberries, lingo berry and extracts like green tea extract, and ginseng [59, 60].

Although considered as a good model for colon cancer, the APC$^{Min/+}$ mouse model has limitations. The main limitation of the model is that the majority of the tumors are located in the small intestine rather than colon. Given the differences between the small intestine and colon in terms of cell biology (e.g. existence of Paneth cells in small intestine), embryonic origin (e.g. foregut for duodenum, midgut for distal small intestine and proximal colon, hindgut for distal colon), and luminal environment (e.g. low pH and

aseptic in upper small intestine, high pH with a diverse microflora in colon), the $APC^{Min/+}$ mice have some limitations to study some of the events and associated changes of human colon cancer scenario. Another disadvantage is the presence of adenomas rather than adenocarcinomas in the intestines of these mice. Due to complications such as anemia and nutrient malabsorption, which shorten the life span of these mice, the adenomas typically do not have time to progress to adenocarcinomas [61].

In spite of these limitations, the molecular events that happen during the initiation and adenoma development are in line with the molecular changes that happen during human colon carcinogenesis, making it one of the most accepted animal model for colon cancer studies [62].

1.2.6 Colon Cancer Chemoprevention

The long period over which colon cancer develops makes it an ideal disease for chemoprevention. Chemoprevention using aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) is being evaluated. There is data to support the risk reduction of colon cancer in people taking NSAIDs, but the mechanism is unknown [63]. Also, these drugs often have toxic side effects with prolonged use which outweighs the benefit of cancer prevention. The U.S. Preventative Services Task Force (USPSTF), recommended against the routine use of aspirin and NSAIDs for risk reduction. Patients who do require aspirin or NSAIDs for other diseases such as cardiovascular health or analgesia may experience the benefits of risk reduction. Hormonal replacement therapy using estrogen has shown to reduce the risk [64].

Dietary changes are accepted as the best prevention strategy for colon cancer [65]. There is evidence that a diet high in fruits and vegetables will reduce the risk of colon cancer. Diet high in fiber has also been linked to a decreased risk. There is also strong evidence supporting the role of physical activity in the reduction of colon cancer. A meta-analysis using data from 21 such studies showed a 27% risk reduction in physically active people [66]. The American Cancer Society and the centers for Disease Control and prevention recommend the public to engage in at least 150 minutes of moderate exercise each week.

1.2.7 Existing treatment approaches and drawbacks

Currently colon cancer is treated with surgical resection, when feasible, in combination with radiation and/or chemotherapy. 5-Fluorouracil (5-FU) and irinotecan are the most common chemotherapeutic drugs used in the treatment of colon cancer. The former agent is a nucleoside analog that inhibits thymidylate synthase, whereas the latter drug is a topoisomerase I inhibitor. The combination blocks DNA replication in cancer cells. The dose-limiting toxicities of these agents include: nausea, vomiting, diarrhea, alopecia, and myelosuppression. Recently, NSAIDs such as sulindac and celecoxib have also been shown to be effective in reducing tumor size in a genetically predisposed mouse model of colon cancer [67]. When a single drug/treatment is rendered ineffective due to resistance, a combination therapy is used to treat colon cancer, wherein multiple hallmarks are targeted to induce cell death. For example, folinic acid (aka leucovorin) and oxaliplatin are used in combination with 5-FU called FOLFOX. Folinic acid enhances 5-FU function in inhibiting DNA replication whereas oxaliplatin crosslinks

DNA. While this combination improves clinical anticancer efficacy, significant systemic and local toxicity remains because the agents are not specific for cancer cells [68, 69].

Surgery is the mainstay curative treatment for patients with non-metastasized colon cancer. The quality of surgery plays a main role in the outcome. In some advanced stages, surgery might be preoperative and chemotherapy, chemo radiotherapy or radio therapy is used prior to or along with the surgical approach. In stage 0 colon cancers that have not grown beyond the inner lining of the colon, surgery to take out the cancer is often the only treatment needed. In most cases this can be done by removing the polyp or taking out the area with cancer through a colonoscope (local excision). Removing part of the colon (partial colectomy) may be needed if a tumor is too big to be removed by local excision [70, 71].

Stage I colon cancers grow deeper into the layers of the colon wall, but they have not spread outside the colon wall itself or into the nearby lymph nodes. Most of the cases in this stage are dealt with surgically the tumor and any cancerous tissue around it.

Stage II colon cancers are the ones which grow through the wall of the colon, and maybe into nearby tissue, but they have not spread to the lymph nodes. Surgery to remove the section of the colon containing the cancer (partial colectomy) along with nearby lymph nodes may be the only treatment needed. In some cases, chemotherapy might be combined with surgery to reduce the risk of reoccurrence.

Stage III colon cancers spread to nearby lymph nodes. Surgery to remove the section of the colon with the cancer (partial colectomy) along with nearby lymph nodes, followed by adjuvant chemotherapy is the standard treatment for this stage. For

chemotherapy, the above mentioned drugs interventions, either the FOLFOX (5-FU, leucovorin, and oxaliplatin) or CapeOx (capecitabine and oxaliplatin) regimens are used most often, but some patients may get 5-FU with leucovorin or capecitabine alone based on their age and health needs [70, 72].

Stage IV colon cancers spread from the colon to distant organs and tissues. They often spread to the liver, but can also spread to other places like the lungs, brain, peritoneum (the lining of the abdominal cavity), or to distant lymph nodes. Surgery, chemotherapy and radiation therapy are used in combination to treat the Stage IV colon cancer, depending on the age and health status of the patient [73, 74].

1.3 Colon cancer cachexia

1.3.1 Clinical characteristics and prevalence

Cancer cachexia is a devastating, multifactorial wasting syndrome that affects around 50-80% of cancer patients. It results in the involuntary loss of skeletal muscle and body fat. Cancer associated cachexia is not included in any national cancer statistics. However, it is agreed that half of the colon cancer patients develop cancer cachexia syndrome. It is also well accepted that cancer cachexia leads to 30-40% of colon cancer related deaths [75-78]. Orexigenic drugs to stimulate appetite have been developed to counteract low appetite in cachexia patients. Currently, ghrelin receptor agonists, Enobosarm (a selective androgen receptor modulator), Nabilone (a synthetic cannabinoid), and omega-3 fatty acids are in various phases of clinical trials to be used in the treatment of cachexia [75].

1.3.2 Mechanisms and Pathophysiology

1.3.2.1 Altered energy balance

Altered energy balance is an essential aspect of the development of cancer cachexia. Cancer profoundly changes the standard homeostatic control of energy expenditure and stability in the body. In patients with cancer cachexia, an elevated resting energy expenditure increases the negative energy balance and is related to tumor metabolism. Tumors compete with other organs in the body for energy reserves. Due to this imbalance, patients with controlled energy intake still lose weight [75]. Additional contributions to elevated energy expenditure include inflammation and metabolic cycling (that is, increased rates of substrate metabolism involving ATP hydrolysis). For example, rates of whole-body glycolysis increases by >300%, as is triacylglycerol or fatty acid cycling [76].

Although skeletal muscle contributes to more than 40% of the body weight and is the main tissue involved in wasting, recent research shows that other organs such as adipose tissue, brain, liver, gut, and heart are also directly involved in the cachectic process. This makes cancer cachexia a multi-organ syndrome [75].

1.3.2.2 Muscle wasting and atrophy

Muscle wasting and atrophy are invariably associated with cancer cachexia. Muscle atrophy leads to the loss of myofibrillar proteins in the muscle cells, and it leads to muscle weakness and fatigue in the patients. Skeletal muscle loss is considered an essential prognostic factor independent of body weight loss during cancer cachexia [79,

80]. The muscle loss is seen as a result of various metabolic alterations, such as abnormal protein synthesis, degradation, and amino acid metabolism in skeletal muscles. Increased apoptosis in the muscle tissue and impaired regeneration is also seen [81, 82]. Ubiquitin-dependent proteasome degradation pathways are mainly associated with muscle wasting [83]. Inflammatory cytokines secreted by immune cells or the tumors further activate pathways that induce muscle breakdown [76].

1.3.2.3 Adipose tissue wasting

In cancer cachexia, loss of skeletal muscle is accompanied by a profound loss of white adipose tissue (WAT). The continuous dissolution of WAT is the result of three main metabolic alterations. Firstly, the increase in lipolytic activity results in the release of glycerol and fatty acids. Then the decrease in the activity of lipoprotein lipase (LPL) in WAT results in the decreased uptake of fatty acids from the blood into WAT. Finally, the *de novo* lipogenesis in the adipose tissue is downregulated with the decreased availability of fatty acids and reduced lipid deposition [84].

An evident interplay between adipokines and myokines is seen in the adipose tissue of cancer cachexia, supporting the possibility of inter-organ signaling between the skeletal muscle and adipose tissue. Some studies have reported that WAT is converted to brown adipose tissue (BAT), mediated by the increased expression of uncoupling protein 1 (UCP1), which switches the mitochondrial electron transport from ATP synthesis to thermogenesis resulting in lipid mobilization and energy expenditure [85, 86].

1.3.2.4 Tumor driven inflammation and pro-cachexic cytokines

In colon cancer patients, the systemic inflammatory response is seen as a main driving force behind the metabolic alterations [87]. Both the immune cells and tumor cells release cytokines, chemokines, and other inflammatory mediators. Interleukin (IL) 6 is one of leading players in driving the progression of cancer cachexia by increasing muscle and fat loss. In the skeletal muscle, chronic IL-6 exposure induces proteasome and autophagy protein degradation pathways that lead to wasting. IL-6 is also indirectly associated with AMP-activated kinase (AMPK) and nuclear factor kappa B (NF-κB) activation. AMPK is one of the central regulators of cellular and organismal metabolism in eukaryotes, and it plays critical roles in regulating growth and reprogramming metabolism, and recently has been connected to cellular processes including autophagy and cell polarity [88]. NF-κB is a transcription factor that regulates the expression of genes related to inflammation and cell survival. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines and plays a key role in regulating the immune response to infection [89]. Several mouse cancer models have clearly demonstrated that blocking IL-6 and associated signaling can attenuate cachexia progression [90]. Additionally, pharmaceuticals targeting IL-6 and associated signaling can relieve some cachectic symptoms in cancer patients. Research with cachectic mice has demonstrated that exercise and nutraceutical administration can

interact with chronic IL-6 signaling during cachexia progression. It is also known to play a main role in tumorigenesis and the production of liver acute phase proteins [87, 91].

1.3.2.5 Gut barrier dysfunction

Although the tumor and related inflammatory responses are the driving factors of cancer cachexia, gut is seen to have an important role [92]. Gut barrier dysfunction and bacterial translocation are associated with cancer and its associated cachexia. The release of bacterial lipopolysaccharide (LPS) and other endotoxins from the bacteria lead to increased release of cytokines by immune cells. Cytokines, in turn, activate the transcription factors involved in wasting of both adipose and skeletal tissues [91].

1.4 Table grapes, grape compounds, and colon cancer

1.4.1 Crop

Grapes are one of the most widely cultivated and consumed fruits in the US and around the world. Table grapes are grapes intended for consumption while fresh, as opposed to grapes grown for wine or juice production. *Vitis vinifera* grapes are the most widely cultivated species of table grape and California accounts for 99% of the US commercial table grape production [93]. Table grape production in the US dates back to 1800s.

US commercially grown table grapes and table grape vineyards cover more than 80,000 acres in the state of California. The dominant US variety of table grape has been the Thompson Seedless, created by Scottish immigrant William Thompson in 1876. The green Thompson Seedless is utilized not only for the table grape market, but also for

raisin production and in some cases wine production. Now a days several varieties are grown widely such as Autumn king, C51-63, Flame seedless, Red Globe, Princess, Sheegene, Scarlet Royal, and Sugraone to name a few. In 2017, more than 7.36 million tons of grapes were grown commercially in the United States. California accounted for nearly 6.48 million tons, or 88%, of these grapes. Other top grape-growing states include Washington and New York.

U.S. per capita consumption of table grapes has increased over the last three decades from 2.9 pounds in 1970 to 8.2 pounds in 2018. The promotion of fruits and vegetables as a healthy dietary choice has helped to increase consumption. In addition, the California Table Grape Commission, established by the state legislature in 1967, provides for research, international trade and marketing, education and advertising. Recent marketing efforts from the California Table Grape Commission, in conjunction with funds from the USDA Market Access Program, have focused on expanding world demand for U.S. table grapes through increased promotion and encouraging retailers to handle more varieties. U.S. table grapes are marketed mainly from May through December with Southern California accounting for much of the early season crop and Central California accounting for the later supply.

1.4.2 Phytochemicals and putative health benefits.

1.4.2.1 Nutritional composition

Fresh grapes contain ~82 % water, 12–18 % sugars, and 0.2–0.8 % organic acids, mainly tartaric and malic acid. Grapes are an excellent source of vitamins C and K and

contain around 50 calories per serving [94]. Consumption of grapes has been reported to be associated with positive health benefits due to the presence of large number of different phytochemicals.

Over 1600 compounds have been identified in grapes, including anthocyanins, catechins, ellagic acid, lutein, lycopene, quercetin, and other potent antioxidants. These phytochemicals are found in the skin, flesh and seeds of the table grapes. In grapes phenolics are the third most abundant compounds after carbohydrates and acids. Their distribution in juice, pulp, skin and seeds is around 5%, 1%, 30% and 64 % respectively. The phytochemical composition differs among different colored grapes. Red grapes can produce anthocyanins while green grapes cannot. Indeed the total phenolic content in the red grape skins is higher than that of green grapes. The phenolic content depends on and is largely influenced by a variety of factors such as genetic factors, environmental conditions, and the stage of development [80].

1.4.2.2 Polyphenols

Polyphenols are the most abundant phytochemicals in table grapes. Polyphenols are divided into several classes depending on their structures such as phenolic acids, flavonoids, and stilbenes. The most common phenolic acids occurring in *Vitis* usually comprise benzoic acid derivatives and cinnamic acid derivatives. Natural phenolic acids in grapes, occurring either in the free or conjugated forms, generally appear as esters or amides. Some important phenolic acids include caffeic acid, coumaric acid, and ferulic acid [93].

Table grape flavonoids are mostly conjugated in glycosylated or esterified forms but can also occur as aglycones. The flavonoids in grapes are grouped into flavonols, including quercetin, kaempferol, and myricetin, flavones such as luteolin and apigenin, flavanols like catechins, epicatechin, epigallocatechin, and epicatechin gallate, flavanones such as naringenin, anthocyanidins, or isoflavonoids consisting of genistein, daidzein, dihydrodaidzein, and equol.

Anthocyanins are one of the most widely distributed phytonutrients in the plant kingdom. They are natural, non-toxic water-soluble flavonoid pigments. They are responsible for the red and black colors found in the skins of blue, red, or black grapes. Cyanidin, peonidin, malvidin, petunidin, and delphinidin are the major anthocyanins found in table grapes [93].

Quercetin is an important flavonol present in glycoside form in grape skins. Other simple flavonoid aglycones, myricetin, isorhamnetin and kaempferol also exist in grapes. Flavanols, found in the seed and skin, are the most abundant class of flavonoids in grapes. The five common flavan-3-ol monomers found in grape skins are catechin, gallocatechin, epicatechin, epigallocatechin, and epicatechin 3-gallate [93].

Stilbenes are phenolic compounds that structurally exhibit two aromatic rings linked by an ethylene bridge. Resveratrol is one of the most studied phytochemicals for its health benefits. It appeared in more than 2000 publications over the past few years and has been linked to a wide variety of health benefits including cancer prevention. It is synthesized in response to pathogenic attack and environmental stress such as injury, UV

irradiation, or fungal infection. It is synthesized in the leaf epidermis and the skin of grape berries, but not in the flesh, it exists in two isomers: the *cis* and *trans* [93].

With having such a wide array of health benefiting phytochemicals, regular consumption of grapes has been linked to reduced risk of cardiovascular disease. Grapes have also been shown to have a protective effect over atherosclerosis by lowering the plasma lipid levels. There is strong evidence of the anti-cancerous effect of grape compounds like resveratrol and grape seed extract. Other putative health benefits include protective effect on neurodegenerative disorders, improved cognitive function, anti-viral effect and improved immune function [93, 95-99].

1.4.3 Colon cancer chemoprevention by grape polyphenols

There is epidemiological evidence that long-term intake of polyphenols can reduce the incidence of cancers and chronic disease. Over the past two decades, polyphenols are being increasingly investigated for their ability to reduce the risk of chronic diseases, because of their free radical scavenging capacity, which, among other biological effects, increases antioxidant activity and prevents cellular damage to DNA. Since oxidative damage to DNA is considered as one of the crucial steps to onset of cancer, the anti-oxidant effect of polyphenols led to many studies investigating their chemo preventive properties [97].

Resveratrol and grape seed extract (GSE) have been studied for their anticancer activity. The anti-cancer activity of resveratrol was first reported by Jang *et al*. Since then there have been many studies investigating anticancer and cancer chemo preventive efficacy in numerous cancer models in cell culture. Chemopreventive potential of GSE, a

widely used dietary supplement has been studied extensively in a variety of cancer types. GSE (> 50 μg/ml) suppressed proliferation of LoVo and HT-29 human colon cancer cell lines. GSE induced G1 phase arrest and mitochondrial/caspase mediated apoptosis in cancer cells. Additionally, we have also shown that GSE induced apoptosis in HCT-116 human colon cancer cells. GSE might thus exert its beneficial effects by suppressing proliferative and elevating apoptosis pathway [93, 97, 100].

There are several *in vivo* studies looking at the anti-cancer effect of grape compounds such as GSE, red grape extract, and grape fiber. Velmurugan *et al*, evaluated the effect of GSE supplementation in $APC^{Min/+}$. Supplementation with 0.5% GSE (wt/wt) mixed AIN-76A diet for 6 weeks reduced the number of intestinal polyps by 40 % compare to control mice. GSE also decreased polyp growth where the number of polyps of 1 to 2 mm in size decreased by 42% and greater than 2 mm in size by 71%, without any significant change in polyps less than 1 mm in size. Immunohistochemical analyses of small intestinal tissue samples revealed a decrease (80%–86%) in cell proliferation and an increase (four- to eight-fold) in apoptosis. GSE feeding also showed decreased protein levels of cyclooxygenase-2 (COX-2) (56%–64%), inducible nitric oxide synthase (iNOS) (58%–60%), and β-catenin (43%–59%) but an increased Cip1/p21-positive cells (1.9- to 2.6-fold). GSE also decreased cyclin D1 and c-myc protein levels in small intestine. Together, these findings show the chemopreventive potential of GSE against intestinal polyp formation and growth in $APC^{Min/+}$ mice, which was accompanied with reduced cell proliferation and increased apoptosis together with down-regulation in COX-2, iNOS, β-catenin, cyclin D1, and c-myc expression, but increased Cip1/p21[101].

Sanchez *et al*., assessed the chemopreventive efficacy of lyophilized red grape pomace containing proanthocyanidin-rich dietary fiber [grape antioxidant dietary fiber (GADF) in $APC^{Min/+}$ mice. Mice were fed a standard diet (control group) or a 1% (w/w) GADF-supplemented diet (GADF group) for 6 weeks. GADF supplementation greatly reduced intestinal tumorigenesis, significantly decreasing the total number of polyps by 76%. They also observed the impact of GADF on the size of the polyps similar to the GSE study. Comparison of microarray expression profiles of GADF-treated and non-treated mice revealed the putative molecular mechanisms underlying the inhibition of intestinal tumorigenesis. GADF supplementation was mainly linked with the induction of G1 cell cycle arrest and the downregulation of genes related to the immune response and inflammation [102].

Another study looked at the impact of anthocyanin-rich red grape extract containing oenocyanin. $APC^{Min/+}$ mice supplemented with 0.3 % oenocyanin for 12 weeks had 50% reduction in overall tumors. They found that the proliferation index in colonic adenomatous crypts, as reflected by Ki-67 staining, was significantly decreased from 88.14% in control mice to 75.6 ± 4% in mice on oenocyanin ($P = 0.014$). Expression of Akt in small intestinal adenomas in mice on oenocyanin was reduced by 54% ($P = 0.003$) when compared to controls [103].

1.5 Purpose and significance

Colon cancer is the 3rd most prevalent cancer and it kills over 50,000 Americans every year. Conventional chemotherapies can significantly reduce tumor growth but have serious adverse side effects. Current chemotherapeutic approaches are designed to target

only a single hallmark of cancer cells or a combination therapy are used to target multiple hallmarks. Despite the use of surgical resection and chemotherapy, nearly 50% of patients develop recurrent disease, highlighting the need for improved therapies. Further, the cost of treating colon cancer is estimated to cost around $150,000 per person per year.

Emerging evidence suggests that phytonutrient plant foods reduce the risk of colon cancer. Grapes, one of the most widely consumed fruits in the world and the US with a per capita consumption of 8.2 pounds. They contain a variety of phytonutrients such as anthocyanins, proanthocyanidins, stilbenes, flavan-3-ols, and flavonols. These phytochemicals are found in the skin, flesh and seeds of table grapes. We have recently shown that grape compounds (resveratrol and GSE) can target cancer stem cells *in vitro* and *in vivo* using a carcinogen-induced rodent model of colon cancer. There were also studies looking at the anti-tumorigenic effect of grape fiber, red grape extract in animal models. However, no information is available on anti-colon cancer activity of whole multi-colored grapes *in vivo.* The main purpose of this study is to investigate the table grapes (freeze dried grape powder – FDGP) efficacy against intestinal tumorigenesis in $APC^{Min/+}$ mice, a rodent model of genetically-induced colon cancer. The other purpose of the study is to assess the efficacy of FDGP in suppressing the progression of cachexia, which is a devastating side effect of cancer driven by tumor with a drastic impact on a patient's life. The results from this study would provide first evidence of anti-tumorigenic and anti-cachectic effect of table grapes in an animal model. It would be valuable for future clinical investigations to study the cancer prevention potential of grapes.

1.6 Hypothesis and objectives

I hypothesize that table grapes will inhibit colon cancer stem cells *in vitro*. I further hypothesize that that dietary supplementation with FGDP will reduce the intestinal tumorigenesis in an animal model and it will suppress the progression of cancer associated cachexia. In order to test the hypothesis, I propose the following specific aims:

1) To investigate the anti-cancer properties of FDGP extract on colon CSCs *in vitro* (Chapter 2).

2) Determine whether the dietary supplementation of 3% or 6 % FDGP will reduce the intestinal tumorigenesis in a human relevant rodent colon cancer model the $APC^{Min/+}$ mice and to further determine the molecular pathways effected (Chapter 3).

3) To evaluate the efficacy of FDGP on the progression of colon cancer cachexia in $APC^{Min/+}$ mice and to look at the impact on body weight, endotoxemia, gut barrier dysfunction and inflammation of the animals (Chapter 4).

Chapter 2

Freeze dried grape powder extract (GPE) exhibits anti-cancer activity against colon cancer stem cells

2.1 Abstract

Cancer stem cells (CSCs) have been shown to be responsible for the initiation and progression of tumors in a variety of cancers. We have previously demonstrated that the grape bioactive compound resveratrol (RSV) potentiates grape seed extract (GSE) induced apoptosis in colon cancer cells *in vitro* at physiologically relevant concentrations. However, the anti-cancer efficacy of whole table grapes is not known. So, we tested the *in vitro* anti-cancer efficacy of the freeze-dried grape powder (FDGP) against isolated human colon CSCs. Colon CSCs were treated with various doses (10-75 μg/mL) of grape powder extract (GPE) made from FDGP. GPE suppressed proliferation, sphere formation, and elevated apoptosis in colon CSCs. Furthermore, shRNA-mediated knockdown of p53, a tumor suppressor gene, in colon CSCs did not alter the efficacy of GPE, which indicates that these anti-proliferative and pro-apoptotic effects are independent of p53. These findings demonstrate the potential colon CSC inhibitory efficacy of FDGP and support the development of future pre-clinical studies to examine the colon cancer preventive effects of table grapes.

2.2 Introduction

Colon cancer is the second leading cause of cancer-related deaths in the United States. For the year 2018, the American Cancer Society estimated that there would be about 157,803 new cases and 50,310 deaths due to colon cancer [104]. Colon cancer is caused by the stepwise accumulation of mutations in tumor suppressor genes and oncogenes, resulting in the formation of benign adenomas which can ultimately progress to adenocarcinoma. There is increasing evidence that most cancers including colon cancer derive from cancer stem cells (CSCs). CSCs, including colon CSCs, mimic the functionality of healthy adult stem cells maintaining their un-differentiated state while dividing asymmetrically. Compared to bulk tumor cells, CSCs are resistant to conventional therapies, and thus lead to relapse of cancer in most patients. Agents that target CSCs could be more efficacious cancer treatment approaches and are more effective in preventing recurrence [42].

Geographic differences in colon cancer rates and temporal changes in risk among immigrant populations suggest that diet and lifestyle strongly influence the occurrence of colon cancer. Current evidence indicates that higher intake of certain diets including those high in fat or red meat and a lower intake of fruits and vegetables is linked to a higher risk for colon cancer. Colon cancer has a long latency period before it is detected There is increasing evidence that dietary bioactive compounds from fruits, vegetables, and herbs have preventive effects against a variety of cancers including colon cancer. A meta-analysis of case-control studies suggests that fruit consumption was associated with a 13% decrease in colon cancer risk [105, 106].

Approximately 74 million tons of grapes are consumed worldwide each year [107]. Table grapes contain high amounts of a large number of phenolic compounds, including phenolic acids, stilbenes, flavanols, flavonols, and anthocyanins [108]. Red grapes are rich in resveratrol (RSV), a stilbene that has been shown to have anti-cancer properties in a variety of models, and human studies. We have previously reported that RSV and grape seed extract (GSE) suppressed proliferation and induced apoptosis via p53 activation in HT-29 and SW-480 human colon cancer cell lines [100]. However, no *in vitro* or *in vivo* studies done to date have assessed the anti-cancer effect of whole table grapes against colon cancer. In the current study, we examined the anti-cancer activity of an extract of freeze-dried table grapes (GPE) against human colon cancer stem cells (CSCs).

2.3 Materials and methods

2.3.1 Freeze dried grape powder and grape powder extract

The freeze-dried grape powder (FDGP) used in this study was supplied by the California Table Grape Commission. FDGP was produced from fresh red, green, and black seeded and seedless table grapes (*Vitis vinifera*) that are mixed in proportion to their annual production and consumer consumption. Due to the presence of large amounts of sugar, fiber, and other insoluble material in FDGP, an extract was prepared for cell culture experiments. FDGP was extracted in 6 volumes of water by stirring for 3 h. The supernatant was collected by centrifugation and the solids were re-extracted twice more. The supernatant was applied to a column containing Diaion resin (Sigma Aldrich, St.

Louis, MO) to remove sugars. The column was washed with water to elute sugars (based on the cloudiness of the eluant). The grape bioactives fraction (purple-colored band) was eluted with methanol. The methanol was removed in a rotary evaporator, and the residual water removed by using a freeze-dryer to yield grape powder extract (GPE). Approximately 0.8 g of GPE was obtained from 250 g of FDGP. The stock of 10mg/ml was made in DMSO for use in *in vitro* assays.

2.3.2 Chemicals

Fetal bovine serum (FBS) was purchased from HyClone (Pittsburgh, PA). All other chemicals and reagents were purchased from Sigma Aldrich (St Louis, MO).

2.3.3 Cell lines

Colon CSCs, positive for the cancer stem cell markers CD 133, CD 44, and ALDH1b1, were obtained from Celprogen (San Pedro, CA). To maintain the cells in their undifferentiated state, colon CSCs maintenance media and special coated cell culture flasks obtained from Celprogen were used. Cells were maintained in incubation at 37 ^{0}C and 5 % CO2 as described earlier. Cell cultures at approximately 80 % confluence were used for all *in vitro* experimental procedures.

2.3.4 Lentiviral shRNA-mediated attenuation of p53 in colon CSCs

Colon CSCs were infected with lentiviral particles encoding shRNA targeting p53 obtained from Santa Cruz Biotechnology (Santa Cruz, CA) according to the manufacturer's protocol. Briefly, colon CSCs were infected at a multiplicity of infection of 10 in CSC growth medium containing 5 μg/mL of polybrene (for selection of cells

with successful lentiviral induction) at 37 °C and 5 % CO_2. After 24 hours, the spent media was replaced with fresh media and the cells were cultured for 2 days. The transduced cells were selected in the presence of puromycin (7.5 µg/mL) for 5 days.

2.3.5 Cell viability

Resazurin Cell Viability

The cellular viability was evaluated using the resazurin assay (Cell Signaling Technology, MA). Briefly, 10,000 colon CSCs were seeded in 96- well plates, and after 24 hours, cells were treated with GPE at 10, 25, 50 and 75 µg/mL. DMSO was used as solvent control. After 24 hours the media was replaced with 100 µL fresh media, and 10 µL of resazurin solution was added to each well. The plate was incubated at 37°C for 2 hr, and the relative fluorescent units (RFU) were measured at 60 min and 120 min, using a plate reader (Clariostar, BMG tech) at parameters: Ex=530-570 nm, Em=590-620 nm.

BrdU assay

Cell viability was also assessed by using the BrdU (5-bromo-2'-deoxyuridine) assay kit from Cell Signaling Technology (Danvers, MA). Briefly, cells were plated at a density of 1 X 10^5 per well in 12-well plates. Media was replaced after 24 hours with colon CSCs media without serum (Celprogen) and dosed with GPE at 10, 25, 50 and 75 µg/mL. DMSO was used as solvent control. After 24 hours, BrdU incorporation was assayed as per the manufacturer's protocol.

2.3.6 Apoptosis

Apoptosis was assayed using the Caspase Glo 3/7 assay kit (Promega, Madison, WI). Briefly, 100,000 colon CSCs were seeded in a 12-well plate and incubated for 24 hours. They were dosed with GPE at 10, 25, 50 and 75 µg/mL. DMSO was used as solvent control. After 24 hours, cells were trypsinized, and approximately 20,000 cells from each treatment were incubated with 100 µL of Caspase Glo 3/7 reagent (Promega, Madison, WI) for 30 minutes in a 96-well plate. The luminescence of each sample was measured using a Clariostar plate reader (BMG lab tech, NC). The experiment was performed in triplicate.

2.3.7 Sphere formation

Briefly, colon CSCs (10,000 cells per well) were cultured in stem cell specific serum free media (2 mL) in an ultra-low attachment six well plates (Costar) for 10 days. GPE at concentrations of 10, 25, 50 and 75 µg/mL was added after 6 hours of seeding. At the end of 10 days, the number of spheres was assayed using a phase contrast microscope.

2.3.8 Statistical analysis

All experiments were performed in triplicate. Data are expressed as the mean ± standard error of the mean (SEM). Data were analyzed by one-way ANOVA using Tukey least square difference (LSD) with IBM SPSS software v22.0 (Armonk, NY).

2.4 Results

2.4.1 GPE suppressed proliferation

We evaluated the anti-proliferative effect of GPE using the resazurin cell viability assay and the BrdU incorporation assay. GPE caused a dose- dependent suppression of cell proliferation in colon CSCs (**Figure 2-1 A**). Similar results were observed using the BrdU assay results, there was a dose dependent suppression in proliferation of CSCs by GPE (**Figure 2-1B**).

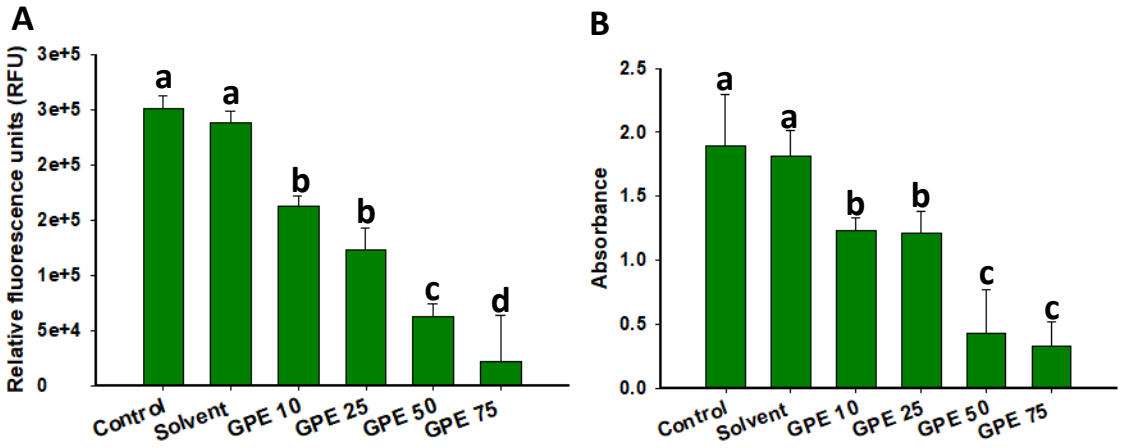

Figure 2-1: Effect of Grape powder extract (GPE) on colon CSCs proliferation. (A) Colon CSCs were treated with GPE for 24 hours and proliferation was measured by Resazurin assay. (B) Colon CSCs were treated with GPE for 24 hours and their proliferation was measured by BrdU assay. Values are in means ± SE (n=3). Values that do not share a common superscript letter are different ($p<0.05$) by one-way ANOVA and Tukey least square difference post-test.

2.4.2 GPE Induced Apoptosis in Colon CSCs

Apoptosis is an important barrier to developing cancer. In our study, we evaluated the pro-apoptotic effects of GPE against colon CSCs using the Caspase 3/7 Glo assay, which measures the activity of caspases 3 and 7. GPE caused a dose-dependent increase in apoptosis in colon CSCs ($p<0.05$, **Figure 2-2**). At the highest dose, GPE increased apoptosis by 6-fold compared to control.

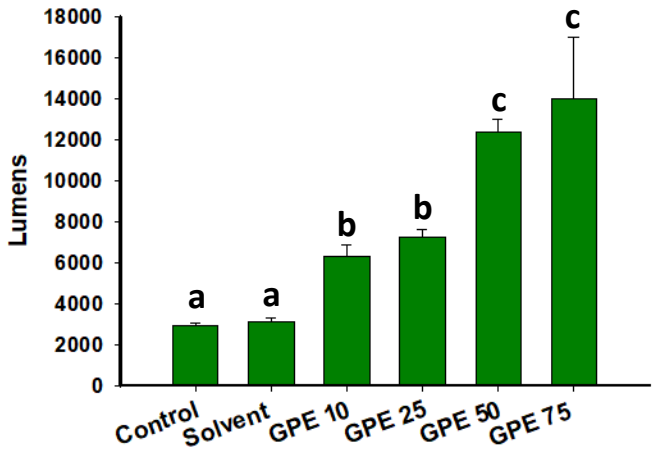

Figure 2-2: Effect of Grape powder extract (GPE) on induction of apoptosis in colon CSCs. The cells were treated with 10, 25, 50 and 75 µg/mL conc of GPE for 24 hours and the apoptosis induction was assayed by Caspase 3/7 Glo assay. Values are in means ± SE. Values that do not share a common superscript letter are different ($p<0.05$) by one-way ANOVA and Tukey least square difference post-test.

2.4.3 GPE suppressed sphere formation ability in colon CSCs

Self-renewal is a crucial property of CSCs. The sphere formation assay is used to measure the self-renewal of CSCs. We treated colon CSCs with GPE at 10, 25, 50 and 75 µg/mL, respectively. At concentrations greater than 25 µg/mL, GPE prevented the formation of spheres (**Figure 2-3A**). Figure 2-3B shows the representative images from

the sphere formation assay demonstrating complete suppression in comparison to the control.

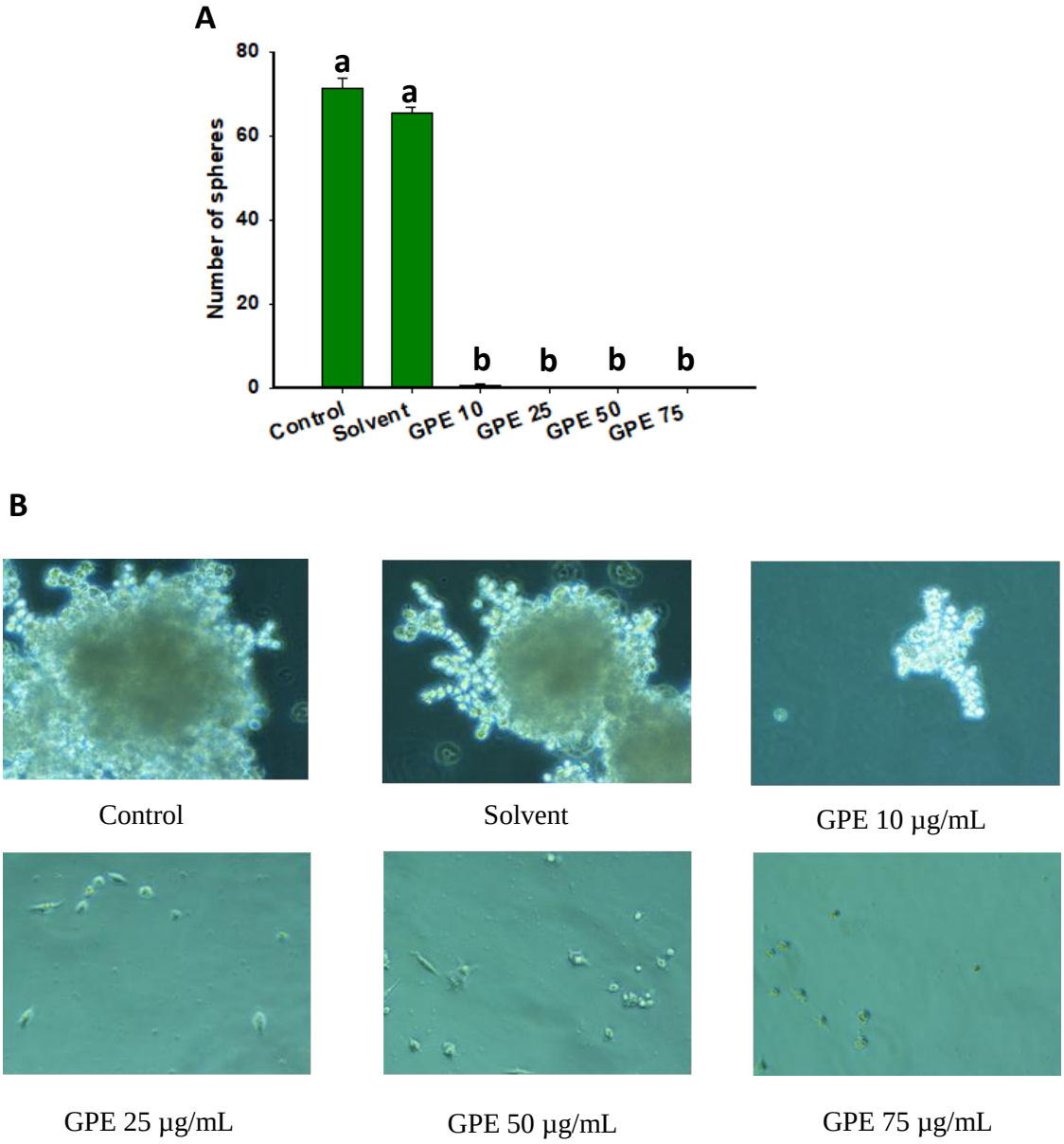

Figure 2-3: Effect of grape powder extract (GPE) on colon CSCs sphere formation ability. (A) The spheres were counted after 10 days post treatment with GPE at respective doses. (B) Representative pictures taken at 100x magnification are shown for Control, Solvent and GPE doses. Values are in means ± SE. Values that do not share a common superscript letter are different ($p<0.05$) by one-way ANOVA and Tukey least square difference post-test.

2.4.4 GPE efficacy in the absence of p53

To determine the requirement of p53 in the CSC inhibitory effects of GPE, we used a lentiviral p53-shRNA construct to attenuate p53 expression. Reduced p53 expression had no impact on GPE -mediated suppression in the proliferation or the induction of apoptosis in the colon CSCs. BrdU assay was performed after dosing the colon CSCs with shRNA attenuated p53 with 25, and 50 µg/mL of GPE for 24 hours. At both doses there was significant suppression in the proliferation of the colon CSCs with shRNA attenuated p53 compared to control ($p<0.05$, **Figure 2-4 A**). Induction of apoptosis was assayed by using Caspase 3/7 Glo assay after treating the cells with 25 and 50 µg/mL of GPE for 24 hours. Both 25, and 50 µg/mL of GPE significantly elevated the induction of apoptosis in p53 attenuated colon CSCs compared to the control ($p<0.05$, **Figure 2-4B**). These results indicate that GPE induced suppression in proliferation and inducted of apoptosis in the colon CSCs occurs via a p53-independent mechanism.

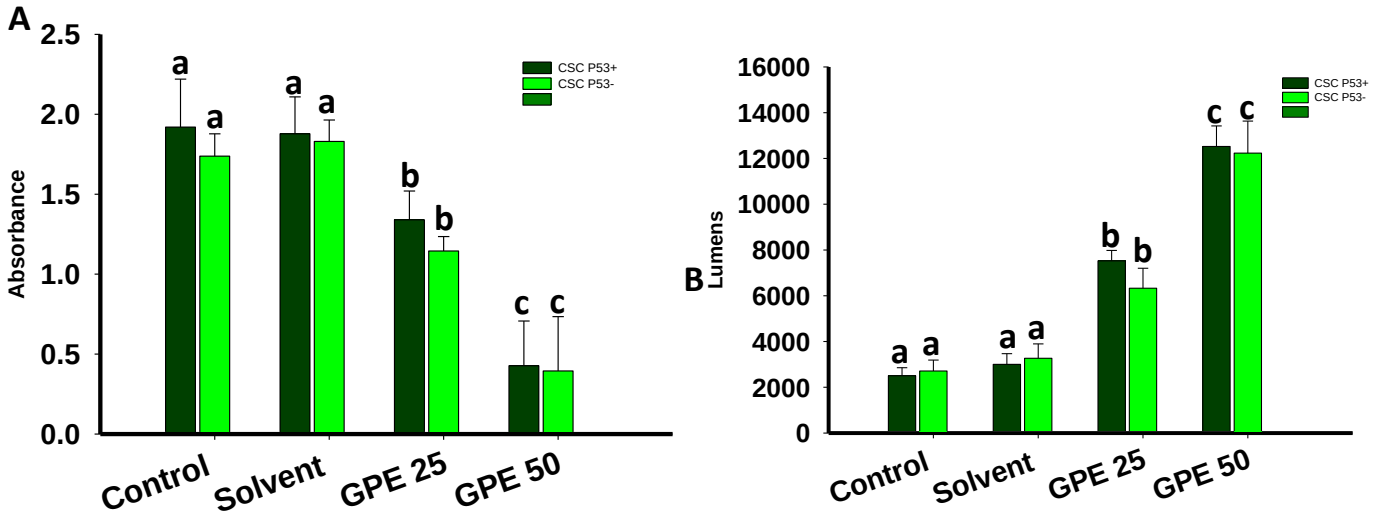

Figure 2-4: GPE suppressed proliferation and induced apoptosis in colon cancer stem cells (colon CSCs) independent of p53. (A) Colon CSCs were treated with 25 and 50 ug/ml of GPE for 24 hours, and the proliferation was assessed by BrdU assay. (B) Colon CSCs were treated with 25 and 50 ug/ml of GPE for 24 hours and the apoptosis induction was evaluated by Caspase 3/7 Glo assay. Values are in means ± SE. Values that do not share a common superscript letter are different ($p<0.05$) by two-way ANOVA and Tukey least square difference post-test.

2.5 Discussion

The main objective of present study was to evaluate anticancer efficacy of GPE on CSCs, so that it can be used to translate into *in vivo* preclinical models. In this study, we found that the grape powder extract made from freeze dried whole table grape powder exhibit anti-cancer properties by suppressing the proliferation and inducing apoptosis in a dose dependent manner in colon CSCs. The quantitative analyses showed that the FDGP contains several bioactive compounds including flavanols (catechin, epicatechin), anthocyanins (cyanidin, malvidin, peonidin), flavonols (isorhamnetin, kaempferol, quercetin) and stilbenes (resveratrol). The suppression of proliferation by GPE in colon CSCs can be attributed to the presence of these phytochemicals, which have been previously shown to suppress proliferation in colon cancer cells individually and in combination in *in vitro*, *in vivo*, and in human studies [109]. It is also possible that these compounds could work together and lead to a greater effect.

Beneath the complexity of every cancer lie critical events including deregulated cell proliferation and suppressed apoptosis that provides a platform necessary to support further neoplastic progression. Also, the loss of apoptotic function is a major contributor to resistance of cancer cells to cytotoxic chemotherapeutic agents. GPE showed a dose dependent effect on suppressing the proliferation and inducing the apoptosis in colon CSCs. The results from the sphere formation experiments support that GPE attenuates the sphere forming ability, an important self-renewal capability of CSCs. It can be due to GPEs effect on stemness related pathways.

We also show that this anti-cancer effect of GPE is independent of p53 protein. This is particularly important because in late/metastatic stages of colon cancer p53 is mutated, and some anti-cancer drugs like sulindac are dependent on functioning p53 for exerting their anti-tumor effect.

This study provided, valuable *in vitro* data that would help for future pre-clinical testing of grape based cancer prevention treatments. Additional mechanistic studies are needed to ascertain how GPE inhibits CSC proliferation and induces CSC apoptosis. In addition, a dose-dependent *in vivo* study with FDGP would demonstrate the relevance of these *in vitro* findings to the *in vivo* situation.

Chapter 3

Freeze-dried grape powder (FDGP) supplementation suppresses intestinal tumorigenesis in $APC^{Min/+}$ mouse

3.1 Abstract

Colon cancer is the 3rd most prevalent cancer and it kills over 50,000 Americans every year. Conventional chemotherapies can significantly reduce tumor growth but have serious adverse side effects. Emerging evidence suggests that phytonutrient plant foods reduce the risk of colon cancer. Grapes, one of the most widely consumed fruits in the world, contain a variety of phytonutrients such as anthocyanins, proanthocyanidins, stilbenes, and flavanols. We have recently shown that grape compounds (resveratrol and grape seed extract) and grape powder extract can target cancer stem cells *in vitro* and *in vivo* using a carcinogen-induced rodent model of colon cancer. However, no information is available on anti-colon cancer activity of multi-colored grapes *in vivo*. Herein, I have investigated table grapes (Freeze dried grape powder – FDGP) efficacy against intestinal tumorigenesis in $APC^{Min/+}$ mice, a rodent model of genetically-induced colon cancer model. We found that dietary supplementation of FDGP (at 3 or 6% w/w) suppressed the total number of intestinal polyps (early markers of colon cancer) by 55%. This is significant because the aspirin (200 ppm; human equivalent dose), a drug used for the prevention of colon cancer in human, decreased intestinal polyps by 39% in this study. FDGP supplementation also decreased polyp growth, and treated mice had fewer polys with diameter > 1 mm compared to control mice. Reduced tumorigenesis was associated with downregulation targets in the Wnt /β-Catenin pathway, such as cyclin D1, c-Jun, and c-Myc expression. Expression of genes linked to angiogenesis, VEGF and HIF-1α, were

also lower in FDGP-treated mice. In conclusion, the present study suggests the potential usefulness of table grapes for the chemoprevention of intestinal/colorectal cancer.

3.2 Introduction

Chemoprevention has emerged as a pragmatic approach to reduce the risk of various cancers including colorectal cancer one of the most common malignancies in the US. Familial adenomatous polyposis a hereditary colorectal cancer (CRC) predisposition syndrome is caused by mutations in adenomatous polyposis coli (*APC*) gene and is characterized by the progressive development of numerous adenomas in colon progressing to CRC during later stages [56]. Animal models of intestinal tumorigenesis are useful to study the pathogenesis and to develop the strategies to control the malignancy including chemoprevention. $APC^{Min/+}$ mice, one of the most studied models of intestinal tumorigenesis, carry a germ line nonsense mutation at codon 850 of the mouse homolog of the human *APC* gene and spontaneously develop multiple polyps in the small and large intestines at the age of 10 to 12 weeks. The *APC* gene is a tumor-suppressor gene, and its mutation has been directly implicated in the development of both human familial adenomatous polyposis (FAP) and sporadic colon cancer. Hence, $APC^{Min/+}$ mice are a useful model for analysis and prevention of human CRC. Therefore, in this study, we used the $APC^{Min/+}$ mouse model to investigate the inhibitory activity of FDGP against intestinal tumorigenesis and the possible mechanisms involved in the inhibitory action [110].

Grapes are consumed worldwide and contain a variety of polyphenols such as anthocyanins, proanthocyanidins, stilbenes, and flavanols. The chemopreventive activity

of quercetin, cyanidin, epicatechin, and resveratrol found in table grapes have been studied extensively [111-115]. We have recently shown that grape compounds (resveratrol and grape seed extract) can target cancer stem cells *in vitro* and *in vivo* using AOM induced rodent models of colon cancer. We observed a significant reduction in colon tumors in the mice supplemented with RSV-GSE. However, no information is available on the efficacy of table grapes for their anti-cancer effects in prevention modality *in vivo* [100].

The overall goal of this project was to determine whether freeze-dried multi-colored table grape powder (FDGP) consumption can suppress colon tumorigenesis in the $APC^{Min/+}$ mice, and to gain insight into the underlying mechanisms of action.

3.3 Material and methods

3.3.1 Freeze dried grape powder

The freeze-dried grape powder (FDGP) used in this study was supplied by the California table grape commission. FDGP was produced from fresh red, green, and black seeded and seedless table grapes (*Vitis vinifera L.*) that were mixed in proportion to their annual production and consumer consumption. Due to the hygroscopic nature, the powder was tightly stored in moisture proof containers at -70 ^{0}C. Nutritional analysis was conducted at the (National Food Laboratory, Livermore, CA). Table 3-1 show the phytochemical composition of FDGP

Table 3-1. Phytochemical composition of FDGP

Class	Compound	Level (mg/kg grape powder)
Flavan-3-ols		
	Catechin	77.4 ± 12.5
	Epicatechin	58.9 ± 10.2
Anthocyanins		
	Cyanidin	266.7 ± 27.1
	Malvidin	219.3 ± 31.3
	Peonidin	47.63 ± 8.62
Flavonols		
	Isorhamnetin	13.95 ± 1.84
	Kaempferol	7.38 ± 0.62
	Quercetin	148.7 ± 10.5
Stilbenes		
	Resveratrol	13.6 ± 1.1

3.3.2 Animal study and experimental diets

Four-week-old male $APC^{Min/+}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained in the animal house facility at the Pennsylvania State University, University Park. Animals were maintained in 12-hour light/dark cycles with free access to water and food (AIN-93G diet from Harlan Diets). After one week of acclimatization, animals were randomly divided into four groups of 12 animals each and fed AIN-93G or 6% w/w FDGP, 3% w/w FDGP, or 200 ppm aspirin. The animals were weighed weekly and food intake was monitored. The Institutional Animal Care and Use Committee at Pennsylvania State University approved all experimental procedures involving the use of mice.

3.3.3 Tumor counting and tissue collection

At 19 weeks of age, after 12 weeks of dietary intervention, the animals were killed by CO_2 asphyxiation followed by cervical dislocation; the intestine was spread onto filter paper, opened longitudinally with fine scissors, and cleaned with sterile ice-cold RNAse-free PBS using a soft brush. The small intestine was divided into three equal parts (proximal, middle, and distal) and the large intestine consisting of cecum and colon. All intestinal polyps were counted under a dissecting microscope, and their sizes were measured with a digital caliper. For future immunohistochemistry, about 1 cm of the duodenum, jejunum, ileum, proximal and distal colon tissues were Swiss rolled and were fixed with 10 % buffered formalin. For gene expression analysis parts of the duodenum, jejunum, ileum, proximal and distal colon tissues (about 1 cm) were collected in RNA later (Invitrogen, Carlsdad, CA). Tissues were collected from normal looking tissue (without any tumors) and tissue having tumors (3-4 tumors). Parts of liver, cecum, serum, digesta were all flash frozen in liquid nitrogen and were stored at -70^{o}C for future analyses. Any remaining tissues (heart, kidneys, testis, stomach were weighted and fixed with 10 % buffered formalin.

3.3.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qPCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA was quantified using Clariostar plate reader (BMG Labtech, VA) and reverse-transcribed to cDNA using an RT^{2} HT First Strand Kit (SA Biosciences, Valencia, CA). Quantitative PCR was performed using a Roche light cycler PCR system (Basel, Switzerland) and PerfecTa SYBR Green

Supermix from Quanta bio (Beverly, MA). The reactions included either 10 µL SYBR® Green PCR Master Mix, 0.2 µL of both the forward and reverse primers and 2 µL diluted cDNA (10 ng/reaction). Reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60 °C for 10 min, 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Data were recorded and analyzed with Roche light cycler analysis software (Basel, Switzerland). Relative gene expression was determined using the $2^{-\Delta CT}$ method (ct-cycle threshold) where ΔCT = (CT, target − CT, reference), with β-actin as the reference gene. The results were presented as a fold change normalized to the control values.

Table 3-2. Primers used in this study

Gene product	Forward (5'→ 3')	Reverse (5' → 3')
Cyclin D1	GCAAGCATGCACAGACCTT	GTTGTGCGGTAGCAGGAGA
C-myc	GCTCAAAGCCTAACCTCACAA	AAAGAAAGAAGATGGGAAGCA
C-jun	GGCTGTTCATCTGTTTGTCTTCA	TTCTTTACGGTCTCGGTGGC
Cox-2	CAGACAACATAAACTGCGCCTT	GATACACCTCTCCACCAATGACC
β-Actin	GGCACCACACCTTCTACAATG	GGGGTGTTGAAGGTCTCAAAC

3.3.5 Statistical analysis

Data are expressed as means ± SD for *in vivo* data. Significance was determined by one-way ANOVA with Tukey's post hoc test using the mixed procedure in SAS v9.4 software (Cary, NC). The p values < 0.05 were considered significant.

3.4 Results

3.4.1 FDGP suppresses the total number of intestinal tumors

At the end of the 12-week feeding study, total tumors were counted in the small intestine (duodenum, jejunum, and ileum) and colon (proximal and distal) in all animals. There was a significant dose-dependent reduction in total number of intestinal tumors in FDGP-treated mice ($p<0.05$). Mice treated with aspirin also had 39% fewer tumors than control animals (**Figure 3-1A**). We also saw significant suppression of colon tumor numbers by FDGP treatment (**Figure 3-1B**). This suppression in the tumorigenesis by FDGP was seen across all the sections of the intestine (**Figure 3-1C**). Figure 3-2 shows the representative images of ileum tissue of $APC^{Min/+}$ from each treatment.

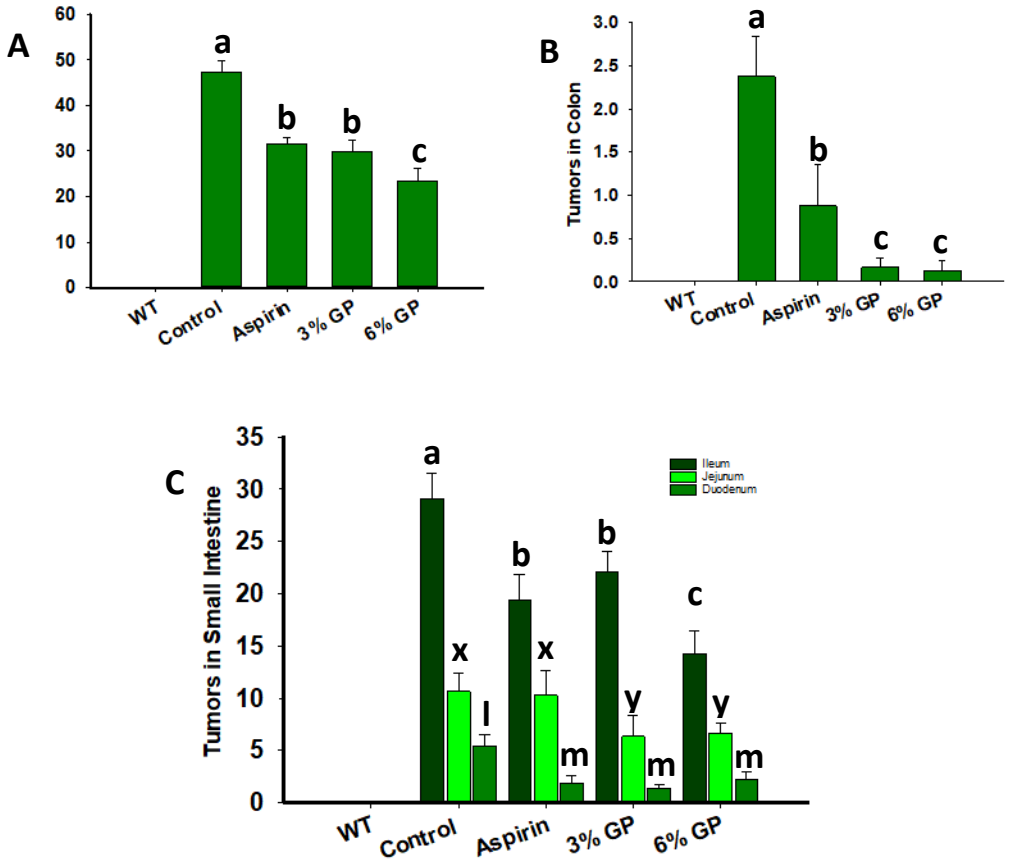

Figure 3-1. Effect of dietary feeding of FDGP on intestinal tumorigenesis in $APC^{Min/+}$ mice. (A) Total number of tumors across all treatments groups. (B) Total tumors in colon tissue. (C) Distribution of tumors across three sections of the small intestine. Data represent the mean ± SD (n=8). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

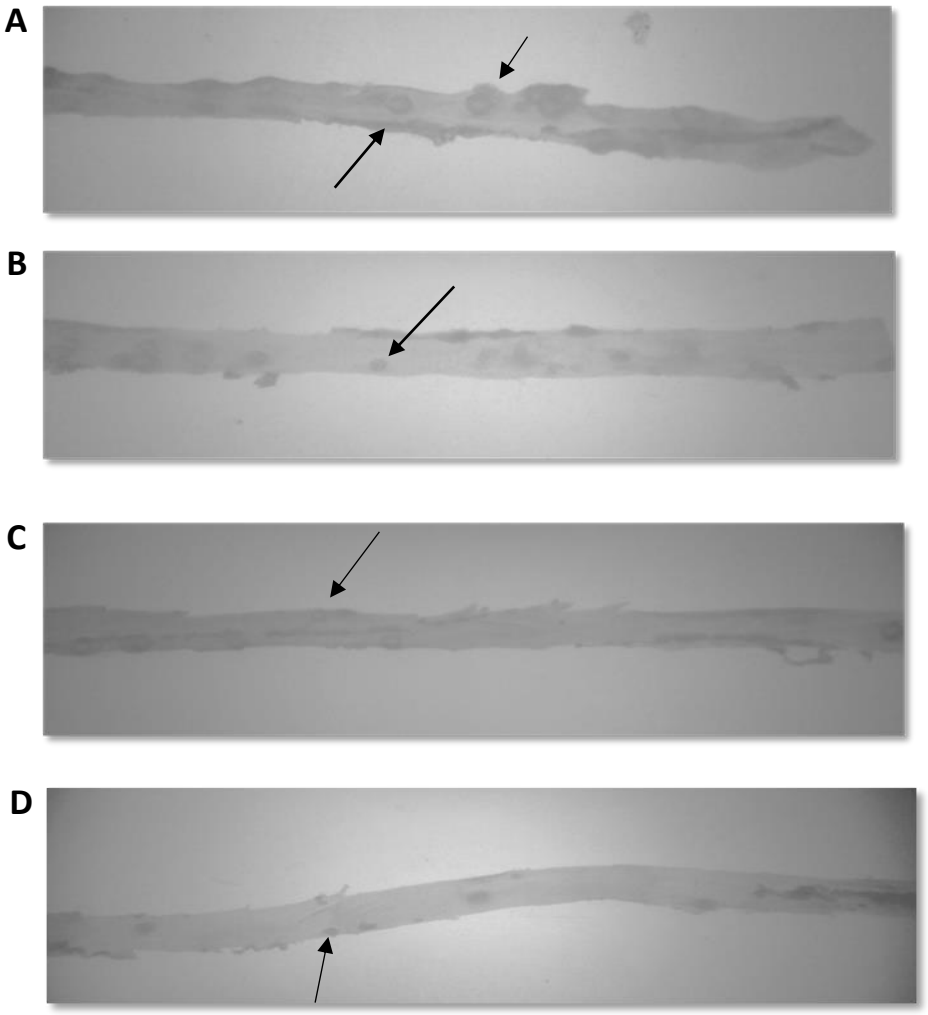

Figure 3-2: Representative images of ileum tissue cut open to show the tumors across all four treatments. (A) Control diet (B) Aspirin 200ppm (C) 3% GP (D) 6% GP.

3.4.2 FDGP suppresses the size of tumors

The size of the tumors in the small intestine were measured by a digital caliper and grouped as tumors <1mm, between 1mm – 2 mm, and >2 mm. FDGP dose-dependently decreased the number of large size tumors (2.5 fold by 6% FDGP, 2 fold by 3 % FDGP) and medium-sized tumors (72.7 % by 6 % FDGP) and (50% by 3 % FDGP) compared to control ($p<0.05$, **Figure 3-3A and B**). The number of tumors (< 1mm) were also

significantly lower in the FDGP treated animals, with 50% reduction in both 6% and 3% FDGP ($p<0.05$, **Figure 3-3C**). Aspirin treatment also significantly suppressed the size of tumors in compared to control animals in 1mm – 2 mm, and >2 mm groups but not <1mm group.

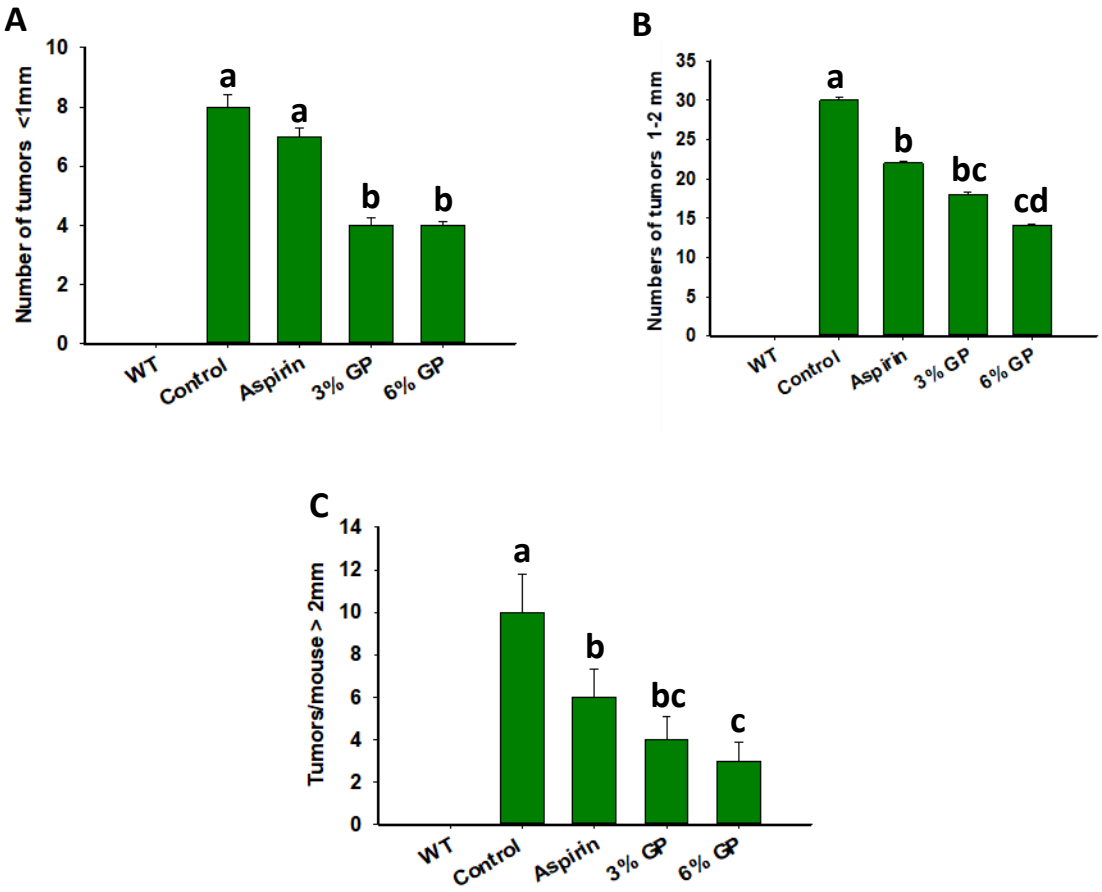

Figure 3-3. Effect of dietary feeding of FDGP on the size of intestinal tumors in $APC^{Min/+}$ mice. (A) Total number of tumors <1mm. (B) Total number of tumors between 1mm-2mm. (C) Total number of tumors >2mm. Data represent the mean ± SD (n=8). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

3.4.3 FDGP feeding reduces the expression of Wnt/β-catenin pathway target genes and COX-2 in tumor tissue

To assess FDGP effect on the Wnt/β-catenin pathway, the expression of the down-stream transcriptional targets cyclin D1, c-Myc and c-Jun in ileal tumor tissue was quantified using Real time PCR. FDGP significantly reduced the expression of all three β-catenin pathway targets compared to control ($p<0.05$, Figure 3-4 A-C). Interestingly aspirin downregulated the gene expression of cyclin D1 but not c-Myc and c-Jun compared to control (**Figure 3-4 A-C**). We also assessed the gene expression of COX-2 in the tumor containing ileum tissue. A positive correlation between COX-2 and β-catenin pathway during colon cancer development has been reported. FDGP-treated mice had reduced COX-2 gene expression ($p<0.05$, **Figure 3-4 D**). In contrast to the other genes, aspirin had a stronger effect on tumor expression of COX-2 in ileum tissue than FDGP treatment.

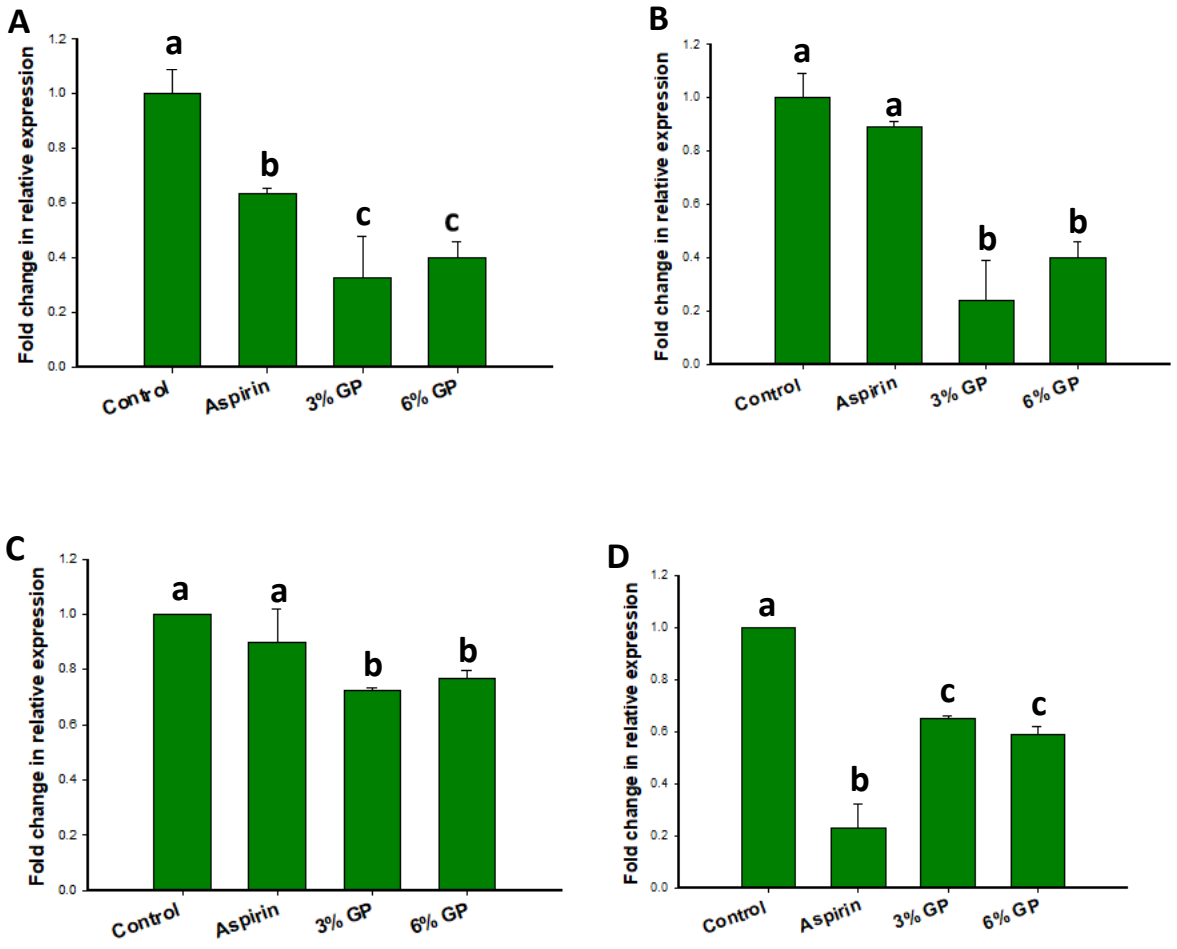

Figure 3-4. FDGP modulates the relative gene expression of β-catenin pathway downstream targets and COX-2 in the ileum tumor tissue (A) cyclin D1. (B) c-MYC. (C) c-Jun and (D) COX-2. Data represent the mean ± SE (n=5). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis

3.4.4 FDGP feeding modulates the transcriptional activity of genes in wnt β-catenin pathway

The data form the Wnt/β-Catenin pathway gene expression array containing 84 gene panel showed that FDGP supplemented downregulated and upregulated several genes playing a role in Wnt signaling, Wnt signaling negative regulation, and genes important in cellular homeostasis, cell migration and developmental processes in the ileum tumor tissue. **Figures 3.5** shows the expression differences in several Wnt pathway genes through a heat map. The gene expression of several genes in the canonical Wnt signaling were differentially expressed in FDGP group compared to control group. The Wnt signaling targets Axin2, Btrc (b-TrCP), Ccnd1, Ccnd2, Dab2, Fosl1 (fra-1), Jun, Mmp7, Myc, Pitx2, Ppard were differentially expressed (3 fold down regulated) across FDGP and control groups showing the FDGP modulated the Wnt signaling and targets in in $APC^{Min/+}$ mice. Genes that play an important role in cell cycle regulation such as Btrc (b-TrCP), Ep300, Fosl1 (fra-1), Jun, and Myc, were differentially expressed as well between FDGP and control groups. Follow-up studies using qRT-PCR are needed to validate the gene expression changes we see in this study.

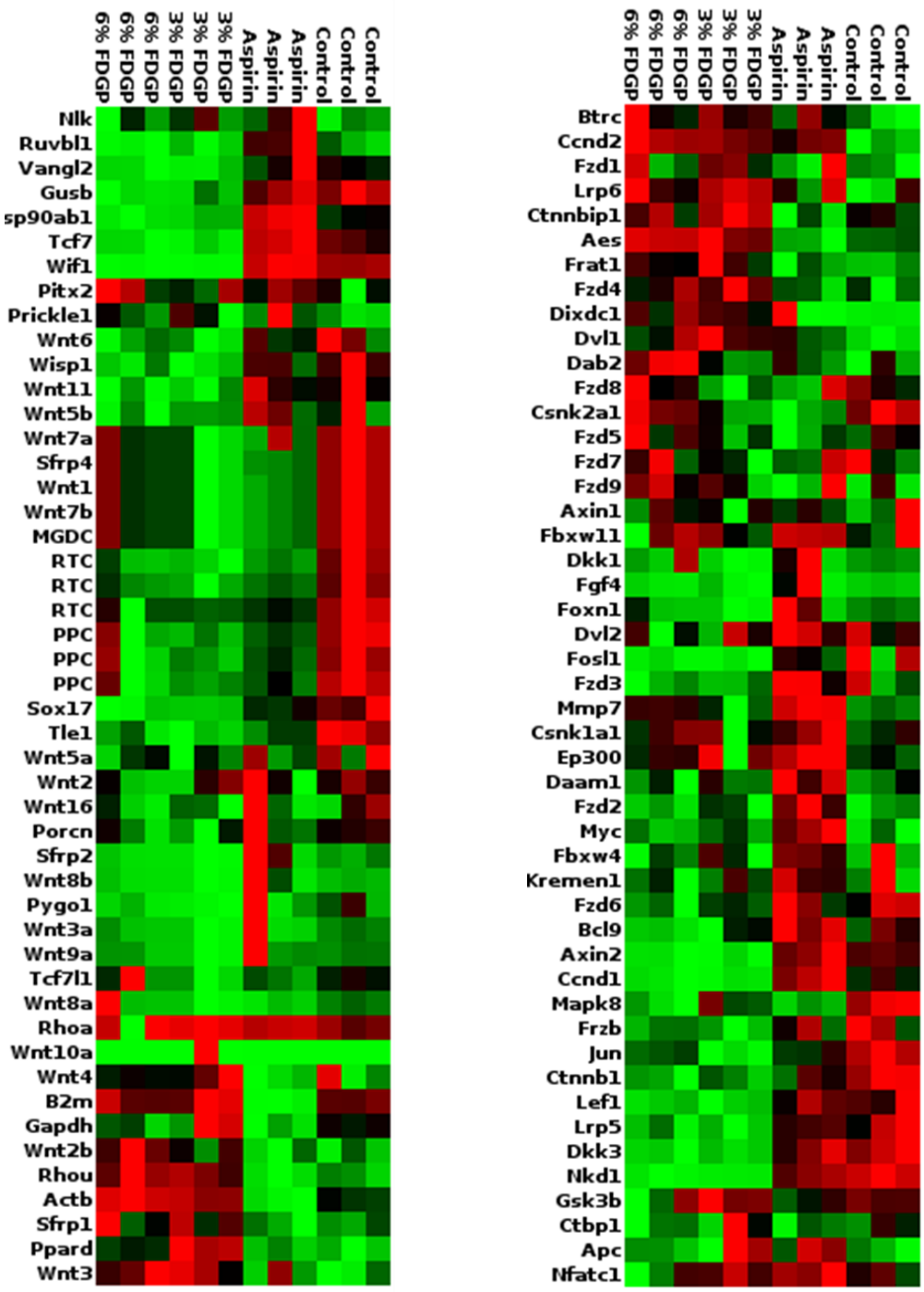

Figure 3-5. FDGP modulates the relative gene expression of β-catenin signaling pathway targets (A & B) Heat map showing the gene expression range of 84 genes linked to Wnt pathway. Green is minimum and Red is maximum gene expression (n=3).

3.4.5 FDGP inhibits angiogenesis in the ileum tissue containing tumors in $APC^{Min/+}$

Neoangiogenesis which is the formation of new blood vessels in tumor, plays a critical role in the growth and progression of the tumor. Both hypoxia and vascular endothelial growth factor (VEGF) have been reported as most important factors that stimulate neoangiogenesis. Hypoxia-inducible factor (HIF)-1α, which is induced under hypoxic condition, is considered a primary regulator of VEGF expression and angiogenesis. Since we observed a strong decrease in size with FDGP and aspirin treatment we wanted to assess the impact on the angiogenesis of tumor. We assessed the gene expression of HIF-1α and VEGF in the ileum tissue containing tumors. Both FDGP and aspirin significantly downregulated the gene expression of HIF-1α and VEGF compared to control animals (**$p<0.05$, Figure 3-5A, B**).

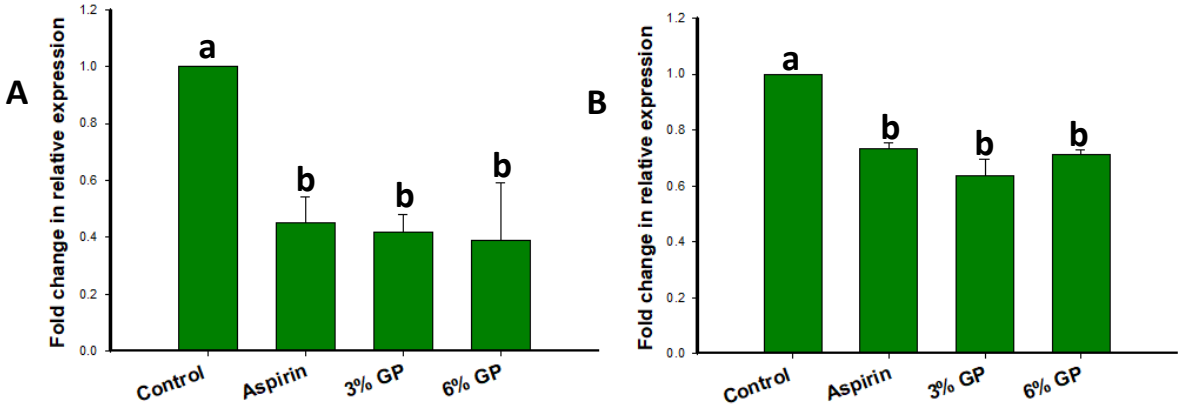

Figure 3-6. FDGP modulates the relative gene expression of important markers of angiogenesis (A) VEGF (B) HIF-1α. Data represent the mean ± SE (n=5). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

3.5 Discussion

$APC^{Min/+}$ mice are heterozygous for a truncating mutation in the *APC* tumor-suppressor gene and provide a model of FAP and sporadic colorectal cancers. Designated as a gatekeeper in colorectal tumorigenesis, mutation of APC leading to the deregulation of the Wnt signal transduction pathway is one of the earliest events in colorectal neoplasia. Normally, in the absence of Wnt stimulation, β-catenin is targeted for degradation by a cytoplasmic complex containing APC, axin, and glycogen synthase kinase 3β. Therefore, mutation of *APC* gene increases cytoplasmic accumulation and nuclear translocation of β-catenin. In the nucleus, the transcription factor, T-cell factor/lymphoid enhancer factor, are trans activated by β-catenin leading to an increased expression of genes that regulate cell proliferation and apoptosis. Hence, the $APC^{Min/+}$ mouse model is unique in which tumors appear spontaneously in the gastrointestinal tract, rather than induction by a carcinogen. Therefore, this model is particularly advantageous for testing chemo preventive agents targeted against early-stage lesions and relevant to the design of human chemoprevention clinical trials because an adequate number of adenomas grow to a grossly detectable size within a few months.

The concept of dietary chemoprevention is usually applied in the context of protecting normal cells from initiating events that introduce oncogenic mutations. It is a well-known fact that carcinogenesis represents a progression of cellular changes, and agents that disrupt this progression at any point can be considered chemo preventive. In recent years, cancer prevention by dietary phytochemicals has received considerable attention.

The present study, using $APC^{Min/+}$ mice, for the first time demonstrated that dietary administration of 3 % AND 6 % table grapes –FDGP suppressed intestinal tumorigenesis, especially the large tumors in the small intestine. The tumorigenesis suppression in the study is comparable to the data generated from other *in vivo* studies with other grape compounds such as grape seed extract (GSR), red grape extract, grade antioxidant dietary fiber (GADF). 6-week supplementation of 0.5 % GSE in $APC^{Min/+}$ resulted in a 40 % reduction in the intestinal tumors [116]. This effect was due to the decreased levels of β-catenin targets cyclin D1 and c-myc. GADF also had a significant anti-tumorigenic effect in $APC^{Min/+}$ and it is linked to the induction of G1 cell cycle arrest and the downregulation of genes related to the immune response and inflammation. However, these studies were done with extract and it is hard to translate these levels to human dietary recommendation.

We also found that decrease in tumor number and size by FDGP was accompanied by down-regulation of cyclin D1, c-Myc, and c-Jun levels which are all important downstream targets of β-catenin. These molecular changes suggest that the inhibition of β-catenin signaling could be one of the possible underlying mechanisms of ant tumorigenic activity of FDGP. The data form the Wnt/β-Catenin pathway gene expression array showed that the FDGP supplemented downregulated and upregulated several genes playing a role in WNT signaling, WNT signaling negative regulation, and genes important in cellular homeostasis, cell migration and developmental processes in the ileum tumor tissue. Follow up studies for the gene array are needed to validate the gene expression changes we see. Moreover, we also saw a significant downregulation of WNT pathway target genes that code cyclin D1, c-Myc, and c-Jun

which play a main role in cell proliferation. We also saw a down regulation of COX-2 expression and important players linked to colon cancer progression. Aspirin had the strongest effect of COX-2 suggesting that it could be the way aspirin suppressed tumorigenesis. FDGP also decreased the size of tumors across all the sections of the intestine and this could be an effect of the FDGP mediated down regulation of angiogenesis markers HIF-1α and VEGF.

In conclusion, the present study suggests that dietary supplementation of one or two servings of table grapes would suppress the tumorigenesis by 48 – 55 %. It supports the potential usefulness of table grapes for the chemoprevention of human intestinal/colorectal cancer.

Chapter 4

The effects of FDGP supplementation on cancer cachexia and its associated morbidities.

4.1 Abstract

Colon cancer is the third leading cause of cancer related deaths in the United States. Cancer induced cachexia is a complex condition of tissue wasting which develops as a secondary disorder in cancer patients and leads to progressive functional impairment, accompanied by chronic inflammation, disrupted energy metabolism, and severe muscle wasting. It accounts for ~30% of all cancer-related deaths. In addition, the radiotherapy used to treat colon cancer can exacerbate cachexia progression. Currently there are no established nutritional approaches to combat cachexia. Several bioactive compounds including the polyphenol quercetin and the omega-3 fatty acid eicosapentaenoic acid are being investigated in animal models for their anti-cachectic properties. The $Apc^{Min/+}$ mouse is an established model of colon cancer and colon cancer-induced cachexia. This mouse model has a heterologous mutation in the *Apc* tumor suppressor gene which predisposes the mouse to intestinal tumor development. The strengths of this cachexia model derive from several features that mimic human cancer, including a gradual increase in tumor burden and chronic inflammation. We recently found that dietary supplementation of $Apc^{Min/+}$ mice with dietary freeze-dried grape powder (FDGP at 3 or 6% w/w) ameliorated weight loss in $Apc^{Min/+}$ compared to control diet-treated mice. FDGP also countered other important markers of cancer cachexia such as endotoxemia, altered gut barrier function, and systemic inflammation in $Apc^{Min/+}$ mice. FDGP supplemented mice had lower levels of circulating endotoxins/lipopolysaccharides (LPS) and LPS binding protein LBP. FDGP also suppressed the systemic and tissue levels of pro-inflammatory cytokines and upregulated the levels of anti-inflammatory cytokines. We also observed that mice treated with FDGP had greater levels of several phyla of

fecal bacteria associated with reduced cachexia, and lower levels of those positive correlated with colon cancer-cachexia. FDGP ameliorated weight loss and other important markers of cancer induced cachexia.

4.2 Introduction

Cachexia has been defined as the progressive, unintended loss of skeletal muscle mass and fat tissue, which leads to functional impairment of the skeletal system. Cachexia leads to difficulties in the performance of everyday tasks and decreases in quality of life. Cachexia incidence is 20 and 80% depending on the cancer type.

Colorectal cancer represents the 3rd most common cancer in the United States and worldwide and is associated with the development of cachexia in up to 50% of the cases. In colon cancer patients, cachexia is generally diagnosed in association with unintentional weight loss of at least 5% of initial weight and is normally accompanied by muscle and fat loss, muscle weakness, fatigue, anorexia, changes in body composition (including lean and fat mass), increased inflammatory state, and chronic anemia. Cancer cachexia can be classified by the degree of body weight loss, metabolic dysfunction, and inflammation present. There are 3 distinct stages of CC, known as precachexia, cachexia, and refractory cachexia. Cachexia can lead to decreased tolerance to radio- and chemotherapy, and overall reduced survival. It is estimated that cachexia is responsible for 25–30% of all cancer-related deaths.

With the rising cost of healthcare and cancer treatment, the development of economically feasible and effective therapeutic interventions has become increasingly

important. Efforts to develop therapeutic interventions have focused on all stages of cancer cachexia. These treatments could improve quality of life, decrease cancer-linked mortality rates, and minimize the economic burden created by cancer cachexia. Dietary components including quercetin (polyphenol) and eicosapentaenoic acid (omega-3 fatty acid) are being investigated in animal models for their anti-cachectic properties and have been found partially effective.

In the previous chapter, we found that dietary supplementation of $APC^{Min/+}$ mice with freeze-dried grape powder (FDGP) at 3 or 6% w/w for 12 weeks decreased the total number of intestinal adenomas by 55% compared to control. FDGP supplemented animals also weighed significantly more than control mice and this prompted us to look at the anti-cachectic effects of FDGP. $APC^{Min/+}$ mice are a good model for cachexia, and there were only few studies that looked the anti cachetic effect of bioactive compounds such as quercetin. However, there is no information on the anti cachetic effect of table grapes, so the aim of this study was to assess the anti-cachectic efficacy of FDGP.

4.3 Materials and methods

4.3.1 Mouse Treatment, Monitoring, and Sample Collection

Male $APC^{Min/+}$ mice were treated with FDGP as described in Chapter 3. Food Intake and body weight were monitored. At the end of the treatment period, mice were euthanized and blood and tissue samples were collected for analysis. Collected blood was allowed to clot at room temperature for 15 min and was centrifuged at 2,000 x g for 10 mins at 4°C to isolate serum. Serum was flash-frozen in liquid nitrogen and stored at -70°C until analysis. To calculate the

hematocrit, during the serum collected step we measured the height of the RBC pellet and divided by the total height of the sample and presented it as a percentage of hematocrit or packed cell volume (PCV).

4.3.2 Fecal Collection and Isolation of Fecal DNA

Fecal samples were collected from each animal on a weekly basis and at final kill. Genomic DNA from the fecal samples was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturers instruction.

4.3.3 Quantification of Endotoxemia and Inflammation

The bacterial endotoxin (LPS) and LBP in serum samples were quantified using an endotoxin detection kit and LBP detection kit from Invitrogen (San Diego, CA) as per the manufacturer's instructions.

Serum levels of the pro and anti-inflammatory cytokines interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and tumor necrosis factor (TNF)-α were quantified using a V-PLEX validated ELISA assay kit from Mesoscale Discovery (Rockville,MD).

4.3.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Intestinal expression of pro-inflammatory genes was measured using qRT-PCR according to the protocol described in chapter 3 using the primers specific to the inflammatory markers of interest.

Table 4.1 List of primers used in this study

Gene Product	Forward primer	Reverse primer
Arg 1	TGGCTTGCGAGACGTAGAC	GCTCAGGTGAATCGGCCTTTT
Mannose Receptor	GCTGAATCCCAGAAATTCCGC	ATCACAGGCATACAGGGTGAC
Trem 2	CTGGAACCGTCACCATCACTC	CGAAACTCGATGACTCCTCGG
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
IL-1β	GCTGAAAGCTCTCCACCTCA	GGCCACAGGTATTTTGTCGT
Cldn2	CGTCACCATCACTCCTTCTTG	GCAGCTCTAGGAGCGCAGCTCT

4.4 Results

4.4.1 Effect of FDGP body weight and epididymal fat deposits.

Weight loss is considered the most significant marker for identifying cancer cachexia. Around the age of 10-11 weeks the $APC^{Min/+}$ on control and aspirin diets started to lose weight and weight loss continued until the end of the experiment (**Figure 4-1A**). Weight loss was mitigated in the FDGP-supplemented animals at both dose levels. At the end of the experiment, animals on FDGP has a 37 - 45.5 % higher body weight compared

to the control-treated APC$^{Min/+}$ mice ($p<0.05$). Fat loss is an important hallmark in cachexia. We found that the epididymal fat stores of FDGP group were 1.5 – 3 times higher than those of control mice indicating that FDGP alleviated cachexia in this model.

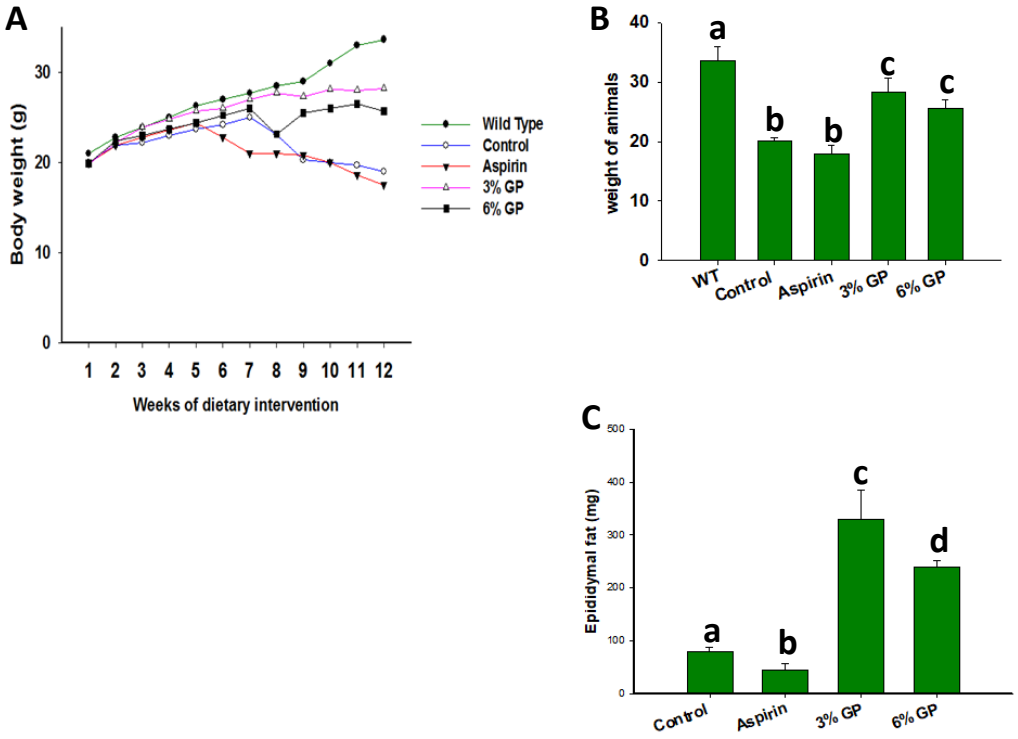

Figure 4-1. Effect of dietary feeding of FDGP on the body weight of APC$^{Min/+}$ mice. (A) Body weight data over the period of 12 weeks. (B) Body weight of animals in grams, at the end of the experiment. (C) Epididymal fat pad mass. Data represent the mean ± SD (n=8). For epididymal fat data (n=4). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

4.4.2 FDGP suppressed splenomegaly, improved the hematocrit

Splenomegaly is often seen in $APC^{Min/+}$ at 12-15 weeks of age and is positively correlated with the increased burden of tumors. Splenomegaly is associated with increased anemia. The FDGP treated groups did not show signs of splenomegaly, whereas the control mice had enlarged spleens ($p<0.05$, **Figure 4-2A**). FDGP treatment at 3% and 6% increased hematocrit by 46% and 30.7%, respectively, compared to control. By contrast, animals on aspirin has 38% lower hematocrit than the control mice. We also observed that the FDGP fed animals had longer colon length compared to control mice indicating reduced colonic inflammation (**Figure 4-1C**).

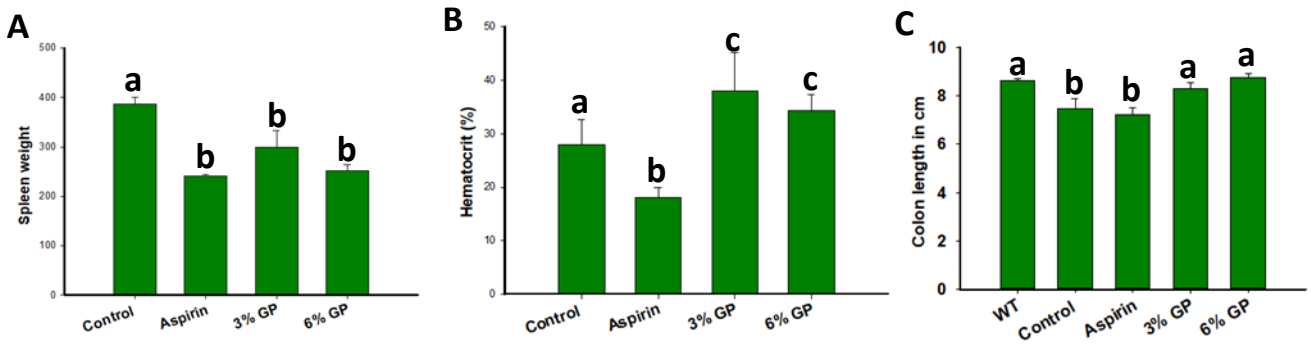

Figure 4-2. Effect of dietary feeding of FDGP on spleen size and packed cell volume (A) weight of spleen in grams (B) Hematocrit in % (C) Colon length at the end of the experiment. Data represent the mean ± SD (n=8). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

4.4.3 FDGP countered endotoxemia and improved the barrier function

Puppa *et al* showed role for gut barrier dysfunction (GBD) and subsequent endotoxemia in the development of inflammation and progression of cachexia in the $APC^{Min/+}$ mouse. We observed a 3-fold decrease in the circulating LPS levels in the animals supplemented 3% and 6% FDGP, respectively, compared to control ($p<0.05$ **Figure 4-3A**). The serum LBP, which is produced by the body in response to LPS was also at significantly lower levels in FDGP compared to control ($p<0.05$, **Figure 4-3B**). Using qRT-PCR, we evaluated the gene expression of claudin 2 (*Cldn2*) in the ileum as a marker of gut barrier function. Claudin 2 is a channel forming protein and increased expression is associated with increased gut permeability. We observed that mice treated with FDGP, but not those treated with aspirin, had decreased ileal expression of *Cldn2* ($p<0.05$, Figure 4-3C).

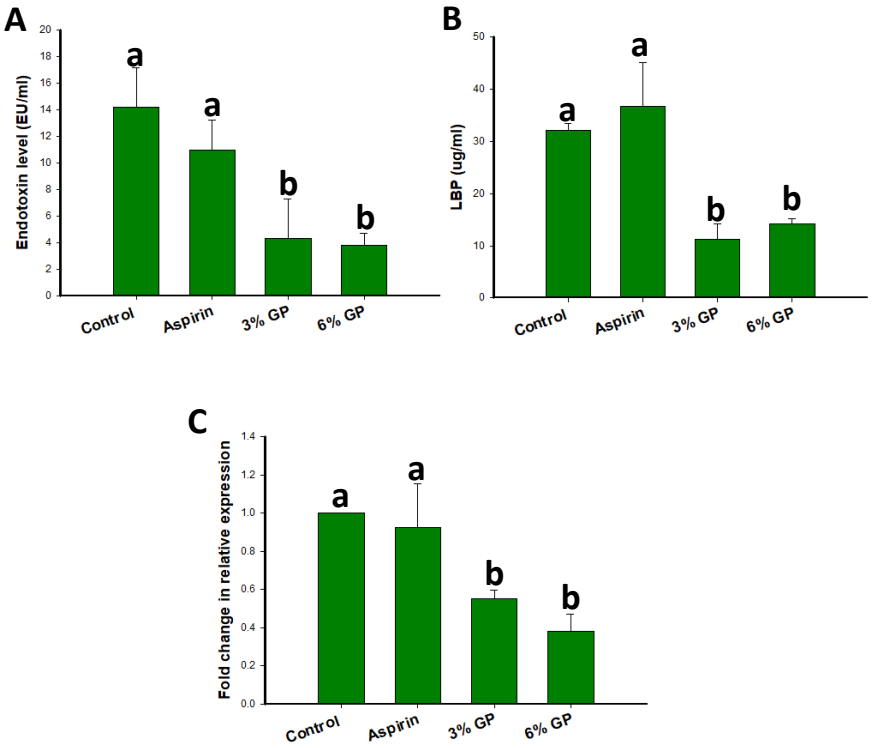

Figure 4-3. Effect of dietary feeding of FDGP on endotoxemia and intestinal barrier function (A) Level of LPS in the serum in EU/ml. (B) Levels if acute phase protein LBP in the serum. (C) Fold change in the relative gene expression of claudin-2 in the ileum tissue. Data represent the mean ± SD (n=8). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

4.4.4 Effect of FDGP on the systemic pro, and anti-inflammatory cytokines

It is known that the tumors drive inflammation in the body through the release of inflammatory mediators, particularly cytokines such as IL-6. It is known that individual members of the intestinal microbiota can markedly alter the inflammatory state of the intestinal immune system. Using a validated multiplex ELISA we quantified the pro and anti-inflammatory cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α in the serum of animals after week 1 and week 12 of the dietary intervention and at the end of the experiments. No significant change ware seen in case of IFN-γ, IL-1β, IL-4, KC/GRO, IL-10, and TNF-α across 6% FDGP, aspirin or control mice. Interestingly we saw a significant decrease in the levels of pro-inflammatory cytokine IL-6 in 6% FDGP group compared to control ($p<0.05$, **Figure 4-4**).). IL-1β, IL-2 and IL-12p70 were below detectable levels.

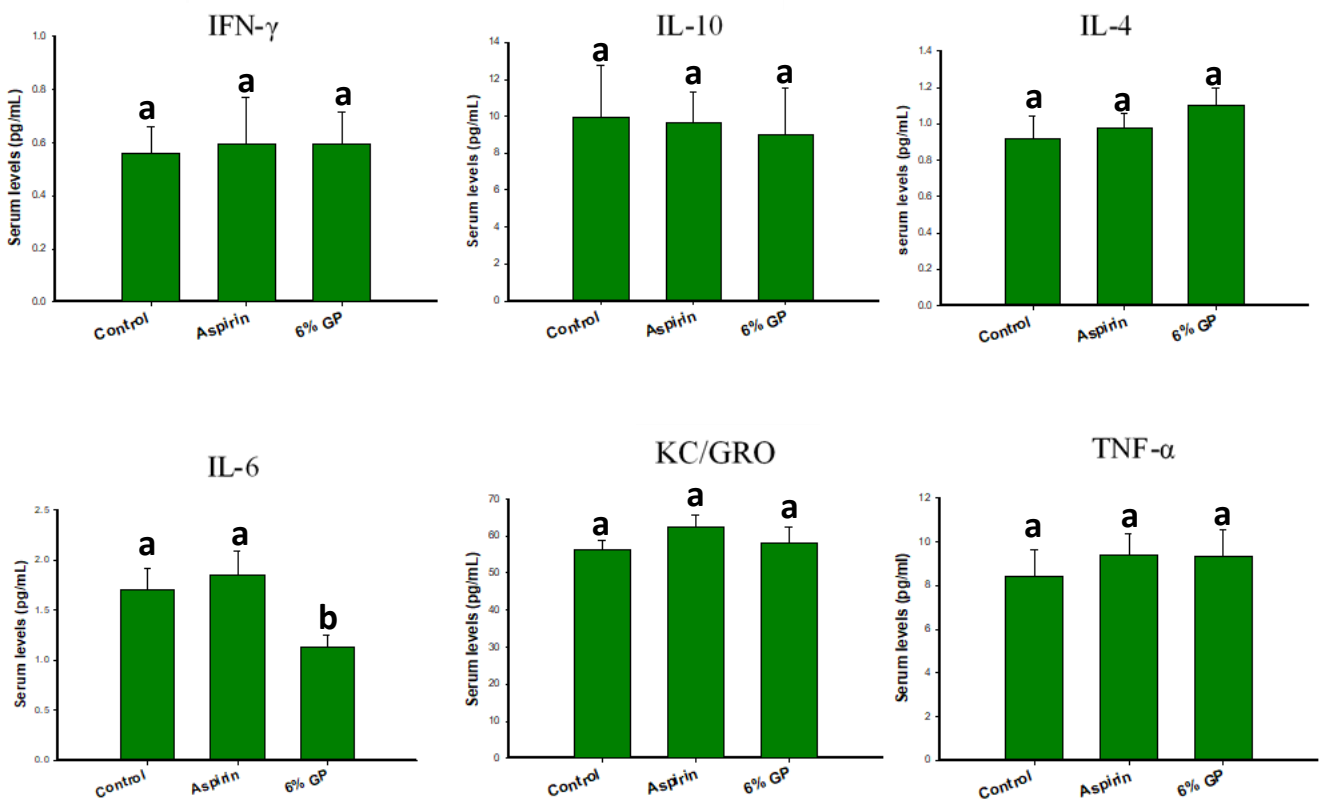

Figure 4-4. Effect of dietary feeding of FDGP on levels of systemic inflammatory pro and anti-inflammatory markers after 1 week. Data represent the mean ± SE (n=4). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

Inflammatory cytokines are known to increase 10-fold during the cachectic stage of $Apc^{Min/+}$ which is around > 15 weeks of age. We evaluated the systemic levels of the pro and inflammatory cytokines in the mice after 12 weeks of FDGP supplementation. The levels of anti-inflammatory cytokines IL-4 and IL-10 were significantly higher in FDGP fed mice compared to control group ($p<0.05$, **Figure 4-5**). On the other hand, FDGP supplementation reflected in significantly lower levels of pro-inflammatory cytokine such as TNF-α and IL-6 ($p<0.05$). These

results show that long term supplementation of FDGP have profound effect on modulating the inflammation an important driver of cancer cachexia.

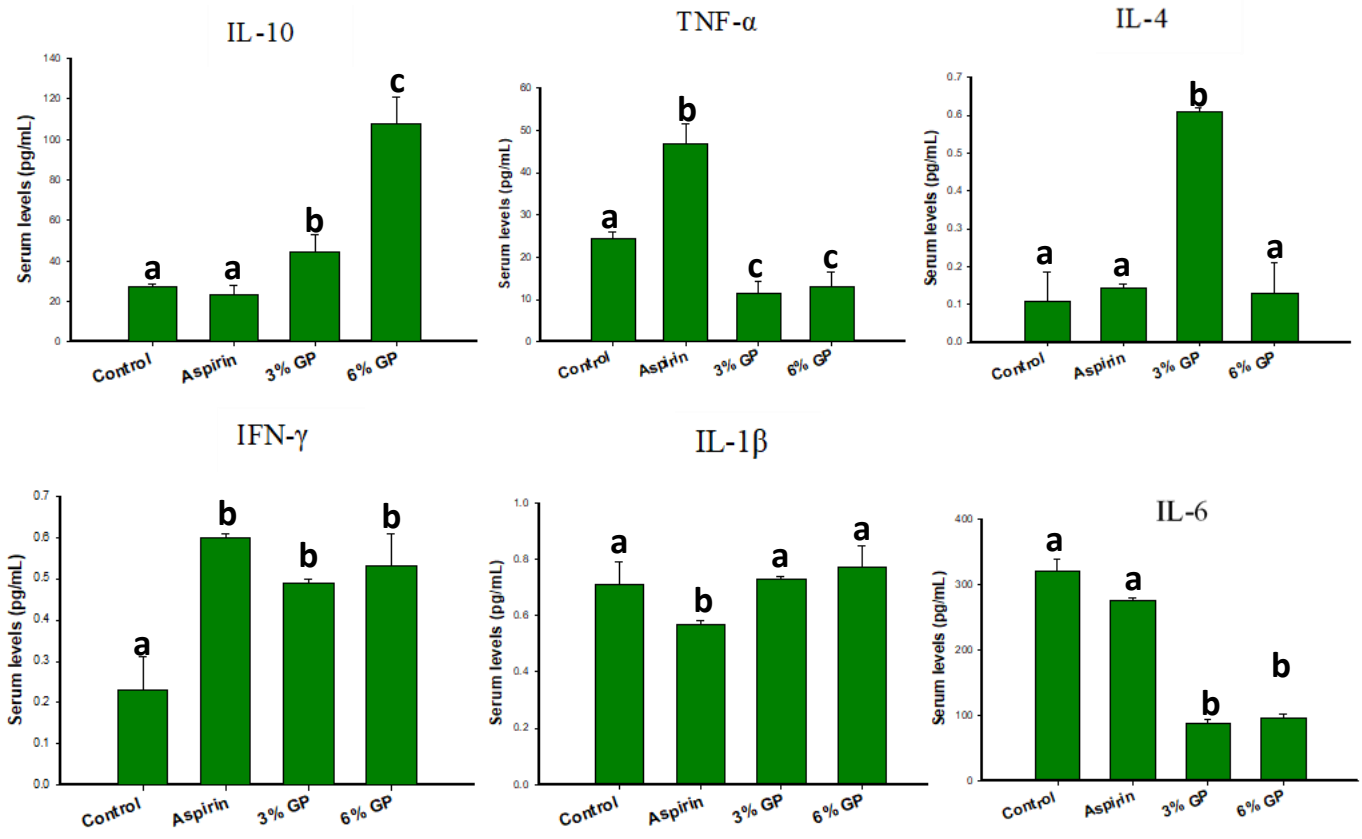

Figure 4-5. Effect of dietary feeding of FDGP on levels of systemic inflammatory pro- and anti-inflammatory markers after 12 weeks. Data represent the mean ± SE (n=4). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

4.4.5 Effect of dietary supplementation of FDGP on gene expression of the pro- and anti-inflammatory markers in the ileum

RT-PCR was employed to evaluate the expression profile of cytokines in the mucosa of the small intestine. We evaluated the expression of M2 macrophage phenotypic markers such as arginase-1, Mannose recptor (MR), and Trem-2 which are known to have anti-inflammatory properties. FDGP upregulated the expression of Arginase 1 (4-fold) and Trem 2 (14- fold) significantly compared to control (**Figure 4-6**). Both FDGP and aspirin increased the expression of other anti-inflammatory marker, mannose receptor. Similar to the systemic data, FDGP supplementation significantly downregulated the expression of IL-6 and upregulated the expression of anti-inflammatory cytokine IL-10 compared to control (**Figure 4-6**).

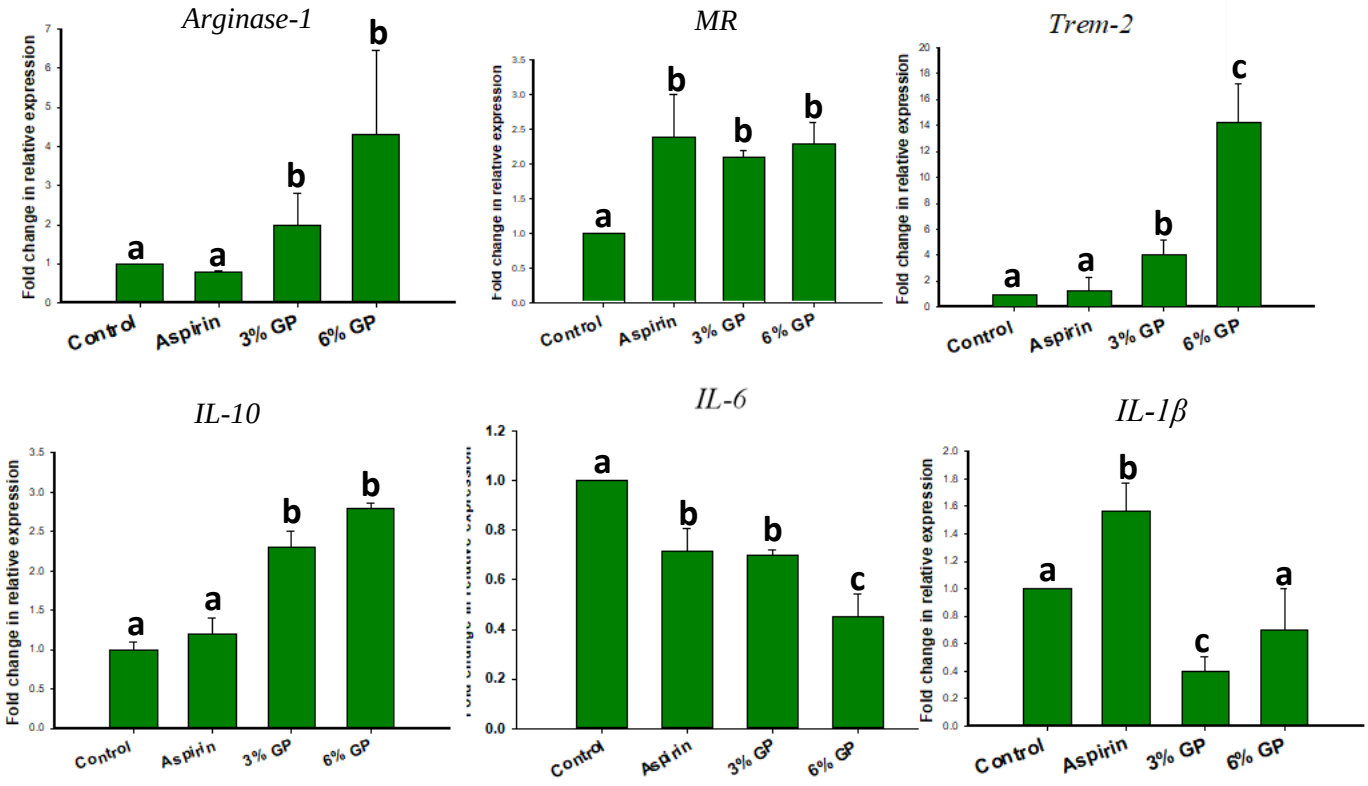

Figure 4-6. Effect of dietary feeding of FDGP on levels of gene expression of pro- and anti-inflammatory markers. Data represent the mean ± SE (n=4). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis.

4.4.6 Effect of dietary supplementation of FDGP on the levels of *Akkermansia muciniphila* and *Helicobacter*

It is known that grape supplementation results in an increased level of *Akkermansia muciniphila* even after a week and we found that the mice supplemented with FDGP had higher levels of *A. muciniphila* which a considered a good probiotic and is linked to reduction in endotoxemia. On other hand the *Helicobacter* species which is linked to the cachexia progression is lower in abundance in FDGP group than the control

group (**Figure 4-7**). Further screening and gut bacterial sequencing is needed to confirm this and find the overall changes in the microbiome and its relation to the FDGP mediated tumor suppression [117].

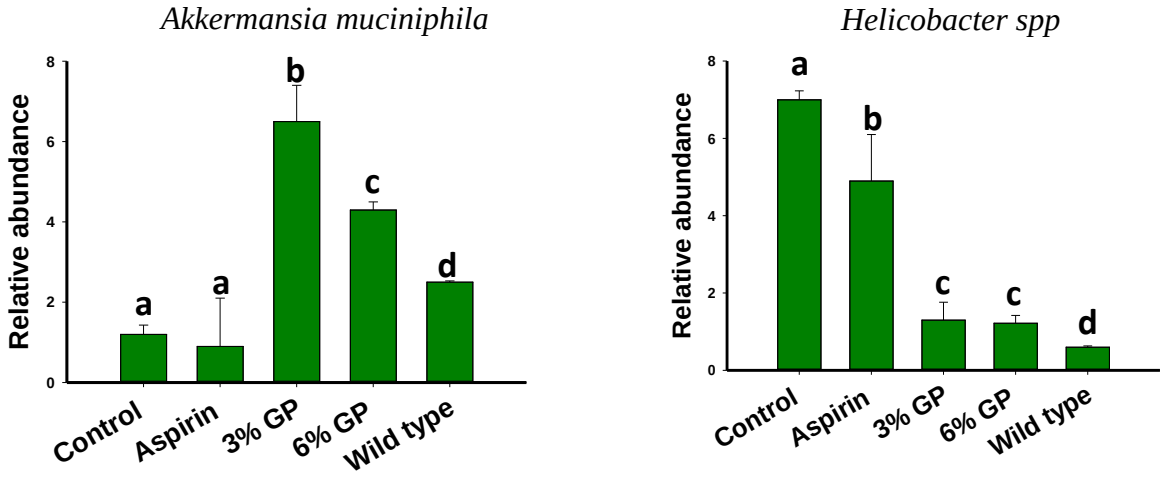

Figure 4-6. Effect of dietary feeding of FDGP on levels of abundance of bacterial species positively and negatively correlated with cachexia. Data represent the mean ± SE (n=4). Values that do not share a common superscript letter are different ($p < 0.05$) by one-way ANOVA with post hoc Tukey analysis

4.5 Discussion

Cachexia, a condition characterized by severe wasting of muscle and adipose tissue, is a common complication of late-stage cancers, especially those of the gastrointestinal system. It contributes to at least 30% of deaths from cancer. Cachexia is commonly associated with increases in acute phase proteins and pro-inflammatory cytokines in plasma, particularly TNF-∝ and IL-6 [76].

Although it is a devastating complication of cancer itself, there are no current proven therapies to counter or to slow down the progression of cancer cachexia. Drugs such as cyproheptadine, hydrazine, metoclopramide, and pentoxifylline proved ineffective in combating cachexia. Drugs with a strong rationale that have failed or have shown equivocal results in clinical trials so far include eicosapentaenoic acid, cannabinoids, bortezomib, and anti-TNF-alpha antibodies.

The $APC^{Min/+}$ mouse is an animal model for colon cancer research, which has a mutation in the *APC* tumor suppressor gene. This mouse develops intestinal polyps, beginning at ~5 weeks of age and begins to develop a progressive cachexia between 12 and ~20 weeks of age, that culminates in a 20– 25% decrease in body weight, The progression of cachexia is associated with an increased concentration of plasma IL-6, and cachexia is inhibited in an IL-6 knockout ($Apc^{Min/+} \times Il6^{-/-}$) mouse despite the presence of intestinal and colon tumors. The cachectic response can be restored by systemic IL-6 over-expression in the $Apc^{Min/+} \times Il6^{-/-}$ mouse, while IL-6 over-expression in C57BL/6 mice does not induce cachexia. Thus, IL-6 is necessary, but not sufficient, for induction of cachexia in mice. Gut barrier dysfunction (GBD), characterized by breakdown and leakage of the gut epithelial barrier, leads to systemic inflammation because of entry of bacterial cell wall components (endotoxin), also known as lipopolysaccharide (LPS), or intact bacteria into the circulation. The resulting inflammation is a common problem in critical care medicine and can lead to multiple organ dysfunction syndrome.

With inflammation and gut barrier dysfunction being the main players in the progression of cachexia, we evaluated effect of FDGP supplementation which was

already shown to be tumor preventive in $APC^{Min/+}$ in Chapter 3. Weight loss is considered the main factor to determine the stage of cachexia and the animals on FDGP has a higher overall body weight with 3% having 45.5 % higher body weight than control, and 6% having 37% higher body weight than control. We found that the epididymal fat stores of FDGP group much higher (121% higher) compared to control. This shows that FDGP countered the cachexia associated fat loss. Aspirin on the other hand had the lowest average body weight and epididymal fat loss across all treatments.

Then we evaluated the impact of FDGP supplementation in the systemic inflammation. After one week of FDGP supplementation no significant difference was found in the serum levels of IFN-γ, IL-1β, IL-4, KC/GRO, IL-10, and TNF-α, but the levels IL-6 were significantly lower in FDGP group compared to control. Increased serum concentrations of IL-6 are associated with the progression of cachexia. Interestingly after 12 weeks of FDGP supplementation the serum levels of pro-inflammatory cytokines TNF-α and IL-6 were significantly lower than the control showing an anti-inflammatory effect. The anti-inflammatory markers IL-4 and IL-10 were significantly higher in FDGP fed mice compared to control group.

I also evaluated gene expression of anti and pro inflammatory markers in the ileum and the FDGP group had higher relative gene expression of anti-inflammatory markers such as of Arginase 1 (4-fold), Trem 2 (14- fold) and MR (2.2 fold) and also had downregulation of inflammation promoting IL-6. With inflammation and gut barrier function being hand in hand, we evaluated the level of endotoxemia, a marker for disrupted barrier function. We observed a significant decrease in the circulating LPS

levels in the animals supplemented with FDGP (114 % in 6% FDGP group; 111 % in 3% FDGP group) compared to control. The serum LBP, which is produced by the body in response to LPS was also at significantly lower levels in FDGP compared to control. Using qRT-PCR we evaluated the gene expression of claudin-2 in the ileum, claudin -2 is a channel forming protein and an important mediator in the GBD during intestinal inflammation. We observed that FDGP the gene expression of claudin-2 in the ileum suggesting a possible way for the improved gut barrier function we saw in the animals consuming FDGP.

Overall the results from this chapter demonstrate the anti-cachectic efficacy of FDGP in $Apc^{Min/+}$ by reducing the systemic and intestinal inflammation and improving the gut barrier function. This could be valuable data for conducting clinical trials to text the anti-cachectic effect in humans.

Chapter 5

Conclusions

5.1 Conclusions

The aim of my dissertation research was to evaluate the anti-cancer effect of FDGP *in vitro* using CSCs and to test the anti-tumorigenic effect and anti-cachectic effect *in vivo* using the $Apc^{Min/+}$ mouse model. This was accomplished via the following objectives.

Objective 1: To investigate the anti-cancer properties of FDGP extract on colon CSCs *in vitro* (Chapter 2)

In this study, we found that the grape powder extract (GPE) made from freeze dried whole table grape powder (FDGP) dose-dependently suppressed the proliferation and induced apoptosis in colon CSCs. GPE also suppressed sphere formation, a marker of stemness. shRNA-mediated knockdown experiments showed that p53, a tumor suppressor gene, did not alter the inhibitory efficacy of GPE against colon CSCs, which indicates that these anti-proliferative and pro-apoptotic effects are independent of p53.

The quantitative analyses showed that the FDGP contains several bioactive compounds including flavanols (catechin, epicatechin), anthocyanins (cyanidin, malvidin, peonidin), flavonols (isorhamnetin, kaempferol, quercetin) and stilbenes (resveratrol). The suppression of proliferation and elevation of apoptosis by GPE in colon CSCs can be attributed to the presence of these phytochemicals, which have been previously shown to suppress proliferation in colon cancer cells individually and in combination in *in vitro, in vivo,* and in human studies. Comparative studies are needed to determine if the interactions between these compounds in GPE enhance the overall anti-cancer activity of the extract.

These findings demonstrate the potential colon CSC inhibitory efficacy of FDGP and support the development of future pre-clinical studies to examine the colon cancer preventive effects of table grapes.

Objective 2: To determine whether the dietary supplementation of 3% or 6 % FDGP will reduce the intestinal tumorigenesis in a human-relevant rodent $APC^{Min/+}$ mice and to further determine the molecular pathways effected (Chapter 3)

The concept of dietary chemoprevention is usually applied in the context of protecting normal cells from initiating events that introduce oncogenic mutations. It is a well-known fact that carcinogenesis represents a progression of cellular changes, and agents that disrupt this progression at any point can be considered chemopreventive. In recent years, cancer prevention by dietary phytochemicals has received considerable attention.

My results demonstrated that dietary supplementation of FDGP (at 3 or 6% w/w) suppressed the total number of intestinal polyps by 55%. This is significant because the aspirin (200 ppm; human equivalent dose), a drug used for the prevention of colon cancer in human, decreased intestinal polyps by 39% in this study. FDGP supplementation also decreased polyp growth, with FDGP-treated mice having significantly fewer polyps with diameter > 1mm compared to control mice. Reduced tumorigenesis was associated with downregulation targets in the Wnt /β-catenin pathway, such as cyclin D1, c-Jun, and c-Myc expression. Expression of genes linked to angiogenesis, VEGF and HIF-1α, were also lower in FDGP-treated mice. In conclusion, the present study suggests the potential usefulness of table grapes for the chemoprevention of human intestinal/colorectal cancer

Objective 3: To evaluate the efficacy of FDGP on the progression of colon cancer cachexia in $APC^{Min/+}$ mice and to look at the impact on body weight, endotoxemia, gut barrier dysfunction and inflammation of the mice (Chapter 4).

Cachexia, a condition characterized by severe wasting of muscle and adipose tissue, is a common complication of late-stage cancers, especially cancers of the gastrointestinal system such as gastric and colon cancer system. It contributes to at least 30% of deaths from cancer. Although it is a devastating complication of cancer itself, there are no current proven therapies to counter or to slow down the progression of cancer cachexia. Drugs such as cyproheptadine, hydrazine, metoclopramide, and pentoxifylline were proved ineffective in combating cachexia.

The current study shows that FDGP countered important markers of cancer cachexia such body weight loss, endotoxemia, altered gut barrier function, and systemic inflammation in $Apc^{Min/+}$ mice. FDGP supplemented mice had lower levels of circulating endotoxins/lipopolysaccharides (LPS) and LPS binding protein LBP. FDGP also suppresses the systemic and tissue levels of pro-inflammatory cytokines and upregulated the levels of anti-inflammatory cytokines. We also observed that mice treated with FDGP had greater levels of several phyla of fecal bacteria associated with reduced cachexia, and lower levels of those positive correlated with colon cancer-cachexia. These results suggest that FDGP ameliorated weight loss and other important markers of cancer induced cachexia. A limitation of our analysis was that we did not look directly at changes in adipose tissue or skeletal muscle, two important tissues in cancer cachexia.

In conclusion, the present study suggests the potential usefulness of table grapes for the chemoprevention of human intestinal/colorectal cancer and its associated cachexia.

Future work

Objective 1

In objective 1, we found that the GPE suppresses proliferation and induces apoptosis in colon CSCs. We also saw that GPE targets CSC stemness. It would be beneficial to look at in-depth molecular mechanisms behind the anti-cancer activity focusing on the hallmarks of colon cancer, such as selective growth and proliferative advantage, anti-apoptotic potential, angiogenesis. This would also help us to unravel the molecular targets of grape compounds. Treating the colon CSCs with different concentrations of GPE and performing RNA-Seq would give the cancer suppression pathways and apoptotic pathways impacted by GPE, Using the gene expression data we would be able to see the effect of GPE on the major hallmarks of cancer cells .

Objective 2

In objective 2, we saw that the dietary supplementation of FDGP (at 3 or 6% w/w) suppressed the total number of intestinal polyps (early markers of colon cancer) by 55%. The FDGP is made from several varieties of grapes, so it would be wise to test the anti-cancer efficacy of 2 to 3 individual grape varieties based on the color of the skin and seed/seedless in the same dosage as I did here, since consumers might have individual preferences for selecting the varieties they eat. The other idea would be looking at the efficacy of grapes in another animal model. Although being an established model,

$Apc^{Min/+}$ mice get their tumors mostly in the small intestine and not colons. Doing a 12-week intervention with the same FDGP doses in another animal model such as the AOM/DSS mouse model would be a good future follow up study. The AOM/DSS mouse, one of the well-studied colon cancer mouse models are known to get most tumors in the colon section. This would avoid the limitation of the current animal model used in this study, which is the distribution of tumors in the small intestine and not colon.

Objective 3

It would be beneficial to perform a follow up animal study with similar design to assess the effect of FDGP on the molecular pathways in the muscle tissue and adipose tissue wasting. This would give information of the molecular events such as increased muscle protein degradation, although impaired muscle protein synthesis and defective myogenesis in muscle tissue of these animals. Analysis of IL-6 mediated muscle degradation and adipose tissue wasting pathways would give useful information, since we see a strong impact of FDGP on IL-6 levels in the $Apc^{Min/+}$ and IL-6 is a known player in the cancer cachexia progression. Also performing a gut microbiome analysis and identifying the changes in the gut bacterial composition and abundance would give invaluable data on the impact of FDGP on the gut environment and its connection to cancer cachexia.

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activation of host genes related to metabolic health. J Nutr Biochem, 2018. **56**: p. 142-151.

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Education

Doctor of Philosophy – Food Science **2015-2019**
Pennsylvania State University, State College, USA.
Master's in science – Veterinary Biomedical Sciences **2010-2013**
Kansas State University, Manhattan, USA.
Bachelor of Science – Chemistry, Zoology and Biotechnology (Honors) **2005-2009**
Andhra University, Andhra Pradesh, India.

Selected Awards and Honors

- M Sahakian Family Endowment Award, College of Agriculture, Penn State **2019**
- H Robert D. and Jeanne L. McCarthy Graduate Teaching Award & Graduate Scholarship, Penn State **2017, 2018**
- The Honor Society of Food Science and Technology, Phi Tau Sigma inductee **2018**
- The Honor Society of Agriculture, Gamma Sigma Delta inductee **2018**
- National Championship, Institute of Food Technologists College Bowl Competition **2017**
- First place, Central Atlantic Area College Bowl competition, IFT **2017**
- Listed in "KSU 2012 Notable Scholarly Graduate Students" list **2013**
- Graduate research fellowship, Kansas State University College of Veterinary Medicine **2012**

Conferences attended (Total Abstracts: 25)

- American Society for Nutrition, Baltimore, MD
- Penn State University Farm to fork Symposium
- Experimental Biology Conference, Orlando, FL
- International Stroke Conference, Honolulu, HI
- 4th Cancer Cachexia Conference, Philadelphia, PA
- American Society for Nutrition, Boston, MD
- Penn State University GSD research symposium
- Protein Structure, Function and Malfunction (PSFaM), Saskatchewan, Canada
- American Society for Rickettsiology, Park City, Utah

Selected Publications

https://scholar.google.com/citations?user=CL6O7g8AAAAJ&hl=en

1. Chuanmin Cheng, Arathy D. S. Nair, **Vijaya V. Indukuri**, Shanzhong Gong, Roderick F. Felsheim, Deborah Jaworski, Ulrike G. Munderloh, Roman R. Ganta (**2013**). Targeted and Random Mutagenesis of *Ehrlichia chaffeensis* for the Identification of Genes Required for In vivo Infection. **PLoS pathogens** 9 (2), e1003171 (**IF: 7.5**).

2. Amr M El Zawily, Behzad M Toosi, Tanya Freywald, **Vijaya V. Indukuri**, Franco J Vizeacoumar, Scot C Leary, Andrew Freywald (2016). The intrinsically kinase- inactive EPHB6 receptor predisposes cancer cells to DR5-induced apoptosis by promoting mitochondrial fragmentation. Oncotarget. **2016** Nov 22; 7(47): 77865–77877 (**IF: 5.3**).

3. Transposon Based Mutagenesis and Mapping of Transposon Insertion Sites within the *Ehrlichia chaffeensis* Genome using semi random Two-step ST- PCR (available online - https://krex.k-state.edu/dspace/handle/2097/16329). (MS thesis).