THE EFFECTS OF DIFFERENT WHEAT TYPES ON COLON CANCER RISK

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Abstract

Colon cancer is the third most common cancer and the second leading cause of cancer-related deaths in the United States (1). A variety of risk factors play a role in the etiology of this disease, including environmental risk factors (2, 3). Because of this, considerable research has gone into finding ways to increase consumption of foods that have an inverse association with colon cancer (4). While several epidemiological studies have shown an association between a diet high in whole grains and a lower risk of colon cancer, experimental studies have been inconsistent. This study looks at the effects of wheat class on colon carcinogenesis during the post-initiation stage of colon cancer development in rats. It also examined whether intermediate wheatgrass (IWG) commercially known as Kernza ™, a perennial grass being developed as an alternate to wheat, modifies colon cancer risk in a way similar to red wheat. A major endpoint of this study was enumeration of colonic phenotypic markers known as aberrant crypt foci (ACF), an early pre-cancerous lesion. Additionally, based on previous findings indicating that the type and amount of mucin production is a marker for dysplasia (5), changes in mucin production was examined as well. Finally, CD44, a putative marker of cancer stem cells, was determined immunohistochemically as an additional indicator of colon cancer risk. It was found that there was a significantly greater number of sialomucin-stained ACF (SIM_ACF), and mucin-depleted ACF (MDF) staining in white wheat and vs. red wheat, indicating a higher degree of dysplasia in white wheat. This shows a greater protective effect of red wheat vs. white wheat in the ACF of the colon. Moreover, staining for CD44 was found to be higher in ACFs of white wheat and IWG vs. red wheat. The correlation between the two, indicated by a dysplasia score, 0.7029

($p<0.0001$) demonstrates a positive relationship between CD44 and dysplasia. The reduced number of dysplastic markers along with a higher dysplasia score in white wheat and IWG vs. red wheat supports a protective effect of red wheat.

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Introduction to Thesis

Colon cancer is the third most common cancer and the second leading cause of cancer-related deaths in the United States (1). Although there has been a decline in mortality from colon cancer in recent years (6), this form of cancer is still a major public health issue. A variety of factors contributes to the development of colon cancer. These include inflammatory intestinal conditions, family history of disease, age, and smoking. However, environmental risk factors play the largest role in the etiology of this disease (2, 3).

A prominent environmental risk factor is diet. It is estimated that over 50% of colon cancer incidence is diet-related (7). Studies have shown inverse associations between high fiber diets, increased fruit and vegetable intake, and increased whole grain consumption and the risk of developing colon cancer (8). In contrast, there is evidence for association between colon cancer and increased red meat consumption, consumption of processed foods, and lack of whole grain consumption (9, 10).

Because environmental risk factors play a large role in disease, much research has been conducted to identify foods that have an inverse association with colon cancer (4). Benefits of whole grains, including wheat, have been examined in detail (11). Wheat is the most consumed grain in the United States (12). There are three classes of wheat – red, white, and durum. The classes are further categorized into groups. The first group includes hard wheats. These wheats have a high protein content; thus, they are typically used in bread making. The second group is soft wheats. In contrast to hard wheat, soft wheats have a lower protein content and are primarily used in making cakes and pastries

(13). Both hard and soft wheat groups exist in both red and white classes. Durum wheat, which does not have groups, is also high in protein, and is mainly used in the production of pasta (14).

Domesticated perennial grains have been investigated in recent years as a replacement for annual grains. Annual grains, such as wheat, are a large contributor to global food security (15), and account for almost half of our agricultural land use. Unfortunately, large-scale wheat production has led to environmental degradation (16, 17), in particular water contamination from fertilizer run-off and soil erosion. Therefore, there is considerable interest in finding alternative crops to wheat whose production would be more environmentally sustainable. Intermediate wheatgrass (IWG; *Thinopyrum intermedium*) is a perennial grass, genetically related to wheat (18), native to Europe and Western Asia, that has been targeted for domestication as an edible grain to replace wheat in breads and other baking products. IWG has a longer growing season compared to seasonal wheats, which may aid in capturing nutrients and water. Because of this, there is less need for fertilizer and pesticides. Further, being a perennial, there is conservation of soils as a result on land not being plowed and left barren for much of the year, which allows more habitat for wildlife (15).

Several epidemiological studies have shown an association between a diet high in whole grains and a lower risk of colon cancer (2, 19). Specifically, the effects of whole grain wheat have shown a positive association with the reduction of colon cancer (20). This is not the case for other whole grains, such as oats or rye. Thus, the epidemiological studies suggest that wheat consumption, not other grains, is responsible for the positive

association with reduced risk of colon cancer, since wheat is by far the most commonly consumed whole grain.

How dietary components such as wheat may reduce colon cancer risk is unknown. One area that may be useful to examine is their effect on cancer stem cells. Within most tissues are relatively undifferentiated cells that give rise to the more specialized cells that comprise the tissue. These are referred to as stem cells. Each division of a stem cell gives rise to two daughter cells. One daughter cell differentiates into a more specialized cell type and the other daughter cell maintains the original stem cell. This is called an asymmetric division.

Consequently, stem cells are long-lived. This longevity, while necessary for stem cells to serve their purpose as the progenitor cell, also allows them to accumulate mutations. If enough mutations accumulate, the cell may turn cancerous; it is then referred to as a cancer stem cell. There is now considerable evidence that for some, perhaps most cancers, mutated stem cells are the cells of origin for the cancer (21). Once transformed into cancer stem cells, they are responsible for growth, resistance to chemotherapy and radiation, and recurrence of tumor cells (22). The cancer stem cell hypothesis proposes that killing of all cancer cells would not be as effective as targeting cancer stem cells. Therefore, selective targeting cancer stem cells, as opposed to all cancer cells, would lead to eliminating the cancer entirely. Although this hypothesis is not yet fully accepted, putative biochemical markers of these proposed cancer stem cells have been identified through research using animal models. In colon cancer, CD44 has been suggested to be a marker of colon cancer stem cells (23).

The purpose of this study was to examine the effect of wheat of different classes on colon cancer during the post-initiation stage of colon cancer development in rats. This study also examined whether intermediate wheatgrass modifies colon cancer risk in a way similar to red wheat. Endpoints of this study included enumeration of colonic phenotypic markers known as aberrant crypt foci, an early pre-cancerous lesion. Some aberrant crypt foci show dysplasia, or abnormal growth, within the tissue. Dysplastic crypts are thought to be associated with a higher rate of malignancy than hyperplastic crypts (5), which are the enlargement of tissue due to the increased reproduction of cells. Therefore, based on previous findings indicating that the type and amount of mucin production is a marker for dysplasia (5), changes in mucin production was examined as well. Finally, CD44, a putative marker of cancer stem cells, was determined immunohistochemically as an additional indicator of colon cancer risk in this experiment.

Chapter 1: Literature Review

Colon Cancer Incidence

Colon cancer is one of the most common cancers in the United States. It is the third most common cancer and the second leading cause of cancer-related deaths (1). Because they are anatomically similar, colon and rectal cancers are often referred together as colorectal cancer (CRC) in many epidemiological studies. According to the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program, in the United States, the 2015 estimate of new cases of colon and rectal cancer cases in both men and women was 132,700, and deaths from these cancers was estimated to be 49,700 (24). This represents approximately 8% of cancer incidence and 9% of cancer deaths in both sexes. Worldwide, the developed world, which includes the United States, accounts for approximately 63% of all cases. A ten-fold variation is seen between countries with the highest rates versus those with the lowest rates. This ranges from more than 40 per 100,000 people in the United States, Australia, and Western Europe to less than 5 per 100,000 in most of Africa and some parts of Asia (25, 26). Thus, internationally, new cases of colorectal cancer vary widely.

Risk factors for CRC can be divided into two major categories, modifiable and non-modifiable. Non-modifiable factors associated with risk include age, family history, race, and the presence of inflammatory bowel disease. Increased age is a prominent risk factor for CRC. There is also much evidence that suggests race conveys a significant disparity on CRC mortality as well. African Americans have a mortality rate 50% higher than Caucasian Americans. This is also double that of Asian and Pacific Islander patients (6).

In addition to age and race, there are other non-modifiable risk factors of this disease, such as heritability and genetics. It has been estimated that 20-30% of colon cancers are an inherited form of the disease, and are likely to be caused by genetic abnormalities with etiologies that are not well understood (27). The most common inherited forms of this disease are familial adenomatous polyposis (FAP) and heredity non-adenomatous polyposis coli (HNPCC). Genetic abnormalities, linked to common single nucleotide polymorphisms (SNPs) are thought to account for only approximately 1.14% (28). Less than 5% of all mutations are highly penetrant mutations with clearly understood etiologies.

In contrast, modifiable risk factors for this disease include lifestyle factors such as physical inactivity and excess body weight. Overconsumption of energy is likely to be one of the major contributors to the high rates of colon cancer in Western countries (29). In addition, epidemiological studies suggest a strong environmental influence on the pathogenesis of CRC (30). Among environmental factors, dietary factors have shown to greatly influence colon cancer incidence. It is estimated that diet is a major factor in over 50% of cases (7). Studies have shown inverse associations between high fiber diets, increased fruit and vegetable intake, and increased whole grain consumption and the risk of developing colon cancer (8). In contrast, there is a possible association between colon cancer and increased red meat consumption. However, epidemiological studies examining the association with red meat have been inconsistent. Although a protective effect of whole grain consumption has been supported through numerous epidemiologic studies, direct evidence through experimental studies still warrants further investigation (31).

Whole Grain and Colon Cancer Risk

Epidemiological Studies

Considerable epidemiological research has suggested an inverse association between whole grain consumption and risk of colon cancer. Jacobs et al., in a review of case control and prospective studies, found an inverse association between whole grain intake and colorectal, gastric and endometrial cancers in thirteen of fourteen studies (32). An expanded review and meta-analysis of this topic was later performed with 40 case-control studies on 20 different types of cancers. In nine of ten case control studies in which whole grains and colon cancer were investigated, odds ratios were found to be <1, indicating lower odds of cancer and colon polyps with intake of whole grains. Even after adjustment for other dietary and lifestyle factors was done, an inverse association was still seen (33). Whole grains that were included in this meta-analysis were brown bread, crisp bread, high-fiber cereal, nonwhite bread, whole-grain bread, whole-grain cereal, whole-grain pasta, and whole meal bread. However, the retrospective studies included in this analysis did not include non-western studies, thus relevant material may have been missed. For example, the author states that whole grains are normally associated with other healthy behaviors. This may not necessarily be the case in non-western areas of the world. For example, in Sub-Saharan Africa whole grain intake is as much as 159.7 grams per day, as compared to Japan and Korea, where it is 9.7 grams per day (34). This intake is unrelated to healthy behaviors such as physical activity level or intake of other food groups in these populations. In addition to this selection bias, recall bias is another limitation of the studies. All case-control studies included in this meta-analysis were retrospective and relied on self-reported dietary intakes. The combination of these elements makes it difficult to draw conclusions.

After the meta-analysis by Jacobs and colleagues, analysis of whole grain and colon cancer was performed in several case-control studies (35-38). For example, Chatenoud and colleagues (35) performed a hospital-based case-control study between 1983-1993. In this study, food intake questionnaires were administered by trained interviewers to 8907 cases of patients with varying types of histologically confirmed cancers, including 828 cases of colon cancer. There were also 7,990 controls participating within the same hospital network, without history of neoplastic disease, similarly matched for age. All questionnaires included inquiries about whole grain intake. Answers were then divided into 3 levels, based on frequency of consumption. In addition to quantifiable whole grain intake, refined grains were also grouped in levels based on consumption. An association was observed between high intakes of refined grains and increased risk of gastrointestinal cancers. An odds ratio of 1.5 in the highest quartile of refined grains consumed indicated a positive association. Conversely, there was an inverse association found with intake of whole grains and colon cancer, (OR=0.5, CI=0.3-0.6) for the highest levels on whole grain consumption vs. the lowest level (OR= 0.9, CI=0.7-1.1). However, there were unmeasured confounders, such as age, gender, and education, that were entered as a group during regression analysis, rather than individually. This could have a major effect on the relationship. Additionally, whole grain intake may have been an indicator of a healthy lifestyle, rather than a having a protective effect against gastrointestinal cancer.

In addition, several cohort studies have also investigated whether whole grains and other dietary sources of fiber have inverse associations with colon cancer (39-43). One model is a prospective cohort study done by Larsson et al. (41) that followed 61,000

Swedish women for 15 years. Dietary information was obtained from food frequency questionnaires at baseline containing questions about intake of whole grain foods such as rye bread and cold breakfast cereal. Questions about refined grain foods included foods such as soft white bread, rice, pasta, and sweet biscuits. Researchers found an inverse association with whole grain intake and colon cancer risk. Participants who consumed more than 4.5 servings of whole grains daily had a risk of colon cancer that was 35% lower than those who consumed less than 1.5 servings of whole grains daily. More recently, in a cohort study, Egeberg and colleagues (43) sent a 192-question food frequency questionnaire (FFQ) by mail to 26,630 male participants and 29,189 female participants in the Diet, Cancer and Health Cohort. Incidence of 461 cases of colon cancer that developed over 10.6 years was compared to intake of whole grain products (rye bread, whole grain bread, and oatmeal) and grams/day of whole grain. Higher intakes of whole grain products were associated with lower risk of colon cancer in men (RR=0.85, 0.77-0.95). However, no consistent association between total or individual whole grain food intakes was found in women.

The epidemiologic studies are not entirely consistent regarding whole grains and colon cancer risk. However, findings from multiple studies, taken together, overall state that whole grains are protective against colon cancer. Both case-control and cohort studies provide important information about associations between whole grain intake in a free-living population and colon cancer. Table 1 summarizes many of the different case-control and cohort studies.

However, not all cohort studies have suggested a strong inverse association of whole grains and colon cancer. For example, in the study by Schatzkin, et al., a dose-

response analysis based on the results of six cohort studies found a 17% reduction in colorectal cancer risk with an increment of three servings (three ounce equivalents or 90 g) of whole grains daily (2). However, in the study by Wu et al., a moderate positive association was seen when looking at Western diets and colon cancer. Overall the findings of these epidemiological studies support a protective effect of whole grain and colon cancer risk.

Although the epidemiology associates whole grain intake with a lower risk of colon cancer overall, caution is appropriate when considering this conclusion. It is widely known that there is a large bias in epidemiological studies in terms of recalling diets and foods within a population. Estimates of intake in these studies may not reflect actual whole grain intake. In these types of studies, the food frequency questionnaire (FFQ) is a commonly used method to assess whole grain intake. Reliability of this questionnaire is dependent on how well subjects can differentiate between whole grain and refined grain products, as discussed below. Other methods, such as the 24-hour dietary recall or a food record, may be better suited to assess whole grain intake, and may have more reliability than the FFQ. In contrast to the FFQ, both the food record and 24-hour dietary recall require a more detailed response. These details include time of day and source of food, portion size of each food and may even use visual aids to facilitate identification of foods and better estimates of portion size (44).

Regardless of the method of estimating intake, proper classification of whole and refined grains is critical when evaluating the effect of whole grains consumption and colorectal cancer risk. When self-reporting, study participants may identify a grain incorrectly based on brown coloring of food. For example, white flour may be reported as

whole wheat flour due to its brown coloring, when in fact this is white flour that has been colored brown. Therefore, though it is generally accepted that epidemiological studies suggest that whole grains are associated with a reduced risk of colorectal cancer, experimental studies are needed to confirm the epidemiology and to determine whether this risk reduction is due to a particular type of grain.

Experimental Studies

There have been numerous studies investigating separate constituents of whole grain on colon carcinogenesis in animal studies, however only a few have observed a positive effect. Zoran et al. reported that a wheat bran diet, as well as wheat bran with 10% added bran oil, reduced the incidence of colon tumors in rats given azoxymethane (AOM) (45). Another study showed that wheat germ significantly decreased aberrant crypt foci in animals given AOM (46). A lower percentage of adenocarcinoma formation as compared to animals without wheat germ diets was seen in this same study (46). Only a handful of studies have examined the effect of wheat flours on colon cancer risk. The first, conducted by Maziya-Dixon et al. (47), investigated the effect of wheat diets on tumor incidence in mice treated with a colon-specific carcinogen, dimethylhydrazine. The two varieties of wheat flours used were hard red and hard white winter wheat. Both wheat varieties were processed into whole and refined wheat flours and wheat bran. Mice were fed diets containing whole wheat flours, refined wheat flours, or wheat bran through the initiation and progression stages of colon carcinogenesis. At the end of the feeding period, histological examination showed mice fed red wheat bran had a lower incidence of colon tumor development as compared to those fed white wheat bran. These finding suggest that red wheat had a greater chemopreventive effect than white wheat.

In another study performed by Yu and colleagues (48), researchers observed the effect of soft white wheat flour (both whole and refined) compared with that of a wheat-free control diet. Different from the previous study, animals were fed either refined or unrefined wheat (both white wheats) and this was compared to a wheat-free control diet. Rats in this study were fed this diet during the initiation and postinitiation (promotion) stages of colon carcinogenesis, which was induced by heterocyclic aromatic amines. Using aberrant crypt foci (ACF) as a marker for cancer risk, these pre-cancerous lesions showed no statistical difference between treatment groups. However, ACF multiplicity, which was calculated as the number of AC per ACF, was significantly greater in the refined soft white wheat diet group in the postinitiation stage compared to the initiation stage of colon cancer development. Interestingly, this did not increase in the diet group that was fed whole wheat during the post-initiation phase. This analysis indicates that the whole-wheat diet had an inhibitory effect on the development of ACF.

In 2014, Buescher and Gallaher (49) investigated the effects of wheat class as well as processing (whole vs. refined) on colon carcinogenesis. Rats treated with a colon carcinogen were fed diets containing whole and refined red wheat or whole and refined white wheat during the initiation phase. After 20 days, they were administered a colon-specific carcinogen and then were fed for another 9 weeks. It was found that animals fed red wheat flour had significantly fewer ACF compared to rats fed white wheat flour regardless of the state of processing. Researchers hypothesized that wheat class but not processing influences colon cancer risk in this animal model.

More recently, a study by Islam and Gallaher in 2015 (50), wheat class (type) was compared in the development of colon cancer risk markers, rather than the state of

refinement. In this experiment, both red and white wheat were compared to a wheat free control diet, in both the early and late postinitiation stages of progression. Although various markers were used, it was found that both red and white wheat diets significantly reduced the number of AC, ACF, and large ACF (more than 4 AC per ACF) as compared to the control diet. This was seen in both early and late postinitiation phases. Additionally, although this marker was mostly lower in red wheat vs. white wheat, this was not significantly different. This study also incorporated the use of a stem cell mutation marker, metallothionein (MT) - positive cells. Elevated MT expression in cancers has been thought to be indicative of microsatellite instability (51), and has been proposed as a stem cell mutation marker. In this study correlation between total MT-positive crypts and both ACF and large ACF was seen. Overall, the effect of hard red wheat was greater than that of soft white wheat in reducing markers of colon cancer risk in the postinitiation phase.

There are several proposed mechanisms to explain a chemopreventive effect against colon cancer risk of whole wheat components. Wheat bran has been suggested to reduce colon cancer development by several different mechanisms, including an increase in apoptosis and a change in colon morphology. In human studies, oligosaccharides derived from cereal grains have been shown to alter the gut microflora (52, 53). Because these oligosaccharides are indigestible, they reach the colon, where bacteria metabolize them. The effects of this metabolism are short-chain fatty acids (SFCAs), which may have a beneficial physiological effect (54). These SFCAs act as signaling agents affecting gut homeostasis. An increase in the number of bifidobacteria and/or lactobacilli resulting

from feeding oligosaccharides has been demonstrated to protect against chemically induced colonic DNA damage in animal models (55).

Thus, experimental studies feeding whole wheat largely support a protective effect. The experimental studies discussed here investigated one specific type of whole grain, that is, wheat (both whole and refined). However, a viewpoint that may have been missed thus far is the class of wheat being used in experimental studies. This means that when epidemiologists are studying whole grain intake, this is typically in the form of breads and cereals. However, they are primarily looking at the intake of red wheat, the class of wheat used in breads, which is the main form whole wheat is consumed.

Wheat – A major whole grain

Worldwide, grains account for approximately 50% of energy intake. In the U.S. it is approximately 40% of energy intake (56). Globally, major cereal grains consumed include wheat, rice, and corn, whereas barley, oat, rye, sorghum, and millet are consumed in minor amounts. In the United States, wheat is the principal cereal grain grown. It also ranks fourth in volume of production and first in the volume of export.

Wheat Kernel

When planted in the soil, the wheat kernel, or wheat berry, provides the plant embryo with food to grow. This part, which is used for human consumption, contains three distinct components: the bran, endosperm and germ. These parts are separated during the process of milling.

Bran: The tough, fibrous outer layer of the wheat kernel is called the bran. The bran comprises approximately 14% of the kernel weight and consists of seven layers. The

largest component of the bran consists of the combined aleurone and pericarp. Aleurone proteins perform a variety of functions to help maintain proper development of the seed, while the pericarp acts as a coat. Together, they compose that bran of whole wheat kernel. This layer is a concentrated source of vitamins, such as B6 and niacin, minerals, like magnesium and calcium, and other nutrients, such as dietary fiber (57). Because the inner part of the wheat kernel is easier to digest, due to less fiber, the bran is often stripped during processing.

Endosperm: The inner part of the wheat kernel, known as the endosperm, is approximately 83% of the kernel by weight. The endosperm, excluding the aleurone layer, contains approximately 50-75% starch, and roughly 8-18% protein. In addition, relatively few vitamins, minerals, fiber, or phytochemicals are found in the endosperm. However, due to its greater size, this portion contains a significant number of bioactive compounds. These include phenolic compounds, flavonoids, and carotenoids. Also included are many B-complex vitamins such as riboflavin, niacin, thiamin, pyridoxine and pantothenic acid (58).

Germ: The germ constitutes about 2.5% of the kernel by weight. The germ contains quantities of high-quality protein, B-complex vitamins, alpha and beta tocopherol, iron, quinine and enzyme inhibitors. The germ is the embryo of the wheat plant, which is separated during the milling process due to its high amount of unsaturated fat. This processing extends shelf life, by preventing lipid oxidation during storage.

Wheat Classes

In the United States, wheat varieties are classified into categories: “winter wheat” and “spring wheat”. Winter wheat, planted in the fall and harvested in the spring or summer, accounts for approximately 80% of US production. Spring wheat varieties are found in northern climates with harsh winters. This wheat is planted in the spring and then harvested in the late summer or fall of the same year. These varieties fall into six major categories, further discussed below.

***Hard Red Winter Wheat*:** This variety of wheat accounts for nearly 40% of the US production. It has a high protein content, with either a hard or soft endosperm. Due to good milling and baking characteristics, it is used to make all-purpose flour and bread flour. The high protein content also yields excellent yeast leavened and artesian breads.

***Hard White Wheat*:** The newest variety grown in the US, this class has a hard endosperm and white bran. It is similar in hardness to Hard Red Winter Wheat, and it is similarly susceptible to harsh weather. Hard white wheat is used in whole-wheat and high-extraction flour applications, such as pan breads, flatbreads, and specialty noodles. Additionally, there is no distinction between winter and spring types.

***Soft Red Winter Wheat*:** With approximately 15% of production, it ranks as the third largest wheat class in US production. It has a lower protein content and soft endosperm. These qualities make this class useful to make flour for cakes and cookies. When mixed with the whole-wheat kernel of hard red wheat, it also creates excellent flatbreads and crackers.

***Soft White Wheat*:** Similar to soft red winter wheat, this class also has a low protein content (8-10%). This low protein/high carbohydrate content makes this wheat class ideal for making pastries, cookies, muffins, cakes and pancakes.

***Hard Red Spring Wheat*:** This wheat class accounts for roughly 20% of the US production and is valued for its high protein content. This makes it desirable in making specialty breads, hard rolls, and bagels. In addition, it blends well with low protein wheat to give good dough handling and mixing characteristics. Thus, this blend produces a wide variety of baked goods.

Durum: This is the hardest of all wheat classes. Its density, along with high protein content, makes durum wheat ideal for making pasta products such as macaroni, spaghetti, and other noodles. A unique property of these amber-colored kernels is their yellow endosperm, which gives pasta its golden hue.

Intermediate Wheat Grass

Not a class of wheat, this perennial grass is genetically related to wheat. It provides sustainable environmental benefits in comparison to annual winter and spring wheats while yielding an edible, nutritious grain. Because it is a perennial, soil erosion is greatly reduced with intermediate wheat grass relative to annual crops, such as wheat (59) and therefore is advantageous in maintaining ecosystems (15). Recently, there has been commercial interest in developing perennial grain crops. This is particularly true in areas with historically low grain production because of a combination of soil, climatic and topographical issues. These unfavorable environmental conditions reduce crop productivity or the capacity for grain to be harvested on time (60). However, the root

systems of perennial plants, such as intermediate wheat grass, help to improve the structure of the soil. Not only this, but with deeper root systems, perennials are able to access nutrients out of the reach of annual plants, like wheat. As well as bringing up nutrients from further down in the soil profile, the root systems of perennial grain crops are also able to draw additional moisture from the ground (61). Intermediate wheatgrass, with a nutritional value similar to that of wheat, is known for its hardy persistence and relatively large seed (62) and shows promise for rapidly improving local soil ecosystem services.

Pathways of Colon Carcinogenesis

Colon carcinogenesis is considered a multiple stepwise process in which normal cells are transformed, or mutated, into malignant cells, with corresponding genetic and biochemical changes. There are several different ways that these mutations can occur.

In the first stage of this process, an initial mutation occurs. This can happen by three different paths. The first pathway to mutation is called microsatellite instability. In this pathway, there are dysfunctional DNA mismatch repair genes, leading to hypermutability. The second mechanism is a CpG Island Methylation phenotype, which leads to hypermethylation. Lastly, major mutations occur via the chromosomal instability (CIN) pathway, which is characterized by accumulation of mutations of specific oncogenes and tumor suppressor genes (63). In the CIN pathway of colon carcinogenesis, mutations in the tumor suppressor gene, adenomas polyposis coli (APC), occur early, leading to adenomas and eventually adenocarcinomas. This is known as the adenoma-carcinoma sequence. It is widely accepted that this represents the process by which most colon cancers arise (64-66). This process begins with dysplasia, or the presence of

abnormal tissue. Subsequently, a benign growth is formed, known as a polyp. This appears on the surface of the colonic mucosa.

The second stage is a multiple step process, and often begins with a mutation in the oncogene KRAS. This leads to conformational changes of the KRAS protein, which is then unable to activate the GTPase enzyme within a cell signaling pathway. This pathway plays an important role in controlling cell growth and proliferation, among other things. As a result, the GTPase enzyme that keeps these processes in check is constitutively active, causing the polyp to grow and protumorigenic effects to be amplified (64).

In the final stages of the process of carcinogenesis, mutations in other tumor suppressor genes, e.g. *TP53* (tumor protein 53) and *DCC* (deleted in colorectal cancer), lead to late adenoma and ultimately, carcinoma. *TP53* is involved in control of the cell cycle by inducing cell cycle arrest and facilitating DNA repair. This occurs prior to the cell committing to the process of DNA replication. Furthermore, *DCC* blocks cell growth in the absence of its ligand. This receptor has three signaling states: receptor on (ligand-bound), receptor off (ligand-unbound) and lack of signal (absence of the receptor). In this case, when the receptor is bound by its ligand, it is in the *on* state, which results in cell survival and growth as usual. When the receptor is un-bound and ligand is not present, the receptor is *off* and apoptosis occurs. This mechanism is part of normal functioning villi in the gastrointestinal tract. In the villi, cell growth occurs at a high rate, and thus the process of the tumor suppressor gene *DCC* must be executed in a precise manner. The division of epithelial cells occurs at the base of the villi; these cells are pushed upwards as part of a normal physiological process. During this process, netrin-1 (the ligand of

DCC) is present and inhibits *DCC*-mediated cell death until the cell reaches the tip of the villi. *DCC* is now unbound from netrin-1 and thus the cell enters apoptosis. However, in the absence of *DCC* availability, tumor growth is favored, and this normal function no longer occurs. In the cancer state, the absence of DCC prevents this normal progression of cell growth and cell death from having an effect. Apoptosis no longer occurs once the cell reaches the tip of the villi, and the cell is more likely to continue to survive.

This sequence of molecular events resulting in dysregulation of conserved signaling networks is one by which most colon cancers arise (64, 66). Figure 1.1 shows genetic mutations that arise within the colon carcinogenesis sequence. The entire process of carcinogenesis typically occurs in three major phases: initiation, promotion, and progression. These stages will now be explained in greater detail.

Initiation

This is known as the first stage of colon cancer and involves a mutation in the DNA. This mutation, which can be inherited or spontaneous, leads to genotypic changes of the cell. Initial damage to the DNA may also be caused by the action of a carcinogen, contributing to damage. However, damage to the DNA may not have long-term consequences, as there are various mechanisms in place to repair or eliminate the damage before it is converted into a permanent mutation (i.e. fixed). Nonetheless, if these mechanisms are dysfunctional or impaired, particularly in the location of a gene that regulates cell growth and proliferation, a mutation occurs. This alters genetic information and the malfunctioning of the cell begins. Thus, it is more prone to become cancerous.

Mutations in the tumor suppressor gene, *APC*, are thought to be the initial gene mutation in the CIN pathway. The *APC* gene, which makes the APC protein, is a tumor

suppressor gene that plays a critical role in cellular processes involved in growth and regulation. Its role as a tumor suppressor is important in keeping cells from growing and dividing too fast or in an uncontrolled manner. This protein helps control how often a cell divides and how it attaches to other cells within a tissue. The APC protein accomplishes these tasks mainly through association with other proteins, especially those that are involved in cell attachment and signaling. One protein with which *APC* associates is β-catenin. This protein consists of several domains that form a multi-protein complex with β-catenin, axin, and glycogen synthase kinase, crucial in regulation and control of uncontrolled growth. Within this multi-protein complex, glycogen synthase kinase phosphorylates β-catenin. This triggers the release of β-catenin from the complex. Subsequently, ubiquitination occurs, and it is tagged for proteasomal degradation. However, with a mutation of *APC* or β-catenin genes, the formation of the multi-protein complex is inhibited. This results in the inability of the β-catenin protein to be degraded. Thus, it accumulates and translocates to the nucleus. Nuclear β-catenin associates with the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors to form a complex, which in turn activates subsequent target genes. This includes activation and expression of cell proliferation, migration, and survival genes such as *c-MYC* and *CyclinD1* (67, 68).

This initial step within the colon carcinogenesis sequence is thought to lead to the formation of aberrant crypt foci (ACF), one of the earliest changes in the colon that is seen within the colon carcinogenesis sequence. It is established that adenomas arise from ACF in the colonic epithelium as part of this sequence (69).

Promotion

In the promotion stage of colon carcinogenesis, this initiated cell becomes pre-neoplastic, or precancerous. In colon carcinogenesis, the first step involved is the initiation phase, often the inactivation of the tumor suppressor gene, APC. In the promotion phase, there is often involvement of the oncogene KRAS. Given the correct circumstances, an oncogene such as KRAS has the capability to transform a cell from its normal function into a tumor cell. Although the tumor suppressor gene and the oncogene have contrasting functions, they work together to inhibit the uncontrolled growth that leads to carcinogenesis. In the case of KRAS, its normal functions of growth regulation are impaired. This protein relays signals from outside the cell to the nucleus of the cell. These signals lead to activation of genes involved in expression of cell proliferation, migration, and survival. With this activation, also called a 'gain of function', a normal cell continues to grow in the absence of normal growth signals (66). Thus, activation of oncogenes, plus inactivation of tumor suppressor genes, allows colon cancer to arise in the promotion stage of colon carcinogenesis. In the case of CRC, this oncogene KRAS is activated and tumor suppressor gene APC inactivated in the same carcinogenesis pathway. The synergistic effects of these two genes promote faster cell division and continuous growth (70). This is known to be an important event in tumor progression in the adenoma-carcinoma sequence (71).

KRAS is one of preliminary events that initiates the activation of various signaling molecules. In other words, it acts like a molecular switch. If KRAS is off, there is no transduction signal coming from the cell surface to the nucleus. However, if KRAS is on, this allows the transmission of transducing signals from the cell surface to the

nucleus (72). Once it is turned on, it works to recruit and activate proteins necessary for the propagation of growth factor signaling and similar receptor signaling. An example of this loss was described by Boutin and colleagues (70) where oncogenic KRAS genes in mice led to cells that had lost their columnar architecture and much of their basal/apical orientation. These functions are consistent with a less differentiated tumor. Another example is the activation of c-Jun, c-Myc and c-Fos. These proteins are involved in enhanced cell proliferation and suppressed apoptosis (73). These proteins have the potential to regulate other genes by interacting directly or indirectly with DNA, thereby influencing gene transcription. All these interactions and abnormal expression leads to the abnormal growth that we see in the second stage of colon carcinogenesis.

Progression

Progression of colon carcinogenesis, as with the initiation and promotion stages, does not have a single defining event. Nevertheless, this stage involves both genotypic and phenotypic changes that ultimately lead to malignancy and metastasis. Additionally, it is more complex than the first two stages, and involves structural changes of the chromosome that may lead to a high rate of invasive activity (66).

One common mutation commonly found in the progression stage of colon carcinogenesis (74) as well as in most human tumors (75) is a mutation in the tumor suppressor protein, p53. This gene is involved in the regulation of the cell cycle and apoptosis. It has been labelled as the ‘guardian of the cell’. This is due to its ability to block cell proliferation in the presence of DNA damage. It has also been shown to stimulate DNA repair and to promote apoptosis if this repair is insufficient (76). In order to carry out this function, it has multiple domains that function to recognize specific

DNA sequences and damaged DNA, such as misaligned base pairs or single stranded DNA. Once there is DNA damage, this triggers the increased synthesis of p53, which is involved in growth arrest and DNA damage-repair. If these functions are insufficient, apoptosis occurs as a final resort (77) in order to avoid proliferation of damaged cells.

Along with the p53 mutation, there is also often a mutation in DCC (deleted in colorectal cancer) during this stage. This cell adhesion molecule is a transmembrane receptor important in development. Traditionally, a receptor is thought to be activated when bound to a ligand. Here, when bound to its ligand, DCC is phosphorylated and interacts with kinases mediating development. However, in the absence of this ligand, there is no phosphorylation and apoptosis occur. In colon carcinogenesis, DCC is thought to be in an active state due to high ligand concentration (78). In the physiological system of the gut, the division of cells occurs at the base of the crypt. Cells are then pushed upwards (with DCC bound to the ligand) by subsequent divisions to the tip where they enter apoptosis (with DCC unbound) and are sloughed off into the lumen. In a cancer state, the absence of DCC prevents the gradient from the bottom of the villi to the tip. There is no unbound DCC and no apoptosis occurs. The cell is more likely to continue to survive. DCC loss indicates that this mutation may be more important in cancer progression than in initiation (79).

In summary, this stepwise sequence, known as the adenoma-carcinoma sequence, involves multiple mutations and aberrations throughout the cell cycle. This ultimately leads to polyp formation and possible invasion into other tissues, i.e. metastasis. The accumulation of these mutations and aberrations is responsible for tumor development and metastasis.

The Cancer Stem Cell Theory

Stem cells have the potential to develop into many different cell types during development and growth. Stem cells normally remain undifferentiated, or unspecialized. However, when a stem cell divides to form a specialized cell, this occurs asymmetrically and results in the formation two daughter cells. Of these, one remains a stem cell, while the other undergoes differentiation to become another type of cell with a more specialized function (80). This specialized function depends on location. In some organs, such as the gut and bone marrow, stem cells regularly divide to replace cells lost to sloughing, as in the gut, or to produce blood cells, as in the case of bone marrow. However, in other organs such as the pancreas and the heart, stem cells only divide under special conditions (81).

There are three primary types of stem cells, embryonic and somatic, or adult stem cells and induced pluripotent stem cells. Embryonic stem cells remain undifferentiated and do not go on to a specialized function unless certain physiologic conditions stimulate them to do so. Once stimulated, they can form muscle cells, nerve cells, and many other cell types. In contrast, an adult stem cell is also thought to be an undifferentiated cell, and can be found among differentiated cells in a tissue or organ. The adult stem cell can renew itself and can differentiate to yield some or all of the major specialized cell types of this tissue. An example of this would be hematopoietic stem cells in the bone marrow. These stem cells give rise to red and white blood cells and platelets. There are also tissue-specific stem cells that replace cells that are lost during normal function or after injury. The last type of stem cell is the induced pluripotent stem cell. These are genetically manufactured from adult stem cells; i.e. reprogrammed. These stem cells undergo

numerous safety tests to ensure they are not mutated or changed in anyway throughout the reprogramming process and are used for expensive and specialized therapies.

Most adult stem cells are multipotent stem cells. Multipotent stem cells can give rise to other types of cells but are limited in their ability to differentiate, like the hematopoietic stem cells that can become a blood cell, but not a brain cell. The colon crypts contain multipotent stem cells, as shown in Figure 1.2.

The intestinal epithelium withstands continuous mechanical, chemical, and biological insults in the form of the food and bacteria. Consequently, there is a need for constant tissue replacement. This tissue replacement is fueled by continuously dividing stem cells that reside at the bottom of crypts (82). The multipotent adult stem cells that are specific to the colon are known as intestinal stem cells. It has been hypothesized that the colorectal cancer may be derived from the malignant transformation of intestinal stem cells that reside at the bottom of these crypts.

This gives rise to the hypothesis that cancer is fundamentally a stem cell disease (83-89)**.** In other words, cancer is thought to arise from a cancer stem cell (CSC), a tumor-initiating cell that has properties similar to those of stem cells. The cancer stem cell hypothesis draws on the concept that cancer cells can be viewed as aberrant cells (90). Similar to a stem cell, a cancer stem cell normally remains undifferentiated. However, during formation of specialized cells or tissues, cancer stem cells divide asymmetrically into two daughter cells. One daughter cell retains the identity of the parental cancer stem cell and the other daughter cell undergoes differentiation to form specialized cells within the tumor. Cancer stem cells are similar to normal stem cells in many ways. Both possess extensive proliferative potential. Additionally, as

aforementioned, cancer stem cells and stem cells have similar self-renewal and differentiation properties. They are both able to proliferate extensively and mechanisms that regulate the self-renewal of stem cells also frequently mediate oncogenesis (91). Figure 1.3 below depicts how cancer stem cells are thought to arise.

According to the concept of the CSC, tumors are not simple monoclonal expansions of transformed cells. Rather, a tumor is the result abnormal growth driven by a small cancer stem cell pool. In order to test the hypothesis that cancer is driven by a small CSC pool, researchers searched for a surface marker that would help to locate these select stem cells. A surface marker could give the ability to provide a molecular marker for targeted therapies in the future (92). There have been several possible markers identified, such as $CD44^{+}$, CD24, and epithelial-specific antigen $(ESA)^{+}$ tumor-initiating cells in breast cancer (93) and $CD133_{+}$ in neural tumors (94). In the colon, possible surface markers include CD 133_{+} (95). The expression of the protein promotes colon cancer proliferation through the activation of WNT/β-catenin (96). Another cell surface marker has been thought to be Lgr5, a specific target gene of WNT signaling activity (97). Lgr5 is expressed in both normal and tumor colon tissue. It is expressed in a small area of the crypt but becomes elevated when the cell transforms into adenoma (98). More discovery and a further understanding of this theory needs to occur in order to find a novel approach to cancer therapy.

Biomarkers of Colon Carcinogenesis

A biomarker is categorized as a substance whose detection indicates a specific disease state, in this case colon cancer. Biomarkers can be categorized as morphological or biochemical. In many cases, specific biomarkers have been well characterized and

have repeatedly shown to correctly predict relevant outcomes, thus their presence can be used as a primary endpoint (99). There are three widely used morphological biomarkers of colon cancer risk. These are aberrant crypt foci (ACF), sialomucin producing ACF (cells within the ACF that produce sialomucin), and mucin depleted foci (foci that are characterized by either absence or limited production of mucin). These biomarkers are thought to detect the early stages of colon carcinogenesis, prior to tumor development. Additionally, detection of biochemical markers can show genetic alterations that also occur within this progression. Both markers can be useful in examining dietary or pharmacological approaches to prevention of colon cancer.

Morphological Markers

Aberrant Crypt Foci (ACF). Aberrant crypts (AC) are focal lesions in the mucosal cells of the colon. They are characterized by abnormally shaped, enlarged crypts, and are thought to be early neoplastic lesions. They were first described by Bird in 1987 (100). AC can be visualized microscopically in carcinogen-treated rodents after methylene blue staining of whole mount colons. The lesions are identifiable as being slightly elevated from the normal surrounding mucosa with an oval slit-like opening and with darker staining. In addition, they are larger than neighboring crypts, with a thickened epithelium. Structures that contain multiple aberrant crypts are called aberrant crypt foci (ACF).

These foci are largely located in the distal portion of the colons of rats and mice (101, 102). This distal location correlates with the most common site of development of tumors (103, 104). Although many studies have shown these ACF to be neoplastic lesions, some studies suggest that ACF number does not correlate with eventual tumor

number (104, 105). Though somewhat controversial, the detection and enumeration of ACF is a frequently used biomarker.

As previously mentioned, various microscopic changes occur with the development of ACF, such as darker staining and abnormal shape and size. However, histological examination shows that these ACF can be either hyperplastic or dysplastic. Distinguishing between these two states can be useful, as dysplastic cells are far more likely to become cancerous (106).

Markers of Dysplasia

*Sialomucin producing ACF***.** Another property of colonic crypts is their mucous-producing nature. Mucous production by crypts is important in the colon for lubricating the transit of intestinal contents (107). In the colon, mucous is formed by gel-like mucins, produced and secreted by goblet cells. Mucin is a high molecular weight, heavily glycosylated protein and has a high content of oligosaccharides. This gives it a high-water retention capacity and has the characteristic ability to form gel. Intestinal mucin has a high degree of sulfate esters and is therefore known as sulfomucin. With dysplasia or abnormality in mucosal cells, the structure and chemical nature of the mucin changes, and goblet cells then start producing sialomucin. Unlike sulfomucin, this mucin contains mostly sialic acid (108).

In the adenoma-carcinoma progression of colon cancer, the production of mucin from goblet cells in ACF changes from sulfomucin (neutral) production and sialomucin (acidic) production. In humans, alterations in sialo- and sulfomucin types have also been observed in patients with colorectal cancer (108). This change is thought to be due to a reduction of oligo-O-acetylation of sialic acids found in normal colonic mucin (109). A

significant decrease in sulfomucins and an increase in sialomucins were found to correlate with disease severity (110).

Sialo- and sulfomucin can both be detected with high-iron diamine alcian blue (HID-AB) staining, which allows the mucous production to be visualized. This is due to the specificity of this stain to both sialo- and sulfoglycoproteins. For example, histochemical visualization can be done using stains that bind to specific functional groups of carbohydrates that are contained in sialo- and sulfomucin. This binding is controlled by the variation in pH seen with either sialo- or sulfomucin (111).

*Mucin depleted foci***.** As crypts become more dysplastic, mucin production begins to decrease. This is thought to be due to their transformation from ACF into more advanced lesions during colon carcinogenesis. Mucin depleted foci, or MDF, share pathologic and molecular alterations with the more advanced lesions seen in the adenoma-carcinoma sequence. This includes abnormal activation of the Wnt pathway and mutations in the *APC* gene (112, 113).

Similar to sulfo- and sialomucin ACF, these are visualized with HID-AB staining. Here, the alteration and virtual absence of mucin production leads to a distortion of the opening of the lumen compared with normal surrounding crypts as well as a washed out and clear-like appearance, compared to normal surrounding crypts. In other words, MDFs identified in HID-AB–stained colons are dysplastic ACFs with defective production or no production of mucins. Moreover, previous experimental studies in rats showed that MDFs are closer to colon cancer in terms of genetic and molecular alterations than ACFs (113-115).

Biochemical Markers

Beta-catenin is a component of the highly conserved WNT signaling pathway, which is an important cellular signaling component in regulation and growth. In several tissues, β-catenin cooperates with several transcription factors to regulate specific genetic targets in order to regulate growth. This activity is summarized below in Figure 1.4.

In the colon, Wnt signaling is primarily known as the principal organizer of stem cell identity and proliferation. Accumulation of β-catenin leads to its translocation into the nucleus, where it binds with the TCF/LEF family of transcription factors and drives the expression of various genes that regulate stemness, cell cycle progression, and cell proliferation. Consequently, Wnt/β-catenin signaling is a hallmark of many adult stem cells, and can be observed in numerous actively dividing or regenerating tissues (116). Because of the pivotal role of Wnt signaling in stem cell control, this pathway is tightly controlled. This is achieved through the expression of a wide array of ligands and receptors and through the secretion of various activators and inhibitors (117).

Aberrant Wnt-β-catenin accumulation leads to colon carcinogenesis, and it is well established that nuclear β-catenin results from this dysfunction (118). The localization of β-catenin to the nucleus functions as a transcriptional activator of several oncogenes. Thus, the expression of nuclear β-catenin is a useful predictive biochemical marker in the adenoma-carcinoma pathway of colon carcinogenesis. Animal studies have shown that accumulation of the β-catenin protein is a consequence of a mutation in the β-catenin gene (119). This mutation occurs early in the adenoma-carcinoma sequence, shown in Figure 1.1. Using β-catenin as a biochemical marker can help ascertain the ability of dietary and pharmacological agents to reduce colon cancer risk.

CD44 as a Stem Cell Marker

The cancer stem cell theory posits that among all cancerous cells, there are some that behave as stem cells. Similar to regular stem cells that reproduce and sustain our organs and tissues, these cancer stem cells renew and sustain cancer. These cancer stem cells have lost regulatory control of cell division, which leads to this over proliferation and uncontrolled growth (120). Fundamental to this theory is the concept that tumors are heterogeneous. This heterogeneity is due to an ongoing differentiation within a tumor. In this heterogeneous population, it appears that only a small subset of cells with self-renewal ability, the cancer stem cells, give rise to all differentiated cells. In the 1990's, Dick and colleagues (84), while investigating the heterogeneity of cells in acute myeloid leukemia (AML), first identified a cancer stem cell. This concept was then applied to tumors at other sites. Subsequently, cancer stem cells were found in the pancreas (88), breast (93) and neural tumor tissue (94).

In the colon, CD44 is an important membrane receptor for hyaluronic acid (HA). Rather, CD44 mediates signal transduction for HA. Through this action, this molecule interacts in the cell-matrix and cell-to-cell interactions (23) by regulating downstream pathways involved in cell maintenance (121). It is expressed in a ubiquitous manner throughout normal colon tissues, and can participate in various inflammatory process through lymphocyte activation (122). Because of these properties, CD44 is thought to be involved in various tumorigenic behaviors, including cell proliferation, differentiation, invasion, and motility (123). For example, inhibition of the interaction between hyaluronic acid and CD44 has led to suppression of tumor growth by disruption of the

PI3/Akt survival pathway (124). In addition to the action between CD44 and the PI3/Akt survival pathway, another example of the involvement of CD44 with cell interactions is during epithelial mesenchymal transition (EMT). This is the process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells. These mesenchymal stem cells then have the ability to differentiate into a variety of cell types. Hyaluronic acid is thought to promote this shift. Moreover, in an HA-rich carcinoma mass, cancer cells that had acquired this EMT-phenotype, were defined by down regulation of E-cadherin and increased nuclear translocation of β-catenin (125). This signifies activation of the WNT signaling pathway, of which CD44 is a target gene. Thus, through interaction with HA, which promotes epithelial mesenchymal transition, there is activation of the WNT pathway, leading to nuclear translocation of β-catenin and higher levels of CD44.

Previous studies have reported that CD44 interacts with several signaling pathways, including the MAPK, PI3K/Akt and Wnt pathways. In cancer, organized function of these pathways is important. MAPK regulates gene expression, cell growth and survival (126), PI3/Akt may contribute to a cell death resistant phenotype by inducing survival signals (127), and Wnt is an important player in regulating development and stemness (128). Thus, CD44 is of functional importance for cancer initiation and progression by interacting with cell differentiation, proliferation and migration. In a study by Du and colleagues, sixty fresh colorectal cancer and matched normal colon samples were collected from the tumor bank in Beijing. In order to determine tumorigenicity of colon cancer cells, cells labeled with or without CD44 markers were injected into nude mice. They found that 100 $CD44^+$ cells were enough to

initiate tumor growth within 28 days of implantation. Researchers further investigated whether a knockdown of CD44 significantly reduced formation of these cells. Through this experiment, it was found that the stemness genes such as β-catenin were down-regulated when CD44 was knocked down. This suggests a crosstalk between CD44 signaling and stemness gene expression (23).

The mechanism by which CD44 intervenes with the way that cell mechanisms occur is not well known. In clinical studies, the clinical significance of CD44 as a prognostic marker for colon cancer has been examined in several studies. For example, in 2014, Liu and colleagues examined the presence of EpCAM/CD44 cells in 80 cases of colorectal cancer and 10 cases of corresponding liver metastases in order to determine the biological behavior of colorectal cancer. Using double immunohistochemical staining to detect the correlation between these cells, researchers found that the expression of EpCAM/CD44 was significantly correlated with degree of differentiation, tumor degree, and invasion and metastasis (129). This finding suggests that targeting cancer stem cells, and specifically EpCAM and CD44, may reduce tumor size. Studies such as this demonstrate the potential use of this kind of strategy.

In 2017, Li, et al. looked at the combination of CD44/CD24/ALDHI (aldehyde dehydrogenase, another putative marker of cancer stem cells due to its role in maintenance, development and differentiation) in breast cancer cells. It was found that the combination of all of these putative CSC markers was the most reliable was to evaluate the stem properties of tumors. This was done by suppression of expression for each specific combination of CSC marker. Since they all had different origins and

properties, researchers were able to demonstrate that the CSC markers performed different functions during tumor progression and metastasis. Thus a combination of a set of CSC is a more reliable way to evaluate the stem properties of tumors (130). The putative CSC markers seen here are similar in function to CSC markers seen in other cancers, such as colon cancer. Therefore, they can serve as biomarkers in order to monitor tumor progression and possibly predict prognosis in colon cancer.

The WNT/ β-catenin pathway has emerged as a promising therapeutic target in colorectal cancer. Most patients with colorectal cancer have mutations in at the least one Wnt signaling cascade gene, such as the β-catenin, of which CD44 is a major target gene. This dysregulation is associated with increased cell proliferation and resistance of the tumor cells to chemotherapy, which implies value as a therapeutic target in treatment of colon cancer. However, the exact role of this molecule in carcinogenesis and tumor biology is unclear. Thus, further study of CD44 as a putative stem cell marker for colorectal cancer stem cells to improve our understanding of how dietary interventions may reduce the risk of colorectal cancer is warranted.

Table 1.1: Case-control and cohort studies investigating whole grain intake and risk of colon cancer

Reference	Study Design	Summary	Results with RR/OR and Confidence Interval
McCullough et al. (131) (2003)	Cohort	Men and women that were part of the Cancer Prevention (CPS) II Nutrition Cohort were given self-administered questionnaires involving demographic, medical and food & lifestyle questions. FFQs included questions of frequency of intake for all major food groups, divided into 5 quintiles of lowest to highest intake. ***Study did not show a strong role for fruits, vegetables and whole grains and fiber in colon cancer risk reduction, however risk may increase with low levels of intake.***	Whole Grains: • Men: ≥11.0 vs. <2.0 servings/wk: *RR=0.95, CI=(0.64-1.42)* • Women: ≥11.2 vs. 2.5 servings/wk: *RR=1.17, CI=(0.73-1.87)]*
La Vecchia et al. (36, 132) (1998, 2003)	Case Control	Men and women with 955 cases of colorectal cancer ages 45-74. All participants were given FFQs with questions about whole grain foods (breads or pastas). Intake was based on level and frequency of consumption. In the population, it was seen that a high intake of whole grains is an indicator of reduced risk for many types of cancers, including colorectal cancer. ***Researchers hypothesized that whole grain intake is generally associated with healthier lifestyle and habits. This may include complex aspects of diet as well as physical activity.***	Frequency of consumption: • High: [OR=0.5 *(CI=0.5-0.6)]* Intermediate: [OR=0.9 *(CI=0.7-1.0)]*
Schatzkin et al. (42) (2007)	Cohort	291,988 men and 197,623 women were given self-administered FFQ's validated by the Association of Official Analytic Chemists. Questions were administered about frequency of intake for fiber (dietary, grain, fruit, vegetable, bean) and whole grains. Levels ranged from 'never' to '≥ 6 times per day' for beverages and 'never to '≥ 2 times per day', along with three categories of food with portion sizes for solid foods. ***Methodological issues need to be considered: range of intake, confounding by other nutritional factors, measurement error - could lead to inconsistent results.***	Researchers observed an inverse association for whole-grain intake.
Wu et al. (39) (2004)	Cohort	561 male cases of colon cancer given FFQ's were separated into prudent vs. western dietary pattern based on frequency of intake of all food groups (i.e. breads, cereals and	Researchers found a moderate positive association between higher western pattern scores and

	starches plus other food groups). Frequency was divided into high vs. low quintiles, combined with medical history and activity level. Additionally, there were 3 separate periods of follow-up with questionnaires: 1986, 1990 and 1994. ***Strengths of this study include: long follow-up period and the ability to have multiple questionnaires. However, the data does not provide consistent evidence for an inverse association between higher pattern scores and overall risk of colon cancer.***	risk of colon cancer. [RR=0.84, *95% CI=0.64-1.10; P_{trend}= 0.37].*

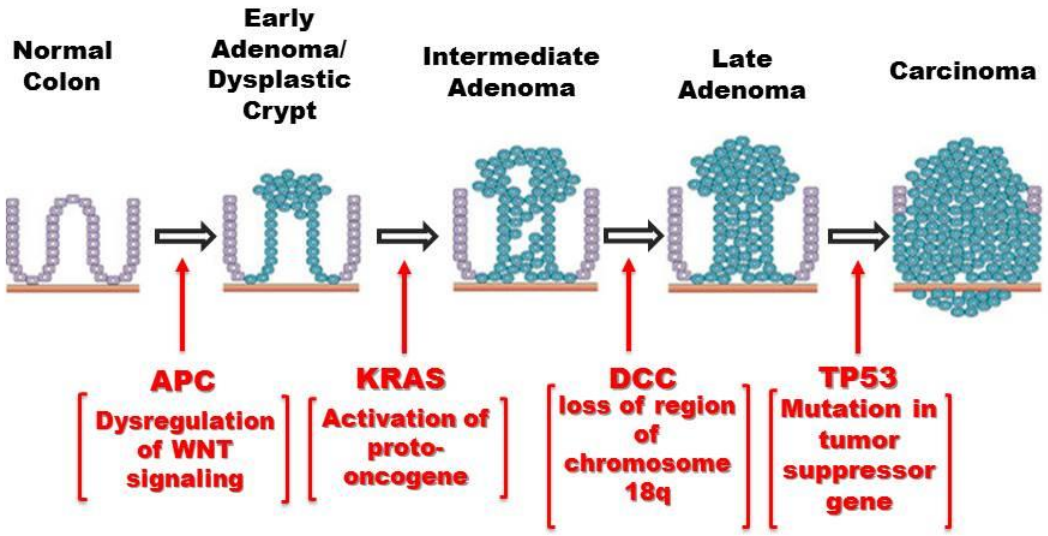

Figure 1.1: Genetic Mutations within the carcinoma-adenoma sequence. Adapted from: Rajagopalan et al. The significance of unstable chromosomes in colorectal cancer.

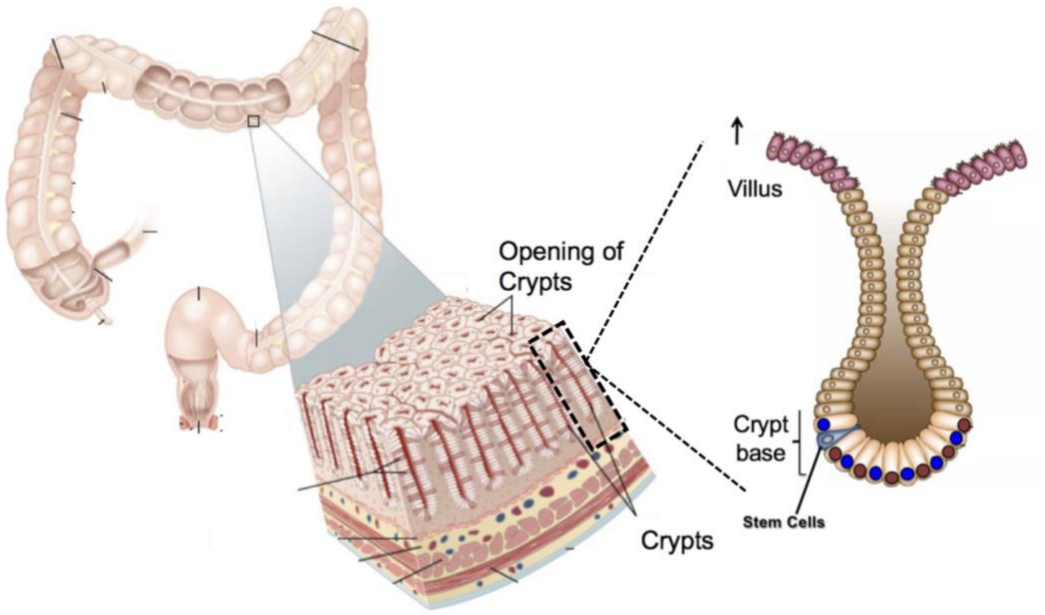

Figure 1.2: Detailed anatomy of the colon, showing the crypt opening. This shows where the intestinal stem cell (a multipotent adult stem cell) resides in the colon. *Adapted from: Clevers, H. Searching for adult stem cells in the intestine*. EMBO Molecular Medicine 2009:1(5), A61-295

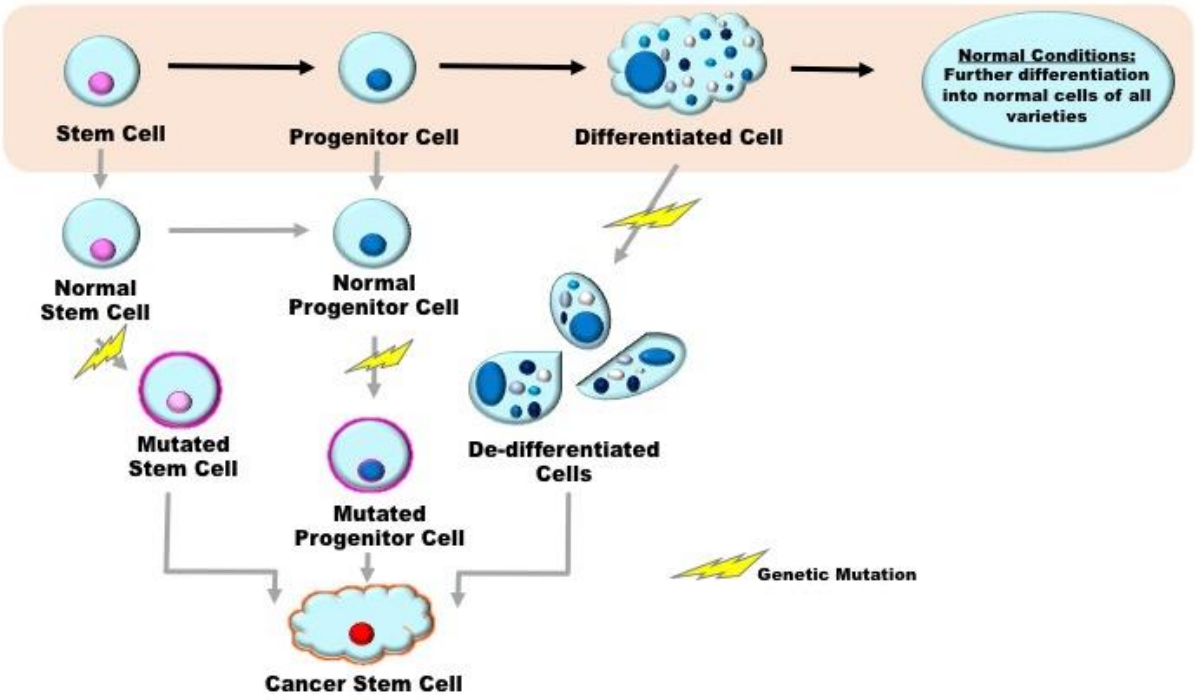

Figure 1.3: How Stem Cells Arise. Figure shows how a normal stem cell creates a new stem cell and a progenitor cell, later maturing to a differentiated cell. An event (i.e. a genetic mutation) make it possible for cells to become mutated or dedifferentiated. Affected cells are thought to be able to produce cancer stem cells. *Adapted from: European Cancer Stem Cell Research Institute - About Cancer Stem Cells (133).*

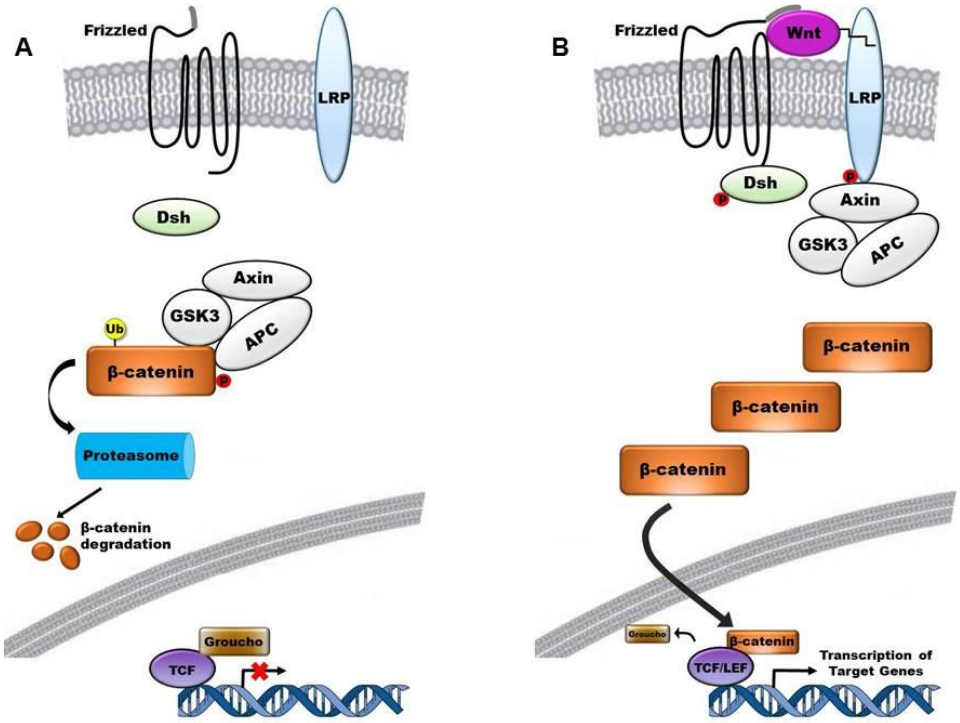

Figure 1.4: Overview of WNT signaling. A) In cells not exposed to WNT, β-catenin associates with and is phosphorylated by the destruction complex, composed of Axin, GSK-3 and APC. Phosphorylated β-catenin is the targeted for ubiquitination (Ub) and subsequent proteosomal degradation. Consequently, T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors are repressed by Groucho in the nucleus. B) In the initiation of WNT signaling, binding of the WNT protein to the Frizzled and LRP receptors induces phosphorylation of LRP and recruitment of Axin. During this interaction, Dsh is also phosphorylated. The Axin/APC/GSK-3 complex inhibits phosphorylation of β –catenin and subsequent proteosomal degradation, leading to accumulation of cytosolic β -catenin. Accumulated β-catenin then translocates to the nucleus, replaces Groucho from the TCF/LEF transcription factor, and controls transcription of target genes. From reference (134)

Chapter 2: The Effects of Different Types of Wheat on Colon Cancer Risk

Introduction

Colon cancer is the third most common cancer and the second leading cause of cancer-related deaths in the United States (1), making colon cancer a major public health issue. There are a number of risk factors that contribute to the development of colon cancer, including inflammatory intestinal conditions, family history of disease, age, smoking, and environmental risk factors such as lifestyle, physical activity, and diet.

Several observational studies have suggested that high intakes of whole grains, fruits and vegetables may lower the risk of colon cancer (2, 19). As seen in many epidemiological studies, types of whole grains such as wheat, rice, oat and barley have been shown to be associated with a reduced risk of colon cancer (32, 33, 35, 132). Although the epidemiology associates whole grain intake with a lower risk of colon cancer overall, caution is appropriate when considering this conclusion. Proper classification of whole and refined grains is critical when evaluating the effect of whole grains consumption and colorectal cancer risk. Most epidemiological studies classify whole grain products as bread, which is made with red wheat. In addition, when looking at refined grains vs. whole grains, most refined grains are categorized as cakes and cookies. These are made with white wheat. Thus, researchers are examining the differences between red wheat and white wheat as well as whole and refined wheat. In other words, the association between whole grain consumption and colon cancer risk is confounded by the type of wheat used in whole grain products. Further, several experimental studies have supported the finding that red wheat is protective against colon cancer. Previous animal experiments in our lab, performed by Buescher and Gallaher (49) and Islam and Gallaher (50), found that wheat, specifically wheat type (class), reduces risk of colon cancer, whereas there is little or no influence of the state of refining of the wheat, i.e. whole versus refined wheat, on colon cancer risk markers.

The domestication of perennial grains has gained interest in recent years. The perennial grass, intermediate wheat grass, is genetically related to wheat. It provides sustainable environmental benefits in comparison to annual winter and spring wheats while yielding an edible, nutritious grain. Because it is a perennial, soil erosion is reduced, and the root systems of these plants help to improve the structure of the soil. This has great impact in areas of historically low grain production. These factors make IGW advantageous as compared to the frequently used annual wheats for the production of foods such as cakes, breads, and crackers. Therefore, we have chosen to study this in addition to red and white wheat in order to determine the efficacy of this in the reduction of risk reduction in colon carcinogenesis.

Colon carcinogenesis involves clonal expansion of mutated colonic crypt cells generally termed as pre-cancerous lesions (135). They have been used by many researchers as an early biomarker of colon carcinogenesis to evaluate the effect of various chemopreventive agents. Evidence is mounting supporting the idea that aberrant crypt foci (ACF) are colon cancer precursors. Their size and numbers directly correlate with the risk of developing colon cancer (136). Therefore, in this study, we chose to examine them as a morphological marker of colon cancer. Another property of colonic crypts is their mucous-producing nature. Intestinal mucin is known as sulfomucin. With dysplasia or abnormality in mucosal cells, the structure and chemical nature of the mucin changes, and goblet cells then start producing sialomucin. Alterations in sialo- and sulfomucin types have also been observed in patients with colorectal cancer (108). Thus, in this study we chose to use several other biomarkers have been considered, such as sialomucin producing ACF (SIM_ACF) and mucin depleted foci (MDF) as markers of dysplasia.

Lastly, CD44, a putative marker of cancer stem cells, was determined immunohistochemically as an additional focus of this experiment. In the colon, CD44 is an

important membrane receptor for hyaluronic acid and found to intervene in cell-matrix and cell-to-cell interactions. CD44 is thought to be involved in various tumoregenic behaviors, including cell proliferation, differentiation, invasion and motility. This is thought to be due to its interaction with the WNT signaling pathway and β-catenin (137).

Materials and Methods

Animals and Diets

Forty-nine male Wistar rats, age 3-4 weeks (average weight 50-75 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). All rats were housed individually in wire-bottomed hanging cages, under standard conditions. Animals were maintained in rooms with temperature at 20 ± 2°C with a relative humidity of 50 ± 10%, and 12-hour light/dark cycle. All diets and water were available *ad libitum* throughout the study period. This study was approved by the University of Minnesota Committee on Animal Care and Use.

Diet ingredients (except wheat flours) were purchased from Dyets, Inc. (Bethlehem, PA). Refined red and white flours were purchased from ConAgra Foods (Commerce City, Co). Whole intermediate wheatgrass berries were a gift from University of Minnesota Food Science and Nutrition Department (Saint Paul, MN). All wheat berries were milled locally. The control diet was a modification of the AIN-93G, a purified rodent diet (138). The three experimental diets contained flours made from the following wheats: refined hard red flour (HR), refined soft white flour (SW) and intermediate wheatgrass (IGW). Wheat flours were stored at -20°C until incorporated into the diets. Table 2.1 shows the proximate analysis of each wheat flour used in the experiment. Flour-containing diets consisted of 55% of wheat flour by weight. The formulation of experimental wheat diets were based on the AIN-93G diet (138) and were balanced for macronutrients. Diets were prepared fresh weekly and kept refrigerated. Table 2.2

shows the composition of the diets.

Experimental Design

Rats were adapted to the control diet for 7 days after arrival. They were then injected twice with the colon-specific carcinogen 1, 2 dimethylhydrazine (DMH) (50 mg/kg body weight) subcutaneously one week apart, a common experimental model used among cancer chemoprevention studies (139). All rats were fed the control diet during and one week after carcinogen treatment. Subsequently, 36 rats were then randomized into the wheat-based experimental diet groups (12 in each) and 12 continued to be fed the control diet. Rats were fed 10 additional weeks prior to sacrifice. Body weights and food intake were recorded weekly throughout the study. Figure 2.1 shows a schematic representation of the carcinogen treatment and feeding regimen.

Preparation of Colon Samples

Rats were anesthetized using isofluorane, opened by laparotomy, and colons excised. Upon removal, colons were flushed with ice cold PBS (pH 7.4). The colons were then gently slid onto a 2 ml glass pipette, starting from the cecal end, and completely submerged in 10% formalin in PBS for 5-7 minutes. Following this, the colons were opened longitudinally with a razor blade and washed with PBS several times. A 2 cm section of the anal end and entire proximal end were removed before final processing. Once these sections were removed, final fixation proceeded in the following manner: colons were separated into 2 cm section of distal end and 5 cm section of distal end above this section, called the mid-distal section. Figure 2.2 shows a schematic representation of the final processing and fixation. In order to fix this colon tissue, the distal colon was placed flat between filter papers saturated with 10% formalin-PBS. These were then individually coded to allow an unbiased enumeration of ACF and stored overnight in an airtight

container at 4 °C. The colon tissue was then transferred to 70% ethanol for storage until ACF enumeration (approx. one month).

Determination of Aberrant Crypt Foci (ACF)

After long-term storage in 70% ethanol, colon tissue was stained with 0.2% methylene blue (Sigma Chemical Co., St Louis, MO) for 2-3 minutes with gentle shaking using a Thermo Scientific rotator. Colon tissue was then washed with PBS to remove excess methylene blue, transferred to a clean dish, and submerged into distilled water to avoid drying. The mucosal side of the tissue was examined under a light microscope at a magnification of 10X (Olympus BX40, Olympus Optical Co, Tokyo, Japan). Using the method introduced by Bird et al. in 1987 (100) to quantify AC and ACF, the total number were counted in both the 2 cm section and the 5 cm section. The 2 cm section of colon was placed in a histology processing tissue cassette and stored in 70% ethanol at 4° C to be used for immunohistochemistry. The 5 cm section of colon was stored at 4° C in 70% ethanol until analyzed for mucin production.

Determination of Mucin Production

Formalin fixed colon tissue was stained with high iron diamine alcian blue (HID-AB) solution to determine type and presence of mucin. Colon tissue was rinsed in distilled water and PBS for 5-10 minutes to remove excess ethanol and then transferred to a staining dish containing freshly prepared iron-diamine solution. This was then incubated in iron-diamine solution in the dark for 8 hours at room temperature. After incubation, colon tissue was rinsed three times in distilled water and stained with 1% alcian blue (Sigma Chemical Co.) in 3% acetic acid solution for 20 minutes at room temperature. Lastly, the colon tissue was rinsed three times in 80% ethanol followed by distilled water and transferred to a clean dish for microscope viewing. HID-AB stained colon tissue was examined at 10X magnification under a light microscope

(Olympus BX40, Olympus Optical Co, Tokyo, Japan). Mucin type and presence was scored according to the criteria described by Caderni et al. (140). These were sulfomucin producing-ACF (SUM-ACF), identified by brown staining, sialomucin producing-ACF (SIM-ACF), identified by bright blue or bright purple staining, a mix of SUM-ACF and SIM-ACF, known as mixed mucin producing-ACF (MIX-ACF), identified by a mixture of brown and blue/purple staining, and mucin depleted foci (MDF), identified by either very little staining or no staining.

Immunohistochemical Determination of CD44

Paraffin embedded 2 cm distal colon sections (fixation method described previously) were heated for 15 minutes in a convection oven in preparation for deparaffinization. Sections were then deparaffinized in xylene three times for 5 minutes, and rehydrated through graded alcohol solutions at room temperature for an additional 30 minutes. Upon complete rehydration, sections were treated with 30% H_2O_2 for quenching endogenous peroxidase and prepared for antigen retrieval. This was carried out by treatment in a decloaking chamber (Biocare Medical, Concord, CA) for 18 minutes at 99° C in freshly prepared 0.01 M citrate buffer. The sections were then washed with protein block consisting of 0.5% casein/0.5% BSA/PBS for blocking of endogenous protein and eliminating non-specific binding. After repeated rinses with PBS and distilled water, they were incubated overnight at 4° C with polyclonal rabbit IgG primary antibody (#PA1021-2, Boster Biological Technology, Pleasanton, CA) at a dilution of 1:850. Following primary antibody incubation, the sections were washed with PBS and distilled water and incubated with the biotinylated secondary antibody, goat anti-rabbit IgG (#ab64632, Abcam, Cambridge, MA) for 20 minutes. Sections were then treated with streptavidin-bound peroxidase for binding to the secondary antibody. This allowed for visualization using diaminobenzidine (DAB) precipitation. Lastly, sections were counterstained with hematoxylin, mounted with 60%

permount solution, and dried overnight in preparation for light microscopy.

Percentage of CD44-positive colonic crypt cells was obtained in each section at 40X magnification under a light microscope (Olympus BX40). Scoring was made based on categories of 0%, 10%, 20%, 40% and >50% of positive staining of CD44 in the colonic crypts. These five categories were based on area of staining of the anti-CD44 antibody vs. non-stained ACF, using methods described by Landini (141), in analysis by Color Deconvolution for Fiji imaging software (National Institutes of Health, Bethesda, MD). These percentage categories were used for statistical analysis.

Statistical Analysis

The data were analyzed by one-way using SAS system for windows, version 9.4 (SAS Institute Cary, NC). One-way analysis of variance (ANOVA) was used to examine the effect of different wheat diets on ACF number, sialomucin production, and percentage of CD44-positive colonic crypt cells. Differences among groups were inspected using Duncan's multiple range test. Differences were considered statistically significant when p value was less than or equal to 0.05. Correlation between dysplasia score and CD44 staining intensity was determined by Pearson correlation.

Results

Food intake and body weight

Initial and final body weights and daily food intake are summarized in Table 2.3. Weekly body weights (data not shown), including final body weight, of the white wheat diet were significantly greater than the groups fed control and intermediate wheatgrass diets. The groups fed red wheat had a significantly greater intake than the control, white and intermediate wheatgrass diets.

Effect of wheat diets on AC and ACF number

Table 2.4 shows the number of AC, ACF and the multiplicity (number of AC per ACF) for each diet group. There were no statistically significant differences among the groups for any of these measures.

Effect of wheat diets on mucin staining of ACF

The effect of the different wheat diets on the degree of mucin staining is presented in Figure 2.3. There was a significantly greater number of SUM_ACF in the red wheat group than the control, white wheat, and IWG groups. This finding was reversed with the MIX_ACF, SIM_ACF and MDF staining. There was a greater number of MIX_ACF in white wheat and IWG groups compared to the red wheat diet. There was also a significantly greater number of SIM_ACF in the control group than the wheat groups, and the red wheat group had fewer SIM_ACF than the white wheat or IWG groups. All three wheat groups had significantly fewer MDF than the control group, and the red wheat group had significantly fewer MDF than the white wheat group. However, IWG showed a trend towards a statistically significant difference from white wheat ($p=0.060$). Figure 2.3 shows the proportion of ACF in each category of mucin expression for each group.

Effect of wheat diets on CD44, a putative stem cell marker, in ACF

Figure 2.4 shows the areas of CD44 stain within ACF. This was separated into 3 categories, depending on the area of staining as previously described. The red wheat group had a significantly greater number of ACF with staining area in the 1-10% stained area than the control diet. No staining in this category was detected with the white wheat and IWG diets. Both white wheat and IWG groups had a significantly greater number of ACF with an 11-15% stained area of the CD44 stem cell marker, as compared to the control group. Additionally, the control group

had significantly more ACF with 11-15% stained area than the red wheat diet. In the 16-20% stained area category, only the control diet group had this degree of CD44 staining in ACF; the wheat diet groups had almost no ACF with 16-20% staining.

Correlation of CD44 staining with mucin staining

A dysplasia score was calculated by assigning a value of 0 to ACF staining for only sulfomucin, a value of 1 for ACF staining for a mixture of sulfomucin and sialomucin, and score of 2 for ACF staining only for sialomucin, and a score of 3 for mucin-depleted foci. A CD44 score was calculated as the number of ACF in each of the three staining categories shown in Figure 2.5. Table 2.5 shows the correlation of 0.7029 ($p<0.0001$) for the association between the dysplasia score and the CD44 score, indicating a high degree of association between them.

Discussion

Several observational studies have suggested that high intakes of whole grains, fruits and vegetables may lower the risk of colon cancer (2, 19). As seen in many epidemiological studies, whole grains such as wheat, rice, oat and barley have been associated with a reduced risk of colon cancer (32, 33, 35, 132). Although the epidemiology associates whole grain intake with a lower risk of colon cancer overall, caution is appropriate when considering this conclusion. Proper classification of whole grains is critical when evaluating the effect of whole grains consumption and colorectal cancer risk. In many epidemiological studies, the primary whole grain product is bread, which is made with red wheat. In addition, when looking at refined grains vs. whole grains, most refined grains are categorized as cakes and cookies. These are made with white wheat. Thus, these researchers are examining comparisons between red wheat and white wheat as well as between whole and refined wheat.

A previous study from this laboratory by Buescher and Gallaher reported that red wheat reduced ACF number relative to white wheat in rats when fed during both the initiation and post-initiation (promotion) stages (142). The state of refinement (i.e. whole versus refined) had no effect on ACF number. However, wheat class (red versus white) was found to be an important chemopreventive characteristic. Another study by Islam and Gallaher further examined the same hypothesis, but did this during the post-initiation stage, which falls after the time of carcinogen exposure. This is thought to be a crucial time in the colon carcinogenesis progression when inactivation of a tumor suppressor gene is followed by the activation of an oncogene. This action induces β-catenin, which leads to an elevation of downstream target genes such as C-myc and cyclin D1 (143). This action can turn normal colon crypts to aberrant crypts and then further to display dysplastic markers.

This study found both red wheat and soft white wheat diets significantly reduced the number of AC, total ACF and large ACF compared to a wheat-free control diet fed during the post-initiation stage (50), using markers of dysplasia. This difference was thought to be due to the timing of the feeding of the different wheat diets and the control diet (i.e. feeding wheat during the post-initiation stage). The study by Buescher and Gallaher examined the combined initiation and promotion stages of colon carcinogenesis. That is, rats were fed before, during, and after carcinogen administration. In contrast, in the study by Islam and Gallaher, rats were fed a control diet before and during carcinogen administration. These animals were then fed diets containing red and white wheats starting one week after carcinogen administration and for 15 weeks following this period. The present study also looked at the post-initiation period. Our finding was consistent with the previous study which showed both red and white wheat significantly reduced AC and ACF during the post-initiation period. The present study confirms

that the timing of the intervention made a difference. Similar to Islam and Gallaher, the red and white wheat showed protective effects when fed to the animals after carcinogen administration. Rather, when red and white wheats are fed to rats during the initiation stage of the colon carcinogenesis process, the protective effects are not seen and there are no significant changes in the carcinogenic process. However, when animals are fed red and white wheats during post-initiation, when genetic mutations have already started taking place in the carcinogenesis process, red wheat has a greater protective effect.

The present study examined markers of dysplasia in addition to the morphological marker of AC and ACF. It has also been hypothesized that the lack of a protective mucus layer might activate the local inflammation, leading to the development of mucin depleted foci during carcinogenesis (144). Here, there was a significant reduction in sialomucin staining of ACF, and mixed (sulfomucin + sialomucin) ACF, in animals fed red wheat vs. white wheat and intermediate wheatgrass. Mucin-depleted foci were also reduced in the red wheat group vs. the white wheat group and IWG group, however this difference was not as large. In contrast, this was not observed in sulfomucin stained ACF when the red wheat diet was compared to white wheat and intermediate wheatgrass. These findings correspond with previous findings showing that ACF changes from sulfomucin (neutral) production and sialomucin (acidic) production during the progression of colon carcinogenesis (145, 146).

Abnormal mucin with predominance of sialomucins is a marker of dysplasia, or the presence of abnormal tissue. This is thought to signify a greater risk of colon cancer. As crypts become more dysplastic, mucin production begins to decrease or stop, resulting in what is referred to as mucin depleted foci. Studies in rodents have demonstrated a correlation between the presence of mucin depleted foci and colon cancer (147, 148). In our study, the significantly

lower number of sialomucin ACF and mucin depleted foci seen within the red wheat groups shows that red wheat was more effective at reducing markers of crypt dysplasia than white wheat.

Intermediate wheat grass, or Kernza™, may provide sustainable environmental benefits in comparison to annual winter and spring wheats while yielding an edible, nutritious grain. Therefore, it was of interest to determine how IWG would compare to traditional wheats. We found that IWG did not differ from red or white wheat in ACF number compared to rats fed with either red wheat and white wheat. IWG was intermediate between the two in MDF number, but numerically was closer to red wheat, and showed a trend towards a difference from white wheat.

Although expression of CD44 occurs in normal tissue, through its participation in cell differentiation, proliferation and migration, high expression of CD44 has been associated with the tumorigenic process in colon cancer (149, 150). This is thought to occur via its role in cell-to-cell adhesion and also through action in inflammatory pathways (151). CD44 promotes resistance to apoptosis and is important in regulating apoptotic genes (152). This protein also plays a role in regulating WNT signaling (137).

In the present study, we found that rats fed red wheat diets expressed less CD44 protein in ACF in comparison to those fed white wheat diets. Further, our study showed a positive correlation between the CD44 score and dysplasia score. At this point it is unknown how red wheat may have reduced CD44 in ACF, or whether the reduction in CD44 is responsible for the lesser degree of dysplasia found in the ACF. Further studies will be needed to disentangle the relationship between CD44 expression and dysplasia.

This study supports previous research in this lab, that both red and white wheat reduced markers of colon cancer risk, but that red wheat was more effective. Thus, this study continues to

support the concept that the association of protection from colon cancer by whole grains may in fact be due to greater consumption of red wheat versus white wheat. Perennial plant domestication in order to enhance food production has been highly popular in the last few years (18, 154, 155). Intermediate wheat grass, commercially known as Kernza™, is one such perennial plant that is beginning to be commercialized. In terms of its effect on colon cancer risk markers, it was more similar to white wheat than red wheat. However, as work on domestication of this perennial grain continues, it will be important to continue to assess its potential health benefits, as further breeding may well alter its chemopreventive character. Finally, our work provides additional support that diet can influence the development of cancer stem cells in the colon. Further studies of the relationship between dysplasia and cancer stem cell development is warranted.

Table 2.1: Proximate Analysis of Wheat Flours [1,2]

Flour	Carbohydrate (%)	Protein (%)	Fiber (%)	Fat (%)	Ash (%)
Hard Red Wheat	71.2	14.0	3.7	1.39	0.57
Soft White Wheat	76.8	9.87	3.1	1.44	0.57
Intermediate Wheatgrass	68.6	17.3	16.4	2.3	2.3

[1] Proximate analysis of wheat flours performed by Medallion Laboratories (Golden Valley, MN)

[2] Proximate analysis of intermediate wheatgrass performed by University of Minnesota Food Science and Nutrition Department (Saint Paul, MN)

Table 2.2: Diet Composition (g/kg)

	Control (Modified AIN-93G)	Refined Hard Red Flour	Refined Soft White	Whole Intermediate Wheatgrass
Flour	0	550	550	550
Sucrose	100	100	100	100
Cornstarch	449.5	17	10	56
Soybean Oil	120	82.5	79	79
Casein	200	140	150	104
Cellulose	80	60	60.5	60.5
Mineral Mix	35	35	35	35
Vitamin Mix	10	10	10	10
L-Cystine	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5
Butylated Hydroxytoluene	0.014	0.014	0.014	0.014
% CHO	54.5	51	54	53
% Protein	20	22	20	20
% Fiber	8	8	8	8
% Fat	12	9	9	9

Table 2.3: Body weight and food intake of animals [1]

Diet Group	Initial Body Weight (g)	Final Body Weight (g)	Food Intake (g/day)
Control (Modified AIN-93G)	84.3 ± 2.41	435.8 ± 16.78 b	20.5 ±1.16 b
Hard Red Wheat	85.5 ± 1.72	467.2 ± 10.98 ab	22.0 ± 1.27 a
Soft White Wheat	79.0 ± 1.83	478.6 ± 10.38 a	21.5 ± 0.88 a
Intermediate Wheatgrass	81.4 ± 1.94	430.3 ± 10.53 b	19.8 ± 0.79 b

[1] Values are reported as mean ± SE, n=12 per group. Values with different letters are statistically significant

Table 2.4: Effect of diets on AC and ACF in rat colon[1]

Variable	Control	Red Wheat	White Wheat	Intermediate Wheatgrass
AC, n/cm2	18.7 ± 2.2	22.8 ± 2.8	17.4 ± 3.0	24.5 ± 2.9
ACF, n/cm2	8.5 ± 0.7	10.3 ± 1.1	7.5 ± 1.2	9.8 ± 1.2
Multiplicity	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.4 ± 0.1

[1] Values are reported as mean ± SE, n=12 per group. AC, aberrant crypts; ACF, aberrant crypt foci; Multiplicity, AC/ACF.

Table 2.5. Correlation between CD44 scores vs dysplasia scores[1]

	Dysplasia Score
CD44 Score	0.7029 <0.0001

[1]Upper value is the correlation coefficient. Lower value is probability. CD44 Score, Percent area of CD44 within ACF, determined by the intensity of staining; Dysplasia Score, degree of mucin staining, with 0=sulfomucin (SUM), 1=sulfo-sialomucin (MIX) 2= sialomucin (SIM), 3=mucin depleted foci (MDF).

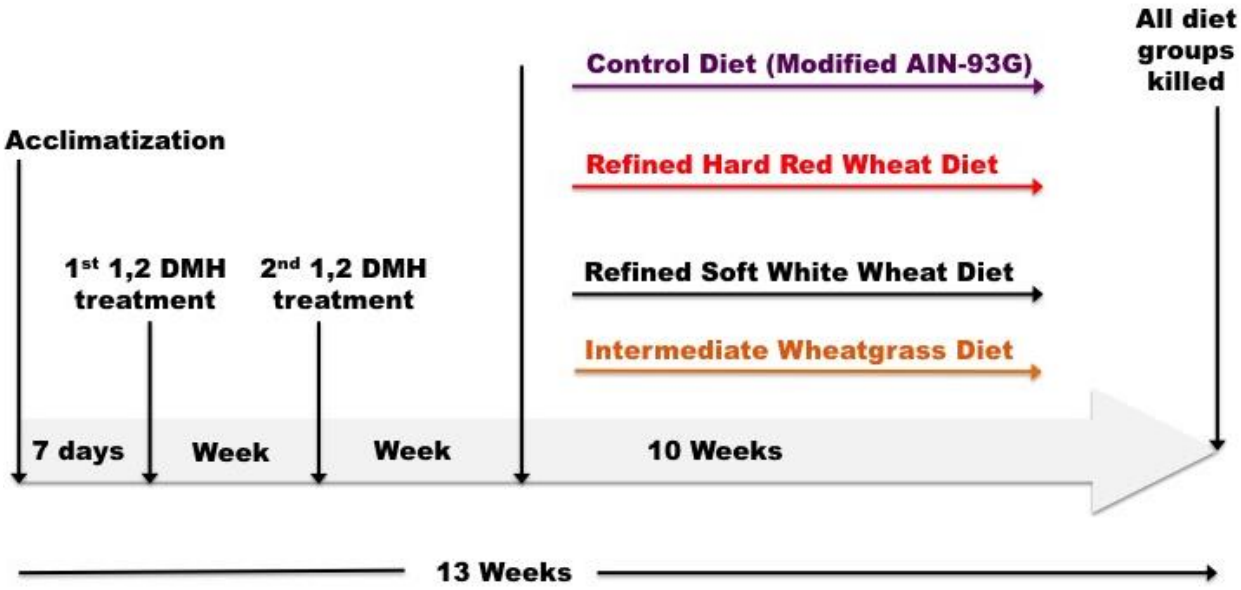

Figure 2.1: Schematic representation of carcinogen treatment and feeding regimen.

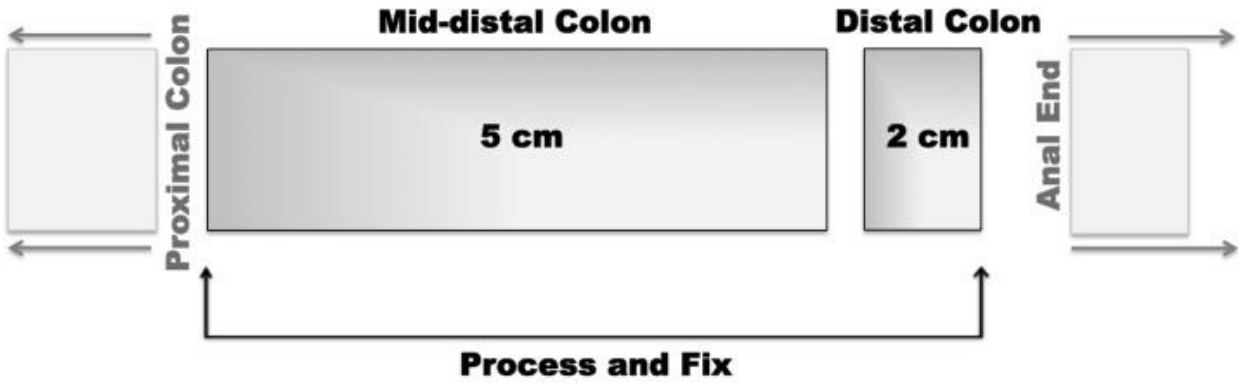

Figure 2.2: Schematic representation of the final processing and fixation of the rat colon

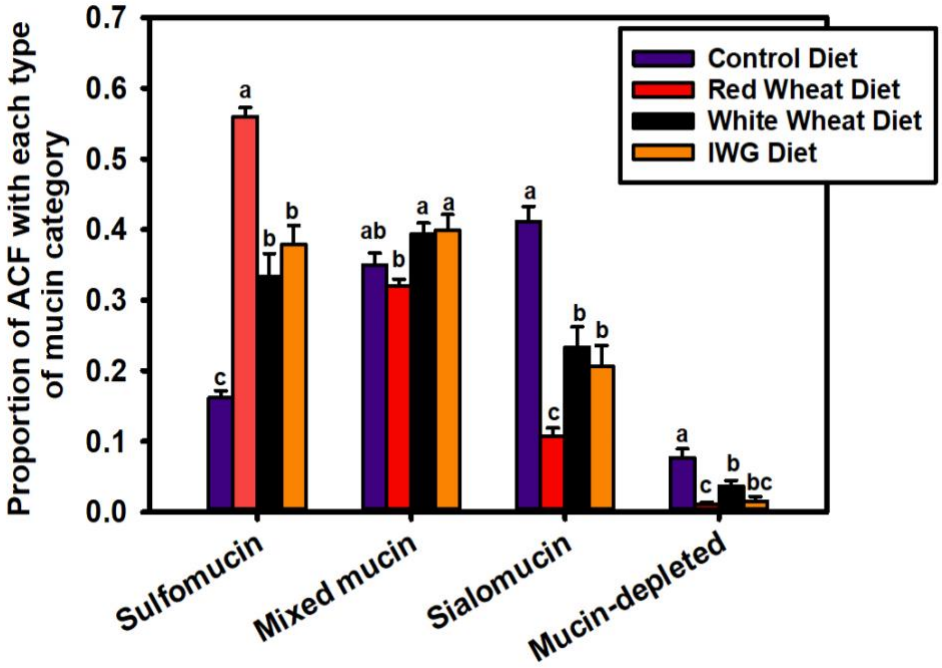

Figure 2.3. Mucin staining of ACF of rat colon tissue during early promotion stage of colon carcinogenesis

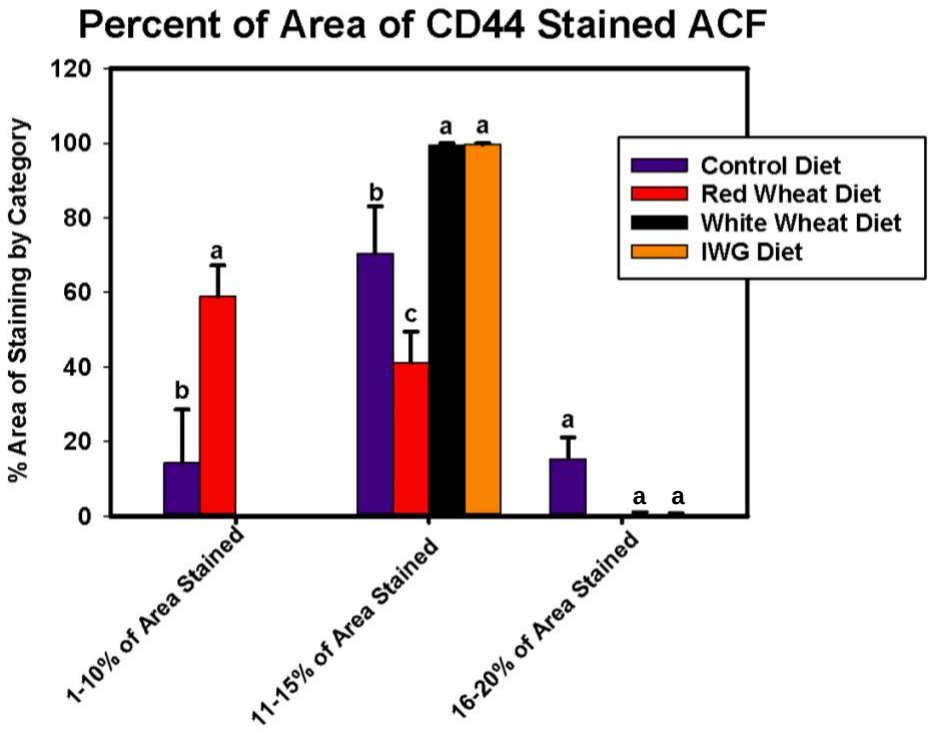

Figure 2.4: Percent area of CD44 staining within ACF in distal colon tissue

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