THE EFFECT OF GENERATIONAL FEEDING OF SOY AND GENISTEIN-SUPPLEMENTED DIETS ON PROGRESSION OF PRECANCEROUS LESIONS IN COLON OF SECOND GENERATION MALE RATS

BY

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THESIS

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Abstract

Colon cancer is the third leading cause of cancer related death in the United States. Epidemiological evidence has shown that populations who consume a traditional East Asian diet high in soy isoflavones have a reduced incidence of developing colon cancer. In previous studies, soy and isoflavones have also been shown to have an effect on reducing colon cancer growth and progression in vitro and in vivo. This study used Sprague Dawley male rats from the second generation of a multiple generation model, which were fed a control, soy, or genistein-supplemented diet. Soy and genistein fed rats had dietary exposure from two previous generations, consumed the same diet and therefore were exposed through gestation, lactation, and post-weaning. Control group rats were obtained at six weeks of age. At ten weeks of age, the rats were injected with azoxymethane (AOM) and were sacrificed at fifteen weeks. Descending colon tissue was collected for physiological, protein, and genetic analysis. Identification of aberrant crypt foci (ACF) was used to determine physiological effect and stage of precancerous progression. Rats fed soy or genistein diets showed significant decrease of total numbers of ACF and reduced multiplicity. Established members of the Wnt signaling pathway, including inhibitors and targets, were analyzed using mRNA expression. Wnt16, Wnt8b, Wnt1, sFRP5, and Dkk1 were significantly increased in the genistein group compared to control and Cyclin D1 was significantly decreased in soy and genistein groups compared to control. β-catenin was used as the protein indicator of Wnt pathway activation. Rats fed a soy or genistein diet showed a significant reduction of nuclear β-catenin. In rats fed soy and genistein diets, advanced stages of ACF decreased, as well as Cyclin D1 expression and nuclear β-catenin also decreased – indicating a reduction in precancerous progression.
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"You enter the forest
at the darkest point,
where there is no path.

Where there is a way or path,
it is someone else's path.

You are not on your own path.

If you follow someone else's way,
you are not going to realize
your potential."

- Joseph Campbell

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CHAPTER 1
INTRODUCTION

1.a Significance

Colon cancer is the third leading cancer death in the United States, with approximately 12.3 to 21.1 deaths per 100,000 Americans, which translates to a near one-third mortality rate per incidence of 34.3 to 56.9 per 100,000 diagnosed each year according to the CDC 2006 records (Centers for Disease Control and Prevention, 2010). The American Cancer Society estimates 140,000 newly diagnosed cases of colon and rectal cancer in 2011, and expects it to cause nearly 50,000 deaths during 2011 (American Cancer Society, 2011). It is also one of the most preventable forms of cancer, with occurrence stemming primarily from chronic poor lifestyle choices (Bloch, Grant, Hamilton, & Thomson, 2010). Early screening has aided in detection at a more treatable stage, leading to decreased mortality (Centers for Disease Control and Prevention, 2010). Yet with a cancer that is largely preventable, incidence is still higher than necessary. Development of environmentally focused preemptive measures could play a major role in identifying a more permanent solution to reducing the incidence and impact of this disease.

One of the most direct environmental interactions with the colon lies with the food that is consumed and passes through it. Previous studies have mainly focused on the use of diet and dietary components ranging from blueberries to calcium supplementation as a treatment after the establishment of a cancerous state (Lappe et al., 2007; Latifah et al., 2010; Paul et al., 2010; Reynoso-Camacho et al., 2011). The origination of this direction in treatment was based on assessments of dietary patterns of people with reduced incidence.

Populations consuming a traditional East Asian diet high in soy have been found to have a lower risk or incidence of developing colon cancer (Moshkowitz & Arber, 2005). It should be noted that any particular protective factor does not remain in later generations who venture away from the traditional diet. Their risk becomes similar to other populations that consume a traditional Western style diet (Akhter et al., 2008; Butler, Wang, Koh, & Yu, 2008; Center, Jemal, & Ward, 2009; Cordain et al., 2005; Kuriki, 2006)).
This points to diet being a primary factor in development of colon cancer, not only a predisposed genetic condition.

Emphasis placed on soy in cancer research is due to its prevalence in traditional East Asian dietary patterns and various functional components found in soy, predominately the isoflavones, one being genistein. Extensive studies have been conducted with isolated isoflavones and other soy components, most of which have been conducted in vitro in colon cancer cell culture models or within one lifecycle of an animal (Cooke, 2006; Daly et al., 2007; Min, Sung, & Choi, 2010; Raju et al., 2009; Wang, 2010). Success of these studies have yielded promising results, however the dosage may seem excessive and such treatment of a preexisting condition does not explain the phenomenon of decreased incidence and risk in populations who routinely consume higher quantities of soy. Lifetime exposure ultimately emerges as the foremost option for advancement of study - to determine how a given diet, over a lifetime and beyond, to later generations, will impact the incidence of colon cancer even under subjection to a powerful carcinogen.

It is known that the Wnt signaling pathway plays an important role in the progression of colon cancer (Katoh & Katoh, 2006; Katoh & Katoh, 2007; Katoh, 2008; Vermeulen et al., 2010). A number of previous studies, to be examined further in Chapter 2, have investigated the response of the Wnt pathway to genistein treatment in colon cancer cell culture. The results are of an inverse correlation – to a degree, the Wnt signaling is suppressed as the genistein content increases. This same correlation has yet to be observed in any animal trial of multiple generation distributions.

A downstream regulation site of cell growth via the Wnt signaling pathway is β-catenin. β-catenin has been studied with colon cancer development, examples of which may be found in Chapter 2, and often linked to cell cycle control. Ultimately this results in a physiological effect that can be seen through cancerous tumors or precancerous lesions such as aberrant crypt foci (ACF), which will be discussed further in Chapter 3.b.

Another significant component of soy that has displayed functional characteristics are saponins. Soy saponins have been studied in various cancer related trials (Bachran, Bachran, Sutherland, Bachran, & Fuchs, 2008; Ellington, Berhow, & Singletary, 2005; Ellington, Berhow, & Singletary, 2006; Fuchs et al., 2009; Gurfsinkel & Rao, 2003; Hu, Reddy,
Hendrich, & Murphy, 2004; Hu, Zheng, Hyde, Hendrich, & Murphy, 2004; Kang et al., 2008; Kim, Yu, Kim, Kim, & Sung, 2004; Oh & Sung, 2001; Su & Simmen, 2009; Tsai, Chen, Chien, Huang, & Lin, 2010). However at this time, little is known about how this soy component specifically acts or interacts – whether it affects the cellular structure or takes a more signalistic approach. The appendix will include preliminary data collected on the effect of soy saponins on colon cancer cells in vitro and bioavailability.

1.b Research Goals

Based on previous work, the goals of this study are to determine whether a high soy diet over generations confers a benefit between generations, and to determine any effects of soy and genistein on precancerous neoplasia and the potential molecular targets. Ultimately, results produced may establish a starting point for prevention of colon cancer.

We hypothesize that a diet containing soy protein isolate or genistein supplement will reduce the presence or progression of precancerous lesions through an inhibition of the Wnt signaling pathway.

1.c Specific Aims

Aim #1: To assess the physiological effect of a diet containing soy protein isolate and genistein on precancerous/neoplastic conditions in rat descending colons. This will be addressed in Chapter 3.b.

Aim #2: To identify the potential target genes that are being stimulated or suppressed by the diet of soy protein or genistein in the Wnt pathway. This will be addressed in Chapter 3.c and 3.d.

Aim #3: To examine the effects of soy saponins, in a colon cancer cell model, on cell proliferation, apoptosis, and potential genes involved in rat colon. This will be provided in the appendix.
1.d References


CHAPTER 2
LITERATURE REVIEW

2.a EPIDEMIOLOGY OF COLON CANCER AND SOY

2.a.1 Colon Cancer in the United States

As the third most commonly diagnosed cancer in the United States, the second leading cancer death in both men and women, and largely preventable with the proper screening and treatment, colorectal cancer is a popular topic for discussion in both treatment and prevention (Espey et al., 2007; Rim, Seeff, Ahmed, King, & Coughlin, 2009). Precancerous polyps may be in the colon for ten years without progressing to a cancerous state and can be easily removed. Therefore, with early detection there is nearly a 90% 5-year survival rate (Centers for Disease Control and Prevention, 2010). Due to its high degree of preventability, focus has been placed on diet as a potential contributing factor, notwithstanding genetic predisposition and heredity.

It is well observed and documented that a Westernized lifestyle, as compared with a traditional East Asian type lifestyle (Figure 2.1), increases the risk of developing colorectal cancer when the primary focus is placed on differences in diet alone. A Western diet has been loosely defined as incorporating higher-glycemic-load carbohydrates such as simple starches and refined sugars, excessive saturated and trans fats, lower poly and mono unsaturated fats, higher contents of dairy products, lower fiber content, and lower lean protein intake (Cordain et al., 2005). Based on direct contact with colonal epithelium, including food types as well as duration of exposure, type of diet is generally viewed as being significant relative to prognosis for development of colorectal cancers.

2.a.2 Lifestyle and Demographics

The term “lifestyle” conveys with it a host of potential variables that could contribute to disease, including activity level, air quality, smoking, alcohol, diet, and age. A number of studies as well as reviews (Rim et al., 2009; Siegel, Jemal, & Ward, 2009; Soto-Salgado et al., 2009; Wirfalt et al., 2009) have considered these factors along with race, ethnicity, and sex. It has been established that men are at higher risk for colorectal cancer than women (Rim et al., 2009; Siegel et al., 2009; Wirfalt et al., 2009), non-Hispanic blacks
are at higher risk than non-Hispanic whites or Asians (Soto-Salgado et al., 2009), while smoking and alcohol in all races significantly increases risk for development of colorectal cancer (Rim et al., 2009; Siegel et al., 2009; Soto-Salgado et al., 2009; Wirfalt et al., 2009).

A “lesser” risk for developing CRC has been noted in East Asian populations (Umar & Greenwald, 2009). However, it should be pointed out as countries advance economically, their risk for CRC correspondingly increases. The Japanese government has been tracking this trend for several decades and has noted an increase in colorectal cancer in their population, with CRC risk for Japanese men increasing >90% (Center, Jemal, & Ward, 2009; Kuriki, 2006) along with an increase in obesity (Matsushita et al., 2008). As their lifestyle behaviors observably changed, they tended to adopt Western style dietary patterns, including increased quantities of meat, dairy, and fat.

### 2a.3 Epidemiological and Soy Influence

Focus has been shifted toward what these particular Asian populations specifically consume that is not part of a traditional Western diet. One staple that has been identified is the high quantity of soy products that are traditionally consumed by East Asian populations (Butler, Wang, Koh, & Yu, 2008; Center et al., 2009; Kim, Sasaki, Otani, Tsugane, & Japan Public Health Center-based Prospective Study Group, 2005; Yang et al., 2009; Yee et al., 2009). Studies completed were long term, lasting from three (Yang et al., 2009) to seventeen years (Sanjoaquin, Appleby, Thorogood, Mann, & Key, 2004), with individuals being excluded if they had previously been diagnosed with colon cancer or any other non-melanomic cancer. Developments of colorectal cancer or precursors such as polyps were monitored over the course of the studies. Studies recorded frequency of soy product consumption including: soy milk, tofu, fried tofu, dried or pressed tofu, fresh soy beans, dry soy beans, soy sprouts, miso, and other soy products (Akhter et al., 2008; Sanjoaquin et al., 2004; Yang et al., 2009).

Epidemiological investigations have identified phytoestrogens, both lignans and isoflavones, to be associated with reduction in CRC (Cotterchio et al., 2006). It has been further hypothesized that high levels of isoflavones may have a promising impact on risk reduction, (Yee et al., 2009) particularly using genistein (Akhter et al., 2008; Bennink, 2001; Spector, Anthony, Alexander, & Arab, 2003). An analysis of six studies, involving
Western populations, found that isoflavone consumption was associated with an approximately 16% reduction in colorectal cancer risk, while in contrast with traditional Asian based population studies, there appears to be no significant risk reduction with additional isoflavone supplementation in those already consuming a soy based diet (Yan, Spitznagel, & Bosland, 2010).

2.a.4 Correlation of Soy and Sex in CRC Development

Increased intake of soy products has been associated with decreased colorectal cancer risk primarily in postmenopausal women, with no significant effect in men (Oba et al., 2007; Yang et al., 2009). This response is hypothesized based on hormonal effects mediated by estrogen receptor binding (Cotterchio et al., 2006) and interaction of soy isoﬂavones. A study done by Akhter et al. with a Japan Public Health Center, including a 5-year follow-up, noted that while intake of isoﬂavones was not clearly associated with overall increase or decrease in CRC in either men or women, it did have a dose-dependent inverse association with CRC risk, particularly with fermented soy foods - potentially due to the changed isoflavone bioavailability effect of fermentation (Akhter et al., 2008; Oba et al., 2007). In most studies, however, a limiting factor was the questionable validity of wording used in the questionnaires. While they did include food frequency, there were gaps in the serving size, genistein content, and form in which the genistein or genistin could be found in each item (Akhter et al., 2008).

2.a.5 Summary

Colon cancer is one of the leading causes of cancer deaths in Western cultured populations. Epidemiological data clearly indicates a relationship between the consumption of soy foods and a decreased risk of developing colon cancer. Because of this, studies have been done to determine the most active components of soy in fighting cancer, of which isoﬂavones have appeared at the forefront. While sex does appear to be a factor when working with isoﬂavones such as genistein, it appears to have an effect beyond its phytoestrogen characteristics.
Figure 2.1 Incidence of colon and rectum cancer in males in a world population. As indicated by the darkening red color, populations consuming an increasingly Westernized diet show higher incidence of colon cancer. (Cancer & Bowel Research Trust, 2011)
2.b SOY AND ISOFlavONE BIOAVAILABILITY

2.b.1 Soy Isoflavone Structure

As the health and nutraceutical values of soy become more popular, attention has been focused on a subclass of flavonoids known as isoflavones. While isoflavones may be found in smaller quantities in other plant foods, soybeans contain the most nutritionally significant amounts of isoflavones. The principal isoflavones found in soy are genistein (4’,5, 7-trihydroxyisoflavone) and daidzein (4’-7-dihydroxyisoflavone) each with four isomeric forms: Aglycones and three glucoside conjugates, the β-, acetyl-, malonyl- and glucoside-(Larkin et al., 2008; Xu et al., 1994; Xu et al., 1995a; Xu et al., 2000). The aglycones have no sugar side group residues and are the most bioavailable forms (Kwon et al., 2007; Larkin et al., 2008). A third type found in lesser amounts is glycine (7,4’-dihydroxy-6-methoxyisoflavone). Differentiation between structures and sugar side groups are notated as - aglycones daidzein, genistein, and glycine and their glucosidic conjugates daidzin, genistin, and glycitin (Rekha & Vijayalakshmi, 2010).

2.b.2 Activities Due to Structure

The basic chemical structure of phytoestrogen isoflavones is similar to that of mammalian estrogens; therefore isoflavones may bind to estrogen receptors and alter estrogen-regulated gene expression (Larkin et al., 2008; Setchell & Cassidy, 1996). Although similar in estrogenic structure, soy isoflavones are considered weak receptor ligands. However, in high concentration this weakness is compensated for and can exert a physiological response. Recent studies found that genistein can bind with exceptional affinity to ERb, an estrogen receptor, – almost as well as 17b-estradiol (Kuiper et al., 1998). Isoflavones bind tightly to serum proteins, increasing their availability to select tissues (Nagel et al., 1998). It is hypothesized that their estrogenic or possible antiestrogenic effect is not the only focus of activity with signal transduction, but they might also possess antioxidant activity (Larkin et al., 2008; Ruiz-Larrea et al., 1997).
2.b.3 Soy Isoflavone Bioavailability

To determine potential effectiveness of any dietary component, it is important to first understand the bioavailability of the component and also its forms available to the general population. For analysis of soy products, these tasks may be challenging, especially when examining isoflavone content, which can vary in amount by up to 70% (Larkin et al., 2008; Murphy et al., 1999). For example, in a Western population where the variety of soy products consumed is fairly low, with the majority of intake coming from soy protein products rather than whole soy, the amount of isoflavone ingested is approximately 1 mg/day for a Western population, as compared to a traditional Asian Diet which may contain 25-100 mg/day of total isoflavones (Larkin et al., 2008; Setchell et al., 2001b).

Bioavailability of isoflavones often may be determined by whether the form is in its conjugated glycosidic form, which is the main form found in foods, or in unconjugated aglycone form (Barnes et al., 1994; Setchell et al., 2002). The majority of their activity is suggested as resulting from their aglycone form (Kawakami et al., 2005). In humans, glycosidic isoflavones are hydrolyzed to their aglycones by the intestinal bacteria and intestinal and hepatal hydrolysis (Setchell, 1998). Therefore, in humans, isoflavone bioavailability depends upon the relative ability of gut microflora to degrade these compounds. Variations in diet, age, and illnesses that have an influence on the intestinal bacteria population will therefore induce variable effects on glucoside de-conjugation to release aglycones (Rekha & Vijayalakshmi, 2010). In cell culture studies, and to eliminate reliance on microflora in animal studies, unconjugated isoflavones are often used.

Another important element of bioavailability is the duration for which the body is exposed to a particular component. For direct contact in the colon, it was found that shorter or longer durations of “stool digestion” correlated with differing levels of isoflavones present in the body (Dobrinska, 1989; Setchell et al., 2001a). The levels found in plasma are also important. Setchell et al. (2001) conducted a study which documented concentrations of various isoflavones found in the plasma, including times required for levels to reach their peaks as well as time elapsed to reach their half-lives. They determined that a peak for genistein occurred 1.33 to 9.33 hours after dose administration and the half-life elimination to be 0.84 to 6.78 hours (Setchell et al., 2001a).
2.b.4 Genistein and Genistin Absorption – is it location or form that matters most?

Kwon et al. conducted an animal trial with male Sprague-Dawley rats giving them a dose of genistein and genistin to estimate bioavailability. They found that the genistein was absorbed up to four times faster than the genistin when administered orally (Kwon et al., 2007). It is hypothesized that the faster absorption is due to its higher hydrophobicity and lower molecular weight. The glycosylate form of genistin or daidzen must proceed to the large intestine to be cleaved to the more absorbable form.

Research in one rat study found that only about 15% of administered genistin is converted to its genistein form in the large intestine (Andlauer et al., 2000; Kwon et al., 2007). Thus, they made a general assumption that less than 50% of genistin administered in their study was converted to genistein. A secondary peak of genistein that was found in plasma analysis is thought to be the result of the enterohepatic recirculation, i.e. the cycling of metabolites after excretion into the biliary system, which are then reabsorbed by the intestine. Enterohepatic recirculation would increase the duration of exposure in the body and thus provide a possibility to prolong pharmacological action (Dobrinska, 1989; Kwon et al., 2007). The reason for low showing of genistein in the genistin-fed animals is thought to be due to the very slow continuous transformation from genistin (Kwon et al., 2007).

Although not a genistein metabolite, equol, has gotten attention for potential health benefits as well. In humans, equol is a soy isoflavone metabolite produced by human intestinal bacterial flora from daizein. Equol can bind (ligand) to ERβ with more affinity in its S form (Setchell, 2010). Equol is touted by some to be the key to soy food efficacy, although only 30-50% of the human population may have the bacteria, including Lactococcus, Eggerthella, and Slakia strains, present in their intestine capable of producing this metabolite (Lampe, 2009). Guinea pigs and sheep also the bacteria present to produce equol, as do rats inoculated with human intestinal microflora (Setchell, 2010).

2.b.5 Influence of Microbiota on Isoflavone Digestion and Absorption

Since a large majority of isoflavone from food comes in the glycosylated form, and therefore not readily absorbed in the small intestine, most ends up in the large intestine. The use of antibiotics has demonstrated the essential role of gut bacteria in isoflavone
metabolism by dramatically decreasing plasma isoflavone concentrations and urinary excretion of bacterial metabolites (Adlercreutz, 1998; Rowland et al., 1999; Winter, 1987). “Studies using “germ-free” rats show the absence of isoflavone absorption, with such absorption being reactivated after inoculation of the animals with human gut flora.” (Bowey et al., 2003) Intestinal bacteria, including Lactobacilli, Bacteroides, and Bifidobacteria, produce β-glucosidase enzymes which aid in glycoside hydrolysis and aglycone absorption, while removing sugar moieties for energy (Izumi et al., 2000; Parodi, 1999; Setchell et al., 2001a; Steer et al., 2003; Xu et al., 1995b). “The relative contribution of intestinal bacterial enzymes has not been established; however, both appear to play an important role in isoflavone bioavailability.” (Larkin et al., 2008)

Metabolism of the glycoside forms not only produces genistein and daidzein, but also additional metabolites, of which equol and OMDA are the most important - derived from daidzein by certain bacterial populations. Relative to its use in research, equol is a nonsteroidal estrogen and functions as a blocker of the androgen DHT (Dihydrotestosterone) as well as a potential treatment in prostate cancer and post menopause (Akaza et al., 2004; Frankenfeld et al., 2004).

2.b.6 Bioavailability Based on Food Matrix

In analysis of bioavailability, Cassidy et al. gave human subjects three types of soy products: Soymilk, TVP rolls, and Tempeh burgers, all with relatively high levels of isoflavones. Evaluation of plasma samples led to a conclusion that type of food matrix plays an important role in bioavailability and pharmacokinetics, with a liquid matrix having the faster absorption rate and higher plasma concentrations than a solid matrix. Aglycones in fermented products were more rapidly absorbed than glucoside conjugates. (Cassidy et al., 2006)

2.b.7 Summary

Isoflavones are not bioavailable in glucoside conjugate form (Cassidy et al., 2006; Izumi et al., 2000). They are hydrolyzed by both intestinal mucosal glucosidases and bacterial B-glucosidases in the product fermentation process, releasing the aglycones (Cassidy et al., 2006; Izumi et al., 2000; Setchell et al., 2002) that are either absorbed
directly or further metabolized by intestinal microflora in the large intestine into other metabolites, including equol and ODMA. Multiple studies have concluded that following ingestion, soy isoflavones attain maximal plasma concentrations within 4-8 hours and are then eliminated from the body through the bile and kidneys (Cassidy et al., 2006). Although the fraction of ingested daidzein absorbed is greater than that of genistein, the ratio of daidzein to genistein is reversed in blood when equal amounts of the two compounds are ingested, resulting in greater bioavailability of genistein than daidzein in the systemic circulation (Cassidy et al., 2006). However, data does suggest that the food matrix plays an incredibly important role in altering the pharmacokinetic profiles. Solubility in the intestine influences the rate of absorption; stomach emptying occurs later after ingesting solid foods compared to liquid food. There is no evidence of difference in bioavailability due to frequency of ingestion (Cassidy et al., 2006).
2.c STUDY DESIGN

2.c.1 Model Characteristics

To further analyze the potential impact of soy on colon health, animal models have been designed to determine whether or not soy may impact the colon relative to prevention and/or treatment of colon cancers. As clinical trials are typically conducted to provide concrete, predictable results when addressing high mortality outcomes such as cancer, due to compounding factors being more difficult to control, animal models are commonly used to determine effectiveness of a treatment within more complex systems.

There are a number of models involving short-term cancer induction, which study the effect of a soy type diet. Due to cost, length of study, adequate sample size, and reduced genetic variability, a small rodent model is the customary animal model used in diet studies involving a cancerous model, specifically a colon cancer model. For the majority of studies involving soy and isoflavones as the diet treatment, rats were regularly involved.

Sprague-Dawley (Kennedy et al., 2002; Min et al., 2010; Raju et al., 2009b; Xiao et al., 2008), F344 (Daly et al., 2007b; Vis et al., 2005), and Wistar (Seibel et al., 2009; Vis et al., 2005) rats are the most widely used. In a study conducted by Vis, et al. (2005) both Wistar and F344 were used and compared, showing no significant difference between the two. When using soy, the content of isoflavones must be considered if not specifically looking at the estrogenic effect. To eliminate a possible interaction involving estrogen-binding receptors, many studies use males only (Kennedy et al., 2002; Min et al., 2010; Raju et al., 2009b; Seibel et al., 2009; Vis et al., 2005; Xiao et al., 2008).

2.c.2 Chemical Induction

The most established and commonly used chemicals for colon cancer induction are dimethylhydrazine (DMH) (Kennedy et al., 2002; Min et al., 2010) and its metabolite azoxymethane (AOM) (K. Daly et al., 2007a; Xiao et al., 2008). The carcinogenic action of the metabolite is responsible for methylation of DNA of epithelial cells in “proliferative compartment of the crypts.” This results in loss of colonic apoptosis, increased proliferation, and increase of cell mutation (Chang, 1984). After treatment, tissues are analyzed for intermediate biomarkers of colon carcinogenesis, termed aberrant crypt foci.
(ACF), (Perse & Cerar, 2011). As reliable producer of cancerous states, AOM has therefore been regularly used in colorectal cancer trials involving a rodent model treated with various food matrices, including soy (Table 2.1).

2.c.3 Physical Marker – ACF

The use of ACF (aberrant crypt foci), preneoplastic lesions, as a biomarker for colon carcinogenesis did not occur until R.P Bird developed the method in 1987, which microscopically identifies these growth abnormalities via methylene blue staining (Bird, 1987; Perse & Cerar, 2011). These lesions are the first in line for development of colon cancer and have been identified in rodents and humans (Bird, 1987; Perse & Cerar, 2011; Pretlow et al., 1991; Pretlow & Pretlow, 2005). “ACF have to date been used as an endpoint in identifying and assessing the preventive or promotional role of natural and pharmacological compounds, as well as dietary and environmental factors, in the process of colon carcinogenesis (Corpet & Tache, 2002; Wargovich et al., 2000).” (Perse & Cerar, 2011)

It is important, however, to remember the stage of carcinogenesis at which the study is or will be conducted. The total number of ACF is a valid indication only at the very early stages, while in advanced cases ACF with higher crypt multiplicities may be a more specific indication of progression rather than total number. And at even more advanced stages, ACF may not be a reliable biomarker after tumors have become present (Bird & Good, 2000; Perse & Cerar, 2011; Raju, 2008).

2.c.4 Brief Review of Recent Studies

Xiao’s study mimics that of an Asian vs. Western type risk factor prevalence for colon cancer, starting in gestation, then lactation, and then AOM induced at adult life. The diet was an AIN93 based diet with protein composed of casein for a Western type diet and soy protein isolate for an East Asian type diet. Their report showed a decrease in circulating insulin levels of the soy protein isolate group and lowering “of insulin mediated colonic DNA damage, and reduction in FASN-repressed apoptosis in response to carcinogen.” (Xiao et al., 2008). As an inhibitor of apoptosis, insulin signals FASN (fatty acid synthase) to stop apoptosis and trigger DNA damage leading to carcinogenesis. Soy protein
isolate fed animals had decreased circulating insulin levels, reducing damage. Overall the study showed that dietary SPI inhibited colonic cancer in AOM treated rats (Xiao et al., 2008).

A study by Kennedy et al. (2002), found that soy molasses, composed of carbohydrates, protein, minerals, and lipids, did not suppress nor enhance colon carcinogenesis in rats. They did find, however, that body weight for the soy fed group was significantly lower than the other groups (Kennedy et al., 2002).

A variety of research has indicated a potential positive effect of soy, as mentioned previously. It has been hypothesized that it is the isoflavones in soy that carry the majority of the positive benefit. Daly et al. (2007) used an AIN93 diet supplemented with 1.9 mg isoflavone/g diet using Novasoy composed of 47% concentration of isoflavones - genistin, daidzin, and glycitin in their glycoside, malonyl, and acetyl forms. Their particular aim focused on the ACF (aberrant crypt foci), which they found there to be no significant effect due to diet on ACF number or multiplicity. However, the groups fed soy had a statistically lower body weight than other groups, which suggests a correlation between a soy protein diet and weight gain. Raju et al. (2009) also used an AIN93 diet with Novasoy 400 which consists of a ratio of 1:1:0.2 genistein, daidzen, and glycitein respectively. Their results showed neither a control diet nor low isoflavone diet had any effect on tumor incidence, with 100% of AOM-induced rats developed tumors, while rats fed a high isoflavone diet had a 95% development rate. In the low dose isoflavone group tumors were smaller than control. (Raju et al., 2009a)

Min et al. (2010) fed rats one of the five graduated diets based on AIN93 diets containing 1, 10, 50, 150, or 500 mg/kg of soy isoflavones. Their results demonstrated that, while total numbers of ACF and AC statistically decreased from control for the 10 mg and 50 mg groups, the 500 mg level group, while showing a decrease, was not significant from the control group. Cox-2 protein expression, a marker for inflammation, was decreased in all the soy isoflavone groups with no significance amongst them. Seibel et al. (2009), while primarily focusing on issues of colitis and soy-based diets with added genistein administered to the treatment group, also reported a reduction in Cox-2 expression, normally elevated in tumors and other inflammatory conditions.
2.c.5 Soy and Isoflavones in Aforementioned Studies

In the aforementioned trials researching soy and soy isoflavones effect on colon health, colon cancer or precancerous conditions were the primary focus of investigation in primarily male rat populations. Reports in several cases found animals in the soy fed groups did not eat as much and had a lower body weight than control and other group rats (Daly et al., 2007a; Kennedy et al., 2002). Markers of inflammation were reduced (Min et al., 2010; Seibel et al., 2009), ACF and/or tumor size was reduced (Daly et al., 2007a; Min et al., 2010; Raju et al., 2009a), but increased levels of isoflavones and genistein in the diets did not correlate linearly with the effects observed on prevention of ACF or tumor.

2.c.6 Summary

Small rodent animal trials, particularly rats, are the most common models in studies for colon cancer development. In these studies, it is standard practice to use physiological markers to verify cancer progression. In long-term studies, tumors are used as a positive indicator. However in short-term studies that do not leave time for full cancer progression, an earlier marker must be used, such as ACF. Studies involving soy and soy isoflavones as dietary treatment have shown decreased number of ACF along with reduction of inflammation markers associated with cancer.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Model</th>
<th>Soy-based Diet Treatment</th>
<th>Aspects of Study</th>
</tr>
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<tbody>
<tr>
<td>Zhang, 2012</td>
<td>46 Male Sprague-Dawley Rats, 13 week trial</td>
<td>AIN-93G with maize oil, 3 diets: control with casein, soy protein isolate, and genistein (control plus 140 mg/kg of genistein).</td>
<td>ACF identification, β-catenin presence via immunoflourscence and immunoblotting, and mRNA Wnt signaling pathway expression. Genistein significantly decreased the number of ACF. Soy protein isolate and genistein decreased presence of nuclear β-catenin, Wnt1 showed no change in any groups, however downstream markers of Cyclin D1 and c-Myc had decreased expression due to reduced presence of the downstream Wnt target, β-catenin.</td>
</tr>
<tr>
<td>Rondini, 2012</td>
<td>95 Male F344 Rats</td>
<td>AIN-93G diet, &gt;85% protein in diet from casein, black beans, or soy flour</td>
<td>Distal colonic mucosa; microarray analysis of genes differentially expressed by diet and AOM induction. Genes affected by both related to inflammation and the extra cellular matrix: Pla2g2a, Rat NP-3, Col1a1, Fn1, Sstr2</td>
</tr>
<tr>
<td>Gourineni, 2011</td>
<td>54 Male F344 Rats</td>
<td>AIN-93G diet with soymeal (10%) and/or Synergy1 (5%)</td>
<td>Incidence of ACF; higher in distal colon than proximal. Diet groups fed both Synergy1 and soy meal had the greatest reduction in number of ACF.</td>
</tr>
<tr>
<td>Raju, 2009</td>
<td>Generational Model of 60 Sprague-Dawley Male rats</td>
<td>AIN-93G diet, AIN-93G with SPI, and AIN-93G with 40mg or 1000mg/kg diet of Novasoy 400 isoflavone mixture; fed same diet through gestation, lactation, and after weaning.</td>
<td>Incidence of ACF and/or colon tumors; expression of ERβ and β-actin. Animal fed soy isoflavones showed reduction in tumor size but not incidence. Animals fed soy isoflavones also displayed augmentation of ERβ expression.</td>
</tr>
<tr>
<td>Nochera, 2009</td>
<td>165 Male F344 Rats, 11 week trial</td>
<td>11 different diets: control AIN-76A modified to a high fat diet, addition of varying combinations and amounts of wheat bran, flax, and soy fiber.</td>
<td>Identification of ACF. All groups with added fibers showed decreased ACF number and multiplicity. Diet which included soy had an additional reduction, although not statistically significant.</td>
</tr>
<tr>
<td>Faris, 2009</td>
<td>60 Male F344/NHsd Rats</td>
<td>6 Experimental groups: AIN-93G control diet; and AIN-93G +5% of either raw whole lentils, cooked whole lentils, raw split lentils, cooked split lentils, or raw soybeans.</td>
<td>Incidence of ACF and glutathione-S-transferases activity in the liver. Rats fed lentils or soy had fewer ACF and reduction in multiplicity. GST data was inconclusive for affecting ACF incidence.</td>
</tr>
<tr>
<td>MacDonald, 2007</td>
<td>Female C57Black/J mice, 35 week trial</td>
<td>Diets containing soy protein depleted of isoflavone (IF) and saponin (SAP), soy+IF, soy+SAP, or soy+IF+SAP.</td>
<td>Tumor incidence. Slight reduction in tumor incidence in groups consuming soy saponin, however the data did not show any diet to be statistically superior.</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Diet Description</td>
<td>Findings</td>
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<tr>
<td>Xiao, 2008</td>
<td>50 Male Sprague-Dawley Rats</td>
<td>AIN-93G control with casein or AIN-93G with soy protein isolate (SPI)</td>
<td>Animal section of experiment: p53 phosphorylation within days post-AOM treatment and circulating insulin. Rats fed a SPI based diet had fewer numbers of phosphorylated p53 per cell, per crypt compared to casein-fed. SPI diets also reduced circulated insulin levels within days post AOM induction.</td>
</tr>
<tr>
<td>Daly, 2007</td>
<td>14 young, 14 mature, and 14 old F344 Female Rats, 15 week trial</td>
<td>AIN-93 control diet or AIN-93 isoflavone diet with 4g Novasoy/kg AIN-93 powder diet.</td>
<td>Identification of ACF, serum estrogen quantification, serum isoflavone quantification, ERβ mRNA and protein detection. At the end of the trial, all groups fed a soy isoflavone diet had statistically lower body weights than the control groups. Young rats had a greater number of ACF than mature or old rats although diet showed no affect. Serum estrodial increased with age for rats fed soy isoflavones. Serum isoflavones showed no difference amongst age groups. ERβ expressed and protein levels were not different between groups.</td>
</tr>
<tr>
<td>Boateng, 2007</td>
<td>40 Male F344, 17 week trial</td>
<td>AIN-93G diet control and AIN-93G containing 20% dry: pinto beans, black-eyed peas, or soy beans.</td>
<td>ACF identification and GST (glutathione-S-transferase activity in the liver. Decreased number of ACF and crypt multiplicity found in animals fed dried beans. Increased GST activity in rats fed dried bean diets.</td>
</tr>
<tr>
<td>Dias, 2006</td>
<td>A/J mice, 32 week trial</td>
<td>Diets containing undenatured whey protein concentrate, Immunocal™, soy protein isolate, or a commercial casein as the primary protein source.</td>
<td>Colon cancer development and immune stimulation. ACF was confirmed as a reliable marker in colon cancer development. No conclusive decision was drawn for immune function.</td>
</tr>
<tr>
<td>Ohuchi, 2005</td>
<td>91 Male F344</td>
<td>Commercial normal control MF diet or a diet containing 10% either: 2 year, 180-day, or 3-4 day fermented miso.</td>
<td>ACF and tumor identification. Tumor incidence and number decreased in relation to increased length of miso fermentation. Tumor size was reduced in 180-day miso fed rats compared to those fed miso fermented for 2-years.</td>
</tr>
<tr>
<td>Xiao, 2005</td>
<td>Male Sprague-Dawley (from previous trial)</td>
<td>Lifetime consumption of control casein diet, soy protein isolate diet, or whey protein hydrolysate. 40 week trial.</td>
<td>Microarray analysis using total RNA from rat colonic tissue of 8799 rat genes. Cyclin D1 was down-regulated in SPI and whey protein diets. SPI diet showed affect including cell adhesion, cell cycle and growth control, detoxification, defense and immunity, signal transduction, transcriptional regulation, metabolism, ligands and carrier proteins, cell death proteins,</td>
</tr>
<tr>
<td>Reference</td>
<td>Animal Model</td>
<td>Lifetime Consumption or Dietary Treatment</td>
<td>Methodology</td>
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</tr>
<tr>
<td>Linz, 2004</td>
<td>53 Male Sprague-Dawley Rats</td>
<td>Diets containing 20% casein or SPI energy content with isoflavone content of SPI 3.7-3.98 mg/g protein. Fed diets for gestation, lactation, and half were rotated to opposite diet at 21 days.</td>
<td>Identification of ACF, TUNEL assay, and dual energy x-ray absorptiometry. SPI feeding throughout trial led to fewer ACF and reduction in multiplicity. Rats fed SPI diets had statistically lower fat percentage.</td>
</tr>
<tr>
<td>Murillo, 2004</td>
<td>20 Female CF-1 mice, 10 week trial</td>
<td>Diets of either control (Teklad-4% Mouse/Rat Diet), 10% garbanzo, bean flour, 10% soy flour and 10% mixed flours (5% of soybean and garbanzo bean flours). All diets were based on casein diet of 26g protein/100g diet.</td>
<td>Identification of ACF. Reduction of ACF was observed in all groups fed garbanzo beans and/or soy.</td>
</tr>
<tr>
<td>Hakkak, 2001</td>
<td>Male Sprague-Dawley Rats, 43 week trial</td>
<td>AIN-93G control diet or AIN-93G with SPI protein substitute containing 430mg isoflavone/kg diet. Fed through gestation, lactation, and post weaning.</td>
<td>Abnormal colon mass classifications benign or invasive adenocarcinoma. Soy fed rats gained less weight than control group rats. Soy-fed rats had fewer colonic tumors (12%) than control casein-fed rats (~50%).</td>
</tr>
<tr>
<td>Azuma, 2000</td>
<td>60 Mature Male F344 Rats</td>
<td>Diets with protein sources from: milk casein, soybean HMF, Wheat gluten, yolk protein, codfish meat, casein (-DCA). 10-34 week trial with intermittent euthanization.</td>
<td>Quantification of fecal bile acid secretion and endoscope observation. Tumor incidence and plasma bile acid concentrations were reduced in soy fed groups and increased in milk casein, wheat, and cod fish fed groups.</td>
</tr>
<tr>
<td>Davies, 1999</td>
<td>114 (6-week old) Male F344 Rats</td>
<td>Formulated diet containing 37% starch, 21% soy protein (isoflavones removed), and 42% lard; fed to animals one week prior to AOM cancer induction.</td>
<td>Number of ACF and aberrant crypts per focus. Animals not treated with AOM displayed no tumor or ACF growth.</td>
</tr>
<tr>
<td>Thiagarajan, 1998</td>
<td>105 Male F344 Rats</td>
<td>AIN-93G SPI control diet; AIN-93G SPI with alterations for addition/substitution of: soy flakes, soy flour, genistein.</td>
<td>Identification of ACF, diet isoflavone analysis. Diets with genistein and calcium showed the most reduction in ACF, followed closely by soy flour.</td>
</tr>
</tbody>
</table>

**Table 2.1** Review of colon cancer studies using a rat or mouse model with AOM (azoxy methane) cancer induction and soy dietary treatments in the past 15 years.
2.d WNT IN COLON CANCER

2.d.1 Overview

Wnt proteins belong to a family of highly conserved, secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis and in newly “produced” cells such as – stem cells. In normal cell progression of colon epithelial cell maturation and differentiation, stem cells are located at the bases of regularly spaced crypts. Cells migrate from the base of the crypt to the upper edges, becoming enterocytes, enteroendocrine cells, and goblet cells. (Figure 2.2) (Schneikert & Behrens, 2007) Activation of the Wnt signaling pathway disrupts this progression of differentiation, causing the cells to remain undifferentiated as they reach the top of the crypt.

Mutation of adenomatous polyposis coli (APC), a classified tumor suppressor gene, or β-catenin is an early event in the transformation of colonic epithelial cells leading to potential colorectal cancer. Moreover, established colorectal cancers also depend critically on Wnt signaling (Groden et al., 1995; Vermeulen et al., 2010). APC mutation generally results in an imperfect β-catenin degradation complex, leading to accumulation of β-catenin in the nucleus and the continuous transcription of Wnt target genes (Liang et al., 2003; Qi & Zhu, 2008; van de Watering et al., 2002; Vermeulen et al., 2010).

Wnt signaling is crucially important in maintaining stemness, the genetic factors “identifying” or denoting a stem cell, in normal colon stem cells and is a common pathway deregulated in most colon cancers. In a similar manner to normal intestinal stem cells (Yen & Wright, 2006), Wnt activity is not merely a cell-intrinsic feature that can be used to define cancer stem cells in a series of colon tumors carrying a variety of mutations, but it is also regulated by extrinsic or environmental factors. (Vermeulen et al., 2010).

2.d.2 Wnt Signaling Pathways (Canonical and Non-canonical)

Wnt proteins constitute a large family of at least nineteen secreted small glycoproteins, all of which contain 23-24 cystein residues (Miller, 2022; Qi & Zhu, 2008). Wnt signals are transduced to two regulated pathways, the canonical for cell fate determination, and to the noncanonical for control of cell movement and tissue polarity.

Signaling via the canonical pathway, often referred to the Wnt/β-catenin pathway, is
transduced through Frzzled (FZD) receptors and LRP-5,6 coreceptors on the cell surface to the β-catenin signaling cascade (Bhanot, Brink, & Samos, 1996; Katoh & Katoh, 2007; Pinson, Brennan, Monkley, Avery, & Skarnes, 2000). “β-catenin in the nucleus forms a complex with T-cell factor/Lymphoid enhancer factor (TCF/LEF) family transcription factors and also with Legless family docking proteins (BCL9 and BCL9L) associated with PYGO family coactivators (PYGO1 and PYGO2)” (Katoh M, 2003; Katoh & Katoh, 2007; Kramps et al., 2002). (Figure 2.3) Activation of the canonical Wnt pathway, such as in cancer, promotes cell survival and inhibits cell death.

There are two non-canonical pathways: the Wnt/planar cell polarity, and the Wnt/calcium pathway (Papkoff, 1996; Veeman, 2003; Qi & Zhu, 2008). Non-canonical Wnt signaling is also transduced through FZD receptors and coreceptors, such as ROR2 and RYK (Lu, Yamamoto, Ortega, & Baltimore, 2004; Lu et al., 2004; Oishi et al., 2003). Small G proteins (RHOA, RHOU, RAC, and CDC42) and c-jun NH2-terminal kinase are the DVL-dependent effector molecules of the noncanonical pathway (Boutros et al., 1998; Tao et al., 2001), whereas Nemo-like kinase (NLK) and nuclear factor of activated T cells (NFAT) are the Ca2+-dependent effector molecules of noncanonical pathway (Dejmek et al., 2006; Ishitani et al., 2003). Noncanonical WNT signaling pathway, transduced to a variety of DVL- or Ca2+-dependent cascades, is overlapping with the planar cell polarity-signaling pathway (Kawano & Kypta, 2003). (Katoh & Katoh, 2007) (Figure 2.3)

2.d.3 Wnt Inhibitors and Targets of the Canonical Pathway

Secreted-type WNT signaling inhibitors include: SFRP1, SFRP2, SFRP3, SFRP4, SFRP5, WIF1, DKK1, DKK2, DKK3, and DKK4 (Katoh & Katoh, 2006; Katoh, 2006; Katoh & Katoh, 2007). SFRP family members and WIF1 are WNT antagonists that inhibit the upstream signaling of WNT binding to FZD family receptors (Qi & Zhu, 2008). DKK family members interact with LRP5/LRP6 coreceptor and trigger its endocytosis to prevent formation of the WNT-FZD-LRP5/LRP6 complex for the canonical WNT signaling (Katoh & Katoh, 2007). APC, AXIN1, AXIN2, CKIa, GSK3h, NKD1, NKD2, ANKRD6, and NLK are intracellular-type negative regulators of the canonical WNT signaling pathway. APC, AXIN1, and AXIN2 are scaffold proteins of the β-catenin destruction complex, whereas CKIa and GSK3h are serine/threonine kinases that phosphorylate β-catenin to trigger degradation (Katoh &
Katoh, 2007).

Genetic predisposition, environment, and aging are risk factors of human cancer (Katoh, 2006; Katoh & Katoh, 2007; Qi & Zhu, 2008). Transcriptional activation of canonical WNTs occurs during tissue regeneration associated with chronic persistent inflammation, and up-regulation of Wnt1, Wnt3, or Wnt10b (Katoh & Katoh, 2006; Katoh, 2006; Katoh & Katoh, 2007; Lee et al., 1995).

Genes encoding canonical WNT signaling inhibitors are down-regulated during carcinogenesis due to epigenetic silencing and genetic alteration, such as epigenetic silencing of SFRP1, SFRP2, DKK1, WIF1, and AXIN2 genes occurs in premalignant tissues associated with chronic inflammation or in human cancer, whereas mutation of APC, AXIN1, and AXIN2 genes occurs in human cancer (Katoh, 2006; Katoh & Katoh, 2007; Nishisho et al., 1991; Satoh et al., 2000; Suzuki et al., 2002).

Primary targets of the canonical Wnt pathway are targets of β-catenin and include genes associated with cell cycle signals, predominately Cyclin D1. Cyclin D1 is responsible for the progression of the cell cycle through G1 to S phase; over-expression is predominantly associated with tumorigenesis. β-catenin target genes, which will be discussed further in section 2.e, have important functions pertaining to regulation of cellular processes such as cell proliferation, cell cycle progression, apoptosis, differentiation, tissue invasion, and angiogenesis (Qi & Zhu, 2008).

2.d.4 Wnt Response to Isoflavones and Genistein

Wnt signaling has been widely tested in an array of tissues using various treatments, including the effect of soy isoflavones. A number of studies have been conducted to determine any specific isoflavone effects. Activated Wnt signaling mediated by β-catenin is known to promote cell growth and inhibit differentiation with higher levels of β-catenin protein in response to a genistein environment (Su et al., 2007; Clevers, 2006; Su & Simmen, 2009). Therefore, it is thought that genistein will induce growth arrest favoring cells to differentiate (Su & Simmen, 2009). “Canonical Wnt antagonists, such as Wnt5b, secreted frizzled-related protein sFRP1, and dickkopf homologue Dkk2 were down-regulated by up to 1.5 to 2.6 fold expression in cultured human adipose tissue cells exposed to genistein” (Kim et al., 2010). And in mammary tissue culture, expression of Wnt5b and
downstream target, *Cyclin D1*, were decreased, whereas *sFRP2* was increased (Sun, 2007).

In DLD-1 colon cancer cells, *Wnt5a* was significantly increased after treatment with Novasoy or genistein when compared to the control vehicle of DMSO. In this same study, SW1116 cells treated with genistein elicited the highest response of *Wnt5a* expression; *Wnt7a* and *Wnt3a* were not affected (Wang, 2010). *Wnt5a* can act through different membrane receptors and participate in both canonical and non-canonical Wnt pathways (Katoh, 2005; Mikels & Nusse, 2006; Widelitz, 2005).

In a brief touch on an epigenetic standpoint, *Wnt5a* mRNA expression was up-regulated by genistein in an early-stage colon cancer cell line SW1116. In this particular study, genistein served as a natural demthylation agent and was specifically effective on colon cancer cells from early-stage colon cancer. *Wnt5a* down-regulation is correlated with hypermethylation of its promoter region in human colon cancer patients (Hibi et al., 2009; Wang, 2010; Ying et al., 2008).

2.d.5 Summary

The Wnt pathway is a highly conserved signaling pathway that regulates cell growth and differentiation, yet can contribute to the process of cancer progression in cases of mutation disregulation of the expression. There are two branches, the canonical and non-canonical pathway, that may be stimulated to signal cascades of β-catenin or Ca\(^{2+}\) both of which trigger abnormal cell growth and movement indicative of cancer. Treatments with soy or genistein have inhibited differentiation of colon cancer cells through regulation of the β-catenin cascade in the canonical Wnt pathway.
Figure 2.2 Schematic of normal colon epithelium. The flat surface of the epithelium forms crypts at regular intervals. The stem cells produce proliferating precursor cells that migrate toward the top and differentiate to become enterocytes, enteroendocrine cells, and goblet or Paneth cells. (Schneikert, 2007)
Figure 2.3 Regulation of the Wnt pathway. WNT signals are transduced to the canonical pathway for cell fate determination, and to the noncanonical pathway for control of cell movement and tissue polarity. Transduction through the Frizzled receptors and LRP5/LRP6 coreceptors signals the β-catenin cascade in the canonical pathway. In the noncanonical pathway, Wnt signals are transduced through Frizzled family receptors and ROR2/RYK coreceptors to the DVL-dependent of the Ca^{2+} dependent cascade. (Katoh, 2007)
2.e β-CATENIN

2.e.1 Actions of β-catenin

β-catenin is a protein encoded by the CTNNB1 gene and an integral participator in the Wnt signaling pathway, as mentioned in the previous section 2.d. It functions as a transcriptional activator when the protein is complexed with members of the T cell factor (TCF) family of binding proteins that are necessary for the formation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells (Behrens et al., 1996; Latifah et al., 2010; Molenaar et al., 1996). β-catenin may also be responsible for transmitting the contact inhibition signal that causes cells to stop dividing (Morin et al., 1997). Accumulation of β-catenin activates genes that are responsive to transcription factors of the TCF/LEF family (Giese et al., 1995; Molenaar et al., 1996; Tetsu & McCormick, 1999).

Regulation of β-catenin via Wnt signaling is important in adenomatous polyposis coli (APC) mutations associated with human and rat colon tumors that repress the degradation process (Takahashi et al., 1998; Vogelstein & Kinzler, 1998). If β-catenin is not degraded, it will accumulate in the cytosol as well as nucleus, leading to activation of the oncogenic β-catenin/TCF pathway (Morin et al., 1997; Yamada et al., 2000). Highlighting its involvement in the initial stage of colon carcinogenesis is the stabilized β-catenin, which enters the cell nucleus and associates with TCF/LEF transcription factors to activate the transcription of Wnt target genes (Schneikert & Behrens, 2007).

2.e.2 Targets of β-catenin

An important concentration of current cancer research is β-catenin activation of the transcription of Cyclin D1 via TCF/LEF binding sites, acting as an important regulator of cell cycle progression (Tetsu & McCormick, 1999). Particularly at the G1 phase, it is often regarded as an oncogene that induces malignant transformation; highly expressed in patients with adenomatous polyps, primary colorectal adenocarcinoma (Arber et al., 1996; Tetsu & McCormick, 1999), and familial adenomatous polyposis (Sola et al., 1999; Tetsu & McCormick, 1999; Utsunomiya et al., 2001). It was found that when compared to noncancerous epithelium, β-catenin and Cyclin D1 were overexpressed in nearly 50% of
biopsied cases. Of these cases, detection of Cyclin D1 expression was present in 74% of cases with increased nuclear β-catenin, whereas only detected 11% of the time without increased β-catenin expression (Utsunomiya et al., 2001). Transcription of Cyclin D1 is activated by β-catenin through TCF-binding sites within the promoter. Induction of Cyclin D1 by β-catenin is likely to play an important role in the development of colon cancer and other malignancies involving cell cycle regulation (Tetsu & McCormick, 1999).

Another target of β-catenin is c-Myc, which is a proto-oncogen commonly overexpressed at mRNA and protein levels in colorectal cancer. (Sikora, 1987; Kolligs, 2002) Like Cyclin D1, c-myc is also involved in cell cycle regulation at the G0/G1 and G1/S transitions (Freytag, 1988; Dang, 1999). Deregulation is thought to take place due to targeting by activation of the β-catenin/TCF complex, linking it to APC and β-catenin (He, 1998; Kolligs, 2002)

2e.3 Activators and inhibitors of β-catenin

As indicated in the previous section relative to Wnt, Wnt signaling molecules in the canonical Wnt pathway activate β-catenin. Several in vivo experiments in mice have shown that the Wnt pathway is essential for the maintenance of the proper architecture of the intestinal epithelium, where normal proliferation of the precursor cells depends on proper activation of the pathway. Nuclear β-catenin accumulates at the bottom of normal crypts, indicating a permanent stimulation of the Wnt pathway in the crypt area cells (Clevers & Batlle, 2006; Schneikert & Behrens, 2007).

The close regulation of β-catenin by the Wnt signaling pathway means that inhibitors of the β-catenin activation are primarily also inhibitors of Wnt signaling. When Wnt signaling is not activated, a multiprotein complex is signaled to stimulate degradation of β-catenin. In this process, β-catenin becomes phosphorylated via glycogen synthase kinas 3β (GSK-3β), ubiquinated and then degraded by proteasomes (Biological Databases et al., 2010). The resulting minimal level of β-catenin keeps the TCF/LEF targets at proper level (Biological Databases, Urbach, & Wingender, 2010).

Wanitsuwan et al. analyzed tumor tissue from 163 colorectal cancer cases using immunohistochemistry (Wanitsuwan et al., 2008). The difference between this particular study and most others was the examination at overall expression of β-catenin rather than
only nuclear accumulation. This overall expression of β-catenin in colon cancer predicted better survival with higher overall staining density, indicating that increased β-catenin correlated with poorer prognosis. It was determined that an inverse relationship was present between higher overall staining density and clinical staging and differentiation. Based on this information, they inferred that "the role of β-catenin in colon cancer may not be explained solely through the Wnt-signaling pathway, that may be an alternative role related to tumor cell differentiation.” (Wanitsuwan et al., 2008) Results indicate that beta-catenin overexpression in the cytoplasm may promote malignant transformation by triggering *Cyclin D1* expression in colorectal cancers.

**2.e.4 Influence of Isoflavones on β-catenin**

Soy and genistein treatment has been studied in various types of tissue to elicit a response related to the Wnt/β-catenin pathway. In one study, conducted by Kim et al (Kim et al., 2010), Canonical *Wnt3* and β-catenin had 1.5-fold higher levels of expression in human adipose cells treated with genistein, but not with daidzein, than those treated with a control (Kim et al., 2010). Genistein treated mammary epithelial cells also showed higher levels of β-catenin protein than control cells (Su & Simmen, 2009). Treatment of prostate cancer cell line PC3 with soy protein isolate or genistein showed that *Wnt4* and β-catenin were reduced in soy treatment samples (Liss, 2010).

**2.e.5 Summary**

β-catenin is closely associated with the canonical Wnt pathway, which is often termed the Wnt/β-catenin signaling pathway. Upon activation of Wnt, β-catenin remains in an active state. As a regulatory factor of the cell cycle, an uncontrolled accumulation of β-catenin in the nucleus and the resulting signal cascade can lead to uninhibited cell division, which is indicative of cancer. An important target of β-catenin is *Cyclin D1*, which is a key gene involved in cell cycle control at the G1 phase and is often at elevated levels in colon cancer. Treatments of cell tissues with soy isoflavones have shown results with higher levels of β-catenin when compared to a control.
2.f References


CHAPTER 3
THE EFFECT OF GENERATIONAL FEEDING OF SOY AND GENISTEIN-SUPPLEMENTED DIETS ON PROGRESSION OF PRECANCEROUS LESIONS IN COLON OF SECOND GENERATION MALE RATS.

In various epidemiological studies of factors affecting cancer development, an increased consumption of soy products is associated with a decreased risk of developing colon cancer (Butler et al., 2008; Center, Jemal, & Ward, 2009; Umar & Greenwald, 2009; Yang et al., 2009; Yee et al., 2009). With the use of a soy and isoflavone genistein based diet, this trial explores the difference in cancer risk between a population that does not consume soy on a regular basis and a population that does. To demonstrate and explore what is seen in epidemiological studies, this trial focuses on whether or not there is a decreased risk of developing colon cancer when rats are fed a traditional East Asian diet typically containing high amounts of soy when compared to a Western style diet where soy ingestion is much lower.

3.a Animal Trial Design

3.a.1 Introduction
The model used in this study incorporates the sampling of one generation within a larger trans-generational trial. For the design of the generational trial, all rats were fed the same diet over a total of three generations, with the focus of this study placed on the second generation. The purpose of this trial is to use the established rat model to mimic populations who consume a traditional East Asian diet for more than a generation. Emphasis is placed on the importance of the question: What is the effect of diet, over generations, through gestation, feeding from mother’s lactation, puberty, and into adult years? In the second generation used in this study, offspring have influence from mother and grandmother’s diets.

As observed in previous studies, a soy-based diet may help reduce the risk of colon cancer. Per discussion in sections 2.a and 2.b, populations that consumed a high soy diet were found to have a reduced risk of developing colon cancer. Cell culture study has also implicated activity of genistein in reduction of growth and viability (Cotterchio, 2006; Liss, 2003; Raju, 2009; Wang, 2010). As an isoflavone that has been studied and compared to its
counterpart, daidzein, genistein is a potent compound, which shows potential to be used in cancer prevention and treatment trials. This particular study provides an examination of the effects from the second generation exposed to soy and genistein. Moreover, with later compilation of data from all the generations under examination, a broader picture of the sum effects of genistein supplementation may become apparent.

3.a.2 Materials and Methods

Study Design. Sprague-Dawley rats were taken from the first generation of rats born from a group of pregnant Sprague-Dawley rats obtained from Charles River Laboratories. Rats were fed an AIN93 genistein-supplemented diet (casein, corn oil, 140 mg/kg of genistein) or AIN93 soy protein diet (isolated soy protein, corn oil) with ad lib access for ten weeks (Table 3.1). Diet with casein or soy protein isolate contained 20% protein, 200g/kg of diet. Male and female rats from the same diet group were put in the same cages to mate, which were continued on the same diet treatment throughout lactation until pups were weaned. Male pups were weaned at 3 wk of age and continued on the same diet treatment as parental generation. Additional control animals from Charles River Laboratories were obtained at 6 wk of age and given a control diet (casein, corn oil). Body weight and food intake were recorded weekly. At 10 wk of age, rats were given a subcutaneous Azoxymethane (AOM) injection of 15 mg/kg of body weight. Food intake and body weight were gathered for an additional five weeks. (Figure 3.1) At wk 15, animals were euthanized using CO2 asphyxiation and tissue samples were collected for future analysis. Male animals were used for this study to reduce the potential interaction of the soy isoflavone phytoestrogens with estrogen receptors which are more prevalent in females.

Dietary Treatment. A modified AIN-93 diet containing corn oil in place of soybean oil was used as the control and base diet for all groups (Dyets, Bethlehem, PA). In the soy group, soy protein isolate was used as the protein source in place of casein, 200g/kg of diet. The genistein supplementation group used the same as the control diet with the addition of 140 mg/kg genistein based on sample analysis represented in Table 3.1.

Cancer Induction. Azoxymethane (AOM) injection was used as the induction method for colon neoplasia, as both AOM and dimethylhydrazine (DMH), of which it is a metabolite,
produce reliably high presence of colon cancer, more specifically colon cancer in the distal colon in a relatively short period of time (Perse & Cerar, 2011). Early biomarkers that can be used with AOM/DMH are preneoplastic lesions called aberrant crypt foci (ACF), which will be discussed further in the following section 3.b, and appear before the usual six to eight months at which the presence of tumors may be examined.

**Sampling.** Male rats were sacrificed at 15 wk of age by carbon dioxide asphyxiation. Blood was collected after decapitation for analysis not included in this study. Internal organs were then removed and immediately flash frozen in liquid nitrogen. The entire colon was opened longitudinally and all fecal matter was removed by washing twice in ice cold PBS. The colon was then divided into ascending, transcending, and descending sections. And each section was further split in half length-wise. Half of each portion was saved for ACF counting, see details below, and the remaining half of each was stored for later gene analysis. Colon epithelial cells were collected by using a glass slide to scrape the mucosa; scraped colon epithelia were then folded in labeled pieces of foil and flash frozen in liquid nitrogen before being stored at -80°C.

**Statistics.** Results are shown as mean ± SEM. Body weight and food intake per gram of body weight data were tested comparing information by using repeated measures 1-way ANOVA and Tukey's Studentized Range test (Statistical Analysis Software). Differences were considered significant at p < 0.05.

**3.a.3 General Observations**

Analyses of dietary groups using Tukey's Studentized Range Test revealed that the body weight of the soy group to be less than that from the control and genistein groups. However, body weight did not differ significantly between control and genistein groups ([Figure 3.2](#)). Therefore, diet did influence overall growth.

Food intake per gram of body weight was determined based on the basis of food offered and food remaining at the beginning and end of each week, then divided by the weight of the corresponding animal and averaged for each week and each diet. **Table 3.2** separates each diet by week. Analysis, with each diet compared directly to the control group, indicated no statistical significance. Difference in food intake per gram of body weight between treatment and control groups, the soy group eating less per grams of body
weight, was significant based on statistical analysis (not shown) with a p-value of 0.04.

3.4.3 Discussion

Possible outcomes and influences of the trial design and differences in food intake and body weight on the overall physiological and molecular results from this study will be discussed further in Chapter 4. It is well documented that populations consuming a traditional East Asian diet have a lower occurrence of colon cancer and are at lesser risk of developing colon cancer when compared with other diet practices. It has also been observed that these populations have a lower body weight or BMI (World Health Organization, 2004). The occurrence of lower body weight in animals fed a soy protein based diet is not unique to this study. Nearly 20% of rodent colon cancer research studies, using AOM as the carcinogen and a soy protein diet as one of the treatment groups, reported animals fed the soy protein diet weighed less or had less body fat than their casein or whey control comparisons (Xiao et al., 2008; Daly et al., 2007; Linz et al., 2004; Hakkak, 2001). As noted in other colon cancer trials involving rodents with soy protein diet treatments (Table 2.1), in this trial lower body weight of rats in this trial fed a soy protein based diet was also observed, being in agreement with the epidemiological data. From this comparison, we believe that the present animal models used in this study generally represent some of the characteristics of a portion of the human population consuming a traditional Asian diet. The specific effects of this diet relative to colon cancer development will be presented and discussed in the sections to follow.
Figure 3.1 Animal trial design with detailed description of generation two. Upon arrival, generation zero pregnant rats were fed a soy or genistein-supplemented diet, which was kept consistent through all stages of generation one and generation two. Control animals were obtained at six weeks of age and put on an AIN93 diet upon arrival. All diet groups were given an AOM injection during ten weeks of age and sacrificed at fifteen weeks of age.
Figure 3.2 Average body weight in grams for male rats fed soy, genistein-supplemented, and control diets. Animals were injected with AOM at week ten. In a group comparison analysis, (*) indicates a significant difference of p < 0.05 in body weight of soy protein isolate fed rats from control AIN93 fed rats.
### Table 3.1

<table>
<thead>
<tr>
<th>Sample</th>
<th>DZI (mg/g)</th>
<th>GNI (mg/g)</th>
<th>DZE (mg/g)</th>
<th>GNE (mg/g)</th>
<th>DZI (mg/g)</th>
<th>GNE (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-1</td>
<td>0.052</td>
<td>0.183</td>
<td>0.283</td>
<td>0.59</td>
<td>0.314</td>
<td>0.7</td>
</tr>
<tr>
<td>PS-1</td>
<td>0.041</td>
<td>0.18</td>
<td>0.294</td>
<td>0.597</td>
<td>0.319</td>
<td>0.705</td>
</tr>
<tr>
<td>PS-2</td>
<td>0.045</td>
<td>0.126</td>
<td>0.28</td>
<td>0.604</td>
<td>0.307</td>
<td>0.679</td>
</tr>
<tr>
<td>PS-2</td>
<td>0.048</td>
<td>0.079</td>
<td>0.284</td>
<td>0.635</td>
<td>0.313</td>
<td>0.683</td>
</tr>
<tr>
<td>PS-3</td>
<td>0.042</td>
<td>0.143</td>
<td>0.302</td>
<td>0.636</td>
<td>0.328</td>
<td>0.721</td>
</tr>
<tr>
<td>PS-3</td>
<td>0.046</td>
<td>0.184</td>
<td>0.284</td>
<td>0.635</td>
<td>0.311</td>
<td>0.745</td>
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</table>

**Total Aglycones Equivalents**

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<tr>
<th></th>
<th>DZI</th>
<th>GNI</th>
<th>DZE</th>
<th>GNE</th>
<th>DZI</th>
<th>GNE</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>PS-1</td>
<td>0.052</td>
<td>0.183</td>
<td>0.283</td>
<td>0.59</td>
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<td>0.7</td>
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<tr>
<td>PS-2</td>
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<td>0.721</td>
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<td>0.184</td>
<td>0.284</td>
<td>0.635</td>
<td>0.311</td>
<td>0.745</td>
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</tbody>
</table>

**STATS**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
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<td></td>
<td>0.315</td>
<td>0.007</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>0.705</td>
<td>0.025</td>
<td>3.5</td>
</tr>
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</table>

**ratio of isoflavone to diet amount**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1.00</td>
<td>2.26</td>
</tr>
</tbody>
</table>

**Table 3.1** Isoflavone content of supplemented diet. PS = powder sample. Samples were analyzed by Dr. Juan Andrade (Helferich Lab, Department of Food Science and Human Nutrition, University of Illinois). Daidzin (DZI) and genistin (GNI) were included along with daidzein (DZE) and genistein (GNE) in the total equivalents. Results were averaged for final isoflavone content. 0.705 mg/g (mg of genistein in 1g diet) was used for the determination of 140 mg/kg of genistein in the final diet formulation.
### Table 3.2

Average food intake (g) per gram of body weight taken every 7 days. Analysis of group comparison indicated no significant difference in food consumption from both soy protein isolate fed rats and genistein–supplemented fed rats from those fed a control AIN93 diet.

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Soy</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 3</td>
<td>-</td>
<td>1.39 ± 0.021</td>
<td>1.19 ± 0.044</td>
</tr>
<tr>
<td>Week 4</td>
<td>-</td>
<td>1.16 ± 0.025</td>
<td>1.14 ± 0.038</td>
</tr>
<tr>
<td>Week 5</td>
<td>-</td>
<td>0.98 ± 0.034</td>
<td>0.87 ± 0.026</td>
</tr>
<tr>
<td>Week 6</td>
<td>0.40 ± 0.008</td>
<td>0.75 ± 0.023</td>
<td>0.70 ± 0.020</td>
</tr>
<tr>
<td>Week 7</td>
<td>0.81 ± 0.033</td>
<td>0.63 ± 0.019</td>
<td>0.58 ± 0.014</td>
</tr>
<tr>
<td>Week 8</td>
<td>0.63 ± 0.013</td>
<td>0.52 ± 0.010</td>
<td>0.50 ± 0.010</td>
</tr>
<tr>
<td>Week 9</td>
<td>0.45 ± 0.008</td>
<td>0.45 ± 0.005</td>
<td>0.44 ± 0.013</td>
</tr>
<tr>
<td>Week 10</td>
<td>0.37 ± 0.005</td>
<td>0.37 ± 0.005</td>
<td>0.37 ± 0.008</td>
</tr>
<tr>
<td>Week 11</td>
<td>0.29 ± 0.015</td>
<td>0.34 ± 0.006</td>
<td>0.31 ± 0.004</td>
</tr>
<tr>
<td>Week 12</td>
<td>0.39 ± 0.010</td>
<td>0.34 ± 0.008</td>
<td>0.35 ± 0.007</td>
</tr>
<tr>
<td>Week 13</td>
<td>0.37 ± 0.008</td>
<td>0.34 ± 0.003</td>
<td>0.31 ± 0.005</td>
</tr>
<tr>
<td>Week 14</td>
<td>0.34 ± 0.006</td>
<td>0.32 ± 0.008</td>
<td>0.32 ± 0.005</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.1747</td>
<td>0.2463</td>
</tr>
</tbody>
</table>

Table 3.2: Average food intake (g) per gram of body weight taken every 7 days. Analysis of group comparison indicated no significant difference in food consumption from both soy protein isolate fed rats and genistein–supplemented fed rats from those fed a control AIN93 diet.
3.b Identification of ACF

3.b.1 Introduction

Aberrant crypt foci (ACF) are groupings of abnormal cell growth consisting of large thick crypts (Figure 3.3), a potential precursor to colon polyps that were first discovered by Dr. R.P. Bird in 1987 (Bird, 1987). Since that time, identification of ACF has been used as an early detection method for colon cancer as the occurrence, along with increasing size and numbers, correlates with risk of developing colon cancer (Roncucci et al., 2000). The use of ACF as an early detection practice involves a fairly simple procedure of staining with methylene blue and viewing under a microscope, their presence having become useful biomarkers for screening compounds with chemopreventive activities (Raju, 2008; Wargovich et al., 2000)

In pioneering work conducted by Dr. R.P. Bird, treatment of rodents with AOM induced growth of colonic crypts to be “larger, thicker, and darker staining than normal crypts when visualized with methylene blue” (Bird, 1987). It was also observed that by increasing the dose of carcinogenic substance, the size and number of crypts per focus increased (McLellan & Bird, 1988), normally appearing a few weeks after AOM injection. The crypts were also categorized as having distorted, slit-like luminal openings, and thicker epithelia (Bird et al., 1989).

It is important to note that ACF are a heterogenous group of lesions and that the changes in morphology of colonic crypts can occur in both cancerous and benign bowel diseases that have the potential to progress to a cancerous state, therefore ACF incidence alone may be used as a biomarker for risk of disease states such as cancer (Wargovich et al., 2000). Additionally, ACF are not distributed evenly among the ascending, transcending, and descending colon, the majority developing in the transcending and descending colon (Ghirardi et al., 1999; Park et al., 1997; Perse & Cerar, 2011; Raju, 2008; Rodrigues et al., 2002). Use of ACF total numbers may be useful as a biomarker only at early stages of carcinogenesis, while ACF with crypt multiplicities greater than three are more indicative of further cancer progression. In more advanced stages of colon carcinogenesis, tumors become a more reliable biomarker rather than ACF. (Bird & Good, 2000; Raju, 2008)

Additional characterization of ACF has included further analysis of size, shape, and
topography, and more recently - overexpression of β-catenin (BCAC) and mucin-depleted foci (MDF) (Marnett, 1992; Melchior et al., 1999). With these new discoveries, there is indication that other variations of histopathological lesions may be associated with future risk factors for colon cancer. (Wargovich et al., 2000)

3.b.2 Materials and Methods

Sample preparation. One vertical half of each colon section was fixed in 10% formaldehyde solution after colon collection, which is described in 3.a.2. The sample sections were flattened by stapling each to a piece of clear film allowing the topography of the colon to be clearly visible (Kochevar et al., 1993; Vivona et al., 1993; Wargovich et al., 1995). After 24 hr of fixation, samples were transferred into 10% Ethanol. Following staining by 0.1% methylene blue, samples were ready for ACF counting.

Identification and quantification of ACF. Colon sections were stained with methylene blue to determine the total number and multiplicity of ACF under a dissection stereoscopic microscope. Identification criteria for ACF included: 1) two to three times larger than normal crypts, 2) microscopically elevated, 3) have slit-like openings, 4) thick epithelial lining that stains darker than normal crypts, and 5) have a large pericryptal zone (Bird, 1987).

Total numbers of ACF were determined in the fixed section of male rat descending colons by counting with a single blind method. Average number of ACF per section, regardless of the multiplicity, was determined as the mean number of total ACF per colon section. Crypt multiplicity indicates the number of crypt(s) per focus. The average number of foci with multiplicity (n=1, 2, ≥3), where n = the number of crypt(s) per focus, was determined. Incidence was based on complete counts of all ACF and analyzed based on whether ACF of any multiplicity were present in an animal.

Statistics. Analysis was conducted using Statistical Analysis Software (SAS) using a basic t-test for total ACF. ACF multiplicity was analyzed by repeated measures and Tukey's Studentized range test, considered significant at p ≤ 0.05. There was no significance between colon samples in terms of length.
3.3.b.3 Results

As indicated by percent incidence, each rat displayed at least one ACF. In rats fed either soy or genistein-supplemented diets, a trend toward fewer total ACF was observed. With a p < 0.05 considered significant, rats fed a soy diet had significantly fewer total ACF compared to control. Rats fed a genistein diet also had fewer total ACF than control, although not significantly fewer (Table 3.3). After analysis of total ACF, groups were divided further into groups indicating number of crypts per focus.

Multiplicity was based on the overall number of crypts in a given focus, quantified as a focus with one, two, or three or more crypt in that particular focus. (Table 3.3) Multiplicity of ACF based on categorization of n=1, 2, and ≥ 3 indicated a significant reduction of number of ACF in n=2 (p = 0.03) and n ≥ 3 (p = 0.03) in the soy diet group verse control. Animals on a genistein diet showed a significant reduction in ACF multiplicity with n ≥ 3 (p = 0.04) compared to control.

3.3.b.4 Discussion

As indicated by the incidence and total ACF per colon, the AOM treatment was successful in inducing a precancerous (neoplastic) response in every rat by means of using ACF as the indicative biomarker (Table 3.3). McLellan et al., previously demonstrated this finding that an increase in ACF multiplicity, or number of crypts per focus, correlated with the increased dose of carcinogen, tying together the use of ACF as a potential biomarker for cancer identification, in a chemically-induced model (McLellan & Bird, 1988).

A study conducted by Raju et al. found that a soy isoflavone diet did not alter incidence or multiplicity of ACF in an AOM-induced rat model (Raju, 2009). Conversely, Min et al. observed a dose dependent response in reduction of ACF in DMH-induced rats fed a soy isoflavone diet (Min, 2010). Linz et al. also came to a similar conclusion with AOM-induced rats fed a soy protein isolate diet from gestation through post-weaning displaying decreased numbers of large ACF compared to rats fed a control AIN-93 diet (Linz, 2004).

The timeline for this study along with a lifetime diet treatment of either soy or genistein supplementation centers the focus on risk factor prevention and mitigation. With treatment of either a soy or genistein supplemented diet in our trial, a significant decrease in crypts with higher numbers of foci was observed. These observations lend support to
the case of soy and/or genistein acting as a potential risk-reducing factor for colon cancer progression.
Figure 3.3 Aberrant crypt foci stained with methylene blue. As described in Bird’s methods (Bird, 1987), tissue was stained with methylene blue. ACF appear larger than normal crypts, as identified within the red circles, more slit like in structure, and have a darker stain indicating a thicker layer A) Photo from currently study, B) photo from Wargovich, et al. 2000.
<table>
<thead>
<tr>
<th>Total ACF per Section</th>
<th>Diet</th>
<th>Average</th>
<th>p value compared to control</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td></td>
</tr>
<tr>
<td>Soy</td>
<td>8.78</td>
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</tr>
<tr>
<td>Genistein</td>
<td>12.78</td>
<td>0.15</td>
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</table>

<table>
<thead>
<tr>
<th>Number of crypts forming ACF</th>
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<th>n=2</th>
<th>n≥3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiplicity;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACF/Section</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.33 ± 1.02</td>
<td>2.83 ± 0.74</td>
<td>1.00 ± 0.28</td>
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</tr>
<tr>
<td>Soy</td>
<td>4.56 ± 1.07</td>
<td>1.00 ± 0.47</td>
<td>0.33 ± 0.21</td>
<td>0.31 0.03 0.03</td>
</tr>
<tr>
<td>Genistein</td>
<td>6.44 ± 1.86</td>
<td>2.11 ± 0.31</td>
<td>0.33 ± 0.23</td>
<td>0.29 0.22 0.04</td>
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<table>
<thead>
<tr>
<th>Incidence, %</th>
<th>n=1</th>
<th>n=2</th>
<th>n≥3</th>
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<tbody>
<tr>
<td>Control</td>
<td>92</td>
<td>83.3</td>
<td>54</td>
</tr>
<tr>
<td>Soy</td>
<td>88.9</td>
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<tr>
<td>Genistein</td>
<td>100</td>
<td>100</td>
<td>17</td>
</tr>
</tbody>
</table>

All p values based on differences from control.

Table 3.3 Total ACF, Incidence, and Multiplicity. N= number of sections (one section per rat) in control, soy, and genistein groups. Total ACF was significantly reduced in rats fed soy and genistein diets (p < 0.05). Multiplicity is indicated based on n=number of crypts in a focus, higher multiplicity is significantly reduced (p < 0.05) in the soy and genistein groups. Incidence of ACF in each rat and sample collected indicates a 100% incidence rate, in that every rate had at least one ACF, although not every rat had ACF with 1,2, 3 or greater crypts per focus. Incidence percentage indicates the percent of rats from the given group that had foci with 1, 2, 3 or greater crypts.
3.c Wnt Signaling Pathway and Related Gene Expression

3.c.1 Introduction

As described previously in Chapter 2.d, the Wnt signaling pathway is intricately tied to cancer initiation and progression when deregulated, especially in colon cancer. There are two pathways that are identified in Wnt signaling: canonical and non-canonical. For the purpose of this study, the focus was on the canonical pathway that is transduced through receptors on the cell surface, which leads to regulation of β-catenin (Figure 3.4), to be discussed further in section 3.d.

Risk factors for human colon cancer include genetic predisposition, environmental factors, and effects of aging. The activation of canonical Wnt through transcription occurs during tissue regeneration associated with chronic or persistent inflammation and up-regulation of Wnt1, Wnt3, or Wnt10b (Katoh & Katoh, 2007). Previous research has been done with tissue cell culture, showing both increases and decreases of Wnts in colon cancer (Hibi et al., 2009; Nishisho et al., 1991; Qi & Zhu, 2008; Schneikert & Behrens, 2007; Wang & Chen, 2010; Ying et al., 2008). Among the treatments, use of soy isoflavones has been observed as a potential inhibitor of the Wnt signaling pathway (Kim et al., 2010; Wang & Chen, 2010).

An important downstream target of the Wnt signaling pathway is Cyclin D1, which is involved in a complex with CDK4 and 6, and is required for cell cycle transition from the G1 phase to S phase. Increased gene expression of Cyclin D1 has been observed in colon cancer tumors and contributes to tumorigenesis (Arber et al., 1997; Motokura & Arnold, 1993; Tetsu & McCormick, 1999; Utsunomiya et al., 2001). Cyclin D1 expression is induced by an increased level of β-catenin protein in the nucleus which then complexes with CBP and TCF/LEF-1 causing increased transcription of Cyclin D1.

3.c.2 Material and Methods

RNA isolation. Five weeks after chemical induction with AOM, rats were sacrificed and tissue samples were collected and processed according to procedure described in section 3.a. Sections of descending colon from 5 male rats in soy, 5 in genistein, and 6 in control groups were homogenized, by grinding with a mortar and pestle, individually in liquid nitrogen and divided for use with either mRNA or protein, and placed in storage at -
80°C. Total RNA was isolated using TRIzol (Invitrogen) according to manufacturers instructions. After isolation, RNA concentration was determined using a SmartSpec Plus spectrophotometer (Bio Rad).

**Gene expression analysis using RT-PCR.** cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and a thermal cycler (Applied Biosystems 2700). To complete real-time PCR analysis, 5 μL of 5 ng/μL of the resultant cDNA template was mixed with RNase-free water, 10 μL SYBR Green PCR Master mix (Quanta), and 5 μmol/L of each of the forward and reverse primers. RT-PCR was performed using the following program: 95°C for 10 min, followed by 35 cycles of 95 °C for 15 sec and 60 °C for 1 min. After PCR, melting curves were acquired stepwise from 55 to 95 °C to ensure that a single product was amplified in the reaction. L7a, the gene encoding a ribosomal protein, was used as the internal control for normalization of individual values. (Strakovsky et al., 2011)

Primers used in this study are as follows: *Cyclin D1*, sense 5'-AGG GAG ATT GTG CCA TCC ATG C-3', antisense 5'-TCC TCT TCF CAC TTC TGC-3'; *Wnt1*, sense 5'-CC GTG GAC CTC TCT GTG TAT CAC-3', antisense 5'-TGA AGC CCA GGT GTG GTG GTT-3'; *Wnt8b*, sense 5'-CGC TGG AAT TGC CCT GAG AGA G-3', antisense 5'-CCG GTT AGC ACT CCG AAG TCC A-3'; *Wnt16*, sense 5'-TGG AAC TGT ATG GTC GCC ACT G-3', antisense 5'-TCA TAG CCA AAG AGG GGA GCT G-3'; *SFRP1*, sense 5'- TTA AGT GAC GCT GGG GTG GAC TG-3', antisense 5'- CCA AGA GCC AGG GTC ATT CAC AC-3'; *DKK1*, sense 5'- ATG CCC TCT GAC CAC AGC CAT T-3', antisense 5'- CAC CGT GGT CAT TGC CAA GGT-3'; *L7a*, sense 5'- GAGGCCAAAAAGGTGGTCAATCC-3', antisense 5'- CCTGCCCAATGCGAAGTTTCT-3'. Additional genes tested are listed in Appendix A. (Table 3.4)

Statistics. Statistical Analysis System (SAS) was used to calculate statistical significance. Gene expression results were then analyzed using Levene’s Test of Equal Variance and Tukey’s Studentized Range Test with differences considered significant at p < 0.05.

**3.c.3 Results**

RT-PCR was carried out and standard curves determined. RT-PCR was used to quantify expression levels of chosen genes (Table 3.4) and expression levels normalized by a ratio to *L7a*. According to statistical analysis, Wnts and a number of inhibitors showed
elevated expression levels in rats fed the genistein-supplemented diet. However, this same pattern was not observed in the downstream target, Cyclin D1.

Wnt 1, Wnt 8b, and Wnt 16 were over expressed in animals fed on the genistein diet compared to the control group. Expression of Wnt16 mRNA had a slight, but not significant, decrease in rats fed a soy diet compared to control. (Figures 3.5, 6, & 7) In rats fed the genistein diet, an increase of nearly 60 percent was seen in Wnt16 mRNA expression level compared to control. Wnt8b mRNA expression showed a similar pattern to Wnt16, with a slight decrease with rats fed a soy diet, and a nearly three fold increase in Wnt8b expression with rats fed a genistein diet compared to the control diet. Wnt1 mRNA expression once again displayed a similar pattern of slight decrease in rats fed with a soy diet, while in rats fed a genistein-supplemented diet, a two and a half fold increase of mRNA expression was seen in rats from the group fed a genistein diet.

mRNA expression of Wnt signaling pathway inhibitors sFRP5 and DKK1, sFRP5 was not significantly different from control in rats fed a soy diet. Rats given a genistein-supplemented diet displayed an increase of two fold in sFRP5 mRNA expression. DKK1 mRNA expression did not differ between rats fed control and soy. The rats in the genistein group displayed an almost one fold increase in DKK1 mRNA expression when compared to control rats. (Figures 3.8 & 3.9)

For downstream genes regulated by the Wnt signaling pathway, rats in both the soy and genistein fed groups displayed one fold decreases in mRNA expression levels of Cyclin D1 when compared to the control group (Figure 3.10). This indicates there is an interaction between Cyclin D1 expression and diets. Cyclin D1 is more closely associated with management via β-catenin, which will be discussed in the next section.

An assortment of other genes were tested including: Cmyc, p21, Wnt5a, Wnt4, Wnt11, Wnt9b, Wnt5b, and SFRP1 all of which showed no statistically significant difference (Figures 3.11-17).

3.c.4 Discussion

The goal of this study was to investigate the molecular and genetic aspects of the effect of soy and genistein on risk factor progression based on observed protection against colon cancer risk with decreased ACF development. Based on previous literature (Groden et al., 1995; Katoh, 2006; Katoh & Katoh, 2007; Katoh, 2008; Kim et al., 2010; Vermeulen et
al., 2010), the Wnt signaling pathway and cell cycle regulators were chosen as a starting point for determination of “what” was acting to decrease the progression of colon cancer risk. A host of Wnt family genes were tested to determine which were or were not affected. Another set of genes known for their inhibition roles in the Wnt signaling pathway was also tested for expression level.

All Wnt family genes that showed any kind of significant difference displayed the same pattern, consisting of a slight decrease in expression in the soy fed group of rats and a significant increase in expression levels for those animals fed a genistein supplemented diet. From what is currently known and previously suggested from research, an increase in Wnt could/or should indicate a disregulation of the cell cycle or eventual cell death, a phenomenon not observed in this study. An increase of Wnt expression did not directly translate to increased or unregulated growth that would be indicative of cancer or precancerous observations. Whether Wnt was increased, decreased, or unchanged showed no patterned connection to downstream regulation of gene expression or protein involved in cancer.

Relative to Wnt signals, the Wnt inhibitors unexpectedly showed an increase in expression with a genistein diet – almost mirroring the pattern of the Wnt expression. Again, no clear pattern was observed between Wnt signaling inhibitors and the pattern observed for gene expression and protein translation associated more directly with growth regulation.

_Cyclin D1_ is a regulator of the transition from the G1 to S phase of the cell cycle. In relation to cancer growth, an increased expression of _Cyclin D1_ is a likely indicator of cancerous activity. As observed in this study, rats fed either soy or geinstein-supplemented diets had decreased gene expression levels of _Cyclin D1_, indicating that this type of diet had more regulation of the cell cycle control. A decrease in _Cyclin D1_ would slow the progression of cells through the cell cycle (Utsunomiya, 2001).

In the genistein fed group, both the Wnts and the Wnt inhibitors showed increased expression when animals fed a soy diet showed no difference from control in this same set of genes. Overall, the gene expression patterns did not completely follow what would be anticipated as Wnt and Wnt inhibitors displaying and inverse relationship. However, the target gene outcome did follow the hypothesis that soy or genistein-supplementation
would decrease cancerous proliferation.

Figure 3.4 Wnt Signaling Pathway. Wnt inhibitors Dkk and sFRPs block the activation of Wnt, which can deactivate degradation of β-catenin in the cytosol, allowing it to complex in the nucleus with TCF/LEF-1. This cascade can then trigger continued expression of cell cycle regulators such as c-Myc and Cyclin D1. (Howard et al., 2003)
**Figure 3.5** Relative mRNA expression of Wnt16 in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C= 6, S=5, and G=5. *Different from control, p < 0.05.

**Figure 3.6** Relative mRNA expression of Wnt8b in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C=6, S=5, and G=5. *Different from control, p < 0.05.
Figure 3.7 Relative mRNA expression of Wnt1 in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C=6, S=5, and G=5. *Different from control, p < 0.05.

Figure 3.8 Relative mRNA expression of SFRP5 in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C=6, S=5, and G=5. *Different from control, p < 0.05.
Figure 3.9 Relative mRNA expression of DKK1 in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C=6, S=5, and G=5. *Different from control, p < 0.05.

Figure 3.10 Relative mRNA expression of Cyclin D1 in rat descending colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n= C=6, S=5, and G=5. *Different from control, p < 0.05.
<table>
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<tr>
<th>Gene</th>
<th>Ensembl Code</th>
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<tr>
<td>Wnt1</td>
<td>ENSRNOG00000014627</td>
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</tr>
<tr>
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</tr>
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<td>Wnt2b</td>
<td>ENSRNOT00000019349</td>
<td>No</td>
</tr>
<tr>
<td>Wnt4</td>
<td>ENSRNOT00000018064</td>
<td>No</td>
</tr>
<tr>
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<td>Wnt5b</td>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td>Dkk1</td>
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<tr>
<td>sFRP5</td>
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</tr>
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**Table 3.4** Ensembl codes for genes included in this study. All listed genes were run and analyzed as described in section 3.c.1. Significance of soy or genistein vs. control was based on a p-value < 0.05.
Figure 3.11 c-Myc mRNA expression in descending colon of male rats. There is no difference in expression levels indicating that genistein and soy do not control or inhibit c-Myc expression, which controls unregulated gene expression.

Figure 3.12 p21 mRNA expression in descending colon of male rats. There is no difference in expression levels indicating that genistein and soy do not regulate p21 to control cell cycle at the G1 phase.
Figure 3.13 Wnt5a mRNA expression in descending colon of male rats. There is no difference in expression levels indicating that genistein and soy do inhibit or stimulate Wnt5a gene expression.

Figure 3.14 Wnt4 mRNA expression in descending colon of male rats. There is no difference in expression levels indicating that genistein and soy do not inhibit or stimulate Wnt4 gene expression.
Figure 3.15 Wnt11 mRNA expression in descending colon of male rats. There is no difference in expression levels indicating that genistein and soy do not inhibit or stimulate Wnt11 gene expression.

Figure 3.16 Wnt5b mRNA expression in descending colon of male rats. Difference in expression was not significant due to high variation amongst the genistein samples for Wnt5b RT qPCR analysis.
Figure 3.17 SFRP1 mRNA expression in descending colon of male rats. Difference in expression level was not considered significant based on high variation in RT-PCR expression quantity.
3.d Nuclear β-catenin

3.d.1 Introduction

In the canonical Wnt signaling pathway, β-catenin is a crucial component encoded by the CTNNB1 gene. It forms a complex with TCF in the nucleus and is key in inhibition of signaling cell growth. In normal circumstances, β-catenin is degraded in the cell cytosol. If that degradation process is disrupted, however, as is the case with increased activity of the Wnt signals, β-catenin accumulates in the cytosol and translocates to the nucleus of the cell. It becomes associated with TCF/LEF transcription factors, which ultimately leads to transcriptions of cell cycle regulation genes and other Wnt target genes. An increase in Wnt activity should lead to higher levels of β-catenin protein found in the nucleus. Nuclear β-catenin is used as a critical indicator of Wnt signal and has been associated with cancerous tumors, including colon cancer (Ishitani et al., 2003; Katoh & Katoh, 2007; Kramps et al., 2002; Liss et al., 2010; Schneikert & Behrens, 2007; Su & Simmen, 2009). It is the accumulation of nuclear β-catenin that activates genes that are responsive to transcription factors from the TCF/LEF family and may regulate cycle control.

3.d.2 Material and Methods

*Nuclear Protein Extraction.* Samples of 100 mg of rat colon scraping were ground, washed twice in ice cold PBS, and centrifuged for 5 minutes at 1600 rpm. Twice the pellet volume of buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 200 mM sucrose, ddH2O, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail, 1x phosphatase inhibitor cocktail 3, 1x phosphatase inhibitor cocktail 2, 0.5% NP-40) was added and centrifuged for 5 min at 1600 rpm. Supernatant was removed and the pellet was resuspended with one times the pellet volume of buffer A, centrifuged, and liquid discarded. The pellet was resuspended in one times the pellet volume of buffer B (1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail, 1x phosphatase inhibitor cocktail 3, 1x phosphatase inhibitor cocktail 2, 1 mM EGTA, PBS), centrifuged as previously described. Pellet was resuspended with three times the pellet volume of buffer B and sonicated for 30 seconds at 30% maximum power. Samples were centrifuged at maximum speed, supernatant collected, pellet discarded, and snap frozen in liquid nitrogen before being stored in -80°C. Positive control to ensure
present nuclear protein was obtained through comparison blotting along side Lamin A, as described in the Western blot methods portion.

*Short Lowry.* To determine protein concentration, 10 μl of sample was added to 90 μl 0.2%SDS/0.2NaOH. Bovine serum albumin (BSA) was used as a standard. Samples were vortexed and 650 μl copper reagent was added to each tube. Samples were vortexed one more time and incubated at room temperature for 10 minutes. Sixty μl of 2N-Folin-Ciocalteu were added to each sample. The mixture was vortexed and incubated for 10 minutes at room temperature. Two hundred μl of each sample were transferred to a 96-well plate and incubated. Absorbance was read at 630nm on an ELx800 Microplate Reader (BioTek). Protein concentration was determined by plotting the value for unknown samples against the standard curve from BSA. Samples were then diluted to a concentration of 1.2 μg/μl.

*Western Blot.* Samples were loaded at 30 μg per well onto 4-20% gradient precast gels (BioRad). Electrophorese was performed at 80 volts for 30 minutes, followed by 110 volts for two hours. Protein was transferred on to a PVDF membrane (BioRad) from the gel at 80 volts for one hour in 4°C. Blotting conditions are as follows: one hour of blocking with ten percent milk, 3.5 hour 1:500 primary Beta-Catenin antibody (Cell Signaling) in 10% nonfat dry milk/TBST, washing 5x5 min in 5% milk/TBST solution, 1:10,00 secondary anti-goat/anti-rabbit antibody for 45 minutes in 5% nonfat dry milk, wash 5x5 min in 1% milk/TBST. Membranes were re-blotted with an antibody against Lamin A (Santa Cruz) to normalize the loading. Chemiluminescent signals were obtained using West Dura Extended Duration Substrate (SuperSignal) and quantified using a Chemi Doc XRS camera (BioRad) and Chemi Doc Imaging software.

*Statistics.* Quantification of protein levels was obtained via ChemiDoc Imaging Software and normalized to Lamin A. Analysis was performed using a Studentized T-test with significance place at p<0.05.

### 3.d.3 Results

Nuclear protein abundance was analyzed by Western blot for β-catenin. Lamin A was used as a control for loading. The band representative of a rat fed a control diet was
the darkest, a soy diet was lighter, and a genistein diet was lightest of all with almost no visually detectable presence for β-catenin. (Figure 3.18) Results showed a decrease in nuclear β-catenin level for soy and genistein diet groups compared to the control diet.

Quantity of nuclear β-catenin protein levels was normalized to Lamin A. Analysis indicated a significant difference between nuclear β-catenin levels of the control group and that of both soy and genistein fed groups (p-value < 0.05) (Figure 3.19). Rats fed a soy protein based diet showed a one-fold decrease and rats fed a genistein-supplemented diet showed a nearly three-fold reduction compared to the control group.

3.d.4 Discussion

β-catenin is a an intracellular mediator of the canonical Wnt signaling pathway; in many cases increased Wnt expression would correlate with increased β-catenin present in the nucleus. This pattern was not directly observed in this study. To the contrary, expression of Wnt and antagonists were all increased while β-catenin was decreased in animals fed genistein. This is in contrast with a study conducted by Su et al. in breast cancer where β-catenin showed increased levels in female rats fed a diet with genistein with inhibited Wnt signaling (Su & Simmen, 2009). Additionally, in a study conducted in prostate cancer, β-catenin showed a slight decrease in the presence of soy treatment (Liss et al., 2010). This is the first report of Wnt signaling in colon by genistein. The seemingly contrasting result between increased Wnt signals and decreased β-catenin level may be due to the fact that genistein diet, at the same time, also induced expression of several Wnt antagonists. The antagonistic effects from increased sFRPs and DKK1 may contribute to the overall down regulation of Wnt signals as decreased nuclear β-catenin. Although there was a difference in expression pattern, β-catenin is the foremost marker representing overall Wnt activity.

Nuclear β-catenin protein expression did, however, follow the same pattern as seen in Cyclin D1 mRNA expression, with both reduced in soy and genistein fed groups. Cyclin D1 is regulated by the β-catenin/TCF complex – with complex being necessary for Cyclin D1 to interact within complex with CDK 4 or 6. β-catenin was less present in the nucleus of soy and genistein fed rat colon than in the control.
Figure 3.18 Western blot of β-catenin and Lamin A in rat colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Lamin A was used as a nuclear protein control and an internal loading control. Its presence remains fairly consistent. Presence of β-catenin is decreased in animals fed a soy diet and nearly undetectable in animals fed a genistein-supplemented diet.
Figure 3.19 Quantification of β-catenin normalized to Lamin A in rat colon samples in AOM induced rats fed a control, soy, or genistein diet during gestation, lactation, adolescence, and continuing into adulthood. Values are means ± SEM, n = 5. *Different from control, p < 0.05.
3.e References


Howard, J., Varallo, V., Ross, D., Roth, J., Faber, K., Alman, B., et al. (2003). Elevated levels of beta-catenin and fibronectin in three-dimensional collagen cultures of dupuytren's disease cells are regulated by tension in vitro. BMC Musculoskeletal Disorders, 16(4),


hepatic phosphoenolpyruvate carboxykinase (pck) expression and histone modification in neonatal offspring rats. *Journal of Physiology, 589*(13)


Along with early detection and treatment, primary prevention of colon cancer is proving to be one of the most effective strategies for reducing mortality. Through this study, which focused on dietary intervention, evidence was found of a reduction in risk factors associated with developing colon cancer in rats fed a soy protein diet or genistein-supplemented diet. The risk factors included for this analysis incorporated the presence and multiplicity of ACF, activation of the Wnt signaling pathway, β-catenin, and the targeting of *Cyclin D1* for cell cycle regulation.

In brief summary, three generations of Sprague-Dawley rats were raised, mated, and fed a consistent control diet of AIN93, AIN93 with soy protein, or AIN93 with soy protein and genistein-supplementation. This trial focused on the second generation, wherein control rats were obtained at six weeks of age and placed on an AIN93 control diet. At ten weeks of age, all rats were injected with AOM for cancer induction. After 15 weeks of age, these rats were sacrificed for tissue collection. Further analysis was conducted relative to ACF development, Wnt signaling pathway expression, and β-catenin protein level.

Several inferences may be concluded from data relative to the difference in body weight of the rats fed a soy diet: the soy diet was less desirable than the other diets; rats fed a soy diet were naturally smaller than control groups; soy protein fed over generations limits weight gain in rats; or there may be an as of yet unknown factor contributing to lesser body weight for this particular group. Due to this unpredicted “turn of events”, body weight must be included as a potential influencing factor on “outcomes”.

Expression of Wnt and Wnt inhibitor genes was increased in rats fed a genistein-supplemented diet, while showing no effect on rats fed a soy protein isolate diet. Detection of increased Wnt expression is widely known and used as an implication of cancerous or precancerous conditions, especially in the colon. However, in this case it is not entirely clear if an increased Wnt is the only or main contributing factor in this cancer progression.

Primary observations from the ACF section suggest that a diet of soy protein or added genistein decreases the progression of precancerous conditions in the colon. The reduced *Cyclin D1* expression pattern is similar to the pattern seen in ACF results. Both soy
and genistein diets showed decreased expression and fewer ACF, while *Cyclin D1* reduction would decrease the rate of cancerous growth and keep cells regulated for death rather than continued growth.

Nuclear levels of β-catenin protein were significantly reduced in rat colons of those fed a soy or genistein diet. This pattern is similar to that observed with the *Cyclin D1* mRNA expression, indicating a regulation of downstream *Cyclin D1* by β-catenin. This similar pattern could also support a decreased progression of ACF and precancerous conditions in the soy and genistein fed groups.

Although conclusions can be drawn with this particular portion of the trial, there are limiting factors affecting the concreteness of any overarching statement made, the awareness of these limitations making it easier to address issues and make plans for future direction.

After combining the mRNA, ACF, and protein data with the body weight and food intake data, the effect of diet would appear to have a more dominant influence than would the difference in weight. That said, the difference in body weight does pose some additional questions: what is the level of influence for each, and what significance does diet factor into this trial for reduction in colon cancer risk? Which factors, the diets of soy or genistein, or a decrease in body weight, or perhaps the combination of both may lead to a reduction in colon cancers?

The US population, as well as others, follows a typical Western diet, one being higher in fats, carbohydrates, and animal protein. These diet and lifestyle choices often lead to obesity and chronic diseases such as diabetes, heart disease, and cancer; each found to be arguably preventable with weight reduction to a given ideal body weight. The mechanism for reaching and maintaining this weight is widely accepted as being through healthy diet and physical activity (American Dietetic Association, 2011). Although it is well documented that populations following a traditional East Asian diet have a lower occurrence of colon cancer and are at lesser risk of developing this particular cancer when compared with other diet practices, it has also been observed that among these East Asian populations, an increased consumption of soy products does not seem to effect over all risk or prevention (Budhathoki et al., 2011; Kim, Sasaki, Otani, & Tsugane, 2005; Minami, 2006; Oba et al., 2007). Rather, other confounding conditions may play a role as well.
It is known that obesity leads to increased risk of colon cancer. Therefore decreased body weight may also decrease the risk of developing colon cancer (Cordain et al., 2005; Jemal, 2008; Matsushita et al., 2008; Moshkowitz & Arber, 2005). Yet a similar decrease in risk factors markers for both the genistein–supplemented as well as the soy diet group lends support that the decrease in body weight may not be the primary contributing factor i.e. it may only aid in the process. It may also be suggested that a decrease in body weight is not significant enough to have a stronger affect than does diet. Understandably, this has yet to be proven, nor in this case investigated. Moreover, it is not to be inferred that all groups fall within “normal” weight groups, and variation within these groups would not indicate any predisposition to cancer or predisposition to reduced risk. To answer these questions, one must initially determine whether or not the other groups are in fact technically overweight or obese. In this particular study, the difference in body weight was significant in overall total ACF, with significantly fewer ACF found in soy protein fed rats, which also had lower body weight. However body weight was not a significant contributing factor with both soy protein and genistein diet groups showing similar outcomes in reduction of ACF with higher multiplicity that could eventually progress to form tumors (Table 3.3). A conclusion can not be drawn at this time as to whether soy protein or genistein would have more effect on stopping further abnormal cell growth to active tumor states.

Apart from the issue of body weight, there are inconsistencies relative to controlling diet and to dietary treatment. There was a difference in food consumption, which could be a contributing factor to various outcomes and which may affect the predicted level of active diet ingredient, genistein. In addition, this particular set of control animals were not kept or exposed to the exact same conditions as the two other experimental groups, since they were obtained at six weeks of age. The composition of the chow diet fed to the control rats during gestation, lactation, and first few weeks after weaning is unknown at this time and may have contained soy or other bone meal component, which according to recent study, calcium may be a contributing factor to colon cancer risk and development (Lappe et al., 2007). For each of these reasons, it becomes difficult for any direct conclusion(s) to be made, and only reportage of what transpired in this particular portion of the trial can be made.
In future research, the trial should continue to the third generation for analysis. From what was observed, analyses of the entire generational studies to note any differences from generation to generation - whether a soy or genistein diet had more impact on generations whose mothers and grandmothers were not exposed to soy or genistein prior to pregnancy. Analysis could also be done on an individual basis in terms of weight, i.e. did the smaller, lighter rats tend to have a greater risk reduction, or were the heavier animals at greater risk, having more pronounced factors associated with risk. In the future, more attention to details and potential shortfalls that could interfere with outcomes – control being an actual control and kept under the same conditions as the experimental groups. Additionally, samples saved for immunohistochemistry that have not already been compromised with rust staining could also be preserved for future analysis. In summary, more attention to detail could offer more options for future research opportunities.

Although limitations exist which prevent this study from concluding with resolute certainty, it can be added the bank of knowledge on the topics of colon cancer, soy isoflavones, the Wnt signaling pathway, and the use of ACF as a physical biomarker. In this study, it does appear that in a second-generation rat population, the risk of colon cancer was decreased in groups fed soy and genistein diets. This reduction in risk is a step in the direction of colon cancer prevention and reduction of mortality for the third leading cause of cancer related deaths.

References


APPENDIX: EFFECT OF SOY SAPONIN ON COLON CANCER CELL LINES
- SW480, SW620, DLD-1

I. Introduction to Soy Saponins

In addition to isoflavones found in soy, saponins are additional functional components that have received attention in recent years. Saponins have been a topic of debate since the late 1970’s when Oakenfull published his first article illustrating the potential health benefits from saponins, especially in terms of cholesterol reduction (Oakenfull, 1979, 1986). Since that time there has been increasing research into whether various saponins possess physical and chemical properties to benefit health. Soy saponins emerged as one of the rising stars of the discussion. There were promising reports early-on of soy diet with increased soy saponin reducing plasma cholesterol (Lee, 2005) however increasing studies have found little impact without the presence of isoflavones (Yamakoshi, 2000; Lin, 2005), indicating a whole soy effect rather than the individual parts.

Saponins are plant glycosides with a steroid/triterpine core, referred to as the aglycone form. Diversity results from differences in this aglycone structure along with the number and composite of sugar side chains (Figure 1.1). Due to their structural formation, they may be able to react with the phospholipids and cholesterol on the membrane of cells, and with the hydroxyl groups on the aglycone moiety. In their membrane permeabilizing properties, the number of side chains influences hemolytic activity and membrane permeability. Soy saponins are found in both the hull and the endosperm, and are primarily found in three groups: A, B, and E with A and B being the most prevalent. Upon removal of their sugar side chains they are in their sapogenol form.

While cholesterol reduction remains a relevant topic among nutrition researchers, due to their lipid like qualities and similarities in cholesterol structure, the current area of soy saponin research has moved toward a cancer or chemopreventive direction. The primary focus has been on colon health due to studies indicating bioavailability – chemical components in forms suitable for use within the body (Hu, 2004), targeting increased cell death or decreased proliferation (Oh, 2001; Kim, 2004). Macroautophagy has had increased attention as a marker of cancer cell death when treated in vitro with soy saponins.
Still others focused more on the change in cell structure in relation to anti cancer properties (Gurfinkel, 2003; Tsai 2010). To a lesser extent, some have looked at a potential link to inhibition of metastasis (Kang, 2008) and other cancer targets (Su, 2009). Most studies on soy saponins have been performed on human colon cancer cells, since soy saponins are expected to primarily influence the gastrointestinal system after they are ingested as part of the diet. (Hu, 2004; Hu, 2004)

While promising in vitro research is present, there still remains the question of how the body can utilize soy saponins in vivo (Hu, 2004). Investigation into that question has yielded the hypothesis that potential benefit could come from the effect on the gut microflora population (Hu, 2004). In the case that soy saponins are not readily degraded until reaching the colon and broken down by the bacterial population, this is a promising avenue for future research.
Figure I.1 Soy saponin structure showing the sterol/triterpenine backbone and sugar side chain attachment. When sugar side chain has been cleaved, compound becomes a sapogenol. (Hu, 2004)
II. Compiled Materials and Methods for Multiple Cell Culture Trials

Cell Culture

Colon cancer cells from line SW480, SW620, or DLD-1 were thawed from previously frozen stock; cells were cultured in MEM 10% FBS plus ABAM media for growth for 7 days or three cell passages with trypsin. On the day of passage three, cells were plated as 1 million cells per 60x15 mm petri dish and calculated to 1 million cells per 5 ml in FBS media; and plated as 5ml per plate. After 12-16 hours allowed for cells to adhere to the bottom of the plate, media was removed and replaced with soy saponin treatment media.

Treatment

Single Soy Saponin Treatment. Group B soy saponins were provided by Dr. Mark Berhow (USDA, Functional Foods Research Unit, Peoria, IL) in an isolated powder form. This sampling of soy saponins measured 1.03 mmol of soy saponin B per gram. To make a 20 mM stock of soy group B saponins, 1 g of saponin was dissolved in 51 ml of DMSO; then aliquoted into 50 µl per tube for easily available stock. The 20 mM stock was diluted with MEM media for treatment concentrations, which included: 500 µM, 250 µM, 100 µM, 50 µM, 20 µM, 10 µM, 2 µM, 1 µM, 0.5 µM, and 0.1 µM. DMSO was used as the control. Each treatment plate was made in quadruplicate. Different cell line trials used different combinations of the concentrations listed.

NEFA Experimental Treatment. DLD-1 cells were plated as per previously described method. Two treatment variables were used in this section of study; NEFA (non-esterified fatty acids) along with soy saponins to determine an interaction between the two, if present. NEFA concentration treatments used were 20 µM and 100 µM concentrations. The level of soy saponin used as treatment was kept consistent, using 250 µM concentrations, the previously identified most significant treatment level (Figure II.2). Treatment plates were conducted in triplicate and included: NEFA 25µM no treatment (NT), NEFA 100 µM NT, NEFA 25 µM + saponin, and NEFA 100 µM + saponin. Control plates included: BSA NT, BSA + saponin, time Zero – collect at 16-18 hours after plating, and time Zero – BSA. On day two, dishes were treated accordingly with BSA or NEFA, on
day three treated with soy saponin or additional NEFA if no treatment was required, and cells were collected for mRNA analysis 24 hours after saponin treatment.

**Cholesterol Experimental Treatment.** DLD-1 cells were plated as previously described. The two treatment methods used in this portion of study were cholesterol and soy saponin to determine an interaction, if present. Crystalline cholesterol was put into solution with serum using units of mg/ml of cholesterol (Chol) with 0, 4, 16, and 32 mg/ml concentration. Once again the level of soy saponin treatment was 250 µM. Treatment plates were conducted in triplicate and included: Chol 4 mg/ml NT, Chol 16 mg/ml NT, Chol 32 mg/ml NT, Chol 4 mg/ml +saponin, Chol 16 mg/ml +saponin, and Chol 32 mg/ml +saponin. Control plates included: BSA NT, BSA +saponin, and BSA time zero along with no-BSA NT at time zero. After plating, cells were treated with cholesterol or BSA on day one, on day two cells were treated with soy saponin or new cholesterol or BSA was added, and cells were collected on day three.

A concentration of 32 mg/ml was used as the highest concentration based on reports of previous study having 25 mg/ml as the highest point used for the majority of data analysis; 4 mg/ml and 16 mg/ml were used to determine the shape of the line.

**mRNA Expression Analysis**

Cells were incubated in soy saponin treatment for two days before RNA isolation. At this time point, media was removed from each dish and 1 ml of Tri Reagent was immediately added. Tri Reagent (Sigma, St. Louis, MO) was used to ‘scrape the cells’ from the dish by repeated pipette washing and deposited into 1.5 ml micro tube. Once all were in tubes, 0.2 ml of 1-chloro-3-bromopropanol was added to each and inverted several times to mix, followed by incubating at room temperature for 15 minutes. Samples were then centrifuged for 15 minutes at 12x1000 rpm in a 4°C conditions. Clear supernatant was removed in volumes of 0.5 ml from each tube, along with the addition of 0.5 ml of 2-propanol. Samples were again allowed to incubate for 5 minutes at room temperature after vortex. Centrifugation was kept at the same speed and temperature as previously mentioned with a time change of ten minutes, after which the 2-propanol was discarded from the pellet formed at the bottom of the tube. As the final was of the pellet, 1 ml of 75%
ethanol as added to each tube and vortexed; a final centrifugation was run with the same conditions for 10 minutes. Ethanol was then discarded and pellets were allowed to air dry in the tubes for 10 minutes, followed by addition of 100 µl of Nuclease Free water (Fisher Scientific) to dissolve the RNA pellet. To control for DNA contamination, 48.5 µl of RNA, 0.5 µl of DNase 1 (Roche Diagnostic), and 1 µl of MgCl2 were combined for each separate tube, incubated at room temperature and reaction terminated in the Thermal Cycler (Applied Biosystems) at 90 C for 10 minutes. This RNA is then ready for concentration analysis, determined for each sample using the BioRad spectrometer; after which RNA was diluted to 200 ng concentration stock. Two-hundred ng RNA stock was used for cDNA protocol. cDNA was diluted to 5 ng concentrated solution with the exception of one DMSO sample which was used for the standard sample with varying cDNA concentrations.

RT-PCR was run with different gene primers (Integrated DNA Technologies), L7a was used as the internal control, as previous experience indicated fairly stable expression levels. Genes of interest included: Pon2, PPARγ, PIK3CA, FOS, MAPK9, Wnt1, Wnt3a, Wnt5a, DKK1, sFRP2, LDLR, HMGCR, HMOX, LNFRSR1, Casp8, Casp9, FAS, FADD, and Cox2 (Table II.1). A two-step real time quantitative PCR was accomplished using a dilution to 5 µg/ul of cDNA. Each sample loaded into a 96 well plate contained a mixture of the specific sample cDNA, iTag Sybr Green Supermix with ROX (Bio-Rad, Hercules, CA), sense and antisense primers for the selected gene, and nuclease free water. Quantitation was run using a 7300 Real-Time PCR System (Applied Biosystem, Foster City, CA).

**Flow Cytometry**

*Apoptosis.* DLD-1 and SW480 colon cancer cells (neither depicted in figures) were plated and treated as previously described. Following 24 hours of treatment, media was removed and cells washed twice with cold PBS. Cells were scraped from dishes in 500 µl of cold PBS, pipetted into 1.5 ml centrifuge tubes, and centrifuged at 5000 rpm for 10 minutes. PBS was discarded and cells were resuspended in 300 µl cold 1x Annexin V Binding Buffer (Beckman Coulter) to a concentration of 1x10^6 to 1x10^7 cells/ml. One hundred micro liters of cells were added to new tubes, along with 10 µl of Annexin V-FITC (Beckman Coulter), followed by incubation in the dark at room temperature for 15
minutes. Next, 380 μl of cold 1x binding buffer was added to each tube, μl of Propidium Iodine (PI) (Beckamn Coulter) was added to each sample tube, and tubes were stored on ice no longer than one hour before being analyzed by flow cytometry using a BD SRL II flow cytometer. Tubes kept for compensation included one with no unstained cells, one containing only Annexin V-FITC, and one with only PI. Flow cytometry was conducted at the University of Illinois Biotechnology Center (Keck Center, UIUC). Data was analyzed using the online FCS Express software. Due to no significant data received with a simple apoptosis test, a more advanced method was used to further distinguish between late and early apoptotic cells.

_Cytotoxicity._ DLD-1 cells were plated as previously described and treated with DMSO, 50µM, or 250µM soy saponin. Cytotoxicity stocks and buffers were prepared according to the Total Cytotoxicity & Apoptosis Detection Kit (Immunochemistry Technologies, Bloomington, MN) instructions. After 24 hours of treatment, cells were trypsinized and spun down at ~750rpm, liquid discarded and remaining cells washed with 1ml/1.5 microtube assay buffer. 100μl of cells from tube was added to 100μl additional assay buffer. 10μl of 20X SR-FLICA working solution, prepared just before use, was added to each tube and additional controls and incubated for 45 minutes in 37°C to detect and stain early apoptotic cells. After, 200μl culture media was added to each tube to terminate SR-FLICA reaction and placed on ice. Just before analysis, 20μl of 21X 7-AAD was added to each tube and gently vortexed and incubated on ice for ten minutes. Cytotoxicity analysis was conducted at the UIUC Keck Center using the SRL II flow cytometer and data analyzed with FCS Express software.

_Cell Cycle._ DLD-1 cells were plated and treated as stated above. For collection, cells were trypsinized, counted, and pelleted with 0.5x10⁶ cells/sample. Media was discarded and cells resuspended in 0.5 ml PBS; 0.5 ml of 100x cold ethanol was added drop wise in addition to vortexing. Samples were stored at -20C for two days. Cells were thawed and pelleted once more at 1000 rpm for 5 min and liquid discarded. Next, 0.5 ml of PBS, 5 μl of RNase, and 10 μl 250 μg/ml PI were added to each sample and mixed well followed by an incubation at room temperature under dark conditions for 20 minutes. Cells were analyzed using the SRL II at the UIUC Keck center and data analyzed by FCS Express.
Statistics. Analysis was run using Tukey's Studentized T-test and Repeated ANOVA with Statistical Analysis Software (SAS). Significance was considered at $p < 0.05$. 
Figure II.1 Cell viability of SW620, SW480, and DLD-1 colon cancer cell lines treated with increasing levels of soy saponin. Determination of desired cell line, DLD-1 cell line showed greatest level of response to treatment. Results are expressed as means ± SEM.

Figure II.2 Time course for DLD-1 treated with soy saponin. A higher concentration of soy saponins showed a significant reduction in growth and proliferation. Results are expressed as means ± SEM. *p < 0.05
Figure II.3 DLD-1 cell viability at differing treatment levels. As a trend, cell viability decreased as concentration of soy saponin treatment increased. At both 250µM and 500µM there was a significant decrease in viability; 250µM was identified as the highest treatment level needed based on observation that 500µM did not significantly differ in effect from 250µM. Results are expressed as means ± SEM. *Significant (p < 0.05) from DMSO control.
Figure II.4 Cell cycle phase of DLD-1 cell line after treatment with soy saponin. Significant increase in cells treated with 250μM of soy saponins detected at G2 phase and significant decrease in the same groups of cells detected at G1 phase, indicating that high levels of soy saponin treatment elicit an arrest of the cell cycle at the G2 phase. Results are expressed as means ± SEM. *p < 0.05

Figure II.5 Cytotoxicity of DLD-1 cells treated with soy saponin. In cells treated with 250μM soy saponin, essentially equivalent to 250ppm based on molecular weight (1.03mmol/g soy saponin B in isolate powder), there is a significant decrease in the number of live cells detected and a significant increase in the number of cells detected at the early apoptotic phase, while numbers of necrotic and late apoptotic cells did not differ. This indicates early apoptosis as the target death reaction for cells treated with soy saponins. Results are expressed as means ± SEM. *p < 0.05
Figure II.6 Cytotoxicity scatter plot representative sample from DLD-1 cells treated with DMSO as control. Upper left indicates necrotic cells, lower left indicates live cells, upper right indicates late apoptotic cells, and lower right indicates early apoptotic cells.
**Figure II.7** Cytotoxicity scatter plot representative sample from DLD-1 cells treated with 250 μM soy saponin. Upper left indicates necrotic cells, lower left indicates lives cells, upper right indicates late apoptotic cells, and lower right indicates early apoptotic cells.
Figure II.8 Pon2 mRNA expression in soy saponin treated SW480 colon cancer cells. *Significance is based on p-value < 0.05. Results are expressed as means ± SEM.

Figure II.9 PPARG mRNA expression in soy saponin treated SW480 colon cancer cells. *Significance is based on p-value < 0.05. Results are expressed as means ± SEM.
Figure II.10 PIK3CA mRNA expression in soy saponin treated SW480 colon cancer cells. *Significance is based on p-value < 0.05. Results are expressed as means ± SEM.

Figure II.11 Wnt1 mRNA expression in soy saponin treated SW480 colon cancer cells. *Significance is based on p-value < 0.05. Results are expressed as means ± SEM.
**Figure II.12** Expression of LDLR in Cholesterol and Soy Saponin Treatment of DLD-1 Cells. LDLR expression change with increase between cholesterol plus saponin and cholesterol groups. *p<0.05 when analyzed using Repeated ANOVA. Results are expressed as means ± SEM.

**Figure II.13** Expression of HMGCR in Cholesterol and Soy Saponin Treatment of DLD-1 Cells. HMGCR expression change between cholesterol and cholesterol plus saponin treatment groups. *p<0.05 when analyzed using Repeated ANOVA. Results are expressed as means ± SEM.
**Figure II.14** Expression of Cox2 in Cholesterol and Soy Saponin Treatment of DLD-1 Cells

Cox2 expression change within subject effects of differing cholesterol concentration without saponin, as well as effect between cholesterol and cholesterol plus saponin groups. *p<0.05 when analyzed using Repeated ANOVA. #P<0.05 when compared to 0 concentration control using Two Factor Factorial ANOVA. Results are expressed as means ± SEM.

**Figure II.15** Expression of PPARG in Cholesterol and Soy Saponin Treatment of DLD-1 Cells.

PPARG expression change within subject effects of differing cholesterol concentration independent of saponin, where as addition of saponin maintained a steady expression rate of PPARG. #p<0.05 when compared to 0 concentration control using Two Factor Factorial ANOVA. Results are expressed as means ± SEM.
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Table II.1 Complete list of genes tested in colon cancer cell lines.
III. Preliminary Animal Trial – Soy Saponin Presence in Rat Colon

To progress beyond cell culture and move toward analysis using an animal model, ten Sprague Dawley female rats were obtained from a concurrent low-protein animal trial. Rats had previously been fed a powder diet, therefore isolated B group soy saponin provided by Dr. Mark Berhow (USDA, Peoria, IL), was added to a control diet mixture to create a 1% soy saponin diet mixture. Three rats were put in the control group and fed the plain mixture; the remaining seven rats were fed the 1% soy saponin diet. For seven days, rats body weight and food intake was taken every day. After one week, rats were sacrificed with samples taken from: blood, liver, feces, digesta, and the colon. Samples were all snap frozen in liquid nitrogen and then packaged in dry ice for shipment to Dr. Berhow for further analysis of saponin presence in samples and form of saponin presence. Samples have not been completely tested or analyzed at this time.
Figure III.1 Average body weight per day of female rats fed either a control or soy saponin diet. No significance was determined between groups.

Figure III.2 Average food intake per day of female rats fed either a control or soy saponin diet. No significance was determined between groups.
IV. Discussion and Future Direction for Soy Saponin Study

Research was first conducted using the SW480 cell line and upon initial analysis, a significant difference ($p < 0.05$) in expression of *Pon2, PPARG, PIK3CA*, and *Wnt1* in samples treated with a low dose of soy saponins compared to DMSO control (Figures II.8-10). However, repeated testing of significant concentrations yielded results indicating no difference from control samples. The same was then repeated using the SW620 cell line and finding similar results.

Based on previous cell culture studies involving soy saponins, the DLD-1 cell line was treated side-by-side with SW480 and SW620 to determine cell viability (Figure II.1). DLD-1 had significantly ($p < 0.05$) more cells than SW480 or SW620 and was therefore chosen to continue with further treatment. Further time course evaluation and treatment with a soy saponin gradation indicated that 48hr was the better time point for analysis and 250$\mu$M was the highest effective treatment, with 500$\mu$M soy saponin showing no difference from 250$\mu$M (Figure II.2 and Figure II.3).

To determine cell arrest and cell death, flow cytometry was used for cell cycle and apoptosis analysis. Cell cycle results indicated an arrest at the G2 phase (Figure II.4). With use of the cytotoxicity kit, the cell death was indicated to be due to early apoptosis (Figure II.5). Results obtained from DMSO control samples are represented in Figure II.6 and results from a sample treated with 250$\mu$M soy saponin are shown in Figure II.7.

For an added level of stress for DLD-1 cells, cholesterol and NEFA were added as treatments in addition to soy saponins. For these treatments, results were viewed based on differences between groups. *LDLR, HMGCR, and Cox2* were significantly ($p < 0.05$) increased in samples treated with both cholesterol and soy saponins (Figures II.11-13). *PPARG* showed no difference overall in the presence of soy saponins, however it was significantly ($p < 0.05$) increased in samples treated with the highest level of cholesterol without saponin treatment – yet with the addition of soy saponins there was no change in expression (Figure II.14).

Based on previous research and research presented in aforementioned studies, a move towards an effect on colon health is clearly the best direction. Through trial and error, it was observed that certain cell lines are more responsive than others yielding
mixed results. The DLD-1 cell line seems to respond more sensitively to the treatment and was used for the majority of later study with NEFA and cholesterol treatments, NEFA treatment was not found to be significant.

Further research should focus on the event of early apoptosis as a way to trigger cell death. Use of the cytotoxicity kit helped to separate out early apoptotic cells that had previously been viewed as necrotic cells in more archaic apoptosis analysis. A focus using the addition of cholesterol as an antagonist may also be a new direction to pursue.

In the small trial conducted with rats, there was no difference in food intake or body weight between the soy saponin fed group and the control fed group (Figure III.1 and Figure III.2). Once data from Dr. Berhow has been completely processed from this short-term trial, there may be a better determination of which direction to take. Based on early reports from the samples analyzed by Dr. Berhow, it appears that the saponin form is changed through digestion. Looking at microbiota responsible for cleavage of side chains that may have an affect on gut environment and play an influential role in colon health is a potential direction to be explored.
V. References


molecule E-cadherin expression and attenuates b-catenin signaling in mammary epithelial cells. *Carcinogenesis, 30*(2), 331.
