THE DEVELOPMENT OF A MODEL OF PHIP-INDUCED COLON CARCINOGENESIS IN CYP1A2-HUMANIZED MICE

By

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ABSTRACT OF THE THESIS

The Development of a Model of PhIP-Induced Colon Carcinogenesis in CYP1A2-Humanized Mice

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The dietary carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is considered one of the most abundant heterocyclic amines formed during the cooking of meats and fish. PhIP is metabolically activated to its carcinogenic form primarily by the human Cytochrome P450 enzyme, CYP1A2; whereas in rodents, PhIP is detoxified by the Cyp1a2 mouse ortholog. This metabolic activation is followed by esterification to produce activated esters that can bind to DNA to form adducts. PhIP-DNA adducts have been identified in various tissues of mice and in the colon of humans. PhIP has also demonstrated carcinogenicity in rodents, preferentially in the colons of rats and lymphatic system of mice. Through the use of a recently generated transgenic mouse model consisting of a human CYP1A1/1A2 gene insert on a mouse Cyp1a1/1a2 knockout background, we have developed a PhIP-induced humanized model of colon carcinogenesis. Male and female hCYP1A2 mice were given various combinations of PhIP (100mg/kg or 200mg/kg) by oral gavage without or with DSS (1% or 1.5%) in the drinking water for one week. We observed a single dose of PhIP (200mg/kg) followed by one week administration of dextran sodium sulfate (1.5%) in the drinking water of the hCYP1A2 mice to be the most effective treatment combination to induce colonic adenocarcinomas, with cancer formation in 6 weeks. In other treatment groups, ACF's were found to be present in the colons of mice treated with

100mg/kg of PhIP + 1% DSS as early as 6 weeks and in mice treated with 200mg/kg of PhIP without DSS as early as 12 weeks. In contrast, colonic neoplasms were not observed in C57BL/6J wild-type mice in any of the treatment groups. Pathological analysis of colonic tissue from hCYP1A2 mice treated with 200mg/kg of PhIP + 1.5% DSS revealed severe inflammation, a hyperplastic epithelial lining and an infiltration of leukocytes in the submucosal layer of the colon. Immunohistochemical analysis indicated that colonic neoplasms were associated with nuclear accumulation of β-catenin resembling mutations commonly found in human colon neoplasms. These results suggest that PhIP in combination with DSS in the hCYP1A2 mice is a good short-term animal model to study colon carcinogenesis.

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LIST OF ABBREVIATIONS

ACF	Aberrant Crypt Foci
AFAP	Attenuated Familial Adenomatous Polyposis
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
BCA	Bicinchoninic Acid Assay
BRAF	v-raf murine sarcoma viral oncogene homolog B1
COX	Cyclooxygenase
CYP	Cytochrome P450
CYP1A1	Cytochrome P450, Group 1, Subfamily A, Gene 1
CYP1A2	Cytochrome P450, Group 1, Subfamily A, Gene 2
CYP1B1	Cytochrome P450, Group 1, Subfamily B, Gene 1
CYP3A	Cytochrome P450, Group 3, Subfamily a, Gene 1
DSS	Dextran Sodium Sulfate
DMSO	Dimethyl Sulfoxide
EGCG	Epigallocatechin Gallate
Ephx1	Epoxide Hydrolayse 1Gene
FAP	Familial Adenomatous Polyposis

H&E	Hematoxylin and Eosin
HAA	Heterocyclic Aryl Amine
hCYP1A2	Humanized Cytochrome P450 1A1/1A2 model
HLA	Human Leukocyte Antigen
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IBD	Inflammatory Bowel Disease
ICR	Imprinting Control Region
IL-10	Interleukin 10
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline
MSH	Melanocyte Stimulating Hormone
P53	Protein 53
PhIP	2-amino-1-methyl-6-phenylimidazol[4,5-*b*]pyridine
PIN	Prostatic Intraepithelial Neoplasia
RAS	Rat Sarcoma Protein
TG	Transgenic
TGF-β	Transforming Growth Factor Beta
WT	Wild-Type (C57BL6/J)

γ-TmT	Gamma Tocopherol-Rich Mixture of Tocopherols

I. INTRODUCTION

1. Cancer

It is estimated that nearly 1.5 million men and women will be diagnosed with cancer this year in the United States (1). In 2009, the Center for Disease Control listed cancer as the second leading cause of death behind heart disease. The loss of cellular regulation such as the mechanisms governing the control of growth and proliferation of cells often gives rise to cancer. Causes of cancer can be genetically inherited such as genetic mutations and immune deficiencies, but also involve external factors such as tobacco use, alcohol consumption and exposure to radiation.

The National Cancer Institute suggests that there are five main categories of cancer: carcinoma, sarcoma, leukemia, lymphoma and myeloma, and cancer of the central nervous system. Carcinoma is cancer arising from skin and other epithelial cells and tissue that encase organs in our body and is the most common type of malignant tumor (2). In contrast to carcinomas, sarcomas arise from our connective tissue such as bone, muscle and fat. Blood cancers such as leukemia arise from blood and bone marrow cells and promote the abnormal proliferation of blood progenitor cells such as leukocytes (2). Lymphoma and myeloma are also cancers targeting the immune system and mainly target lymphocytes and white blood cells. Lastly, cancers of the central nervous system target the brain and spinal cord and are thought to be some of the most deadly forms of this disease.

The development of cancer is characterized as a multistep process. Animal and human studies have suggested that initiation, promotion, and progression are the three main phases of tumor formation (3). Cancer can be initiated in a single normal cell such as a stem or progenitor cell by external carcinogenic factors or genetic mutations, which often leads to cellular DNA damages. Promotion of this disease involves the initiated cells, or premalignant cells, becoming susceptible to further growth stimulation or mutations. In the majority of cancer cases, the original initiation

event is only one of the events in the development of cancerous cells; several other mutations or epigenetic events are often involved in cancer development as well. Promotion refers to the conversion of initiator cells into neoplastic cells (3). It is thought that endogenous elements such as hormones, growth factors, nutrients, cell growth, and other common cellular processes advance the promoted cancerous cell further. Once these cells are promoted into neoplastic cells, they can be characterized by six fundamental cellular properties: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis (4). These properties allow for the continued growth and development of the cancerous cells in our bodies.

Treatment options after the diagnosis of cancer include a combination of chemotherapy, radiation, surgery, and drug options that are specifically targeted to a single type of cancer. New screening technologies have been able to detect malignancies much earlier, thus making the above treatment options more effective. Genetic screens are also becoming a popular option for those individuals at a higher risk for certain cancers. Inhibition of factors that promote the onset of disease is a more effective method for eradicating cancer before it develops. Therapy regimens and drugs that specifically target early stages of carcinogenesis (pre-malignant diseases) are becoming a beneficial option for cancer prevention and treatment.

2. Colorectal Carcinogenesis: Natural History, Clinical Presentation and Epidemiology

Colorectal cancer is referred to as cancer of the colon and rectum and begins as a benign polyp, a growth of tissue that starts in the lining and grows into the center of the colon or rectum. The benign polyp can develop into an advanced adenoma with high-grade dysplasia eventually progressing to an invasive cancer (5). The location of colon cancer is generally centralized in the large intestine whereas rectal cancer is located in the last six inches of the distal portion of the colon (2).

While there is not an exact known cause of colorectal cancer, known risk factors include smoking and alcohol consumption, age, genetics, personal history of colon polyps, family history of cancer, dietary components, and lack of exercise. Colon polyps and early colon cancers generally reside without symptoms until they are more advanced, thus this disease is often not diagnosed until symptoms are present. Symptoms of colon cancer are generally presented as changes in bowel habits, rectal bleeding or blood in the stool, stomach pain, and fatigue.

Screening and detection of colorectal cancer includes rectal exams, colonoscopies, barium enemas, and CT colonographies (2). It is possible to detect polyps in the colon at an early stage utilizing these screening methods if symptoms occur or in an individual who is at a higher risk for the disease. Treatment plans may vary depending on the stage of the cancer but include colectomy or segmental resection with removal of nearby lymph nodes, radiation, chemotherapy, targeted therapies or a combination of more than one of these treatments.

2.1. Epidemiology of Colorectal Cancer

According to data reported by the American Gastroenterological Association, in 2009 there were over 100,000 new cases of colon cancer and nearly 50,000 deaths from this disease making colon cancer the 3rd most common cancer found in men and women in the United States and the second leading cause of death from cancer among American adults (1).

The incidence of colorectal cancer between males and females is slightly higher in males, specifically for rectal cancers, and there is also considerable variation of this disease among racially and ethnically defined populations (6). The age of colon cancer risk begins around 40 years with a median age of presentation occurring around 70 years (2). The incidence of colon cancer varies geographically from country to country, however western countries such as North America, Australia and Europe seem to have a slightly higher incidence of the disease (6). Ethnic

and geographical studies suggest that environmental factors may play an important role in colorectal cancer.

2.2. Pathological Progression of Colorectal Cancer

In almost all cases, adenomatous polyps are precursors to colon carcinomas and the sequence from adenoma to carcinoma takes several years and can be influenced by factors such as the environment and genetic alterations. There are five distinct stages of colon cancer that are important in predicting curability and survival. Stage 0 is the earliest stage of colon cancer and includes the formation of polyps in the innermost lining or mucosa of the colon, this stage is also referred to as carcinoma *in situ* (2). When the polyp has progressed through the inner lining of the colon to the submucosa, the cancer is considered Stage 1. A large portion of colon and rectal cancers found at the two earliest stages are considered curable (3). Stage 2 involves the cancer in the colon invading into the surrounding tissue of the colon, the muscle layer, but not to the lymph nodes or surrounding organs. Cancer that has spread to the lymph nodes is considered a Stage 3 colon cancer and once it has invaded other organs in the body, it can be classified as a Stage 4 diagnosis and has the lowest rate of survival (2). Over 95% of colon cancers are considered adenocarcinomas (2).

3. Etiology of Colorectal Cancer

Although the exact origin of colon cancer is unknown, there are several risk factors involved in the incidence of this disease. Some of the most significant risk-factors demonstrated to be involved in the incidence of colon cancer are described below.

3.1. Inflammatory Bowel Disease

Colitis and Crohn's disease, which are two major subsets of inflammatory bowel disease (IBD), are considered increased risk factors for colorectal cancers. Colitis is recognized as inflammation of the colon, more specifically the large intestine and rectum, and can be caused by a variety of diseases and infections. Ulcerative colitis, a variation of colitis, is similar to the characteristics of colitis but with the presence of ulcers and open sores in the colon (2). Crohn's disease, often considered chronic inflammation, is observed as a constant relapse of inflammation in the colon and mainly occurs in the intestines but can affect any area of the gastrointestinal tract (2). Crohn's disease is an autoimmune disease thought to possibly have an inheritable link. The risk of colon cancer increases with the duration of IBD and the degree of inflammation of the bowel (7). Population based studies have suggested that the increase for colon cancer in patients with IBD is approximately 2-3 fold higher than in patients without inflammatory bowel disease (7). IBD patients with a family history of colorectal cancer also have a 2-fold higher risk of acquiring this disease (7). The age of onset of colorectal cancer often occurs earlier in patients with IBD as well, therefore earlier screening for colon cancer is recommended to patients with IBD to detect early mucosal dysplasia (7).

3.2. Adenomatous Polyposis

Familial adenomatous polyposis (FAP) affects approximately one out of thirteen individuals and is a genetically inherited disease of the colon that is diagnosed when a patient develops over 100 benign colon/rectum polyps (2). Polyps can begin developing from the early teen-years through late adulthood. If this disease goes untreated, it is almost certain that the patient will develop colon cancer. Mutations in the adenomatous polyposis coli (APC) tumor suppressor gene are thought to be the general cause of FAP, thus genetic testing in families with a known risk of the disease is often recommended. Because APC is located on chromosome 5 and not on the sex chromosomes, it can be inherited maternally or paternally.

Attenuated familial adenomatous polyposis (AFAP) is another form of FAP where patients develop fewer than 100 polyps in the colon/rectum. The polyps that do form often arise on the right side of the colon whereas in the classic form of the disease they occur on all sides of the colon (8). Health problems associated with AFAP are similar to those of FAP but with an increased risk for cancer.

3.3. Hereditary Nonpolyposis Colon Cancer

Hereditary nonpolyposis colon cancer (HNPCC) is also referred to as Lynch Syndrome. As mentioned previously, family members with a history of colorectal cancer have an increased risk for developing the disease, even more so than those with a history of FAP (9). Lynch syndrome is diagnosed when there are multiple cases of colon cancer on the same side of the family. It is thought that mutations in mismatch repair genes on chromosomes 2p and 3p are held responsible for this form of colorectal cancer as these mutations appear in almost all colorectal tumors from patients diagnosed with HNPCC (9). As HNPCC is an autosomal dominant mutation, only a mutation in one copy of the gene is required. Generally, 50% of children from a gene carrier will be at an increased risk for developing this disease, and 100% of gene carriers will develop this form of colorectal cancer (10). Although colorectal cancers are the most predominant form of cancer resulting from this mutation, urinary tract, liver, stomach, ovarian and small bowel cancers also occur. Criteria for diagnosing HNPCC, referred to as the Amsterdam Criteria, has been set forth to create a useful diagnostic tool for this disease. The criteria are as follows: 1) At least three or more relatives have HNPCC related cancer, with one being a first degree relative of the other two, 2) Two successive generations should be affected by this disease, 3) In one of the relatives HNPCC should be diagnosed younger than 50 years of age and 4) FAP should be ruled out as the cause of the disease (10).

3. 4. Molecular Mechanisms and the Increased Risk of Colon Cancer

The developmental stages of colon cancer beginning with initiation all require additional mutations to promote and progress to malignant colon cancer. The development of colorectal cancer is a multi-step process involving genetic mutations in mucosal cells, the activation of tumor promoting genes and the loss of genes which suppress tumor formation (6). Mutations in two classes of genes; proto-oncogenes and tumor suppressor genes are often implicated in cancer development.

Mutations in oncogenes often promote cell growth whereas tumor-suppressor genes normally restrain growth (4). Oncogenes that are often involved in various forms of colon cancer are the rat sarcoma protein (RAS) and v-raf murine sarcoma viral oncogene homolog B1 (BRAF) which activate the mitogen activated protein kinase signaling pathway. BRAF mutations can be detected in small polyps whereas RAS mutations are common in hyperplastic lesions, adenomas, and carcinomas (5). Mutations in tumor-suppressor genes such as APC, protein 53 (p53), and SMAD are the most commonly found mutations in colorectal cancer accounting for 80% of sporadic colorectal cancers and often arise due to chromosome instability (5).

Many signaling pathways are involved in the development of colon cancer. The Wnt signaling pathway is thought to be one of the most common mutations in colon cancer and is regarded as the initiating event of this disease (5). Specifically, mutations in the APC protein are the most common mutation in colon cancer. When APC is mutated, β-catenin becomes stuck in the “on” position; therefore Wnt signaling is constitutively activated. Constitutively activated Wnt signaling can often lead to uncontrolled cell proliferation and growth. APC mutations can be genetic as mentioned by the adenomatous polyposis syndrome, or somatic.

A key step for promotion and progression of colon cancer is thought to be mutations in the p53 pathway. Inactivation of p53 is observed in most tumors of patients with colon cancer. This mutation is often recognized with the transition from adenomas to large invasive carcinomas (5). Mutations that are associated with the progression of adenoma to high-grade dysplasia or carcinomas are also suggested to be involved with inactivated transforming growth factor- β (TGF-β) signaling. Inactivated TGF-β signaling is thought to be another important step involved in the progression of colorectal cancer. Mutations inactivating the SMAD family of proteins which act downstream of TGF-β are found in approximately one third of colorectal cancers (5).

Growth factor pathways involving epidermal growth factor and prostaglandin signaling are also activated in several forms of colon cancer. An early step in the development of an adenoma is the activation of prostaglandin signaling. Inflammation is thought to up-regulate cyclooxygenase-2 (COX-2) which is a mediator of prostaglandin E2. Increased prostaglandin E2 activity is strongly associated with colorectal cancers (5).

Mismatch-repair mutations, although not common, are also found to increase the risk of colon cancer. For example, it was previously mentioned that HNPCC which is a hereditary form of colon cancer is acquired through silencing of a gene that encodes a DNA mismatch-repair protein (5). Many of these genes and molecules described above that are involved in various cancer pathways are now being used as markers for colon cancer which may lead to earlier diagnosis and novel targeted gene therapies.

3.5. Dietary Factors and the Risk of Colon Cancer

Population variation in colon cancer incidences suggest that environmental factors, specifically dietary trends, may play an important role in this disease. The incidence of colon cancer in Western societies such as North America and Europe is much higher compared to areas

such as China and Japan. It has been suggested through epidemiological studies that a "Western-style diet" which consists of high-fat, low fiber, and low vitamin D and calcium are some of the major dietary factors involved in the formation of various forms of cancer, specifically cancer of the colon. A recent study involving mice fed a "Western-style diet" demonstrated the activation of oxidative stress in the colon of mice which further promoted severe inflammation in the colon mucosa (11). Conversely, diets rich in fresh fruit and vegetables along with the absence of highly refined sugars have demonstrated a decrease in the risk for colon cancer (12).

A diet high in animal fat and protein has also been suggested to increase an individual's risk for developing colon cancer. Red meat has been one of the most popular food items that have shown a positive correlation to colon cancer in many populations within the United States (12). This correlation is thought to be due to the cooking of red meat which produces heterocyclic aromatic amines (HAAs), dietary chemicals found to be carcinogenic in animal studies, and the hyperproliferative effect of dietary heme (12,13).White meat or fish have not been found to dramatically increase the risk for colon cancer.

Although evidence linking alcohol consumption to colon cancer is often inconsistent, alcohol is also suggested to increase the risk for colon cancer by way of antagonism of methyl-group metabolism (14). A recent review on alcohol consumption and the risk for colon cancer suggested that one or more liquor drinks a day have increased the risk for colon cancer in individuals by 70% (15). However epidemiological studies have also found moderate consumption, specifically consumption of wines, to yield an inhibitory effect against various forms of cancer including colon (15).

4. Colon Carcinogenesis and Chemoprevention

Chemoprevention can be defined as the use of synthetic or natural products to inhibit or prevent diseases such as cancer. The multi-stage process of tumorigenesis in colorectal cancers provides significant opportunities for intervention with chemoprevention agents at any of these stages. Below is a description of specific natural and synthetic agents utilized in the chemoprevention of colon cancer.

4.1. Agents for Prevention

A review on chemoprevention agents for colon cancer has suggested that a daily intake of aspirin or other anti-inflammatory drugs result in a reduced risk of developing colorectal polyps and cancer (16). Nonsteroidal anti-inflammatory drugs are some of the most popular studied chemopreventive agents against colorectal cancer. A clinical trial in humans with sulindac, which is a nonsteroidal anti-inflammatory drug, has demonstrated the regression of colorectal adenomas in patients with FAP (16). A more popular drug marketed under the name of Celebrex has also demonstrated inhibitory effects in FAP patients by methods of targeting COX-2 and has been approved for general care in these patients by the Food and Drug Administration (16). Difluoromethylornithine and hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors are other synthetic agents found to demonstrate chemopreventive activity in colon cancer *in vivo.*

Non-pharmacological agents such as calcium, folate, vitamins and antioxidants have all demonstrated chemopreventive effects against colon cancer and are becoming the more popular choice of agents to use due to their natural availability. High calcium and vitamin D intake have demonstrated a moderate chemopreventive effect in human and animal studies. It is thought that the calcium gradient in crypts of the colon mucosa is disturbed in colon carcinogenesis and that by increasing the level of intracellular calcium, tumor growth is repressed and differentiation of transformed intestine and colon cells is promoted (17). Vitamin D is also involved in restraining

cell proliferation and has been reported to be significant in colon cancer prevention specifically as an apoptosis stimulating factor (17).

Folate has also been demonstrated to have an inverse relationship with colon carcinogenesis. Song et al, reported a decreased incidence of small intestinal and colon adenomas in Apc-mutant min mice that were fed a diet with high folate content (18). Epidemiological and cohort studies have also demonstrated a correlation between diets low in folate and colon polyp formation. However, chemoprevention studies with folate *in vivo* have proven to be contradicting as a recent review has also reported that diets high in folate induced dysplastic and hyperplastic abnormalities in the colon (17). Further studies should be performed to characterize the threshold at which folate may be useful for cancer prevention.

Antioxidants such as vitamins E, β-carotene and polyphenols to name a few, are capable of protecting our bodies from harmful free radicals. It has been suggested that vitamin E and β-carotene decrease tissue damage by preventing lipid peroxidation through trapping and deactivating free radicals that can damage DNA, thus inhibiting carcinogenesis (17). Recently in our laboratory, we have presented evidence that a vitamin E mixture rich in γ-tocopherol inhibits several forms of cancer including colon in animal models (19).

Polyphenols are present in fruits, tea, chocolate, peanuts, olive oils and many more products. Numerous *in vitro* and *in vivo* studies have demonstrated a chemopreventive effect for various forms of polyphenols in colorectal carcinogenesis. Green tea polyphenols have recently become promising agents in the prevention of colon cancer, and are thoroughly studied in our laboratory. For example, green tea polyphenols showed a reduced appearance of aberrant crypt foci (ACF) in the colon of azoxymethane (AOM)-induced F344 rats fed a standardized green tea preparation compared to mice not administered the dietary tea preparation (20). Epigallocatechin gallate (EGCG), which is a potent chemopreventive agent of green tea, has been recognized as an

inducer of apoptosis and has demonstrated suppression of the formation and growth of human colon cancers. The future aim of the current study will be to use the Cytochrome P450 (CYP) 1A1/1A2 humanized mouse model (hCYP1A2) of PhIP-induced colon cancer for chemoprevention studies with natural agents such as tocopherols and EGCG which are currently studied in our laboratory.

5. Animal Models for Colon Carcinogenesis

Much has been documented about human inherited characteristics and molecular mechanisms involved in colorectal cancer. Animal models for colorectal cancer have been generated by mimicking mutations found in various stages of the human disease in various mammalian systems. Chemicals known to have carcinogenic effects in humans have also been used to induce various forms of cancer in laboratory animals. These models are useful in identifying other potential characteristics involved in the activation, promotion and progression of colon cancer as well as testing chemoprevention agents that may be beneficial in human studies.

5.1. Rat Models for Colon Cancer

Rats are a predominant model used for carcinogenicity studies. The most widely used rat colon cancer model has been the AOM-induced model. Because most rodents, including rats, do not spontaneously develop colon cancer, they are generally administered a carcinogen to induce the development of cancer (21). AOM is a potent carcinogen commonly used to induce colon cancer in rodents. AOM, which is a derivative of dimethylhydrazine, is administered to rat strains such as the F344, Sprauge-Dawley and Wistar rats to induce tumors of the colon. This model is relevant to human cancers in that the tumors share many histopathological characteristics with human tumors such as mutations on *K-ras* and β-catenin genes. AOM is generally injected

subcutaneously or through i.p. injections and is administered repeatedly throughout the experiment in order to induce tumors. A recent review of the AOM rat model stated that two injections of AOM in F344 rats have been determined to result in a high incidence of tumor yield (22). Single injections of AOM have been used in rat models but with a much lower incidence of tumors (23).

AOM rodent models are also used in combination with dextran sodium sulfate (DSS), which is a potent inducer of colitis, to promote AOM-induced colon cancer in a shorter time-period. This AOM/DSS model retains features of human colon cancers that are promoted by inflammation such as colitis and IBD. A single injection of AOM followed by a one week treatment of 1% DSS has induced tumors as early as 20 weeks after AOM injection in the F344 rat (24). A recent model using the AOM/DSS method for inducing colon cancer was applied to the Kyoto APC Delta rat carrying an Apc mutant. Colon tumors were reported as being more severe in the Apc mutant rat compared to the generic F344 rat (25).

Heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazol[4,5-*b*]pyridine (PhIP) have also been used as a carcinogen for rat models of colorectal cancer. PhIP-induced colon cancers mimic human colon cancers more closely than AOM-induced rodent models. PhIP is a more naturally occurring chemical abundant in our diets whereas AOM is a synthetic chemical. PhIP-induced colon cancers exhibit mutations mainly in the APC gene which represent the majority of mutations found in human colorectal neoplasms (26). PhIP primarily targets the colon and prostate in male rats, and mammary glands in female rats (27). Administration of PhIP (400 mg/kg) in the diet of rats has resulted in aberrant crypt foci in the colon shortly after exposure to PhIP (28). After 52 weeks, these rats eventually developed colon carcinomas at a frequency of approximately 50% (27). Both the AOM/DSS and PhIP-induced colon cancer models represent

important models in human colon cancer research, and can be beneficial for chemoprevention studies.

5.2. Mouse Models for Colon Cancer

Mouse models are generally easier to maintain physically and are often more cost-effective in terms of diets, chemicals and per-diem charges. The *Apc* $^{Min/+}$mouse model along with the AOM/DSS model similar to that in rats mentioned above, are two of the most important mouse models in colon cancer research. The *Apc*$^{Min/+}$ mouse was the first murine model of intestinal tumorigenesis induced by a germline mutation (29). Adenomas in these mice are predominantly found in the small intestine as early as four months, but after a longer-term study have expressed tumors in the colon as well (29). This model is closely related to FAP associated human colon cancers in that tumors reported harbor mutation or loss of the normal Apc allele. Tumors examined are also observed as being pathologically similar to those found in human colon cancers and exhibit nuclear localization of β-catenin much like human colorectal tumors. Interleukin-10 (IL-10) knockout mice, which are useful models of colitis and IBD related colon cancers, and melanocyte stimulating hormone 2 (MSH2) and MSH6 mutated mice, which are involved in DNA mismatch repair, have also been used in combination with other mutations or tumor promoting agents to induce colon tumors. IL-10 knockout mice begin to develop colon carcinomas around 9 weeks with a 65% incidence around 10-31 weeks (30) whereas MSH mutated mice begin to develop colon carcinomas around one year of age. These two models are not as widely used in colon cancer studies as the *Apc*$^{Min/+}$ mouse model because of the absence of mutations related to humans found in the tumors. Apc, K-ras, and p53 mutations are not common in tumors evaluated in IL-10 and MSH mutated mice (30).

Much like the AOM/DSS rat model, this method is popular in various strains of mice. Suzuki et al, recently developed a mouse model for colitis-related colon carcinogenesis in male imprinting control region (ICR) mice utilizing the AOM/DSS treatment (31). This regime has also been applied to C57BL/6N and Balb/c mice. A current model of AOM/DSS treated mice used in our laboratory results in tumors forming in the colon around week 13 in CD-1 mice after two i.p. injection of AOM (6mg/kg four days apart) followed by 1.5% DSS for 7 days in the drinking water. This model has proven useful in our laboratory to investigate chemoprevention agents against colon carcinogenesis.

5.3. Transgenic and Humanized Animal Models

Transgenic animals prove to be useful models for the study of disease and specific genetic alterations involved in various mammalian processes. A transgenic animal is that in which a foreign gene is inserted into a host animals' genome. In general, transgenic animals are created by isolating a gene for a specific trait or disease and injecting it into fertilized mouse ova. These eggs are then implanted into the uterus of a female surrogate mouse. The implanted genes are then expressed in some of the first generation offspring. Further breeding with the mice expressing the gene of interest will create a larger colony for experimental purposes.

The transgenic mouse was the first transgenic animal model used for biological research and often contains human gene inserts involved in specific diseases such as cancer (32). Numerous mouse models have been designed with tumor promoting genes inserted into the mouse genome for tumorigenesis studies. Genetically engineered mouse models for cancer include mice overexpressing a transgene (oncogene or point mutation) and knock-in and knock-out models utilizing the Cre-lox system (33). For example, a mouse model with a phosphatase and tensin homolog knockout has allowed researchers to study the importance of this lipid phosphatase in

prostate cancer. A c-myc/TGF-α mouse model is another model that has proven to be an ideal model for observing the development of liver cancer (34). Transgenic mouse models for colon cancer have included several molecular mechanisms such as overexpression of various APC mutants and other genes involved in the Wnt signaling pathway. The use of transgenic animals is important in investigating the carcinogenic potential of various genes known to play a role in cancer development.

Humanized animal models are significant to research and development of human genetics and disease. The study of human biology is often limited by technical and ethical constraints. Physiological processes that cannot be closely monitored in humans can be genetically manipulated in mouse models to reach a better understanding of the human system. Although mice are not humans, humanized mouse systems are a more closely adaptive method for studying human processes *in vivo*. Two of the most significant mouse models to human research are the nude mouse and the severe combined immunodeficiency mice which have aided in the ability to study hematopoiesis and the human immune response in experimental systems. These models are often used for xenograft transplant studies of human cells as the lack of immune response does not reject the insertion of foreign cells.

Mouse models with human genetic information inserted into their genome are also ideal for studying the human system *in vivo*. For example, the human leukocyte antigen (HLA)-B27 transgenic rat expresses HLA-B27 and human β2-microglobulin which spontaneously develops inflammatory arthritis resembling that found in humans (35). By replacing the mouse β2-microglobulin gene with the human β2 gene, researchers were able to study inflammatory disorders directly associated to humans and their genes involved (35).

Currently used humanized mouse models of tumorigenesis include transgenic models listed above that produce tumors expressing similar characteristics as those found in humans and

xenograft implant models using cancer cell lines grown *in vitro*. However, xenograft mice do have their limitations as humanized models for cancer. The derangement of the normal tumor architecture and alterations in the tumor microenvironment are crucial features that are disrupted or lost in xenograft tumors (36). Several humanized transgenic mice have been generated with human xenogenic resistance or CYP genes expressed in mice lacking the corresponding murine gene for purposes of drug metabolism studies (37). These mice have demonstrated to obtain metabolic properties more closely related to humans than wild-type mice and other humanized mouse systems. Mice expressing human genes closely related to specific forms of cancer may be a more beneficial model for *in vivo* cancer studies.

Pertinent to our studies, a bacterial artificial chromosome (BAC)-transgenic mouse line with the absence of mouse orthologs Cyp1a1 or Cyp1a2 and containing human CYP1A1 and CYP1A2 and other common human haplotypes have been developed to evaluate function in human CYP1A1/1A2 locus variability with regard to toxicity and cancer (38). Through the use of the Cre-lox recombinase system, Dragin et al, was able to generate mice with the hCYP1A1/1A2 (+) transgene combined with the Cyp1a1/1a2 (-) double knockout allele, now having both human genes replacing the mouse orthologs (39). The use of *in vivo* mammalian model systems with the absence of mouse orthologs should be ideal for investigating various aspects of genetic diseases and the involvement of specific human genes. There is often error associated with extrapolating data from rodent models and comparing it to human populations. Rodent models carrying human genes are often preferred models to study human metabolism and disease. The two above models have been successfully utilized in investigating the effects of various toxic environmental carcinogens such as heterocyclic amines and drug metabolism specific to the human CYP enzymes (39, 40).

6. Heterocyclic Amines and Dietary Carcinogens

Dietary factors such as high fat and low fiber diets have already been described as having a correlation to certain forms of cancer. However, mutagens/carcinogens from cooked foods, plant products, nitrite materials, aromatic hydrocarbons and oxidative agents also play a role in the onset and promotion of tumorigenesis. Arylamines, specifically HAAs, are of great interest because of their natural availability in our daily lifestyle and their demonstrated carcinogenicity in animals and humans as reviewed by Kim and Guengerich (41). The formation of HAAs is the result of a Maillard reaction occurring when sugar, creatine and amino acids are heated (42). Especially high amounts of HAAs can be found in charred pieces of meat and somewhat lower levels can be found in the smoke and juices produced from meat that has been grilled over a long period of time. The resulting HAA product is further metabolically activated to form a mutagen or carcinogen. This activation is generally mediated by CYP and further initiated by esterification, acetyltransferase and sulfotransferase activity. The resulting carcinogen is thought to bind to DNA and form adducts producing changes in DNA sequences (43). These DNA adducts are considered biomarkers of potential mutagenesis and carcinogenesis (41).

Heterocyclic amines were found to be mutagenic in various bacteria strains *in vitro* shortly after their discovery. Specifically, smoke condensates isolated from grilling fish and beef exhibited potent mutagenic activity in S.Typhimurium (43). Heterocyclic amines were further isolated and consist of compounds such as 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), and PhIP, to name a few. *In vivo* analysis of these isolated HAAs from cooked meat and fish has demonstrated carcinogenicity primarily in the liver, however PhIP has been shown to induce colon, lymphoid and mammary neoplasms as well (27, 44). Mutations by HAAs in rodents also demonstrate similar characteristics found in human cancers such as the expression of cancer-related genes and

activation of specific pathways. HAAs are thought to induce mutagenesis by producing mutations in oncogenes and tumor suppressor genes (41). Epidemiological studies in humans have shown a positive correlation between meat intake and carcinogenesis as demonstrated previously (12). Further studies of the carcinogenic potential of HAAs in humans would be beneficial.

6.1. 2-amino-1-methyl-6-phenylimidazole [4,5-*b*]pyridine (PhIP)

PhIP is one of the most abundant HAAs found in cooked meats and fish (45). As previously mentioned, heterocyclic amines are formed in cooked meats by the heating of amino acids and proteins at high levels of heat over long periods of time and are often thought to be potent dietary carcinogens. PhIP has been demonstrated to be mutagenic in both bacteria and mammalian cell genotoxicity assays (45).Human daily intake of PhIP is estimated to be between 0 and 865ng/day with the average amount of PhIP ranging from 0.29 to 182ng/g in cooked meat (46).

PhIP along with other heterocyclic amines such as IQ and MeIQ have been found to be highly mutagenic and carcinogenic in animal studies. Administration of PhIP in rats mainly targets the colon whereas administration of PhIP to mice mainly induces non-epithelial malignancies such as malignant lymphomas and leukemia (27, 47, 48, 73). It has also been recently reviewed that PhIP induces cancer of the colon, prostate and mammary gland in various strains of rats (50). Although most heterocyclic amines primarily target the liver, the site specificity of PhIP being prostate, mammary and colon tissues is interestingly related to the primary sites of human diet associated cancers.

PhIP-DNA adducts have also been detected in the colon, mammary and lymphoid tissue of humans (51). This data strengthens the importance for characterizing the carcinogenetic effects of PhIP and other heterocyclic amines in humans as diet associated cancers are becoming more prevalent in our Western society.

6.2. Cytochrome P450, PhIP Activation and Metabolism

CYP is regarded as a superfamily of heme-containing enzymes that catalyze the oxidative metabolism of diverse chemicals such as exogenous compounds including xenobiotic drugs, pollutants and pesticides and endogenous compounds such as steroids, eicosanoids, and other regulatory molecules (52). CYP is a group of enzymes found in our body that aid in the breakdown of toxins and is extremely important in drug metabolism. Natural products metabolized by CYP include vitamins A and D, fatty acids, cholesterol, steroid hormones, retinoic acid, and bile acid (53). For example, various forms of CYP activate vitamin D substrates as well as degrade vitamin D metabolites to oxidation or lactone products (54). Knockout mice for various CYP genes fed a diet high in vitamin D were observed to have the inability to degrade vitamin metabolites and resulted in hypercalcemia and death in a significant amount of animals (54). In some instances, CYP can be considered dangerous if it fails as a mediating system. For example, intermediate molecules produced by CYP can be more dangerous than the original molecule (53). Carcinogens can often result from this mediating failure and can be activated by a specific compound by CYP. For example, CYP1A1 aids in both the activation and detoxification of benzopyrene which is a carcinogen present in cigarette smoke (55). This has also been demonstrated by the mutagenic CYP activation of aflatoxin, a chemical produced by various molds, which is thought to be one of the major causes of liver cancer (53). Therapeutic drugs are also important in CYP activity as many drugs function to inhibit or promote the activity of CYP. One specific example of the inhibition of CYPs is antidepressants. *In vitro* and human studies have demonstrated that antidepressants such as serotonin reuptake inhibitors work through CYP inhibition (55). A majority of therapeutic drugs are thought to be metabolized by the CYP3A

family. Many drugs currently on the market for HIV treatment as well as calcium channel blockers interact through the CYP3A4 enzymes (55).

The majority of the CYP enzymes are expressed in the liver, but extrahepatic metabolism also occurs in the kidneys, skin, gastrointestinal tract, and lungs (56). The human and mouse genomes carry 57 and 102 functional CYP genes respectively (57). There are 18 mammalian families of CYP all of which have specific functions (53). Our focus is on the CYP1 family of enzymes which are specifically involved in the activation of heterocyclic amines such as PhIP and other compounds such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons such as benzo[a]pyrene to form potent carcinogens. The CYP1 family members consist of CYP1A1, CYP1A2, and CYP1B1 which are all upregulated by dioxin or polycyclic aromatic hydrocarbons (58). These three family members are found in both human and mouse. CYP1A1 and CYP1A2 are oriented heat-to-head on chromosome 15q22 and separated by a 23.3 kb spacer region in humans (38). The mouse Cyp1a1 and Cyp1a2 genes are found on mouse chromosome 9. CYP1A2 has been suggested as an essential enzyme for the bioactivation of PhIP because of its high abundance in both human and rodent livers and its high catalytic activity as a PhIP-N^2-hydrolase but is not exclusively the enzyme responsible (59). Other CYPs or enzymes may contribute to PhIP metabolism.

PhIP is metabolically activated in the liver and is then thought to be secreted in the bile and eventually reabsorbed by the gut. This enterohepatic circulation makes colon epithelial cells receive repeated exposure to activated PhIP (28). After consumption of a meal with a high red meat intake, especially when well done, PhIP is rapidly absorbed. The metabolism of PhIP has been extensively studied *in vitro* and *in vivo* throughout the use of animal models (40,60). PhIP becomes activated by N^2-hydroxylation, mainly in the liver, which is catalyzed by CYP, primarily by the 1A family of enzymes (61). CYP1A2 is believed to be a key enzyme in the activation of

PhIP and has been demonstrated to yield a higher N-hydroxylation activity for HAAs compared to CYP1A1 and CYP1B1 which are found to be much less active (62). Recent reviews have shown CYP1A1 to also be involved in the detoxification of PhIP to 4'-hydroxy-PhIP yielding the highest 4-hydroxylation activity of the 1A family of enzymes (60, 63). When PhIP becomes activated, the N^2-hydroxy metabolites are further metabolized by *O*-esterification by enzymes such as *N*-acetyltransferase and sulfotransferase producing its carcinogenic form; N-acetoxy PhIP (64) (Figure 1). Arylnitrenium ions produced from the mutagenic metabolite react with DNA, mainly at guanine bases, and lead to the formation of PhIP-DNA adducts in the colon and other sites throughout our body (64). The mutagenic metabolite of PhIP is also suggested to react with cellular components other than DNA and form unstable products that are spontaneously degraded to 5-hydroxy-PhIP (65).

Differential metabolism of PhIP in rats and humans has been implied from previous studies *in vitro* and *in vivo*. In rodents, the major metabolic pathway of PhIP results in detoxification of PhIP by 4'-hydroxylation of the ring group followed by conjugation of the exocyclic amino group (60, 66). However, in humans, N-oxidation and esterification of PhIP to form its carcinogenic metabolite is the major metabolic pathway (65) (Figure 1). Rat and human CYP1A2 are only 75% identical in amino acid sequences and this 25% difference is thought to account for the differences in catalytic activities of HAA oxidation (66). *In vitro* analysis of human tissue cytosols and microsomal extracts have demonstrated a greater capacity to N-hydroxylate heterocyclic amines compared to rodents (67). More specifically, the catalytic efficiency of N-oxidation of PhIP by human CYP1A2 was found to be 19-fold greater than that of the rat ortholog (59). Recently, Cheung et al, also demonstrated that PhIP was preferentially metabolized by N^2-hydroxylation through the use of a humanized mouse model consisting of human CYP1A1/1A2 on a mouse Cyp1a2 knockout background. Inversely, wild-type mice containing mouse

Cyp1a1/1a2 preferentially metabolized PhIP through 4'-hydroxylation (40). Further supporting this theory, Turteltaub et al, has also observed significantly higher levels of PhIP-DNA adducts in human tissues and blood compared to rats administered the same doses of PhIP (67).

II. GOALS AND SPECIFIC AIMS

The goal of this thesis research was to create a mouse model for colon carcinogenesis using PhIP; a potent carcinogen of the colon in hCYP1A2 mice. Current animal models utilized in our laboratory such as the AOM/DSS mouse model are not the most ideal models to study colon cancer as AOM is not naturally occurring and is not presented in human colon cancer etiology. Previous studies utilizing various strains of rats and mice have demonstrated the occurrence of cancerous tumors in the colon of rats and other tissues such as mammary and prostate in mice after long-term PhIP feeding protocols (27, 44, 68). The addition of DSS in mouse models such as C57BL/6J and ICR mice has been shown to significantly reduce the time it takes for tumors to form in the colon (48, 69, 70). The addition of DSS, a chemical inducer of colitis, in combination with PhIP will be useful for the induction of colon cancer in the hCYP1A2 mouse model. Because PhIP is activated by CYP, primarily by CYP1A2, we hypothesize that the hCYP1A2 model will result in an increased tumor incidence and the increase in colon inflammation by the addition of DSS will shorten the time frame for tumors to occur. As PhIP is one of the most abundant heterocyclic amines known to cause cancer in humans, the proposed hCYP1A2 mouse model should be more closely related to human colon cancer etiology compared to mouse models currently being utilized. The hCYP1A2 model should be more economical than rat models in diet expenses, chemoprevention agents, and animal per diem charges. A humanized mouse model for colon cancer utilizing a common dietary agent such as PhIP would facilitate numerous studies mechanistically and for chemoprevention purposes. Therefore, in developing the hCYP1A2 PhIP-induced mouse model, we hypothesized that the increased activation of PhIP and addition of DSS in the hCYP1A2 mice would decrease the length of time it takes to develop colon carcinomas compared to wild-type mice (C57BL/6J).

1. Rationale

Several PhIP-induced colon cancer rodent models have been reported. Tsukamoto et al, demonstrated that multiple doses of PhIP could induce large intestinal tumors in F344 rats within 50 weeks (71). PhIP-induced rat colon cancers have been shown to resemble human neoplasms with regard to multistage development and genetic alterations (28, 48, 72). In certain strains of mice, small intestinal tumors are occasionally induced several weeks after PhIP administration in combination with a high-fat diet however; malignant lymphomas and leukemia are the primary observations (73). Tanaka et al, demonstrated that a single dose of PhIP followed by 2% DSS for one week induced colon carcinomas in male ICR mice after only 16 weeks compared to 52 weeks after a single injection of PhIP alone (70). Ochiai et al, observed similar results in C57BL/6J mice given a combination of PhIP and DSS as colon carcinomas appeared as early as week 20 after PhIP administration (73).

A mouse line consisting of human CYP1A1 and 1A2 and mouse Cyp1a1 and 1a2 double knockout has been generated by Dragin et al (Figure 2). Previous studies have utilized the human CYP1A1 and CYP1A2 mouse lines with a knockout of either mouse Cyp1a1 or Cyp1a2 separately to investigate various effects of PhIP. The newly developed hCYP1A1/1A2 mouse line with both the mouse Cyp1a1/1a2 orthologs knocked out should be a much easier model to study the effects of PhIP and human CYP1A1/1A2 without the interference of both mouse Cyp1a1 and CYP1a2 which detoxify PhIP. There are no reports of PhIP-induced carcinomas in the hCYP1A2 mouse model thus far.

2. Specific Aim

To Develop a Humanized Colon Carcinogenesis Mouse Model in hCYP1A2 Mice using PhIP, a Dietary Carcinogen of the Colon The insertion of human CYP1A1/1A2 genes on a mouse Cyp1a1/1a2 null background is expected to increase the metabolic activation of PhIP, a dietary carcinogen. The increased activation of PhIP in combination with DSS, a potent inducer of colitis, is expected to result in earlier tumorigenesis and a higher tumor yield in the hCYP1A2 mice compared to wild-type (C57BL/6J) mice. This should enable us to develop a model that more closely mimics human colon carcinomas induced by dietary carcinogens such as PhIP.

IV. MATERIALS AND METHODS

1. Chemicals and Reagents

A REDExtract N Amp PCR Kit was purchased from Sigma-Aldrich Co. (St. Louis, MO) and PCR primers were purchased from Sigma Genosys (The Woodlands, TX) for PCR amplification and genotyping purposes. The carcinogen administered to the animals, PhIP, was obtained from Toronto Research Chemicals (North York, Ontario, Canada) in solid form. DSS was purchased from MP Biomedicals (Solon, OH) and was received in powder form (100mg/mL). A T-PER buffer kit for protein extraction and Pierce bicinchoninic acid assay (BCA) kit for determining protein concentrations were purchased from Thermo-Scientific (Rockford, IL). Methylene blue was obtained from Sigma-Aldrich Co. (St. Louis, MO) and dissolved into a 0.2% solution with DMSO for staining of aberrant crypt foci. Hematoxylin was also purchased from Sigma-Aldrich Co. (St. Louis, MO) for hematoxylin and eyosin (H&E) staining of tissues. Antibodies utilized for immunohistochemistry and western blot analysis of proteins are described in Table 1.

2. Research Design for Specific Aim

Three breeding pairs of hCYP1A2 and C57BL6/J mouse strains were obtained from Jackson Laboratories (Bar Harbor, ME). All of the animals were housed in the Laboratory for Cancer Research's Animal Facility at Rutgers University. Five to eight-week old male and female hCYP1A2 and wild-type mice were divided into 10 groups as illustrated below. Wild-type mice were used as controls for the transgenic hCYP1A2 mice. Dimethylsulfoxide (DMSO), which was used to dissolve PhIP, was administered as a vehicle control in both strains of mice.

Group A: C57BL/6J wild-type mice with 200mg/kg PhIP

Group B: C57BL/6J wild-type mice with 200mg/kg PhIP + 1% DSS

Group C: C57BL/6J wild-type mice with 200mg/kg PhIP +1.5% DSS

Group D: C57BL/6J wild-type mice with 20% DMSO vehicle control

Group E: hCYP1A2 mice with 200mg/kg PhIP

Group F: hCYP1A2 mice with 100mg/kg PhIP + 1% DSS

Group G: hCYP1A2 mice with 200mg/kg PhIP + 1% DSS

Group H: hCYP1A2 mice with 200mg/kg PhIP + 1.5% DSS

Group I: hCYP1A2 mice with 20% DMSO vehicle control

Group J: hCYP1A2 mice with 20% DMSO + 1.5% DSS control

3. Animals

All of the mice used were bred in our sterile animal facility in the Department of Chemical Biology at Rutgers University (Piscataway, NJ) and handling procedures complied with the Rutgers Animal Welfare Policy. The breeding animals were housed in plastic cages with hardwood bedding and dust covers in a high efficiency particulate air-filtered, environmentally controlled room (24 ± 1°C, 12/12h light/dark cycle). The diets consisted of lab chow and water *ad libitum* and were monitored by laboratory technicians. One week prior to PhIP gavage, animals were transferred to a non-sterile facility, housed 4-10 per cage and given an AIN-93M diet which is a standard adult maintenance diet for rodents. The animals were maintained on the AIN-93 diet

throughout the entire duration of the study unless otherwise mentioned. The diet was made by Research Diets, Inc (New Brunswick, NJ) and maintained at 4°C. The diet consists of 14.2% protein, 73.1% carbohydrates and 4.0% fat by weight. Prior to PhIP administration, animals were transferred to plastic cages with absorbent alpha-dry bedding and dust covers. One week after PhIP was administered, the animals were transferred back to regular cages with hardwood chips. Animals were weighed and monitored and diets were replenished weekly.

Male and female hCYP1A2 (*Cyp1a2/Cyp1a1*^{tm2Dwn} Tg(CYP1A1,CYP1A2)1Dwn/J) and wild-type (C57BL/6J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) as breeders. Zygosity of the transgenic mice was checked by breeding male hCYP1A2 mice with female wild-type mice. Pups from these litters were genotyped using the method described below. Pups homozygous for hCYP1A1/1A2 and the mouse Cyp1a1/1a2 knockout were bred with one another for several generations until all pups in each litter were homozygous for the transgene. Pups from the wild-type backcross were genotyped for CYP1A1 or 1A2 genes and the mouse Cyp1a1/1a2 double knockout allele. Mice positive for both human CYP1A1/1A2 and mouse Cyp1a1/1a2 knockout were used as new breeders. Transgenic breeding continued for several generations in our sterile animal facility until breeding was approximately 98% homozygous.

4. Treatments

Designated groups of transgenic and wild-type mice were administered PhIP or DMSO by Dr. Guang-Xun Li (Rutgers University) through oral gavage in doses of either 100mg/kg or 200mg/kg of body weight per mouse. PhIP was dissolved into solution with 20% DMSO. Hydrochloric acid was added in 10µl increments to dissolve the PhIP. One week after PhIP gavage, certain experimental groups were given 1-1.5% DSS substituted for their drinking water

for duration of 7 days to induce inflammation. DSS in solid form was weighed and dissolved into solution of 1% or 1.5% with water. Water was replaced as the source of drinking water after the 7th day of DSS treatment and the animals remained on drinking water for the remainder of the experimental period. Animals were monitored on a daily basis while on DSS and body weights were monitored weekly throughout the remainder of the experiment.

5. Tissue Collection

Upon completion of the experiment, mice were euthanized by CO_2 asphyxiation. Blood was collected by cardiac puncture and serum was obtained after centrifugation and stored at -80°C. Liver, lung, mammary, prostate, stomach and spleen tissues were removed, weighed and examined for any abnormalities and then fixed in 10% buffered formalin solution for 24-48 hours at 4°C before being transferred to 70% ethanol. The colon was also removed, flushed with saline solution and cut longitudinally for examination, and fixed on filter paper in 10% buffered formalin as described above. The length of the colon was recorded and if visible tumors were observed, the length, width and height of the tumors were measured. Tumors and other tissues were also frozen in liquid nitrogen and stored at -80°C for future analysis. The formalin-fixed tissues were processed, embedded in paraffin, sectioned and mounted onto glass slides by Miss. Yuhai Sun for pathological analysis.

6. Experimental Sample Preparation

Collected samples were prepared according to the experimental techniques used. Below is a description of various sample preparation procedures utilized throughout the study.

6.1. DNA Extraction

All transgenic mice were genotyped for human CYP1A1or 1A2 genes and mouse Cyp1a1/1a2 double knockout allele. Genomic DNA was isolated from fresh tail biopsies, approximately 0.55cm in length, of hCYP1A2 mice using the REDExtract-N-Amp Tissue PCR kit. Extracted DNA was stored at 4°C.

6.2. Cell Lysate Protein Preparation

Expression of β-catenin in the colon and tumor tissue of the hCYP1A2 mice was analyzed. Select tumor and colon samples were homogenized and protein was extracted using the T-PER buffer kit. T-PER buffer was added with the sample to a glass douncer and homogenized on ice for 5 minutes each. Following homogenization, samples were sonicated 3 times for 15 pulses. Homogenized tumor samples were then centrifuged for 20 minutes at 3,000 rpm. The supernatant, which represents the whole cell lysate, was collected and the protein concentration of the samples was measured using the BCA protein assay kit and stored at -80°C until further analysis.

7. PCR Genotyping

To determine the genotype of the hCYP1A2 mice, PCR genotyping was performed. Two separate reaction mixes were made respectively for the human 1A1/1A2 gene and the mouse 1a1/1a2 knockout allele. The PCR reaction mixtures contained prepared DNA, RED Extract-N-Amp mix and primers specific to that reaction. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with the following parameters for the hCYP1A1/1A2 reaction mix: The initiation cycle began at 94° for 4 minutes, followed by thirty five cycles at 94°C, 54°C, and 72°C for 30 seconds each, one cycle at 72°C for 5 minutes, and

held at 4°C indefinitely. For the 1a1/1a2 mouse knockout amplification, the parameters were carried out as follows: One cycle at 95°C for 5 minutes, thirty three cycles at 95°C, 60°C, and 72°C for 1 minute each, one cycle at 72°C for 10 minutes and held at 4°C indefinitely.

A complete list of primers used for specific genes, concentrations used and expected PCR product size can be found in Table 2. Mouse epoxide hydrolase 1 gene (*Ephx1*) primers served as an internal positive control for amplification.

PCR products were then subject to electrophoresis on a 2% agarose gel containing 0.3µg/mL of ethidium bromide alongside a 50bp DNA size marker. DNA was visualized by a UV light source and Kodak Electrophoreis Documentation and Analysis System 290.

8. Histopathology of the colon

Histopathological analysis was performed by Dr. Guang-Xun Li (Rutgers University). The formalin-fixed colons were made into a Swiss-Roll, paraffin-embedded, and sectioned serially at 4-µm of thickness. The entire colon was evaluated histopathologically using two H&E-stained sections (sections 1 and 10) per colon (Figure 10). Hyperplasia and dysplasia were scored as 0 (normal), 1 (mild hyperplasia: epithelial cells lining the colon appeared normal, but crypts appear 2 to 4 times thicker than normal crypts), 2 (low-grade dysplasia: 2 to 4 times thicker epithelium, hyperchromatic cells, fewer goblet cells, and scattered crypts developing an arborizing pattern), or 3 (high-grade dysplasia:>4 times thicker epithelium, hyperchromasia, few or no goblet cells, highly mitotic cells in the crypts with arborizing pattern, and crypts extending to muscularis mucosa or submucosa) (74).

9. Methylene Blue Staining for Aberrant Crypt Foci

ACF were analyzed in colon tissues using a conventional methylene blue staining method (49). In brief, fixed colon tissues were stained with 0.2% methylene blue for 3 to 5 minutes and visualized under a micro-dissection microscope. The colon was cut into three sections; proximal, middle and distal. The three sections were individually examined for the total number of ACF's, and the multiplicity of crypts per ACF (≥ 4 and ≥ 10 crypts).

10. Specific Staining for β-catenin

Formalin-fixed tumors were prepared in a similar fashion as described in Section 9. Two different serial sections (sections 2 and 11) of tumor samples were evaluated by Dr. Guang-Xun Li for β-catenin expression by immunohistochemistry. In brief, tumor sections were deparaffinized in xylene and rehydrated in distilled water, and the tissue antigens were unmasked in antigen unmasking solution (Vector Laboratories Inc, Burlingame, CA) by boiling the slides for 6 minutes in the microwave. Endogenous peroxidase was quenched using 3% H_2O_2 in distilled water. Sections were then blocked for 1 hour at room temperature in PBS containing 3% normal serum and incubated with β-catenin (1:2000) primary antibody overnight at 4°C. Biotin-conjugated secondary antibody (1:200) and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) was used as the chromogen. Sections were then counterstained with Hematoxylin and mounted with Permount. Negative controls were processed in the absence of the primary antibody. Quantification of the number of total cells and infiltrated β-catenin-positive cells in mucosa and submucosa was performed by using the Image-Pro Plus Image Processing System (Version 5.0)

11. Western Blot Analysis

Prior to performing poly-acrylamide gel electrophoresis, samples containing equal amounts of protein (10-20µg) were heated with sample buffer for 5 minutes at 95°C and centrifuged briefly prior to use. Protein samples were maintained on ice for the duration of the experiment and electrophoresed using a freshly prepared 10% SDS-polyacrylamide gel. Voltage was maintained at a constant 100V. The proteins were then electrophoretically transferred from the gel to a nitrocellulose membrane for one hour at 90 volts. The membrane was blocked with blocking buffer overnight at 4°C followed by incubation of primary antibodies for one hour at room temperature. A list of antibodies and concentrations used can be found in Table 1. The membrane was then washed in TBS containing 0.1% tween-20 three times and incubated with infrared fluorescent-labeled secondary antibody (1:5000) for 1 hour at room temperature. The membrane was washed again and the immunoblots were processed and visualized using the Odyssey infra red imaging system (LI-COR, Lincoln, NB).

12. Statistical Analysis

Data was measured by the Student's t-test using Microsoft Excel. All numeric variables were expressed as mean ± standard error.

V. RESULTS

1. hCYP1A2 Mouse Model for PhIP-Induced Colon Carcinogenesis

1.1. Generation of the hCYP1A2 Mouse Colony

The generation of the hCYP1A2 transgenic mouse colony homozygous for both the human CYP1A1/1A2 gene insert and the mouse Cyp1a1/1a2 knockout allele was established in our laboratory. Jackson Laboratories state that the mice supplied are either heterozygous or homozygous for the human CYP1A1/1A2 allele so breeding with wild-type mice was done to determine zygosity. After backcrossing two of the original male humanized CYP1A1/1A2 mice purchased from Jackson Laboratories to C57BL6/J wild-type females, it was discovered that some of the animals received were not homozygous for the human 1A1/1A2 and mouse 1a1/1a2 knockout. Further brother-sister mating and continuous genotyping allowed us to eventually generate and maintain a completely homozygous colony. As shown in Figure 3, we genotyped for both the human CYP1A1/1A2 gene and mouse Cyp1a2/1a2 knockout allele. Human CYP1A1 results in a band located near 528 base pairs, human CYP1A2 results in a band around 180 base pairs, mouse Cyp1a1/1a2 knockout results in a band around 353 base pairs and the Ephx1control results in a band near 341 base pairs.

An average of 5-7 pups are produced from hCYP1A2 crosses with no observed abnormalities resulting in the transgenic mice. Often, the Cyp1a1/1a2 knockout mice that were also maintained in the sterile laboratory resulted in pups with grey colouring on their fur. Most of the transgenic and knockout mice had slightly shorter life spans and were often smaller in size compared to the wild-type mice.

2. hCYP1A2 Mice with Combined PhIP and DSS Treatment

hCYP1A2 mice bred in our animal facility were administered various combinations of PhIP and DSS to determine if colon carcinomas could be induced in this model. Optimal conditions were chosen based on the ability of the animal to withstand combined treatments and acquire an average of 4-10 tumors per colon in approximately 80% of the animals.

2.1. Animal Conditions

After PhIP gavage, most mice (wild-type and hCYP1A2) survived the initial treatment of PhIP. After the 3rd or 4th day of DSS treatment, rectal bleeding was observed in the majority of mice treated. In most cases, bleeding ceased 3-4 days after DSS treatment was complete. This trend was similar to that found in other PhIP/DSS and AOM/DSS carcinogenesis models (24, 48, 70). However, in certain instances, rectal bleeding was observed again near 6 to 10 weeks after PhIP gavage. When these mice were sacrificed, blood was also present in the colon.

Most wild-type and hCYP1A2 mice survived the combined treatment of PhIP and DSS (73/87= 84%), while the rest died of bleeding or unknown causes (Table 3). The hCYP1A2 mice treated with PhIP and DSS lost an average of 0.48g of body weight due to the stress of the PhIP gavage and prolonged DSS treatment compared to an average gain of 0.74g in the wild-type mice in the same treatment group (Figure 4a vs. 4b). Body weights in the hCYP1A2 mice began to increase one week after the DSS treatment was completed (Figure 4b). The most significant decrease in body weight was evident in the 200mg/kg of PhIP + 1.5% DSS and 20% DMSO + 1.5% DSS treatment group in the hCYP1A2 mice. Diet intake was monitored on a weekly basis and no abnormalities were observed among the various groups.

2.2. Development of the hCYP1A2 PhIP-Induced Colon Cancer Model with DSS

To establish an hCYP1A2 mouse model for PhIP-induced colon cancer, various concentrations of PhIP and DSS were investigated in the hCYP1A2 mice. Our goal was to determine the best combination for inducing tumors in the hCYP1A2 mouse colon. Combinations of 100mg/kg of PhIP + 1% DSS, 200mg/kg of PhIP + 1% DSS, and 200mg/kg of PhIP + 1.5% DSS were used as described in the research design. Based on previous results reported by several groups, colonic tumors in PhIP-induced mice in combination with DSS have occurred as early as 20 weeks, therefore, a few mice in each group were sacrificed starting at week 12 after PhIP gavage and continuing past 20 weeks (Figure 5) (69, 75, 48). The 200mg/kg of PhIP + 1.5% DSS treatment group resulted in 4 tumors in a transgenic female mouse and 10 tumors in a transgenic male mouse at week 12 after PhIP gavage (Table 4). The 100mg/kg and 200mg/kg of PhIP + 1% DSS treatment groups did not result in any tumors or gross abnormalities at week 12 and were maintained past this time point. The 100mg/kg of PhIP + 1% DSS treatment group did eventually result in a single tumor formation in one mouse at week 24 after PhIP gavage, however the 200mg/kg of PhIP + 1% DSS group did not (Table 4). Wild-type mice in the same treatment groups were sacrificed alongside the transgenic mice as controls and did not result in any tumors past week 24 (Table 4). It has been reported that tumors can be observed around 20 weeks after PhIP administration combined with DSS in C57BL6/J mice (48), which we are using for wild-type mice, monitoring these mice past week 24 did not result in any gross abnormalities. This suggests that this animal model has a high specificity towards the human 1A1/1A2 genes.

The 200mg/kg of PhIP + 1.5% DSS treatment group demonstrated to be the best model for tumor formation in the hCYP1A2 mice as the other two treatment groups resulted in little or no tumors past 12 weeks (Table 4). With such a high incidence of tumor formation at week 12,

specifically in the transgenic male, we hypothesized that tumors were forming even earlier than this time point.

Our goal was to determine an optimal time point for sacrifice in the PhIP-induced hCYP1A2 colon cancer mouse model with an average of 4-10 tumors forming in 80-90% of the animals. The next time points that the hCYP1A2 mice were sacrificed at were 6, 8, and 10 weeks after PhIP gavage (Figure 5a). Surprisingly, multiple tumors were formed in the colon of the hCYP1A2 mice as early as week 6 with an average tumor multiplicity of 3.75 ± 0.70 (N=20). This suggests that tumors could be forming even earlier than 6 weeks after PhIP gavage. At weeks 8 and 10 the average tumor multiplicity was recorded as 1.67 ± 0.96 (N=9) and 3.9 ± 0.61 (N=29) respectively (Table 4). Based on these results, we have suggested an optimal time point for sacrifice to be between 6-10 weeks.

At the termination of the experiment, 76 hCYP1A2 mice were treated with 200mg/kg of PhIP + 1.5% DSS. Out of the 74 mice treated, 65 transgenic mice survived (34 males and 31 females). The overall tumor incidence for this group was found to be 83% (54/65). The tumor multiplicity for this treatment group at the various time points of sacrifice were calculated to be 4.86 ± 1.49 at 12 or more weeks (N=7), 3.90 ± 0.61 at 10 weeks (N=29), 1.67 ± 0.96 at 8 weeks (N=9) and 3.75 ± 0.70 at 6 weeks (N=20) (Table 4). The tumor multiplicity between males and females in this treatment group was also calculated to determine if there were any sex differences in this model. The tumor multiplicity for males and females was calculated as 3.2 ± 0.79 (N=10) and 4.3 ± 1.16 (N=10) respectively for mice sacrificed at week 6 after PhIP gavage and 4.1 ± 0.6 (N=15) and 3.8 ± 1.1 (N=14) respectively for mice sacrificed at week 10. These results were not found to be significant suggesting that sex may not play a role in the development of this model.

2.3. Observation of Aberrant Crypt Foci by Methylene Blue Staining

In the 100mg/kg of PhIP + 1% DSS treatment group, only one tumor was observed at week 24 after PhIP gavage. We wanted to further characterize this model by determining if ACF's could be detected in the colon of these mice. It has been reported that ACF's are observed in the colon of rodents shortly after PhIP treatment (28, 73). A continuous treatment of PhIP in the diet results in ACF's around 25 weeks whereas a combined treatment of two-weeks of PhIP in the diet followed by four weeks of a high-fat diet has resulted in ACF's as early as six weeks (73).

In order to examine ACF's as a precursor for colorectal polyps, we used a conventional staining method with 0.2% methylene blue staining solution. ACF's were characterized by their morphology: enlarged crypts and a thicker layer of epithelial cells were observed by a darker-blue staining pattern (Figure 6a). The crypts have irregular lumens and appear to be microscopically elevated compared to adjacent normal mucosa (Figure 6a indicated by arrow). In the 100mg/kg of PhIP + 1% DSS treatment group, ACF's were observed as early as 6 weeks after PhIP treatment. Between 6-16 weeks after PhIP gavage the average ACF per colon was recorded as 8.3 ± 2.2 (N=12), at 20 weeks the average ACF per colon was recorded as 26 ± 4.9 (N=9), and between 24-25 weeks the average ACF per colon was 27.8 ± 10.9 (N=11). The number of ACF's observed appeared to increase with the duration of the experiment suggesting a correlation of ACF formation over the function of time (Figure 6b). Over 200 ACF's alone were observed in the colon with the recorded tumor in the 100mg/kg of PhIP + 1% DSS treatment group suggesting that the ACF's increase with tumor formation and they may be observed as precursors for colon polyps in this animal model. The majority of ACF's were located in the middle to distal sections of the colon which corresponds to the observations of tumor location in both the 100mg/kg of PhIP + 1% DSS and 200mg/kg of PhIP + 1.5% DSS treatment groups

2.4. Histopathological Findings at Weeks 6 to 21 after PhIP Treatment

Normal mouse colon mucosa has an observed healthy epithelial lining, a steady ratio of leukocytes in the mucosa layer, and many visible goblet cells. Inflammation and ulceration were observed in all of the hCYP1A2 colon tissues beginning as early as week 6 after PhIP gavage in the 200mg/kg of PhIP + 1.5% DSS treatment group. Crypts in normal mouse colon tissue are round in shape and are maintained in the mucosal layer of the colon (Figure 7A). Hyperplasia was observed as tissue having many epithelial layers with large nuclei beginning to lose their round appearance and a visible loss of most of the goblet cells. Adenomas were observed when the crypts extended through the mucosal layer into the epithelial lining creating a polyp-like structure (Figure 7B). Adenocarcinomas and severe dysplasia were observed in the tumor samples as having an irregular shaped dark nucleus, dilated lumen and leukocytes were observed infiltrating the submucosal layer of the colon (Figure 7C and 7D).

Of the tumors collected from the 200mg/kg of PhIP + 1.5% DSS treatment group, 15% were scored as adenomas and 85% were scored as adenocarcinomas (Table 4). The score of tumors did not seem to increase with duration of the experiment. The 100mg/kg of PhIP + 1% DSS treatment group resulted in 1 tumor out of the 34 remaining mice in this group. Pathological observations of this treatment group also included enlarged lymph follicles in all areas of the colon suggesting an immune response to the inflammation of the colon region. A thickening of the proximal mucosal layer of the colon was also observed at the time of sacrifice and tumor formation generally occurred near the middle to distal regions in the colon (Figures 8a and 8b).

2.5. Expression of β-catenin

β-catenin is found to be overexpressed or constituently activated in most human colon cancers (5). To observe the expression of β-catenin in the 200 PhIP + 1.5% DSS tumor samples, we performed a Western blot analysis with a β-catenin primary antibody (1:500) for transgenic tumor samples and transgenic normal colon tissue samples. β-catenin was observed to be expressed in both male and female mouse tumor samples at week 6 and not in the normal transgenic colon samples that were not treated with PhIP or DSS (Figure 9). This was further confirmed by immunohistochemistry analysis.

Nuclear localization of β-catenin staining in the colon and tumor tissues of hCYP1A2 transgenic mice treated with 200mg/kg of PhIP + 1.5% DSS at week 10 was observed (Figure 10). Minimal staining of β-catenin was observed in the membrane of the crypts of normal colon mucosa (Figure 10A). Colons with high grade dysplasia demonstrated strong nuclear localization of β-catenin. Adenomas have a lower nuclear localization compared to that observed in the adenocarcinomas which are represented by extremely strong staining patterns (Figure 10 C-D). These results are consistent with the protein expression found in tumors extracted from hCYP1A2 mice treated with PhIP and DSS and further establishes this model as exhibiting characteristics similar to those found in human colorectal cancers.

3. hCYP1A2 Mice Treated with PhIP Without the Addition of DSS

hCYP1A2 mice were treated with a single dose of 200mg/kg of PhIP without the addition of DSS to determine if PhIP alone could induce carcinogenesis in this model. In the treatment groups with DSS, we expected the main target for cancer to be in the colon as DSS is a strong promoter of colitis and in combination with carcinogens can rapidly induce colonic tumors. Although PhIP is mainly considered a colon carcinogen due to its bioavailability to the digestive

tract, we also predict that other forms of cancer may occur in this treatment group as well. As reviewed by Nakagama et al, cancerous malignancies have been reported in other PhIP-induced rodent models after a long-term feeding protocol of PhIP alone without any additional chemical promoters (28).

3.1. Animal Conditions

The survival rate for the 200mg/kg of PhIP treatment group was recorded as 98% (54/55) within the first week after PhIP gavage. The overall survival rate for the entire duration of the experiment was 87% (47/54) (Table 3).

The body weight variation of the 200mg/kg of PhIP treatment group can be seen in Figure 4, and it was noted that the body weights of the hCYP1A2 mice for this group did not have significant weight loss compared to the DSS treatment groups and it was also found to be comparable to wild-type mice in the same treatment group.

A total of 8 out of the 54 mice treated died of rectal bleeding or unknown causes. Initially, the body weight of these mice slightly decreased and in some instances rectal bleeding was observed around 20 weeks after PhIP gavage. At the time of sacrifice, those mice with reported rectal bleeding also had blood in the colon. Enlarged spleens, which may suggest a sign of infection, along with yellow fluid in the cecum and intestines was also observed in certain mice.

3.2. Development of the hCYP1A2 PhIP-Induced Cancer Model Without DSS

Our goal for the development of the hCYP1A2 mice in the 200mg/kg of PhIP treatment group was to observe the physical and biological effects of a single injection of PhIP without the addition of DSS. As previously mentioned, PhIP primarily targets the colon. It has been reported that small intestinal tumors are observed in C57BL6/J mice 52 weeks after PhIP administration without the addition of DSS or other chemical promoters (27).There have been no reports thus far of PhIP alone causing colon cancer in mouse models.

Initially we began by following the same sacrifice time points as we did in the DSS treatment groups starting with 12 weeks after PhIP gavage (Figure 5b). At the first time point of 12 weeks, no abnormalities were noted. We continued past 12 weeks with one month intervals in between sacrifice time points. At week 20 we began to observe enlarged lymph nodes and spleens in the animals, but still no visible tumors or cancerous malignancies were reported. At week 24, rectal bleeding was observed in certain mice suggesting inflammation or lesions in the colon/rectum but upon autopsy, no tumors were observed. During the 30th week after PhIP gavage, low grade prostatic intraepithelial neoplasia (PIN) was observed in the prostate of two transgenic and two wild-type mice. Because PIN is thought to be an early precursor for prostate cancer and we had not visibly observed any cancerous malignancies, we are currently still working on this model and will sacrifice again in 10 more weeks (week 40). As this is an ongoing experiment, results will be reported in the near future.

3.3. Observation of Aberrant Crypt Foci by Methylene Blue Staining

As previously demonstrated in the 100mg/kg of PhIP + 1% DSS treatment group, hCYP1A2 mice develop ACF's as early as week 6 after PhIP gavage. We wanted to utilize the methylene blue staining method to characterize ACF's in this 200mg/kg of PhIP model without

treatment of DSS. The earliest time point that ACF's were observed in this treatment group was at week 12 after PhIP gavage.

3.4. Histopathological Findings

Colon, prostate, mammary and stomach tissues were observed histopathologically in hCYP1A2 humanized and wild-type mice after each sacrifice time point. As mentioned above, at week 30, PIN was observed in the prostate of two transgenic and two wild-type male mice. PIN is thought to be an early precursor to prostate cancer which suggests that a longer time-point after 30 weeks of PhIP gavage may result in the development of prostate cancer in both the hCYP1A2 and wild-type mice. Small signs of necrosis were also observed in the stomach of one hCYP1A2 male at week 30 as well suggesting infection or trauma to the stomach possibly as a result of PhIP ingestion.

VI. GENERAL DISCUSSION AND FUTURE DIRECTION

1. General Discussion

A transgenic humanized mouse model for colon carcinogenesis has been developed in our laboratory utilizing the dietary carcinogen PhIP, in combination with DSS in hCYP1A2 mice. The results discussed in this paper satisfy our aim to create a model suitable for investigating the effects of human colon cancers *in vivo*. The data presented indicates that a single injection of PhIP (200mg/kg of PhIP) followed by one week exposure to DSS (1.5%) in the drinking water of hCYP1A2 mice produces colonic adenocarcinomas as early as 6 weeks after PhIP gavage with an overall tumor incidence of 83%. To our knowledge, this is the earliest time frame that tumors have been reported in PhIP-induced rodent cancer models thus, this should be an excellent model to investigate the carcinogenic effects of PhIP in a short-term study.

Several PhIP-induced experimental animal models have been developed to investigate the carcinogenic potential of PhIP *in vivo* (27, 69, 70, 73). After 52 weeks on a diet containing 400 mg/kg of PhIP, F344 rats have been shown to develop colon carcinomas at a frequency of 50% (27). Ochaia et al, demonstrated a short-term study with PhIP by introducing a high-fat diet into the protocol. After 6 weeks, ACF's were observed in the colons of mice treated with PhIP in combination with DSS and a high-fat diet (73). A mouse model utilizing DSS as a tumor promoter in combination with 300 mg/kg of PhIP in the diet was demonstrated to induce colonic tumors at 20 weeks (69). Recently, Tanaka and colleagues developed an ICR mouse model with a single 200mg/kg dose of PhIP by oral gavage followed by 2% DSS supplemented in the drinking water for one week (70). Colonic adenocarcinomas were observed within 16 weeks at a 56% incidence in the ICR mice (70). Although a high dose of PhIP was used in their model, Tanaka et al, was able to demonstrate the development of a short-term model for PhIP-induced colon

carcinogenesis. In the present study, we demonstrated the induction of colonic adenocarcinomas at 6 weeks after PhIP gavage in the 200mg/kg + 1.5% DSS treatment group. These results represent a possible specificity for PhIP towards the human CYP1A1/1A2 genes as the tumor incidence occurred significantly earlier compared to PhIP-induced carcinogenesis models currently being utilized. Therefore, our model can be used to study PhIP-induced carcinogenesis in humans as the CYP1A1/1A2 phenotype in humans has been associated with increased PhIP metabolism inducing the formation of PhIP-DNA adducts (45).

Various combinations of PhIP and DSS were observed in the hCYP1A2 model. It was interesting to note that the 200mg/kg of PhIP + 1.5% DSS treatment group had the greatest carcinogenic effect at 6 weeks after PhIP gavage, the 100mg/kg of PhIP + 1% DSS treatment group had a very low tumor incidence beyond 24 weeks, the 200mg/kg of PhIP + 1% DSS treatment group had no recorded abnormalities past 24 weeks, and the 200mg/kg of PhIP treatment group alone had very little observed abnormalities past 30 weeks of treatment (Table 4). A recent review on various mouse strain susceptibilities to PhIP and DSS noted that a dose of 200mg/kg of PhIP + 2% DSS results in the greatest incidence of tumors in several strains of mice (48). It is possible that the concentration increase from 1-1.5% of DSS in the 200mg/kg of PhIP treatment regime, along with the increased activation of PhIP by the CYP1A1/1A2 genes in our model could be increasing inflammation in the colon to the point where the inflammatory response can no longer control the effects of the carcinogen, thus rapidly inducing tumors. Further studies should be developed to possibly look at lower doses of PhIP in combination with various doses of DSS. However, caution must be taken as higher doses of DSS in various rodent models have resulted in a higher mortality rate of the animal (31, 48). By observing the survival rates between the 200mg/kg of PhIP + 1.5% DSS and 100mg/kg of PhIP + 1% DSS treatment groups one can suggest that higher doses of PhIP and DSS may be toxic for the animal (Table 3).

Specifically, the hCYP1A2 treatment groups with 1.5% DSS appeared to have the most significant drop in body weight (Figure 4). However, lowering the dose of DSS to 1%, in combination with 200mg/kg of PhIP, did not seem to dramatically affect the mortality rate suggesting that the amounts of PhIP and DSS both need to be lowered to reduce toxicity. It is also interesting to note that the decrease in body weights was found to be different between the wild-type and hCYP1A2 treatment groups. The hCYP1A2 treatment groups had a more significant drop in body weight compared to the wild-type treatment group and it also appears that 1.5% DSS in general had a more profound effect in the hCYP1A2 mice with or without PhIP treatment (Figure 4a vs. Figure 4b). This effect of 1.5% DSS and not 1% DSS could be because of the higher concentration of DSS used, therefore increasing inflammation. It has also been demonstrated *in vitro* and in various DSS-induced colitis rodent models that certain hepatic CYP's are down-regulated during inflammatory processes (75, 76, 77). This could be another possible explanation for the stable bodyweights observed in the wild-type treatment groups with DSS compared to the decrease in body weight in certain hCYP1A2 treatment groups. Our findings demonstrate a powerful tumor-promoting effect of DSS, much like that described by Tanaka et al (70), and a possible threshold for the effects of PhIP in the hCYP1A2 mouse.

Although the 200mg/kg of PhIP + 1.5% DSS treatment group is an interesting model for short-term animal studies in PhIP-induced colon cancers, it would be ideal to utilize this model without the presence of DSS or other non-naturally occurring agents. A colon cancer model with PhIP alone would also be significant as colon cancer in mice has not been induced by a sole treatment of PhIP. Thus far, we have presented data past 30 weeks of 200mg/kg of PhIP treatment. ACF's have been observed in the colons of hCYP1A2 mice and PIN has been observed in the prostate of male hCYP1A2 and wild-type mice. These results suggest that treatment beyond 30 weeks may result in cancerous malignancies as both ACF's and PIN are regarded to be

pre-cursors of cancer. It has recently been demonstrated in a PhIP-induced rat model that PhIP is capable of inducing a rapid and persistent prostatic inflammation (68). Borowsky et al, reported an onset of inflammation followed by PIN and hyperplasia in the prostate of F344 rats fed a diet containing PhIP (400-200mg/kg) (68). After week 30 of PhIP administration (200mg/kg) we observed inflammation and PIN in both wild-type and hCYP1A2 male mice, with the hCYP1A2 having a higher grade of both PIN and inflammation (data not presented). These results could be significant as the pathological development of precancerous stages of prostate cancer have not been closely observed in PhIP-induced mouse models.

We do expect to see malignancies in the 200mg/kg of PhIP model without DSS at a longer period after PhIP gavage as it has been reported in various strains of mice and rats that cancerous lesions appear between 52 weeks to two years after PhIP administration (27, 44). This model may also be beneficial as it initially presents a less-toxic treatment to the animals without the addition of DSS. Although the survival rates between the 200mg/kg of PhIP without and with 1.5% DSS are very similar, bleeding and initial weight loss was not observed in the treatment without DSS (Figure 4). Although DSS is used as a potent inducer of colitis and rapidly induces tumors, it can prove to be significantly harmful to animals (31). A PhIP-induced hCYP1A2 cancer model without the addition of DSS may be an ideal long-term model to study carcinogenesis, however longer treatments are still being analyzed.

Incidences of dietary related cancers in humans are becoming more common with the increase in Westernized dietary habits (28). Therefore, it is important to find models closely related to human colon cancers to investigate the effects of heterocyclic amines such as PhIP and other dietary carcinogens. A benefit for generating this PhIP-induced colon cancer humanized mouse model is to closely mimic the carcinogenicity of PhIP in humans in an *in vivo* system. Translating data generated in animal models *in vivo* to human populations is often prone to error.

By utilizing this hCYP1A2 model, we have eliminated any possible inhibitory effects of specific mouse orthologs (Cyp1a1/1a2) in PhIP-induced cancer development. Therefore, this model is specific to human genes known to be involved in PhIP carcinogenicity in humans. Although the doses of PhIP used in this study are high (100mg/kg-200mg/kg of body weight) compared to the amounts of PhIP consumed on a daily basis in humans (72ng/day), PhIP and other heterocyclic amines have been found to be ubiquitously present in cooked foods and can rapidly form DNA adducts in humans (78). The high doses of PhIP used in this study may be more relevant to PhIP exposure over a significant period of time in humans. For now, this design allows us to investigate the carcinogenic potential of PhIP and the role of human CYPs in a short-term model.

Current models for colon cancer research and chemoprevention studies such as the AOM/DSS model, which is used in our laboratory, although relevant to pathological characteristics of human colon cancer, are not idealistic. AOM is not a naturally occurring cancer agent and is not involved in human colon cancer etiology. PhIP is commonly found in our diets from the consumption of over-cooked meat and poultry, and is considered a dietary carcinogen. We observed the pathological progression of the PhIP-induced hCYP1A2 model to be similar to that found in the AOM/DSS model and human pathology with the presence of ACF's as precursors to colonic neoplasms, adenomatous polyps, high-grade dysplasia and hyperplasia developing into adenocarcinomas. β-catenin mutations have also been found in colonic tumors induced by PhIP + DSS in the hCYP1A2 mice which suggests the involvement of the Wnt signaling pathway in the development of this model. These mutations have been demonstrated to be frequent in PhIP-induced rat neoplasms (79). The activation of this pathway is considered one of the most common mutations and possibly an initiating event in the progression of colon carcinogenesis (5). This data further relates our hCYP1A2 PhIP-induced model of colon cancer to

the development of colon carcinogenesis in humans. The use of a mouse model that closely relates to human forms of colon cancer is an ideal model for chemoprevention studies.

2. Future Direction

As mentioned in Section I, naturally occurring agents such as antioxidants have been found to inhibit various forms of cancer. Specifically in our laboratory, we focus on the chemopreventive effects of tocopherols and EGCG. Based on published data, we expect that dietary tocopherol and EGCG treatment will have a preventive effect on the tumors in our model. Tea is thought to have preventive effects on PhIP-DNA adduct formation in rats by inhibiting the DNA binding of N-acetoxy-PhIP (80). More specifically, EGCG has been found to have the most significant preventive effect against PhIP-DNA adduct formation (80). Recently, it has been suggested that γ-tocopherols may be the most effective forms of vitamin E for cancer prevention because of its strong anti-inflammatory activity (19). Results in our laboratory recently found that a γ-tocopherol-rich mixture of tocopherols (γ-TmT) has cancer preventive effects in prostate, mammary, colon and lung tumorigenesis in animal models (19). This diet may prove to also be successful for the inhibition of colon tumorigenesis in our hCYP1A2 PhIP-induced model.

We have begun preliminary chemoprevention studies in the hCYP1A2 mice treated with 200mg/kg of PhIP + 1.5% DSS. Two diets consisting of a 0.3% γ-TmT and 0.1% EGCG have been supplemented for the regular AIN-93 diet. One week prior to PhIP gavage, hCYP1A2 mice were supplemented with their respective treatment diets and remained on the diets for the duration of the experiment. The experimental design remained the same with PhIP/DSS administration (Figure 5). Based on the results from the 200mg/kg of PhIP + 1.5% DSS treatment

group, animals in the experimental diet groups were sacrificed at both weeks 6 and 10 after PhIP gavage.

Preliminary results from the 0.3% γ-TmT treatment group have been observed. The survival rate in the tocopherol treatment group was interesting in that only 63% (17/27) survived compared to the 86% (65/76) survival rate observed in the AIN-93 control diet group. An increase in rectal bleeding 2-3 days after the start of DSS treatment was observed along with prolonged bleeding after the completion of this treatment, in some cases past two weeks. In the control diet group bleeding usually was observed near the end of DSS treatment and began to cease shortly after the completion of the treatment. We also started a 0.3% γ-TmT treatment group with the hCYP1A2 mice treated with 200mg/kg of PhIP without DSS to determine if the tocopherol diet was interacting with PhIP to cause this high mortality rate. The survival rate in this group was found to be 79% (19/24) which was much higher than the PhIP + DSS treatment group. These mice are currently still being maintained past week 23 of PhIP gavage in our laboratory and will be used to compare the chemopreventive effects in the hCYP1A2 mice treated with PhIP alone beyond 30 weeks of treatment.

It has been reported that vitamin E may interfere with blood clotting. Liu et al, has demonstrated that a γ-TmT diet resulted in stronger inhibition of platelet aggregation compared to α-tocopherol alone in the diet of humans (81). This could be a possible reason for the prolonged bleeding and high mortality rate in the 200mg/kg of PhIP + 1.5% DSS treatment group on the 0.3% γ –TmT diet. As this is a likely possibility, perhaps a more appropriate experimental design would be to start the tocopherol diet after the DSS treatment has stopped and the bleeding or infection has subsided. It is not expected for tumors to form during the course of DSS treatment (as early as 2 weeks after PhIP gavage) so this may be a more plausible design. This design would be useful not only as a prevention treatment, but possibly for treatment after diagnosis.

Male and female hCYP1A2 mice on the 0.3% γ-TmT diet along with the AIN-93 control diet were sacrificed at week 6 after PhIP gavage. The mice in the 0.3% γ-TmT treatment group lost an average of 0.26g of body weight per mouse after PhIP injections and an average of 3.83g per mouse after DSS treatment. The body weights began increasing 1-2 weeks after the completion of DSS treatment which is slightly longer than the control group. The average tumor multiplicity for this group was 2.5 ± 0.79 (N=17) which was not found to be significant between the tocopherol treatment group and the AIN-93 control diet at week 6 after PhIP gavage. Histopathological analysis revealed similar characteristics compared to those reported in the 200mg/kg of PhIP + 1.5% DSS treatment group on the control diet such as severe dysplastic cells in the colons of these mice and adenocarcinomas.

We do not have preliminary results for the 0.1% EGCG treatment group. However, we did not see severe or prolonged rectal bleeding as was observed in the 0.3% γ-TmT treatment group. So far the survival rate for the 0.1% EGCG group is 79% (15/19). These mice will be sacrificed at week 10 after PhIP gavage which will be completed in the near future.

The hCYP1A2 model utilized in combination with PhIP without or with DSS should be an excellent model to study the cancerous effects of PhIP and eventually other carcinogens that are specific to human CYP1A1/1A2. An appropriate use for this model would also be to study the effects of a high-fat western-style diet in combination with 200mg/kg of PhIP. As previously mentioned, PhIP demonstrates preferential carcinogenicity to the colon, prostate, and mammary glands in rats and mice. These sites correlate to common organs affected by dietary cancers in humans found in Western countries where modern dietary habits have an increased fat intake. It has been demonstrated that PhIP with the addition of a high-fat diet has accelerated the induction of colon tumors compared to a control diet (73). Ochaia et al, demonstrated the induction of numerous large ACF's in rats after a two week feeding period of 400mg/kg of PhIP in the diet

followed by a four week period of a high fat diet (73). However, protocols with continuous feeding of PhIP in a high-fat diet have not demonstrated a linear increase in ACF's or tumor formation suggesting that continued exposure to PhIP may have an inhibitory effect on ACF formation (73). This data suggests that a shorter exposure-time to PhIP may be a better model; therefore the single injection of 200mg/kg of PhIP by oral gavage in our hCYP1A2 mice may be a more effective method of administration. The hCYP1A2 PhIP-induced model in combination with a high-fat diet may be an excellent model to study the effect of diet on the carcinogenic potential of PhIP.

As this is a newly established mouse model for human colon cancer research, further analyses need to be performed in order to understand the full potential of the hCYP1A2 mice in combination with PhIP. Microsomal fractions of liver tissue extracted from hCYP1A2 and wild-type treatment and non-treatment groups have been made. These fractions are currently being used to determine CYP expression of various human and mouse CYPs including human CYP1A1/1A2 and mouse Cyp1a1/1a2 (results not presented). It has been reported that PhIP induces CYP1A1 and CYP1A2 in rats, but not in mice (64). The developers of the hCYP1A2 mouse model also reported that human CYP1A1 was not expressed in untreated hCYP1A2 liver samples. These results were further confirmed by Cheung et al, who also did not find expression of human CYP1A1 protein in untreated hCYP1A1 liver samples while investigating the metabolism of PhIP in the hCYP1A2 mice (40). Further analyses of CYPs in this mouse model may help to distinguish whether or not CYPs besides human and mouse 1A1/1A2 could be involved in the carcinogenic potential of PhIP in the hCYP1A2 mice.

Characterizing other genes and pathways known to be involved in PhIP-induced cancer models and human-specific colon cancers such as APC, nitric oxide synthase, and COX-2 expression would be beneficial (82). It has been noted that mutations such as p53 and K-ras are

very rare in PhIP-induced colon cancer models (82). These mutations are often found in various forms of human cancers, therefore it would be interesting to determine their expression in our hCYP1A2 PhIP-induced colon cancer model as it has a higher specificity to human forms of cancer than current PhIP-induced models. Once the chemoprevention studies are complete, it would also be ideal to characterize some of the genes and pathways involved in the prevention or inhibition of PhIP-DNA adducts in these mice to further enhance the development of the hCYP1A2 PhIP-induced colon cancer model for chemoprevention studies in humans.

In summary, we have developed a novel PhIP-induced mouse model for colon carcinogenesis in hCYP1A2 mice. hCYP1A2 mice were found to develop tumors as early as 6 weeks after 200mg/kg of PhIP followed by 1.5% DSS compared to C57BL/6J wild-type mice who did not show any tumors or other malignancies after the same treatment regime. Pathological progression of adenocarcinomas and induction of the Wnt β-catenin signaling pathway observed in tumor samples suggest that this model shares similar characteristics to colon carcinogenesis in humans. The increased activation of PhIP in this mouse model by the addition of human CYP1A2/1A2 genes and knockout of the mouse ortholog, Cyp1a2/1a1, seems to be an excellent model to study PhIP-induced colon carcinogenesis as it pertains to humans. Variations of this model may also prove to be significant in investigating the effects of PhIP in prostate, mammary, liver and other forms of cancer as these sites have been reported in various PhIP-induced rodent models. This hCYP1A2 model of PhIP-induced carcinogenesis is highly relevant to human colon carcinogenesis, thus chemoprevention studies in this model may pertain more to the human condition.

Table 1. Antibodies used for specific staining by immunohistochemistry and protein expression analysis.

Antibody	Description	Concentration Used	Obtained from:
β-actin	Detects endogenous levels of total β-actin protein	(1:10,000)	Cell Signalling Technology, Danvers, MA
β-Catenin	Recognizes rat, human and mouse β-Catenin protein	(1:2000)	BD Biosciences, Franklin Lakes, NJ
Goat Anti-Mouse/Anti-Rabbit IRDye Secondary Antibodies	Binds to primary mouse/rabbit antibodies	(1:5000)	LI-COR Biosciences, Lincoln, NB

Table 2. Primers used for genotyping hCYP1A2, and Cyp1a1/1a2 knockout mice.

Gene	Primer Name	Primer Sequence (5'-3')	Expected PCR Product Size (bp)
Human	NEBH1A1F	GCAGCCCTGTTTGTTCCTG	528
1A1/1A2	NEBH1A1R	AGGCTGGCCTATGTGGTCTA	
	NEBH1A2F	AGGATTGGCATTGTTGAAGG	180
	NEBH1A2R	GGGCACTGGCCATAGTATTC	
Ephx1	MEHFOR	AAGTGAGTTTGCATGGCGCA	341
	MEHREV	CCCTTTAGCCCCTTCCCTCTG	
Mouse	NEBDKOE	GACATAGGAGCTACCTACAC	353
1a1/1a2	NEBDKOF	GTCAAAGTAACCAGACACATCCTGC	
	NEBWTF	GGCTAACCATCTCGTCAGC	417
	NEBWTR	TGCACACGGCACTCTGAG	

Primers were purchased from Sigma Genosys (The Woodlands, TX)

Table 3. Breakdown and survival rates of the various hCYP1A2 and wild-type treatment groups.

Mouse	Treatment	Duration (weeks)	No. Mice Treated	Survival Rate
hCYP1A2	200 PhIP + 1.5% DSS	6-20	76	86% (65/76)
WT	200 PhIP + 1.5% DSS	12-24	11	100%
hCYP1A2	200 PhIP + 1% DSS	10-24	15	80% (12/15)
WT	200 PhIP + 1% DSS	12-24	10	100%
hCYP1A2	100 PhIP + 1% DSS	6-25	35	97% (34/35)
hCYP1A2	200 PhIP	16-40	54	87% (47/54)
WT	200 PhIP	12-40	37	100% (37/37)
hCYP1A2	DMSO	6-24	27	85% (23/27)
WT	DMSO	12-24	20	100% (20/20)
hCYP1A2	DMSO + 1.5% DSS	6-10	20	90% (18/20)

The survival rates in the wild-type treatment groups were much higher than the majority of the hCYP1A2 treatment groups.

Table 4. Incidence and multiplicity of colonic neoplasms induced by PhIP and DSS.

Mouse	Treatment	Duration	Total		Adenomas		Carcinoma	
	(No. of mice)	(weeks)	Incidence	Multiplicity	Incidence	Multiplicity	Incidence	Multiplicity
hCYP1A2	200mg/kg PhIP + 1.5% DSS (7)	12 +	100%	4.86 ± 1.49	0%	0	100%	4.57 ± 1.39
hCYP1A2	200mg/kg PhIP + 1.5% DSS (29)	10	86%	3.90 ± 0.61	20%	0.60 ± 0.16	80%	2.4 ± 0.45
hCYP1A2	200mg/kg PhIP + 1.5% DSS (9)	8	62%	1.67 ± 0.96	33%	1.0 ± 0.32	67%	2.0 ± 1.3
hCYP1A2	200mg/kg PhIP + 1.5% DSS (20)	6	85%	3.75 ± 0.70	5%	0.20 ± 0.13	95%	3.5 ± 0.95
hCYP1A2	100mg/kg of PhIP + 1% DSS (34)	25	2.9%	0.029 ± 0.029	0%	0	100%	0.029 ± 0.029
Wild-type	200mg/kg PhIP + 1.5% DSS (11)	12 +	0%	0%	0%	0	0%	0

hCYP1A2 and wild-type mice were sacrificed at various time points beginning with week 12 after PhIP gavage. Week 6-10 appears to be the best time point for sacrificing the 200mg/kg of PhIP model with 4-10 tumors in 80-90% of the animals. The majority of tumors scored were found to be adenocarcinomas.

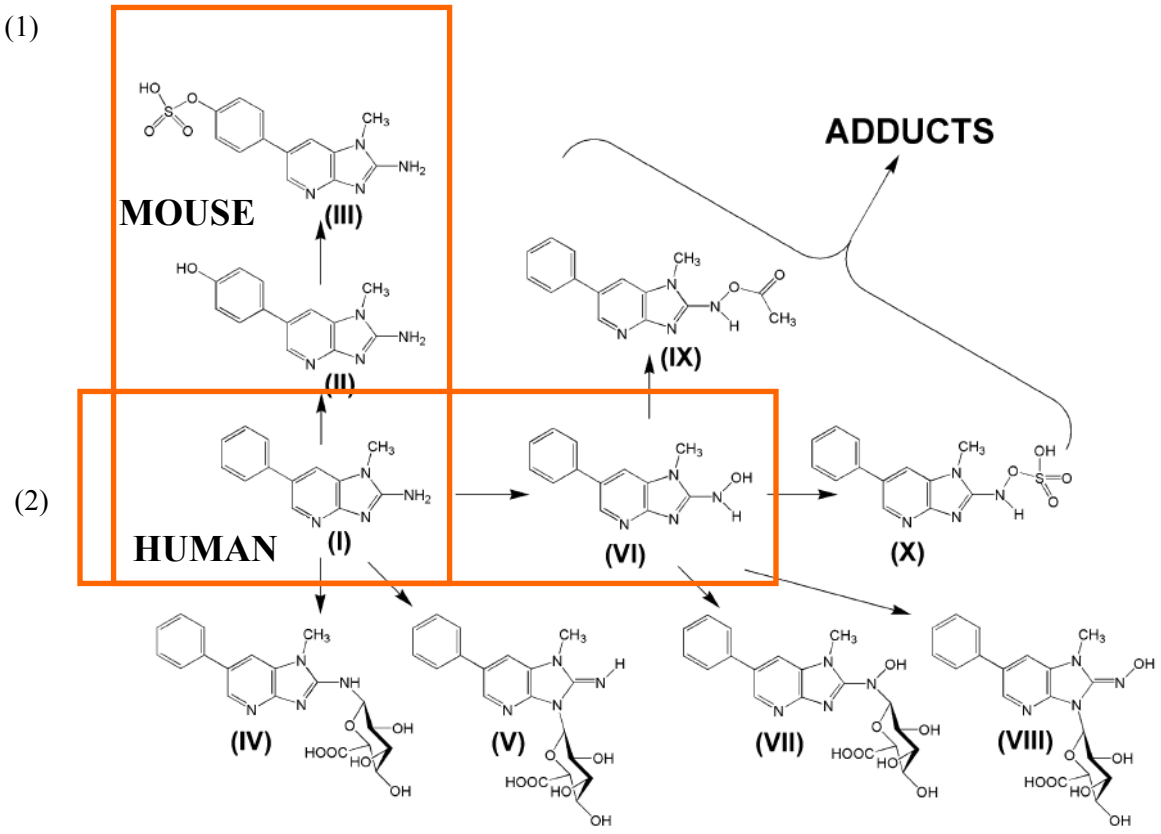

Figure 1. The pathway of metabolic activation of PhIP and species differences in PhIP metabolism (40). (I) The chemical structure of PhIP (II) 4'-hydroxy-PhIP (III) sulfate conjugate (IV) PhIP-N^2-glucuronide (V) PhIP-N^3-glucuronide (VI) N^2-OH PhIP (VII) N^2-OH PhIP N^2-glucuronide (VIII) N^2-OH PhIP N^2-glucuronide (IX) N^2-acetoxy-PhIP (X) N^2-sulfonyloxy-PhIP. Box (1): Primary pathway of detoxification of PhIP to 4'-hydroxy-PhIP and its sulfate conjugate in mice. Box (2): Primary pathway of PhIP activation to N^2-OH PhIP in humans.

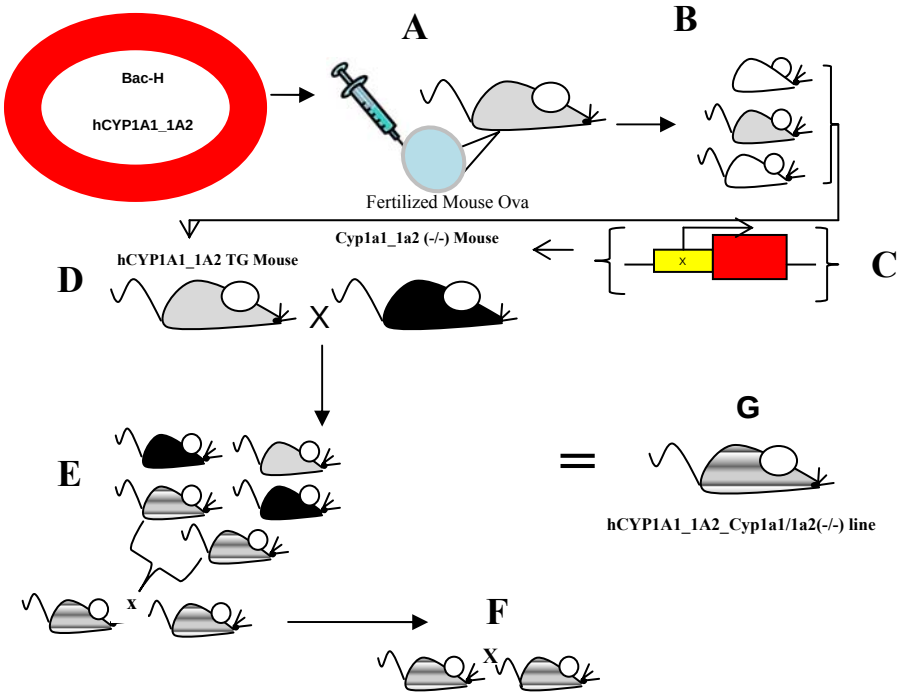

Figure 2. hCYP1A2 humanized mouse model construct (Dragin et al., 2007). A: BAC clone (BAC-H) containing both hCYP1A1 and hCYP1A2 genes were microinjected into fertilized mouse ova; B: Progeny from surrogate mouse were genotyped for the BAC-H insert and backcrossed into B6 background for several generations; C: Cre-recombinase system used for mouse Cyp1a1/1a2 double knockout, backcrossed into B6 background; D: hCYP1A1_1A2 mouse crossed with Cyp1a1_1a2 (-/-) mouse; E: Progeny from founder parents and subsequent F2, F3 generations were found to inherit the hCYP1A1_1A2 genes in the mouse Cyp1a1_1a2 knockout background; F: Breeding pairs purchased from Jackson Laboratories for our purposes, backcrossed into C57BL6/J background to check zygosity, homozygous colony maintained by brother-sister mating resulting in; G: human CYP1A1_1A2 mouse Cyp1a1/1a2 (-/-) line.

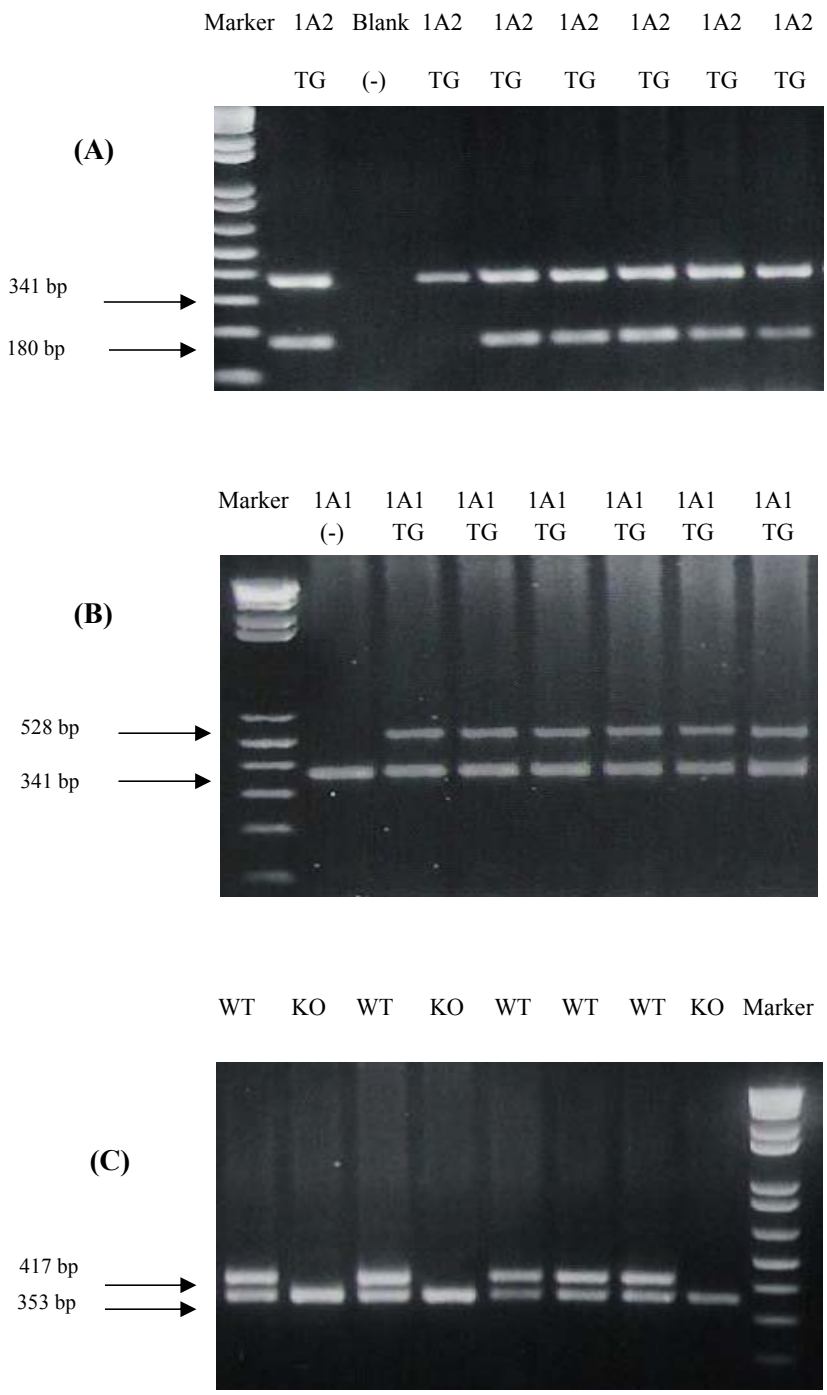

Figure 3. PCR genotyping for hCYP1A1/1A2 genes, Cyp1a1/1a2 knockout and wild-type alleles. (A) hCYP1A1 showed a 180 bp fragment representing a transgenic mouse (TG), Ephx1 which was used as an internal control, showed a 341 bp fragment. (B) hCYP1A1 showed a 528 bp fragment representing a transgenic mouse (TG), Ephx1 again used as an internal control. (C) The wild-type allele showed a 417 bp fragment and the Cyp1a1/1a2 knockout allele was demonstrated by a single band at 353 bp.

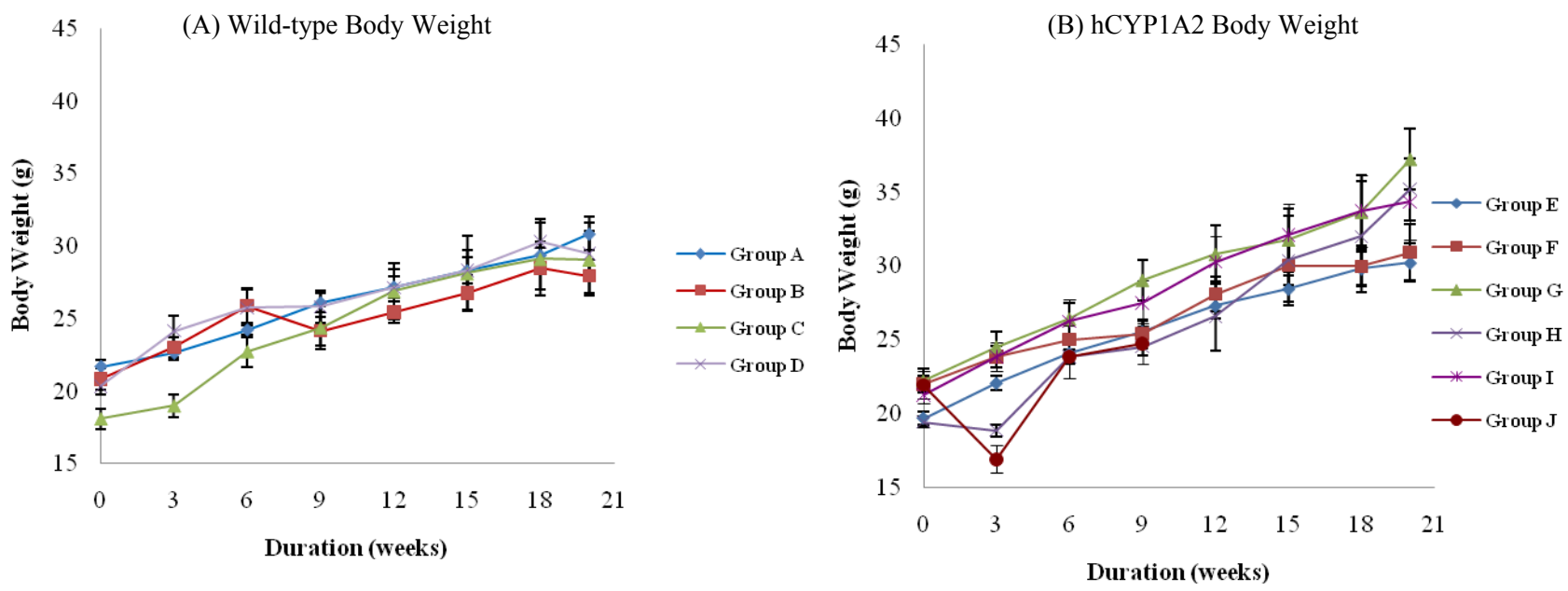

Figure 4. Comparison of the body weight changes between hCYP1A2 and wild-type mice in treatment groups A-J. Graph (A) Group A: Wild-type mice with 200mg/kg of PhIP; Group B: Wild-type mice with 200mg/kg of PhIP + 1% DSS; Group C: Wild-type mice with 200mg/kg of PhIP + 1.5% DSS; Group D: Wild-type mice with 20% DMSO vehicle control. Graph (B) Group E: hCYP1A2 mice with 200mg/kg of PhIP; Group F: hCYP1A2 mice with 100mg/kg of PhIP + 1% DSS; Group G: hCYP1A2 mice with 200mg/kg of PhIP + 1% DSS; Group H: hCYP1A2 mice with 200mg/kg of PhIP + 1.5% DSS; Group I: hCYP1A2 with 20% DMSO vehicle control; Group J: hCYP1A2 mice with 20% DMSO + 1.5% DSS. Error bars represent ± standard error. A slightly larger decrease in body weight after for the hCYP1A2 treatment groups was observed.

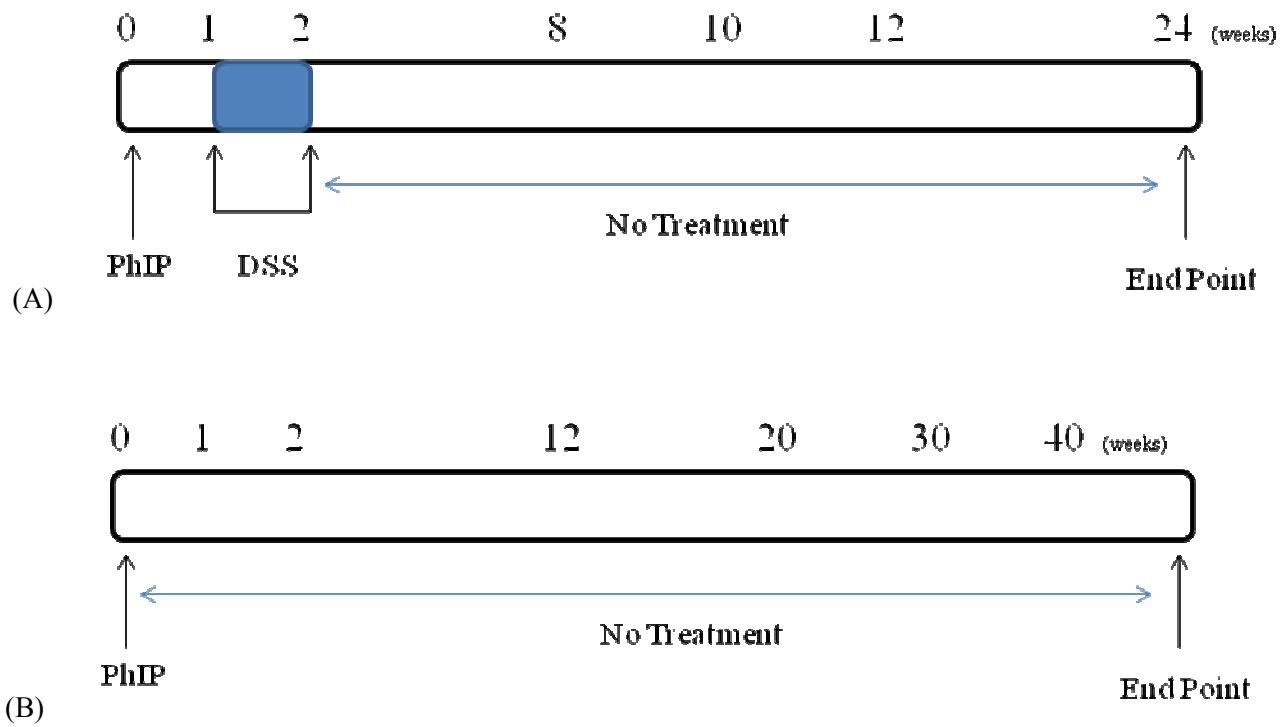

Figure 5. Experimental protocols. (A) Original protocol for PhIP + DSS treatments. Mice were administered a single dose of PhIP (100mg/kg or 200mg/kg) by oral gavage. One week later mice were given 1.0 or 1.5% dextran sodium sulfate (DSS) in their drinking water for 7 days. Mice were sacrificed beginning at week 8, 10 and 12 and the entire duration of the experiment did not go past week 24. (B) Original protocol for PhIP treatment without DSS. Mice were administered a single dose of PhIP (200mg/kg) by oral gavage. Mice were sacrificed beginning at week 12 and approximately every four weeks in between each time-point. This experiment is still on-going and the latest time-point the mice have been sacrificed at is 30 weeks after PhIP gavage.

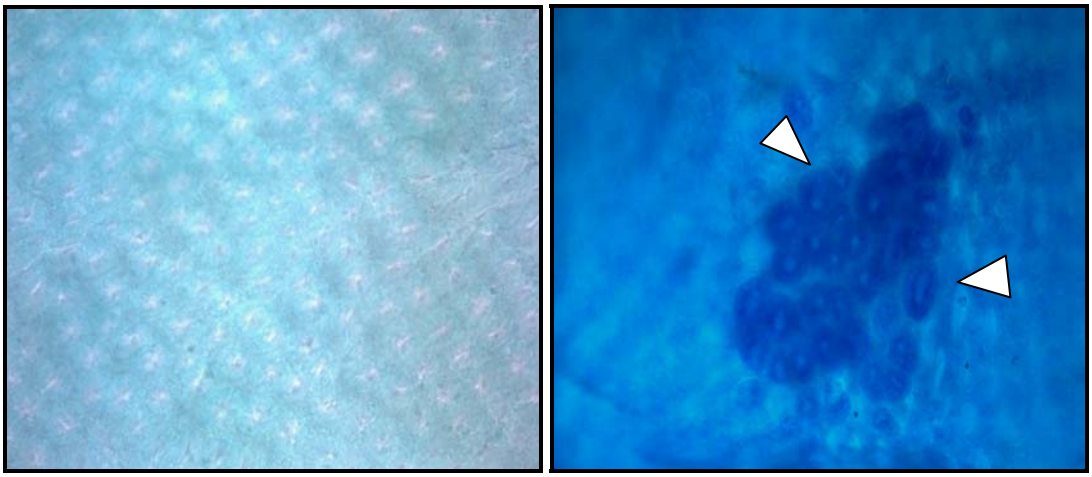

Figure 6a. Methylene blue staining of hCYP1A2 transgenic colons treated with 100mg/kg of PhIP + 1% DSS 20 weeks after PhIP gavage. Arrows indicate sections of dark blue staining of aberrant crypt foci in colon.

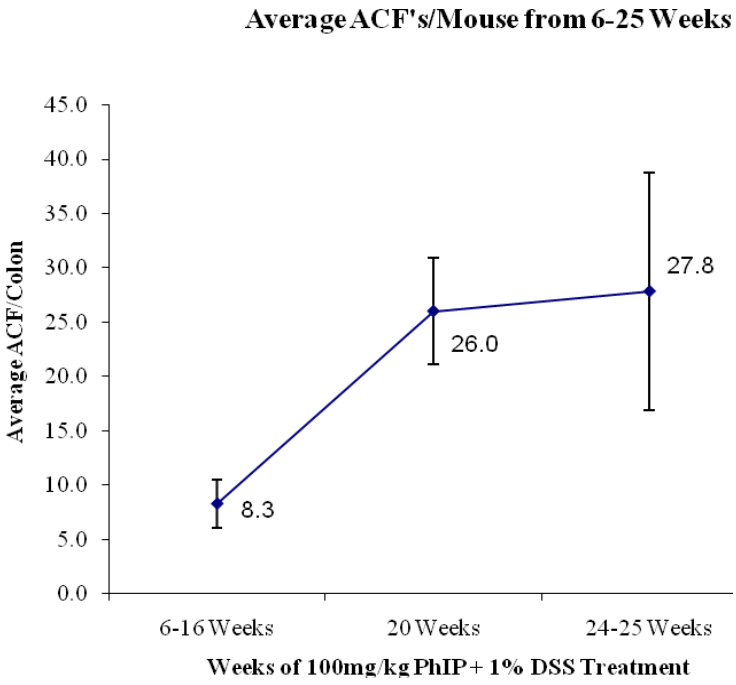

Figure 6b. Distribution of ACF's visualized throughout treatment of 100 PhIP + 1% DSS from weeks 6 to 25. Error bars represent ± standard error of the mean. For 6-16 weeks N=12, for 20 weeks N=9 and for 24-25 weeks N=11. A linear trend can be observed between the number of ACF's and duration of experiment.

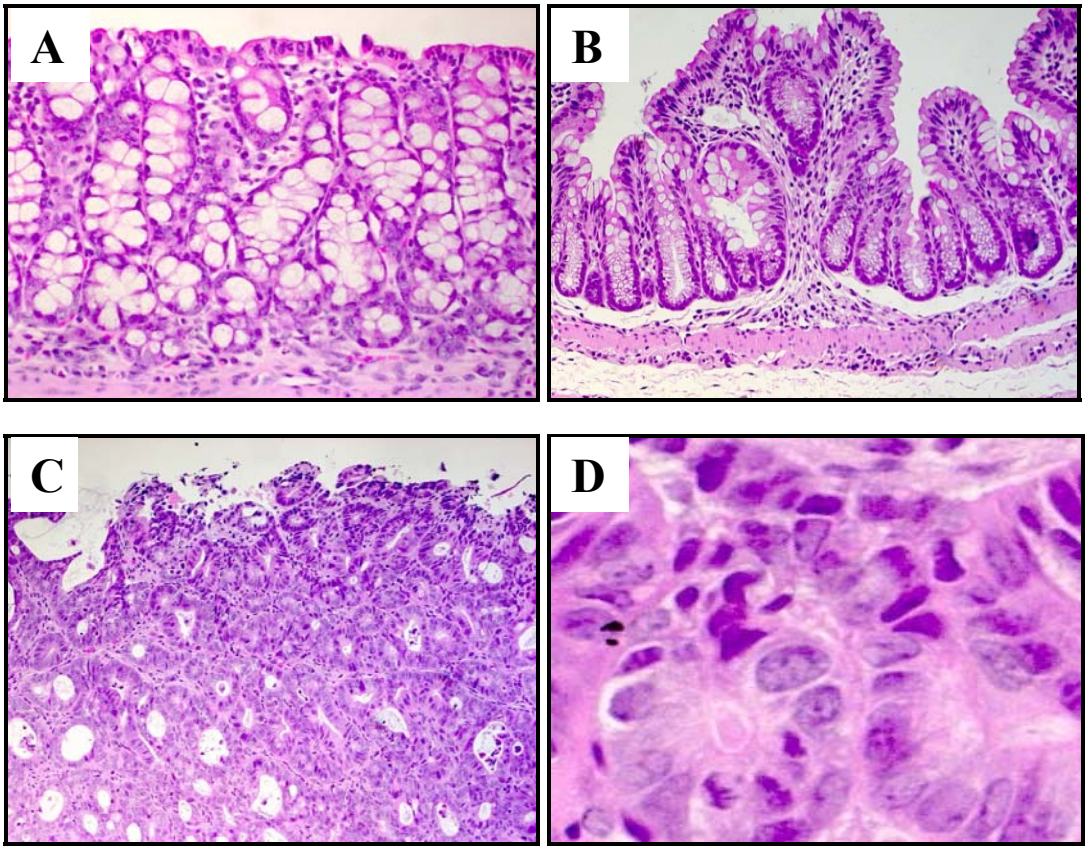

Figure 7. Histopathology of hCYP1A2 transgenic mice treated with 200mg/kg of PhIP + 1.5% DSS (H&E) 6 weeks after PhIP gavage. A: Normal mucosa of transgenic colon (x200); B: Adenomatous tissue of the colon (x200); C: Adenocarcinoma of the colon (x200); D: Adenocarcinoma of the colon (x 400).

(a)

Figure 8(a). Wild-type vs. hCYP1A2 female mice treated with 200mg/kg of PhIP + 1.5% DSS. Left figure represents a wild-type female mouse treated with 200mg/kg of PhIP + 1.5% DSS sacrificed 24 weeks after PhIP gavage. Colon tissue is smooth with no tumors or other malignancies present. Right figure represents a wild-type female mouse treated with 200mg/kg of PhIP + 1.5% DSS sacrificed 10 weeks after PhIP gavage. Colon tissue is very thick with multiple tumors located near the middle to distal end of the colon. Arrows are indicative of select visible tumors.

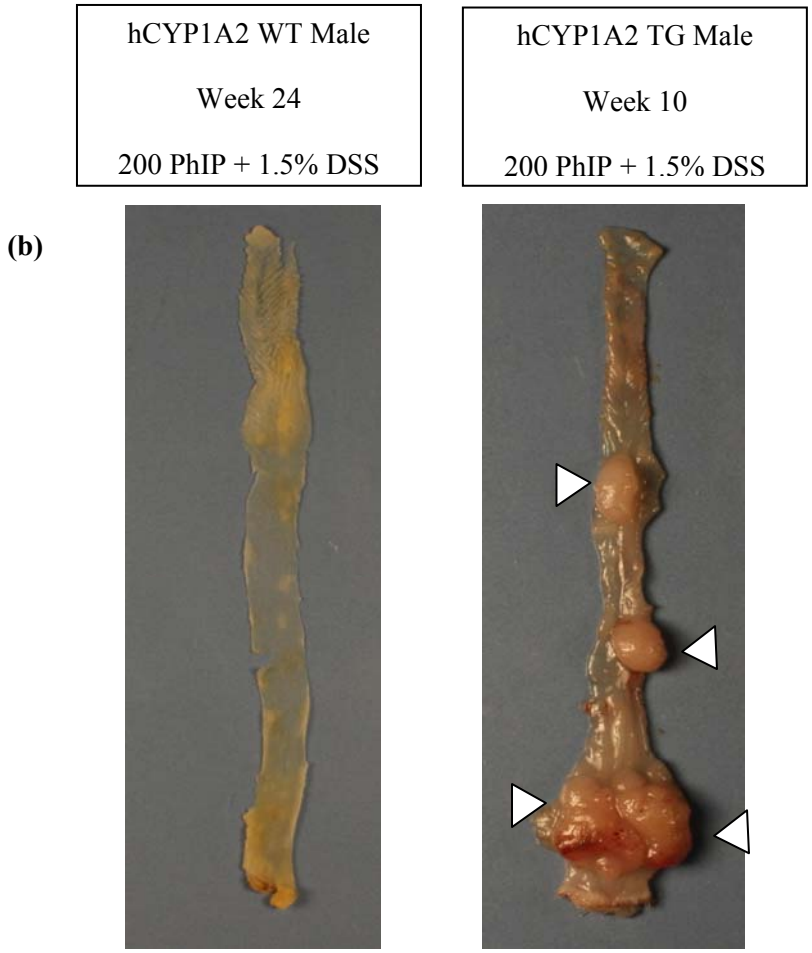

Figure 8(b). Wild-type vs. hCYP1A2 male mice treated with 200 PhIP + 1.5% DSS. Left figure represents a wild-type male mouse treated with 200mg/kg of PhIP + 1.5% DSS sacrificed 24 weeks after PhIP gavage. Colon tissue is smooth with no tumors or other malignancies present. Right figure represents a wild-type male mouse treated with 200mg/kg of PhIP + 1.5% DSS sacrificed 10 weeks after PhIP gavage. Colon tissue is very thick with multiple tumors located near the middle to distal end of the colon. Arrows are indicative of select visible tumors.

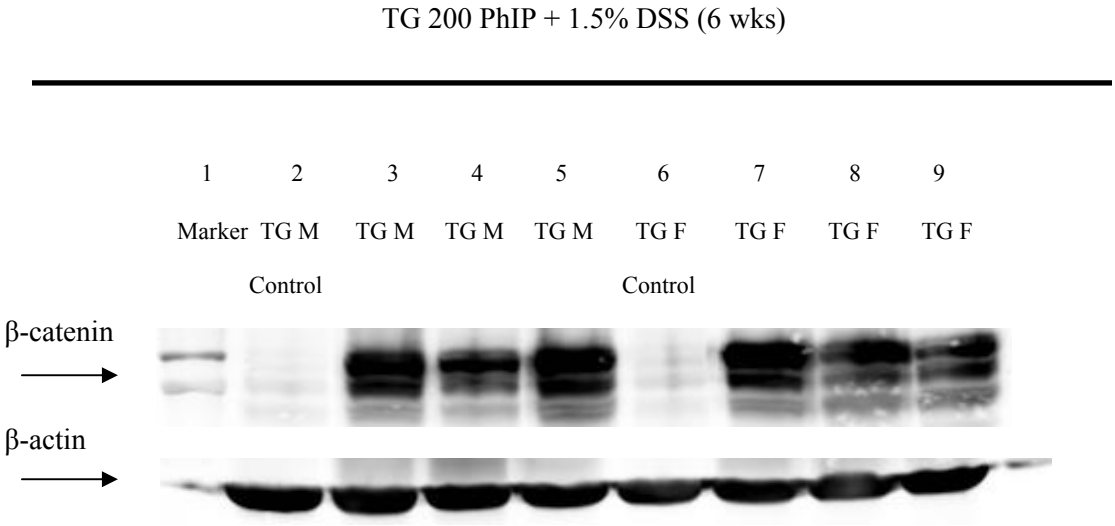

Figure 9. Western blot analysis of β-catenin expression in hCYP1A2 mice treated with 200mg/kg of PhIP + 1.5% DSS 6 weeks after PhIP gavage. Upper blot: Transgenic male and female colon tissue not treated with PhIP were used as negative controls (lanes 2 and 6). Strong β-catenin staining can be observed (around 92 kDa) in the transgenic tissues treated with 200mg/kg of PhIP + 1.5% DSS (lanes 3-5 ND 7-9). Lower blot: β-actin was used as a loading control and was observed around 45 kDa.

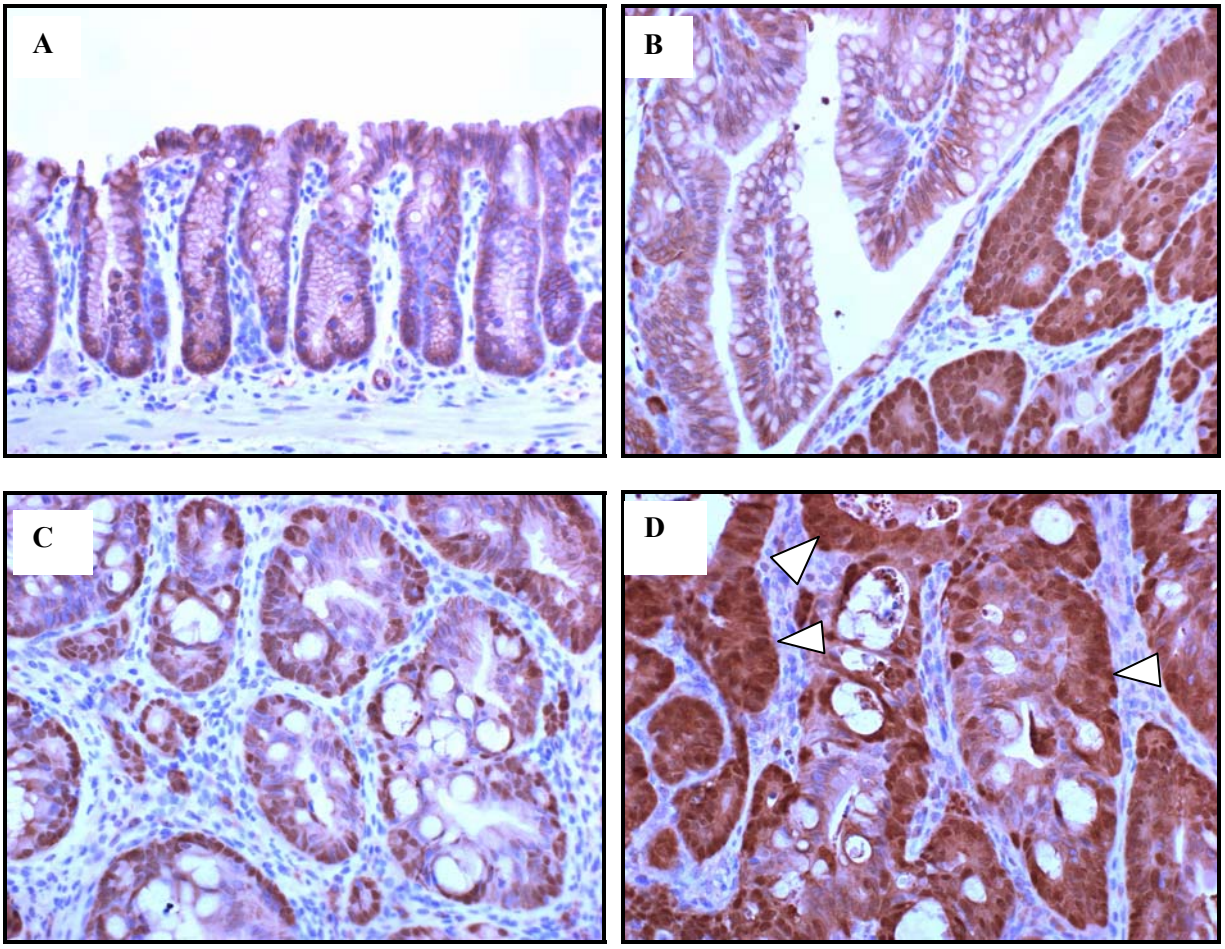

Figure 10. Immunohistochemical analysis of β-catenin in colonic tissue of hCYP1A2 mice treated with 200mg/kg of PhIP + 1.5% DSS 10 weeks after PhIP gavage (x200). A: Normal colon mucosa with very light nuclear localization of β-catenin; B: Comparison of adenoma vs. adenocarcinoma: top left-adenoma observed in hCYP1A2 mouse by lighter membrane staining, bottom right- high grade dysplasia visualized by strong nuclear localization of β-catenin; C: Adenoma visualized by darker nuclear staining compared to normal colon tissue; D: Adenocarcinoma visualized by strong epithelial staining patterns and nuclear localization (certain sections represented by arrows).

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