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# Regulation of obesity-associated inflammation and colon tumorigenesis by resveratrol and adiponectin

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**Regulation of obesity-associated inflammation and colon tumorigenesis by resveratrol  
and adiponectin**

by

**Rebecca Luchtel Boddicker**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
**DOCTOR OF PHILOSOPHY**

Major: Nutritional Sciences

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## ABBREVIATIONS

ACC	acetyl CoA carboxylase
ACF	aberrant crypt foci
AdipoR	adiponectin receptor
AMPK	AMP activated protein kinase
AOM	azoxymethane
AP-1	activator protein 1
APC	adenomatous polyposis coli
APPL1	adaptor protein containing PH domain, PTB domain and leucine zipper motif1
BMI	body mass index
C/EBP	CCAAT/Enhancer binding protein
COX-2	cyclooxygenase 2
DsbA-L	disulfide-bond A oxidoreductase-like protein
DSS	dextran sodium sulfate
ERK	extracellular signal-regulated protein kinase
ERp46	endoplasmic reticulum protein 46
FFA	free fatty acid
GLUT4	glucose transporter 4
HMW	high molecular weight
IFN	interferon
IGF-1	insulin-like growth factor-1
IL	interleukin
IMC+IC	intramucosal carcinoma and invasive carcinoma
iNOS	inducible nitric oxide synthase
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	macrophage chemotactic protein-1
MDF	mucin-depleted foci
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κ B
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
PAI-1	plasminogen activator inhibitor 1
PGC-1α	PPARγ CoActivator 1α
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferator-activated receptor
RACK1	receptor for activated protein kinase C1
ROS	reactive oxygen species
RSV	resveratrol
SFA	saturated fatty acid
SIRT1	sirtuin 1

STAT3	signal transducer and activator of transcription 3
SVC	stromal vascular cells
TGF- $\beta$	transforming growth factor $\beta$
TLR	toll like receptor
TNF $\alpha$	tumor necrosis factor $\alpha$
TNFR	tumor necrosis factor $\alpha$ receptor
VEGF	vascular endothelial growth factor

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**ABSTRACT**

Obesity is characterized by decreased production of the anti-inflammatory hormone, adiponectin, increased secretion of pro-inflammatory cytokines and chemokines, and increased risk of colon cancer. Although adiponectin has been negatively associated with colorectal cancer development in human population, the role of adiponectin in colon tumorigenesis is unknown. The anti-inflammatory dietary polyphenol, resveratrol (RSV), increases circulating adiponectin concentrations *in vivo*, and has been shown to be effective in prevention of colorectal cancer and obesity-related morbidities. However, the effect of RSV supplementation on obesity-linked colorectal cancer had not been previously reported.

We first used an adiponectin knockout mouse model to investigate the role of adiponectin in obesity-associated colorectal cancer. In this study, we showed that in the azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced mouse model of colorectal cancer, adiponectin genotype, high fat diet, and gender regulate colon carcinogenesis. Specifically, development of early lesions (aberrant crypt and mucin-depleted foci) was dependent on adiponectin genotype whereas the development of advanced lesions (intramucosal and invasive carcinomas) was dependent on diet and sex of the mice. Interestingly, adiponectin wildtype (Wt) mice had a greater number of total lesions than the knockout (KO) mice suggesting that under pro-inflammatory conditions, the presence of adiponectin promotes colonic tumorigenesis.

We next investigated the interaction between RSV and adiponectin in early colon tumorigenesis under obesigenic conditions. In this study, we showed that low-dose dietary RSV (20 mg/kg diet) had a tendency to decrease the number of aberrant crypt foci in Wt but not KO mice. Similarly, RSV had a tendency to reduce circulating pro-inflammatory

cytokine and insulin concentrations, prevent adipocyte hypertrophy, and increase lean mass in Wt mice only. Taken together, RSV had a tendency to interact with adiponectin to attenuate tumorigenesis and the metabolic effects of obesity *in vivo*. We further investigated the interaction between adiponectin and RSV *in vitro* through the use of adipocytes derived from adiponectin KO and Wt mice and a colon cancer cell line. Here we showed that in adipocytes but not colon cancer cells, the anti-inflammatory effects of RSV are dependent on adiponectin.

Finally, we compared the effects of Sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK) inhibitors in the attenuation of LPS-induced inflammation by RSV among human adipocyte, monocyte, and colon cancer cell lines. Results obtained from this series of cell culture experiments, suggests that RSV acted through different mechanisms to inhibit chronic LPS-induced inflammation among cell types. In adipocytes, RSV inhibited LPS-induced inflammation and lipolysis, and Sirt1 and AMPK inhibitors reversed this, respectively. In HT29 colon cancer cells, RSV inhibited ROS and proliferation, and the Sirt1 inhibitor, but not the AMPK inhibitor reversed this. Conversely, RSV's inhibition of LPS-induced inflammation in U937 monocytes was not reversed by Sirt1 or AMPK inhibitors. Thus, while Sirt1 appears to be important in the effects of RSV in adipocytes and colon cancer cells, we did not identify a role for Sirt1 or AMPK in RSV's anti-inflammatory action in monocytes.

Collectively, our data suggests that adiponectin, diet, and gender interact in the development of obesity-associated colon tumorigenesis under pro-inflammatory conditions. Moreover, we showed that RSV has a tendency to interact with adiponectin to attenuate tumorigenesis and improve the metabolic and inflammatory profile in obesity. These findings

support the potential use of RSV as an anti-inflammatory dietary supplement aimed at prevention of obesity-associated colon tumorigenesis through anti-inflammatory function in adipose and colon tissues. Continued research is warranted to further elucidate the role of adiponectin and the cell- and tissue-specific actions of RSV in obesity-associated colon cancer.

## CHAPTER 1. GENERAL INTRODUCTION

### Introduction

The prevalence of obesity in developed countries is approaching epidemic levels with over one third of the United States population now classified as obese (1). Obesity poses serious health and economic concerns in the treatment of co-morbidities such as colorectal cancer (2,3,4). The American Cancer Society estimates 102,900 new cases of colon cancer and 51,370 colon cancer related deaths in the year 2010 (5). Of these, overweight and obesity account for an estimated 14% of cancer deaths in men and 20% in women (6).

Obesity-associated co-morbidities are attributed to chronic inflammation characteristic of an obese metabolic state. As adipocyte size expands in obesity, increased adipocyte necrosis and subsequent inflammation signals the infiltration of macrophages into the tissue (7). The adipocytes and immune cells in the dysfunctional adipose tissue together produce a host of pro-inflammatory mediators. This obese adipokine profile is characterized by increased expression of pro-inflammatory adipokines, interleukin-6 (IL-6), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), macrophage chemotactic protein 1 (MCP-1), and decreased expression of the anti-inflammatory adipokine, adiponectin. Furthermore, saturated fatty acids (SFAs) released by hypertrophic adipocytes provide additional inflammatory stimulation through interaction with toll like receptor 4 (TLR4) and subsequent nuclear factor- $\kappa$  B (NF- $\kappa$ B) and activator protein 1 (AP-1) activation in multiple cell types (8). Collectively, the adipokine profile contributes to obesity-associated tumorigenesis through increased oxidative stress, proto-oncogene activation, cell proliferation, and immune cell activation.

In the colon, TLR4 expression is elevated in obesity and is associated with adenocarcinoma and increased risk of disease progression (9,10). Increased TLR4 expression

in multiple cell types allows for transcription of pro-survival and pro-inflammatory genes such as IL-6 and TNF $\alpha$  that in turn directly propagate tumorigenesis (11,12). It has been suggested that the obesity-associated decrease in circulating adiponectin concentration also plays a role in colon cancer development. This has recently been supported by epidemiological studies reporting a negative correlation between circulating adiponectin concentrations and risk of colon cancer (13,14). However, adiponectin knockout mouse models have yielded controversial results regarding the role of adiponectin in colon tumorigenesis (15,16,17). The discrepancies are likely dependent on the different tumorigenesis models, inflammatory stimulus, and dietary conditions.

Resveratrol (RSV) is a polyphenol found in grapes and red wine that has been shown to have a preventative role in metabolic dysregulation associated with obesity as well as colon tumorigenesis. These functions of RSV are attributed to activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1). In mice fed a high calorie obesigenic diet, RSV supplementation improved metabolic profile and decreased reactive oxygen species (ROS) and inflammation (18). Furthermore, dietary RSV has been shown to increase circulating adiponectin concentrations (19). The effects of RSV on colitis and colon tumorigenesis have been well identified in rodent models. To this end, RSV has been demonstrated to inhibit colon cancer cell proliferation and macrophage infiltration through attenuation of iNOS expression and NF- $\kappa$ B activity (20,21,22).

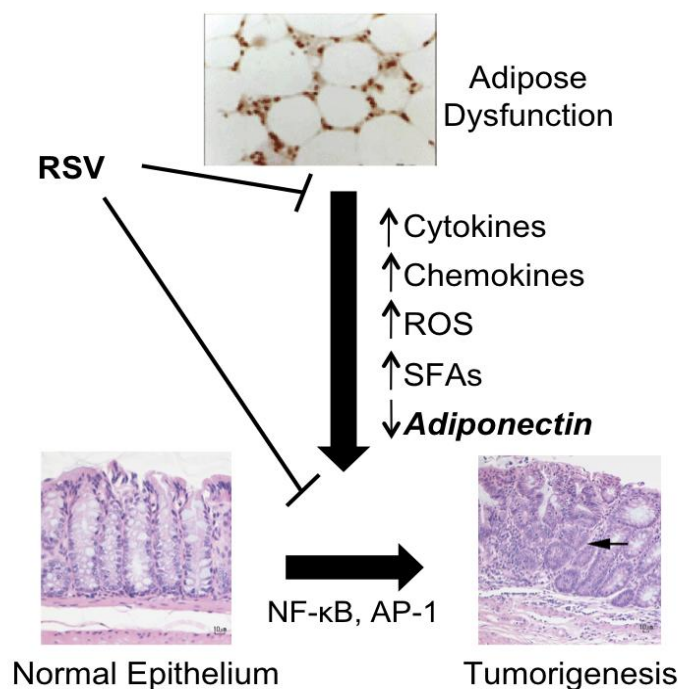
Taken together, this body of literature suggests that obesity-associated colon cancer is initiated in part through the pro-inflammatory adipokine environment that includes decreased adiponectin expression. Moreover, literature suggests that RSV is an effective means of attenuating both the metabolic dysregulation associated with obesity and the development of



colon cancer under normal diet conditions through inhibition of NF- $\kappa$ B activity. However, the role of RSV in obesity-associated tumorigenesis has not been reported. Furthermore, the mechanism of RSV action in cell types involved in obesity-associated colon carcinogenesis has not been fully elucidated. *A mechanistic understanding of the interaction between adipose dysfunction and colon tumorigenesis and the role of RSV in these tissues is critical for the development of therapies targeting obesity-associated colon cancer.*

### **Objective and Specific Aims**

Our *objective* is to understand how adiponectin and RSV and their interaction regulate obesity-associated colorectal cancer. Our *central hypothesis* is that adiponectin regulates colon tumorigenesis through inhibition of inflammation in colonic epithelial cells. We further hypothesize that dietary RSV attenuates colon tumorigenesis in part through increased adiponectin concentrations and subsequent disruption of NF- $\kappa$ B and AP-1 transcription factor activity (**Figure 1**). The *rationale* for our work is that an understanding of the mechanisms by which RSV regulates obesity-associated colon tumorigenesis will promote the development of future therapies targeting pathways involved in RSV signaling. Our *specific aims* are to i) identify the role of adiponectin in obesity-associated colon tumorigenesis, ii) determine the importance of adiponectin in attenuation of obesity-associated colon tumorigenesis by RSV, and iii) elucidate mechanisms by which RSV acts to reduce inflammation in cell types involved in obesity-associated colon cancer.



Adipose image adapted from Davis, et al. 2008, *Obesity*

**Figure 1.** *The proposed roles of RSV and adiponectin in attenuation of colonic tumorigenesis.*

Adipocyte hypertrophy results in increased production of cytokines and chemokines and decreased production of the anti-inflammatory adipokine, adiponectin. Concurrently, macrophages are signaled to infiltrate the tissue to clear necrotic adipocytes, which release SFAs and ROS in addition to inflammatory mediators. The systemic pro-inflammatory environment characteristic of adipose dysfunction promotes activation of TLR4 and the transcription factors NF- $\kappa$ B and AP-1 in colonic tissue. This in turn drives tumorigenesis through production of pro-survival and pro-inflammatory gene products. We hypothesize that RSV inhibits this process through action at adipose and colon tissues. In adipose tissue, we hypothesize that RSV increases production of adiponectin and decreases pro-inflammatory gene expression thus attenuating NF- $\kappa$ B and AP-1 activation in the colon. Furthermore, we hypothesize that RSV signals directly at the colon to inhibit these pathways by Sirt1 and AMPK activation.

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## CHAPTER 2. REVIEW OF LITERATURE

### Obesity and Co-Morbidities: Role of Chronic Inflammation

#### Obesity prevalence and implications

The incidence of obesity continues to affect a growing proportion of the United States population. In 2007-2008, the prevalence of adult obesity (BMI  $\geq$  30) in the United States was estimated to be 32.2% for men and 35.5% for women (1). Combined with obesity, the prevalence of overweight adults (BMI  $\geq$  25) was a staggering 68% for the same time period. This high prevalence of overweight and obesity poses serious burdens both on the health and economies of developed countries (2). While the populations of developing countries have a lower incidence of obesity than those of developed countries, the rate of obesity is also climbing in these countries with highest prevalence in women and rural communities (3,4).

Visceral or central adiposity is highly correlated with metabolic syndrome (5). The third Report of the National Cholesterol Education Program Expert Panel defines metabolic syndrome as the presence of at least three of the following criteria: waist circumference greater than 102 cm in men and 88 cm in women, 150 mg/dL serum triglycerides, high-density lipoprotein cholesterol less than 40 mg/dL in men and 50 mg/dL in women, blood pressure of at least 130/85 mm Hg, or serum glucose of at least 110 mg/dL (6). Using these criteria, the Panel estimated the prevalence of metabolic syndrome to be 23.7% with higher prevalence reported for higher age groups, women, and ethnicities (6). In addition to metabolic syndrome, obesity predisposes individuals to a host of chronic diseases including diabetes mellitus, heart disease, respiratory disease, kidney disease, osteoarthritis, and cancer (7). Alternatively, benign obesity occurs in a small subset of the population whom, although obese, remains metabolically healthy (8).

### **Obesity-associated chronic inflammation and co-morbidities**

Obesity is often characterized by low-grade, chronic inflammation. While acute inflammation is a critical step in a host's immune response to pathogen and injury, chronic inflammation is compromising to overall health by several mechanisms. The main outcomes of an inflammatory response are increased membrane permeability, infiltration of immune cells into tissue, proliferation, and angiogenesis. These functions are accomplished by inflammatory mediators that can have deleterious effects when chronically elevated in the circulation and tissues. Inflammatory mediators include cytokines, chemokines, adhesion molecules, growth factors, angiogenic factors, and reactive oxygen species (ROS) that collectively act to promote development of obesity-related co-morbidities.

### **Adipose expansion and inflammation**

Adipose tissue is composed of an integrated network of adipocytes, endothelial cells, immune cells, and connective tissue that functions as a dynamic endocrine organ. The cell types that make up adipose tissue secrete a myriad of hormones, cytokines, and chemokines collectively referred to as adipokines (9,10). The secretion of adipokines is a highly regulated process that in a normal state has a net anti-inflammatory effect. However, disruption of the normal milieu in obesity results in a shift to a pro-inflammatory adipokine profile. Nearly all co-morbidities of obesity can be attributed, at least in part, to chronic inflammation (11,12).

In obesity, inflammation is initiated by adipocyte expansion and increased cell turnover (13,14). Adipocyte turnover, or remodeling, requires recruitment of macrophages to aide in necrosis through formation of crown-like structures around the necrotic adipocyte (15). Cytokines and chemokines secreted by the activated macrophages along with nucleic acids released from necrotic adipocytes stimulate local inflammation in surrounding adipose

tissue (16). Adipose inflammation is mediated by pro-inflammatory adipokines, which play an integral role in further monocyte and macrophage recruitment (17).

### **Saturated free fatty acids and inflammation**

An increase in circulating concentrations of free fatty acids (FFAs) is also observed in obesity. The cause of this phenomenon is two-fold: 1) down-regulation of FFA clearance from blood and 2) increased FFA release from adipocyte turnover. Saturated fatty acids (SFAs) have been causally linked to stimulation of inflammation in adipocytes and macrophages. Daniel Hwang's group was one of the first to demonstrate an inflammatory response to SFA in RAW 264.7 macrophages (18). In this study, the SFA lauric acid, induced inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) expression in macrophages through activation of toll like receptor 4 (TLR4) and nuclear factor  $\kappa$  B (NF- $\kappa$ B). In adipocytes, SFAs cause an inflammatory response through TLR4 and TLR2 that results in activator protein 1 (AP-1), c-Jun N-terminal kinase (JNK), and NF- $\kappa$ B activation (19,20,21). Moreover, inhibition of TLR-mediated signaling blunts basal and palmitate-stimulated pro-inflammatory cytokine secretion and NF- $\kappa$ B activation *in vitro* (22) and partially protects against obesity-induced inflammation *in vivo* (23,24).

Increased FFA release from adipocytes also results in ectopic lipid deposition in non-adipocytes referred to as lipotoxicity. Lipotoxicity describes the cytotoxic accumulation of lipid in liver, pancreas  $\beta$  cells, skeletal muscle, and cardiomyocytes (25). The cytotoxic lipid and ceramide accumulation causes apoptosis of non-adipocytes, thereby contributing to insulin resistance and metabolic syndrome (26,27).

## **Role of Adipokines in Obesity-Associated Inflammation**

### **Pro-inflammatory cytokines**

Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF $\alpha$ ) are cytokines secreted from adipose tissue that act both locally on adipose tissue and systemically on multiple cell types to mediate an inflammatory immune response (28,29). Adipose production of IL-6 and TNF $\alpha$  is elevated in obesity (28,30,31), and decreases with weight loss (32,33). Elevated cytokine concentrations contribute to the pathogenesis of many obesity-associated diseases including insulin resistance, cardiovascular disease, hepatic inflammation, and many cancers (34).

**IL-6.** The transcription of IL-6 can be activated by multiple transcription factors involved in adipose inflammation including NF- $\kappa$ B, AP-1, and CCAAT/Enhancer binding protein beta (C/EBP- $\beta$ ) (35). Adipose tissue is a major source of IL-6, contributing up to one third of circulating IL-6 in healthy adults (29). IL-6 binds its receptor, IL-6R $\alpha$  or soluble IL-6R $\alpha$ , in multiple cell types and complexes with gp130 to activate a downstream signaling cascade beginning with janus kinase (Jak1) binding and subsequent activation of signal transducer and activator of transcription (STAT3) (36). IL-6 is key activator of liver acute phase protein synthesis and contributes to angiogenesis and endothelial dysfunction through increased vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor 1 (PAI-1) expression, respectively (37,38,39).

The importance of IL-6 has been demonstrated in studies investigating the common IL-6 -174G>C polymorphism in the promoter region of the human IL-6 gene. The IL-6 genotype significantly affects circulating IL-6 concentrations and risk of developing insulin



resistance and other obesity-associated metabolic complications (40,41). The role of IL-6 has been further defined through the use of transgenic animal models. Over-expression of IL-6 in muscle of mice led to hyperinsulinemia and impaired glucose uptake despite a reduction in adipocyte size and body weight (42). These mice also had increased F4/80 macrophage infiltration into the liver. The reduction in adipocyte size and weight is due to increased energy expenditure, the opposite of which is also evident in IL-6 deficient mice. IL-6 deficient mice develop obesity and leptin resistance that are reversed with IL-6 injections (43).

**TNF $\alpha$ .** In obese individuals compared to lean, adipose production of TNF $\alpha$  increases 2.5 fold (44). Like IL-6, TNF $\alpha$  is regulated by NF- $\kappa$ B activation, and acts in both an autocrine and paracrine manner. TNF $\alpha$  signals through two transmembrane receptors, TNF $\alpha$  Receptor 1 (TNFR1) and 2 (TNFR2) to activate NF- $\kappa$ B, JNK, and mitogen activated protein kinases (MAPK) such as extracellular signal-regulated protein kinase (ERK1/2) and p38 as reviewed by Cawthorn and Sethi (45). These signaling cascades result in propagation of inflammation, and over time, development of insulin resistance through decreased GLUT4 expression (46). TNF $\alpha$  knockout and TNFR knockout mice have reduced, but not diminished adiposity and are partially protected from loss of insulin sensitivity compared to mice with functional TNF $\alpha$  fed a high fat diet (47). TNF $\alpha$  has also been demonstrated to be responsible for the obesity-associated increase in the adhesion molecule, E-selectin (48).

### **Chemokines**

Chemokines such as macrophage chemotactic protein 1 (MCP-1, also known as CCL2) and macrophage inflammatory protein (MIP1 $\alpha$ , also known as CCL3) are important

mediators of obesity-induced inflammation and co-morbidities. The primary functions of chemokines are recruitment of immune cells to sites of inflammation, activation of inflammatory immune response, and control of cell migration through tissues. Adipose expression of MCP-1 and MIP1 $\alpha$  is elevated in obesity but is not consistently reflected in serum concentrations of these chemokines due to their local action (49,50). In addition to the primary role of chemokines in immune function, chemokines also function in regulation of glucose homeostasis in adipose tissue under conditions of obesity.

Of these chemokines, MCP-1 has been most prominently linked to adipose inflammation and adipose-linked co-morbidities of obesity. Over-expression of MCP-1 in adipose leads to elevated IL-6 and TNF $\alpha$  adipose expression and insulin resistance (51). In adipocyte cell culture, MCP-1 causes decreased glucose uptake through down-regulation of GLUT4 (52). Chronic MCP-1 infusion in mice resulted in development of insulin resistance, macrophage infiltration in adipose tissue, and hepatic steatosis (53). Similarly, over-expression of MCP-1 in adipose tissue of mice using the adipocyte promoter (aP2) resulted in macrophage accumulation in adipose tissue, insulin resistance, and increased circulating pro-inflammatory cytokine and FFA concentrations (51). Conversely, obese mice lacking MCP-1 receptor (CCR2) have reduced macrophage infiltration into adipose tissue, decreased hepatic steatosis, and improved insulin sensitivity (54).

### **Adiponectin**

Adiponectin (also known as Acrp30 and AdipoQ) is an anti-inflammatory and insulin-sensitizing hormone produced primarily by adipocytes. The 30 kDa protein circulates in three forms with distinct biological activity and functions: trimer, hexamer, and high molecular weight (HMW). Agonists of the adiponectin promoter, peroxisome proliferator-

activated receptor  $\gamma$  (PPAR $\gamma$ ) have been shown to increase adiponectin expression (55) and up-regulate the enzyme responsible for adiponectin multimerization, disulfide-bond A oxidoreductase-like protein (DsbA-L), and other endoplasmic reticulum proteins involved in adiponectin processing and stabilization (56). A feed-forward mechanism has also been demonstrated between adiponectin and PPAR $\gamma$  (57). Adiponectin concentrations decrease as adipose tissue expands in obesity. This is accomplished by down-regulation of DsbA-L and PPAR $\gamma$  (58).

Adiponectin signals through two 7-transmembrane receptors, adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) expressed differentially on multiple cell types (59). Several binding proteins have recently been implicated in adiponectin signaling including adaptor protein containing PH domain, PTB domain and leucine zipper motif 1 (APPL1), endoplasmic reticulum protein 46 (ERp46), receptor for activated protein kinase C1 (RACK1) and protein kinase CK2 $\beta$  (60). Downstream signaling pathways from AdipoR1 and AdipoR2 have not been fully elucidated. However, targeted disruption of AdipoR1 and AdipoR2 revealed preferential downstream activation of AMPK and PPAR $\alpha$ , respectively (61). Additionally, both receptors regulate ERK 1/2, p38, JNK, and STAT3 signaling pathways (62,63). The involvement of these signaling pathways in obesity and its co-morbidities makes adiponectin a promising therapeutic target.

In a long term mouse obesity study, Bullen et al. reported decreased adiponectin and increased adiponectin receptors after prolonged high fat diet feeding in mice (64). The elevated inflammation that often accompanies obesity is likely due in part to decreased adiponectin production. In adipocytes, adiponectin inhibits lipopolysaccharide (LPS)-induced NF- $\kappa$ B activation and IL-6 and MCP-1 production (57,65). Adiponectin has been shown to

inhibit pro-inflammatory macrophage signaling and promotes activation of M2 macrophages to aid in resolution of a pro-inflammatory response, in part through AMPK and PPAR $\gamma$  signaling (66,67). Obesity-induced decrease in circulating adiponectin concentrations (especially HMW) may also promote insulin resistance. Adiponectin participates in regulation of glucose uptake by increasing APPL1 and Rab5 binding to AdipoRs, resulting in GLUT4 translocation (68,69). Insulin sensitizing effects of adiponectin are mediated by AMPK and PPAR $\alpha$  activation via AdipoR1 and AdipoR2, respectively (70).

### **Pathogenesis and Mechanisms of Obesity-Associated Colon Cancer**

#### **Obesity and colon cancer risk**

The National Program of Cancer Registries reported more than 800,000 cases of colon cancer in the United States from the years 1999 to 2004 (71). This report also revealed a greater gender disparity (males > females) in people older than 65 yr and a greater race disparity (blacks > other races) in people under the age of 65. In the year 2010 alone, the American Cancer Society estimated 102,900 new cases of colon cancer and 51,370 colon cancer related deaths (72). Obesity is associated both with elevated risk of colorectal cancer (73,74) and with colon cancer reoccurrence and mortality (75). Of total cancer deaths in the United States, overweight and obesity account for an estimated 14% of cancer deaths in men and 20% in women (76).

#### **Molecular and pathological progression of obesity-linked colon cancer**

The multi-hit model is widely used to describe the process of spontaneous colon carcinogenesis. The premise of this model is that of accumulated damage. Multiple “hits” or genetic mutations are required to evade clearance by the immune system and allow promotion and progression of a tumor cell. The majority of colon cancer is initiated by a

somatic or germ-line mutation in the Wnt pathway adenomatous polyposis coli (APC) gene or in mismatch repair genes, MLH and MSH (77). Somatic mutations can result as a consequence of inflammation-linked oxidative DNA damage. If not detected, these initial molecular “hits” progress to hyperplasia and formation of small lesions such as aberrant crypt foci (ACF) that are frequently accompanied by depletion of mucin-secreting goblet cells.

The progression from ACF to adenoma is often caused by mutation in a proto-oncogene such as *K-ras*, resulting in proliferation of the tumor cells. Up-regulation of proto-oncogenes and angiogenic factors is stimulated by growth factors and pro-inflammatory cytokines common to the obese serum profile. Inflammation in obesity-induced colonic epithelium may also be derived from increased intestinal endotoxin transport and interaction of saturated FFAs with TLRs (78). Adenomas are highly associated with elevated cyclooxygenase 2 (COX-2) due to increased prostaglandin signaling and are characterized by dysplasia and often display a vascularized stalk-like structure. The transition from an adenoma to a malignant phenotype or carcinoma is often accompanied by additional mutations in the tumor suppressor genes p53 and TGF- $\beta$  Receptor. The metastatic potential of carcinomas often results in invasion of the submucosa layer and entry into the circulation. Colon cancer cells primarily metastasize to the liver via the portal system connecting the intestinal tract and liver. In addition to APC, K-ras, and p53 mutations commonly observed in human colorectal cancer, alterations in DNA methylation and chromatin regulation have recently been identified as a common event in human colon cancer (79).

Evidence now suggests that location of the tumor within the colon may present with different characteristics. Development of tumors in the right vs. left side of the colon is

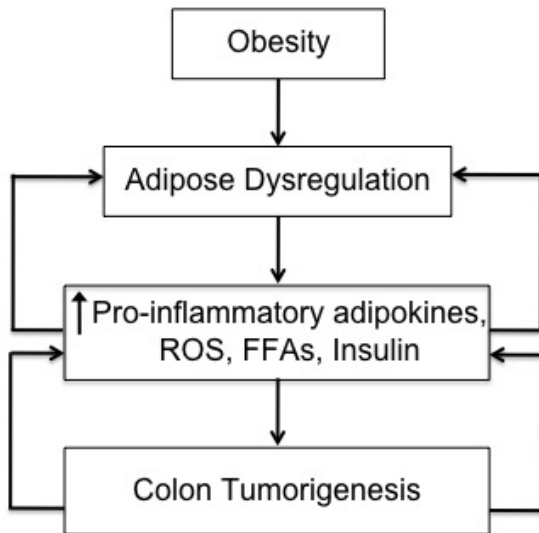
influenced by age, gender, and environmental factors (80,81). Along these lines, histological features and tumor subtype are influenced by colon location (proximal vs. distal and right vs. left) (82). MiRNA profile has also been shown to vary between proximal, distal, and rectal colonic tumors (83). Thus, it is becoming increasingly important to evaluate specific tumor subtypes and locations separately when assessing colon carcinogenesis.

### **Research animal colon cancer models**

Chemical induction of animal carcinogenesis is aimed to imitate spontaneous gene carcinogenesis pathway in humans. The most effective chemical carcinogenesis strategies involve both initiation and promotion. In a rodent model of colon cancer, the most common initiator is azoxymethane (AOM), a stable metabolite of dimethylhydrazine. AOM is a precursor to methylazoxymethanol, a metabolite that initiates PI3K/Akt pathways (84,85). Like spontaneous colon carcinogenesis in humans, AOM-induced carcinogenesis causes  $\beta$ -catenin accumulation in the nucleus mutation of *K-ras*. However, AOM-induced carcinogenesis typically does not cause p53 or APC mutations as are often observed in human cancer (86,87). Irritants such as dextran sodium sulfate (DSS) are often combined with AOM to promote carcinogenesis through inflammation and oxidative stress (88). The choice of rodent strain is important as strains respond differentially to AOM/DSS treatment with Balb/c and C57BL/6 mice developing great number of carcinomas than C3H/HeN and DBA/2N strains (89).

### **Mechanisms of obesity-linked colorectal carcinogenesis**

Obesity contributes to colorectal tumorigenesis through dysregulation of adipokine, cytokine, chemokine, ROS, FFA, and insulin production as summarized in **Figure 2**.



**Figure 2.** *Progression of obesity-associated colon tumorigenesis.* The hypertrophy of adipose tissue in obesity results in dysregulation of adipokine secretion and subsequent transition to a pro-inflammatory milieu characterized by increased production of cytokines, chemokines, ROS, and FFAs. This environment promotes colon distress and tumorigenesis, further contributing to chronic inflammation and propagation of tissue dysfunction.

### Adipokines and colon cancer

The adipokines, adiponectin and leptin, are linked to obesity-associated colon cancer risk. Several human studies have reported an association between decreased serum adiponectin concentrations and increased risk and severity of colorectal cancers (90,91,92). Furthermore, differential AdipoR1 and AdipoR2 expression has been detected in human tumors of the colon compared to normal colon tissue suggesting a possible role for adiponectin signaling in colon tumorigenesis (93,94,95). However, there also exist reports of no association between circulating adiponectin and colon cancer, illustrating the multifactorial nature of metabolic syndrome and tumor development (96).

Adiponectin knockout mouse models have produced equally inconsistent results. The first *in vivo* mouse model of adiponectin deficiency and colitis was reported in 2006 by Nishihara, et al. (97). In this study, authors reported a protective effect of adiponectin against colitis after administration of 0.5% DSS for 15 days. The study was complicated by the use of non-littermate knockout and wild-type mice and the high basal circulating concentration of

TNF $\alpha$  in these mice. The following year, Fayad et al. reported that adiponectin deficient mice were protected from DSS-induced colitis compared to wild-type littermates (98). The authors proposed that adiponectin was inhibiting the activity of epidermal growth factor and heparin binding epidermal growth factor through direct binding, thereby inhibiting their protective activity in DSS-induced colitis. The first study using AOM-induced tumorigenesis and high saturated fat diet-induced obesity in an adiponectin deficient mouse model was reported in 2008 by Fujisawa et al. (99). In contrast to results reported in the DSS-model, adiponectin was protective against obesity associated colon cancer development in the AOM model. This is somewhat surprising considering the pro-inflammatory potential of fatty acids. The investigation of the role of adiponectin in colon carcinogenesis has been complicated by the varied effects of carcinogenic agents, diet, and model. Further research is warranted to identify the specific effects of experimental factors and their interaction with adiponectin.

Mechanistically, adiponectin is a logical molecular target for obesity-linked colon cancer prevention due to its role in AMPK and PPAR activation and inhibition of NF- $\kappa$ B (100,101,102,103). In support of this, two groups have recently demonstrated that adiponectin exerts an AMPK-dependent anti-proliferative effect on human colon cancer cells (102,104). Moreover, PPAR $\gamma$ , the transcription factor responsible for induction of adiponectin transcript, is considered anti-neoplastic in colon cancer (100). Microarray analysis of multiple cancer cell lines treated with full length adiponectin revealed a broad mechanism of action with most differentially expressed genes falling within inflammation, stress response, and proliferation regulation (105). Interestingly, it was recently shown that the effect of adiponectin on cancer cell cancer proliferation is glucose-dependent whereby adiponectin supports DLD-1 colon cancer cell survival in low glucose but inhibits



proliferation under high glucose conditions (106). In contrast to this, pro-inflammatory effects of adiponectin in monocytic cell and colon cancer cells have been reported *in vitro* (107,108). Taken together, adiponectin is an attractive target for obesity-associated colon cancer, but further research is needed to completely identify the potential actions of adiponectin.

Leptin has been implicated in colon cancer cell proliferation and invasiveness through activation of key pathways including phosphoinositide 3-kinase (PI3K), Src, Wnt, NF- $\kappa$ B, and insulin-like growth factor (IGF) pathways (109,110,111). Interestingly, colon cancer cells treated with conditioned media from leptin-deficient ob/ob or Wt adipocytes showed that leptin mediates the proliferative effect of adipocytes (112,113). Conversely, Ealey et al. reported that that leptin does not play a role in early lesion formation using ob/ob and db/db mice (114).

Rather than consider adiponectin and leptin separately as risk factors, the leptin:adiponectin ratio has recently emerged as a positive risk factor for colon cancer (115). In breast cancer cell lines, an interaction was observed between leptin and adiponectin whereby adiponectin treatment caused a decrease in leptin and leptin receptor expression and leptin treatment decreases AdipoR1 expression (116). Moreover, when adiponectin and leptin are administered to breast cancer cells concurrently, the anti-proliferative effects of adiponectin and pro-proliferative effects of leptin are negated. Further interactions have been reported between leptin and adiponectin whereby adiponectin rescued the cancer-promoting effects of leptin on p53, Bcl2, and NF- $\kappa$ B in prostate, hepatic, and colon cancer cells (117,118,119).

### **Inflammation and colon cancer**

Inflammation controls a fine balance between tumor suppression and promotion. An acute inflammatory response can promote immune recognition and clearance of tumor cells whereas chronic inflammation contributes to genomic instability, angiogenesis, and proliferation of tumor cells. A strong causative association between inflammation and colon cancer has been established in inflammatory diseases such as Crohn's disease and colitis (120,121). This is demonstrated by data showing NSAIDs (non-steroidal anti-inflammatory drugs) such as aspirin effectively prevent colorectal adenoma development (122,123).

Expression of the pro-inflammatory cytokines IL-6 and TNF $\alpha$  is up-regulated in adipose tissue in obesity. In addition to autocrine and paracrine effects on adipose tissue, adipose-derived IL-6 and TNF $\alpha$  can signal in an endocrine manner to activate NF- $\kappa$ B in tumor cells. In turn, the tumor cells increase production of cytokines, chemokines, and COX2 leading to a feed-forward propagation of tumor cell proliferation and growth (124,125). This association was demonstrated in obese subjects who underwent diet-induced weight loss (126). Weight loss of on average 10% of initial body weight resulted in decreased colonic expression of MCP-1, TNF $\alpha$ , and IL-1 $\beta$ , a 42% reduction in macrophage number, and downregulation of STAT3 and NF- $\kappa$ B pathways. IL-6 itself drives colon cell proliferation (127) and contributes to damage of colonic tissue through increased expression of MMP, ICAM, and VCAM and increased immune cell infiltration (35). The importance of TNF $\alpha$  in colon cancer has been demonstrated through targeted disruption of TNF $\alpha$  signaling. Anti-TNF $\alpha$  antibody blocked NF- $\kappa$ B activation in epithelial cells (128). In line with this, mice lacking TNF Receptor p55 were protected from AOM/DSS-induced immune cell infiltration, mucosal damage, and tumorigenesis compared to wild-type mice (129).

The effects of pro-inflammatory cytokines on tumorigenesis are mediated in part through TLR4 and NF- $\kappa$ B activation. In obesity, TLR4 expression is up-regulated by increased circulating and colonic endotoxin as well as adipose- and diet-derived SFAs (78,130). TLR4 expression is associated with adenocarcinoma and increased risk of disease progression (131,132). In support of this, mice transgenic for constitutive activation of TLR4 were more susceptible to AOM/DSS-induced carcinogenesis (132). Moreover, amelioration of TLR4 signaling in DSS models of colitis reduced disease severity, decreased MAPK p38, c-Jun, IL-6, and TNF $\alpha$  mucosal expression, and inhibited macrophage and dendritic cell infiltration (133,134). However, TLR4 blockage also inhibited repair of DSS-induced mucosal tissue damage (134), illustrating the importance of inflammation-mediated tissue repair to colonic integrity. The dual roles of TLR4 are explained by transcription of growth factors, cytokines, and chemokines by NF- $\kappa$ B that both promote tumorigenesis and tissue repair.

### **Oxidative stress and colon cancer**

Up-regulation of ROS production occurs upon activation of NF- $\kappa$ B in colonic epithelium, immune cells, and adipose tissue in obesity (135,136,137). When produced in large quantities, ROS overwhelm the oxidant:anti-oxidant balance, resulting in oxidative damage to DNA (138). The persistence of ROS-induced DNA damage leads to genomic instability and ultimately formation of a cancer cell (139). This is supported by multiple human studies showing a strong association between oxidative stress and colon cancer (140,141,142). ROS also activates molecular pathways that play a role in carcinogenesis such as the MAPKs, p38 and JNK (143), and cytokine production by NF- $\kappa$ B (144).

### **Insulin dysregulation and colon cancer**

High insulin and glucose are associated with increased risk for colorectal adenomas (145,146). Moreover, diabetes is an independent risk factor for several cancers types, including colon, with risk increasing across BMI categories (146,147,148). These associations support the hypothesis that obesity-induced hyperinsulinemia and increased active IGF-1 promote colon cancer (149). Hyperinsulinemia promotes colon cancer cell proliferation through activation of the mammalian target of rapamycin (mTOR)/c-Myc pathway and nuclear accumulation of  $\beta$ -catenin (150). Chronic injections of insulin increased ACF multiplicity and tumors in rats after AOM injections (151,152). Blockage of downstream insulin signaling through insulin receptor substrate 1 (IRS-1) inhibition results in attenuation of colon cancer cell proliferation (153). Moreover, mice with muscle-specific insulin receptor knockout develop fewer AOM-induced ACF than mice with functional insulin receptor (154).

IGF-1 Receptor (IGF-1R) is over-expressed in human adenocarcinoma (155), and expression of IGF-1R is positively associated with severity and metastasis of colorectal cancer (156). IGF-1 mediates proliferation and apoptosis signals for many cell types, and as such has been highly implicated in promotion of tumorigenesis (157). The RNAi knockdown of IGF-1R *in vitro* causes chemosensitization and reduced proliferation of human colon cancer cells (158). Liver-specific IGF-1 deficient mice have reduced circulating IGF-1 concentrations and show attenuation of AOM-induced colorectal proliferation and tumorigenesis (159). Furthermore, growth of human colorectal tumors injected intrahepatically (a common metastasis site) in mice was inhibited by IGF-1R antibody (160).

The link between insulin dysregulation and colon cancer is further demonstrated by diabetes drugs that have been shown to attenuate colon tumorigenesis. Pioglitazone, a PPAR $\gamma$  agonist, reduces ACF multiplicity in humans (161). Moreover, Metformin reduces ACF development in AOM mouse model through AMP-activated protein kinase (AMPK)-dependent decrease of IGF-1 (162,163). In a retrospective cohort analysis of human diabetic subjects on various diabetes treatments, those patients receiving insulin therapy had an elevated risk of colorectal cancer whereas those patients taking Metformin had the lowest risk of colorectal cancer (164).

### **Dietary fat and colon cancer**

There is a paucity of human data relating dietary fat and colon cancer risk, and the currently published data is inconsistent. Older epidemiological studies report a positive association between dietary fat and colon cancer (165,166,167,168,169). Conversely, more recent prospective and retrospective human studies have reported little or no association between saturated fat and colon cancer (170,171,172,173). There is yet another set of literature that argues general caloric intake rather than saturated fat contributes to colon cancer risk (165,174,175). Interestingly, dietary fat restriction increased incidence of CRC reoccurrence in patients over a 4 year period (176). These controversial results are the consequence of varied statistical methods for inclusion of genetic mutations, food composition, degree of cancer severity, and collection of subject diet information.

In contrast to human data, a causal association between dietary fat type or amount and development of colon cancer has been reported in several rodent carcinogenesis models. One of the first reports of the role of fat quantity and type in colon carcinogenesis was performed by Reddy et al. in 1976 (177). In this study, diets of 20% corn oil or 20% lard increased

dimethylhydrazine-induced colon tumor incidence compared to 5% fat diets, but there were no differences observed between fat type and incidence. In a follow-up study, Reddy et al. demonstrated that type of fat could modify colon cancer development where replacement of 75% of a 23.5% corn oil diet with fish oil resulted in a significant reduction in development of AOM-induced tumors (178). Rao and colleagues further investigated the effects of high saturated fat, high omega-3 fat, or low fat corn oil diets in an AOM rat model (179). In this study, rats fed the high saturated fat diet developed greater colon tumor multiplicity, decreased colonic apoptosis, and higher COX-2 activity than other diets. A similar role for high saturated fat has been reported in benzo(a)pyrene and tribromomethane models of carcinogenesis and hepatic metastasis (180,181,182). Together, these results suggest that both amount and type of fat are important in regulation of colon tumorigenesis.

Recently, a more mechanistic relationship has been built between dietary fat and colon cancer. Endo et al. showed that a high fat but not low fat diet promoted ACF formation and colon cancer cell proliferation through the JNK pathway in an AOM mouse model (183). In support of this, circulating triacylglycerols are positively associated with colonic oxidative stress and lesion formation (184). The pro-inflammatory effects of saturated fats on adipose tissue discussed above provide support for the data that suggests saturated fats potentiate colon carcinogenesis. It is possible that saturated fat elicits a toll like receptor-mediated immune response in the colon as in adipose tissue. It is also plausible that saturated fat-induced adipokines act on the colon to propagate inflammation and carcinogenesis. In addition to induction of inflammation, a potential mechanism by which high fat diets promote colon carcinogenesis is through increased bile acid synthesis and bile acid-induced DNA oxidative damage. Bile acids have been shown to be carcinogenic (185) and to be

increased under high fat diet conditions (186,187). However, animal studies aimed at this mechanism have not yet been reported. Further investigation is warranted in this field to identify the effects of dietary fat and obesity on cancer risk.

### **Resveratrol Functional Properties and Bioavailability**

#### **Functional properties**

Resveratrol (*trans*-3,5,4' trihydroxystilbene, RSV) is a polyphenol found in grapes and red wine among other plant products. RSV is synthesized in plant species in response to stress by the enzyme RSV synthase (trihydroxystilbene synthase) (188). The polyphenolic and lipophilic structure of RSV account for its antioxidant properties as well as its uptake by tissues. RSV primarily exists in foods as its glucoside, *trans*-piceid (189). However, RSV aglycone is the form most widely studied, commercially available, and most commonly used in supplements.

#### **Absorption, metabolism, and bioavailability**

The bioavailability of RSV was investigated by Walle, et al. through the tracking of 25 mg <sup>14</sup>C-RSV administered orally to six human subjects (190). Walle, et al. reported high absorption (70%) with 2 μM peak plasma metabolite and RSV concentrations occurring at 1 hr. A second peak of 1.3 μM occurred at 6 hr. after intake. Unfortunately, amounts of unchanged <sup>14</sup>C-RSV were not well detected (<5 ng/ml) in the serum. The primary metabolite of RSV detected in the serum was the sulfate-conjugate (although the glucuronide form was also detected). The fate of this metabolite is currently unknown regarding tissue uptake and metabolism. RSV is ultimately eliminated in the urine (191) and is not reportedly toxic (192). A similar study was performed following <sup>14</sup>C-RSV after oral administration in Balb/c mice (193). In consensus with the human study, whole blood circulation concentrations were 1.5

$\mu\text{M}$ . However, the use of mice allowed researchers to evaluate tissue concentrations of RSV revealing high accumulation in the liver and kidney (25  $\mu\text{M}$  and 50  $\mu\text{M}$ , respectively). Taken together, these studies provide extremely valuable information regarding the metabolic processing of RSV and indicate that RSV may indeed be a realistic therapeutic tool.

There are several factors that may affect circulating RSV detection. In light of a recent report showing RSV conjugates are detected better in whole blood than serum (194), RSV may circulate at higher concentrations than previous studies have reported. Additionally, the *cis* isomer of resveratrol is rarely measured specifically, but exists in the circulation. However, the biological activity of *cis*-RSV is largely unknown due to the lack of commercial availability. Recent evidence suggests that RSV bioavailability may also be affected by circadian rhythm and that highest bioavailability occurs in the morning (195).

RSV absorption and metabolism has also been investigated *in vitro* through the use of the Caco-2 intestinal cell culture model (196,197). RSV aglycone is better absorbed than the glucoside *trans*-piceid due to its lipophilic properties allowing for passive diffusion of RSV while *trans*-piceid is transported by SGLT-1 (197). It has been well established that both RSV and piceid are metabolized upon uptake into the enterocyte. Piceid may be hydrolyzed to release RSV by the enzymes  $\beta$ -glucosidase in the cytosol or the membrane-bound lactase phlorizin hydrolase (198,199). RSV is primarily modified by sulfonation and glucuronidation. As demonstrated in Caco-2 cells, these modifications may render RSV less bioactive once absorbed (196). In addition to RSV metabolites, RSV is also found circulating bound to serum albumin or hemoglobin, resulting in a longer half-life (200).



## Resveratrol and Obesity

The promising anti-obesity effects of RSV were introduced in 2006 when Sinclair and colleagues showed RSV supplementation (0.04%) increased survivability in high calorie-fed mice (201). This effect was accompanied by increased insulin sensitivity, decreased hepatic lipid accumulation, increased mitochondrial function, and improved overall metabolic profile. In response to this study, a number of molecular targets have been identified linking RSV with anti-obesity effects.

### Primary molecular targets

**SIRT1.** It is well established that RSV is a SIRT1 activator. SIRT1 is an NAD<sup>+</sup>-dependent deacetylase of histones, transcription factors, and other non-histone proteins located in the nucleus of several cell types including white adipose, skeletal muscle, and liver (202). Borra, et al. proposed that RSV directly binds SIRT1 thereby inducing a conformational change that enables interaction between SIRT1 and its substrate group (203). SIRT1 activates a wide range of genes including PPAR $\gamma$ , PPAR $\gamma$  CoActivator 1 $\alpha$  (PGC-1 $\alpha$ ), and FoxO1. These pathways are critically involved in adipocyte differentiation, metabolism, and regulation of ROS (204). In general, the effects of resveratrol are not negated when SIRT1 is knocked out, but rather diminished suggesting an additional route of action of resveratrol (205).

**AMPK.** In addition to SIRT1, AMPK has recently been recognized as a primary target of RSV. The mechanism by which RSV activates AMPK is currently unknown. It has been proposed that AMPK activation is linked to SIRT1 activation (206). This is supported by evidence that SIRT1 regulates LKB1, the upstream AMPK activating kinase (207).

However, acute RSV-induced LKB1 activation of AMPK has been demonstrated

independent of SIRT1 (208). Moreover, it is now recognized that PGC-1 $\alpha$  activation requires both SIRT1 deacetylation and AMPK phosphorylation (209). The individual effects of AMPK activation are on fatty acid oxidation through phosphorylation of acetyl CoA carboxylase (ACC) and stimulation of glucose transport (210,211).

### **Anti-obesity physiological effects**

#### **Adipokines and inflammation**

The anti-inflammatory properties of RSV are mediated in part through adipokine regulation. Numerous studies have reported increased adiponectin production in response to RSV treatment both *in vitro* (212,213,214) and *in vivo* (215). In an intervention study of participants with elevated serum CRP, subjects given a supplement containing RSV among other antioxidants had a 7% increase adiponectin concentrations and a decrease in the overall inflammation (216). Until recently, the mechanism by which RSV increased adiponectin concentrations was unknown because expression and stability of the adiponectin transcription factor, PPAR $\gamma$ , is reportedly down-regulated by RSV in adipocytes (217,218). Recently, Wang, et al. reported one potential mechanism of adiponectin regulation by RSV in which RSV activates DsbA-L, the enzyme responsible for multimerization of adiponectin to form its HMW structure (214). They further described the activation of DsbA-L as Sirt1-independent, and AMPK- and Foxo1-dependent pathway both *in vivo* and *in vitro*.

Decreased pro-inflammatory cytokine production in adipocytes and macrophages is another mechanism whereby RSV ameliorates obesity-associated inflammation. In 3T3-L1 adipocytes, RSV suppressed macrophage conditioned media-induced IL-6 and TNF $\alpha$  secretion through NF- $\kappa$ B p65 inhibition (213). RSV also suppressed MCP-1, COX2, IL-6, and IL-1 $\beta$  expression in TNF $\alpha$ -stimulated adipocytes through NF- $\kappa$ B inhibition (219,220).

Using human macrophages and adipocytes, McIntosh's group recently demonstrated a role for MAPKs (JNK and p38) and AP-1 in addition to NF- $\kappa$ B in the anti-inflammatory effects of a grape powder extract containing RSV (221). A similar study was conducted using human monocytes wherein RSV inhibited LPS- and TNF $\alpha$ -induced MAPK, NF- $\kappa$ B, and AP-1 pathways in monocytes and lymphatic cells (222). The inhibition of pro-inflammatory cytokine and chemokine secretion by RSV in adipose tissue and adipocytes has been shown to be SIRT1-dependent (205,223).

RSV has also been shown to alleviate inflammation-induced oxidative stress. In TNF $\alpha$ -treated adipocytes, RSV decreased ROS production and increased glutathione peroxidase, superoxide dismutase, and glutathione S-transferase activity (224). Moreover, RSV functions to prevent hepatic steatosis (201,225) and improve endothelial function (226) through attenuation of ROS production.

### **Energy balance and metabolism**

RSV has been demonstrated to improve the obese metabolic profile through increasing fatty acid oxidation, glucose uptake, and insulin sensitivity. Growth of both pre-adipocytes (205) and mature adipocytes (217) was down-regulated by RSV through increased mitochondrial activity and decreased expression of genes involved in adipogenesis and fatty acid synthesis. Similarly, obese Zucker diabetic rats treated with long-term daily RSV supplementation (10mg/kg body weight) had increased liver AMPK phosphorylation (Thr172) and ACC phosphorylation, resulting in increased  $\beta$ -oxidation (215). RSV improved insulin-stimulated glucose uptake in C2C12 myotubes (210) and adipocytes (205,213) through inhibition of IRS-1 serine phosphorylation and increase in tyrosine phosphorylation.

Importantly, RSV has also been shown to activate hepatic PGC-1 $\alpha$  thereby allowing for greater thermogenic potential (201,227).

### **Resveratrol and Colon Cancer**

RSV is a promising chemotherapeutic agent due to its ability to attenuate initiation and progression of carcinogen-induced colon cancer (228,229,230,231). The molecular targets of RSV, SIRT1 and AMPK, function to attenuate colon carcinogenesis by mechanisms similar to its anti-obesity effects: inflammation, oxidative stress, and proliferation.

#### **Inflammation and oxidative stress**

The role of RSV in colitis and inflammation-associated tumorigenesis has been best studied using the irritant, DSS. The addition of 0.03% RSV to the diet of mice resulted in a dose-dependent decrease in DSS-induced colitis and a significant decrease in AOM/DSS-induced tumorigenesis (232). Singh and colleagues showed that RSV (100 mg/ kg body weight) prevented DSS-induced colitis through an increase in SIRT1 and decrease in NF- $\kappa$ B that was accompanied by a reduction of serum IL-6, IL-1 $\beta$ , IFN- $\gamma$  and decreased macrophage number in lamina propria (233). In similar studies of DSS-induced colitis, RSV protected the colonic mucosa through attenuation of iNOS expression and NF- $\kappa$ B activity (234,235). It is noteworthy for the interpretation of studies combining RSV and DSS treatments that the impact of RSV on DSS metabolism is currently unknown. The role of RSV in oxidative stress was further demonstrated by RSV-induced activation of p53 in colon cancer cells in response to NO production (236).

### **Cancer cell proliferation**

In human patients given 0.5 or 1 g RSV prior to undergoing surgical resection of malignant colon tumors, colon cell proliferation was reduced by 5% compared to patients that did not receive RSV (237). Interestingly, tissue accumulation of RSV was higher on the right side of the colon compared to the left in these patients. It has been shown that RSV inhibits HT29 and SW480 colon cancer cell proliferation in part through suppression of IGF-1R protein levels *in vitro* (238). In support of this, daily intake of RSV has been shown to also markedly decrease circulating IGF-1 and IGF binding protein-3 concentrations in mice (201) and humans (239). High dose RSV (100-150  $\mu$ M) has been shown induce apoptosis through activation of p53 *in vitro* (240) and induction of endoplasmic reticulum stress (241). However, this high circulating concentration of RSV is not attainable *in vivo* from dietary RSV, and as such is not a likely physiological mechanism of dietary RSV.

### **Summary**

The prevalence of obesity and co-morbidities continues to rise in developed countries. Hypertrophic adipose tissue contributes to an obese inflammatory profile through down-regulation of adiponectin and up-regulation of leptin, IL-6, and MCP-1. The resulting increase in NF- $\kappa$ B activation and MAPK signaling promote development of colorectal cancer through increased proliferation, proto-oncogene expression, and oxidative stress. Additionally, metabolic dysregulation associated with adiposity contributes to tumorigenesis through stimulation of proliferation and inflammation by hyperinsulinemia and SFAs, respectively. While adipose-derived cytokines and hormones have been suggested to play a role in the pathogenesis of colon cancer, the mechanisms have not been elucidated. The best-studied adipokine in colorectal tumorigenesis is adiponectin. However, the literature is

inconsistent regarding the role of adiponectin in colon tumorigenesis with both protective and tumorigenic roles reported. Furthermore, the interpretation of the current literature is complicated by the varied cancer models and diets used. The characterization of adiponectin function in development of obesity-associated colorectal cancer is important for the development of therapeutic targets aimed at disease prevention.

The polyphenol, RSV, has reported therapeutic potential for prevention of both colon cancer and obesity-related metabolic perturbations. In obesity models, RSV has been shown to attenuate inflammation, improve insulin sensitivity, increase fatty acid oxidation, and alleviate oxidative stress. Moreover, RSV mediates the adipokine profile through up-regulation of adiponectin expression and down-regulation of pro-inflammatory cytokines and chemokines. RSV has also been shown to successfully inhibit multistage colon carcinogenesis and proliferation of colon cancer cells. The preventative role of RSV in obesity and colon cancer makes it a promising dietary strategy for prevention of obesity-associated colon cancer. However, the function of RSV in colon carcinogenesis has not been reported in the context of obesity. Moreover, the interaction between RSV and adipokines in colon cancer is not known. Sirt1 and AMPK have been identified as the primary targets of RSV. However, the specific roles of Sirt1 and AMPK in the anti-inflammatory function of RSV are unknown in the context of obesity-associated colon cancer. The elucidation of the mechanistic action of RSV in cell types involved in obesity-associated colon cancer is important for potential therapeutic target detection. Predicated on this literature base, we hypothesized that adiponectin regulates colon tumorigenesis through inhibition of inflammation in colonic epithelial cells. We further hypothesized that dietary RSV attenuates

colon tumorigenesis in part through increased adiponectin concentrations and subsequent disruption of NF- $\kappa$ B and AP-1 transcription factor activity.

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**CHAPTER 3. EARLY LESION FORMATION IN COLORECTAL CARCINOGENESIS IS ASSOCIATED WITH ADIPONECTIN STATUS WHEREAS NEOPLASTIC LESIONS ARE ASSOCIATED WITH DIET AND SEX IN C57BL/6J MICE**

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**Abstract**

Adiponectin is an anti-inflammatory and insulin-sensitizing hormone that is decreased in obesity. Although controversial, it has been suggested that decreased adiponectin contributes to colorectal cancer risk in obesity. To further investigate the role of adiponectin in obesity-linked colorectal carcinogenesis, we utilized male and female adiponectin knockout (KO) and wildtype (Wt) C57BL/6J mice. Tumorigenesis was induced in all mice with the combined treatment of azoxymethane (AOM) and dextran sodium sulfate (DSS). Following AOM/DSS treatment, mice were fed a low fat, control (LFC) or high fat, lard (HFL) diet for 7½ weeks. We report that KO mice developed fewer total lesions than Wt mice, males developed fewer lesions than females, and mice fed HFL diet developed fewer lesions than those fed the LFC diet. Early lesion multiplicity was influenced by genotype, while advanced lesion development was influenced by sex and diet. Moreover, lesion types were differentially correlated with serum adipokines and colon gene expression of adiponectin receptors, insulin receptor and toll like receptor 4. These data suggest that in the AOM/DSS model of carcinogenesis, adiponectin functions to promote early lesion development whereas sex and diet are important regulators of advanced lesion development through pathways involved in inflammation and insulin signaling.

## Introduction

Adiponectin, an abundant protein secreted primarily from adipose tissue, signals through AdipoR1 and AdipoR2, which are expressed on many cell types (1,2,3). This adipokine functions to increase insulin sensitivity and reduce inflammation in adipocytes and other cells (4,5). As adipose tissue expands in high fat diet-induced obesity, the adipokine profile promotes systemic inflammation and insulin resistance through depression of adiponectin expression and concurrent increases in expression of pro-inflammatory cytokines, including IL-6, lipocalin 2 (Lcn2) and TNF $\alpha$  (6,7). The pro-inflammatory environment resulting from a high saturated fat diet increases risk for colon carcinogenesis (8).

Although low serum adiponectin concentration was recently identified as a potential factor in development of several malignancies, including colorectal cancer (9,10,11), this association is controversial and is not supported in all populations reported (12). In contrast with its anti-inflammatory role in adipocytes, a pro-inflammatory role has been reported for adiponectin in colonic epithelial cells *in vitro*. Administration of globular and full length adiponectin to colonic epithelial cells resulted in stimulation of pro-inflammatory cytokine secretion and proliferation (13). Moreover, adiponectin receptor expression was elevated in colons of patients with colorectal malignancies compared to patients with normal colons, and *in vitro*, adiponectin receptors were activated by adiponectin in colon adenocarcinoma cell lines, suggesting that adiponectin is recognized by the colon and therefore may play a role in colorectal cancer development (14). In addition to adiponectin, several factors related to adipose tissue expansion have been correlated to risk of colorectal cancer including hyperinsulinemia, IGF-1 resistance, leptin resistance, and dyslipidemia (15,16,17,18,19).

The role of adiponectin in colonic inflammation and colorectal cancer pathogenesis has recently been studied *in vivo* through use of adiponectin- and adiponectin receptor-deficient mice. Results of these studies are conflicting and have proposed both protective and promoting roles for adiponectin in dextran sodium sulfate (DSS)- or azoxymethane (AOM)-induced inflammation and colorectal carcinogenesis (20,21,22). In addition to DSS and AOM treatment differences, these discrepancies may be due to treatment duration, lesion endpoint analysis, and high fat vs. low fat diets differences between studies. To further investigate the association between these parameters and adiponectin in colorectal carcinogenesis, we used our adiponectin deficient mouse model coupled with combined treatment of AOM and DSS-induced carcinogenesis to which the C57BL/6 strain is highly sensitive (23). This animal model was designed to elucidate the roles of chronic adipose inflammation characteristic of high saturated fat diet-induced obesity and sex under conditions of adiponectin deficiency in carcinogenesis and neoplastic progression.

### **Materials and Methods**

*Animals.* Adiponectin-deficient mice were developed by Xenogen Biosciences Corporation (Cranbury, NJ) using homologous recombination in mouse embryonic stem cells and subsequent blastocyst injection of the appropriate targeted embryonic stem cells. The mouse chromosome 16 sequence (n.t.# 21,898,000~21,978,000) was retrieved from the Ensembl database Build 32 and used as reference. BAC clone RP23-364K13 was used for generating homologous arms and southern probes by PCR or RED cloning/gap-repair method. The 5' homologous arm (5.3 kb) and the 3' homologous arm (4.8 kb) were generated by RED cloning/gap repair. They were cloned in FtNwCD vector and confirmed by restriction digestion and end-sequencing. The final vector was obtained by standard

molecular cloning method. Aside from homologous arms, the final vector also contains FRT flanked Neo expression cassette (for positive selection of the embryonic stem cells), and a DTA expression cassette (for negative selection of the embryonic stem cells). The final vector was confirmed by both restriction digestion and end sequencing analysis. NotI was used for linearizing the final vector for electroporation. The 5' and 3' external probes were generated by PCR reaction using proofreading LA Taq DNA polymerase (Takara Bio, Madison, WI), and were tested by genomic Southern analysis for screening of the embryonic stem cells. The probes were cloned in the pCR2.1 backbone and confirmed by sequencing. Pups were screened via PCR specific to the Neo gene insertion and four male heterozygote founder mice were shipped to our laboratory at Iowa State University. Heterozygotes were bred to wild type females, and offspring were genotyped. Heterozygotes were then bred to heterozygotes to establish the colony.

Forty adiponectin knockout (KO) and forty wild type (Wt) mice of both sexes were generated for this study. Genotypes were determined by PCR of genomic DNA extracted from tail clips using the following primers: Forward1 CCAACTAAGACACTGATGAAGACCTCCTG, Forward 2 CTTTACGGTATCGCCGCTC, and Reverse CTGGGCAGGATTAAGAGGAA. Mice were housed individually in a climate controlled facility with 12:12 hr light:dark cycle. All experimental protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Iowa State University, Ames, Iowa.

*Study Design.* A complete randomized block study design was used with blocks structured on the basis of age, genotype, and sex. At 6 weeks of age  $\pm$  one week, each block of mice was moved to individual housing and acclimated on AIN-93M diet (Harlan Teklad,

Madison, WI) for one week. At 7 weeks of age, all mice were administered a single subcutaneous injection of 10 mg/kg body weight AOM (Sigma-Aldrich, St. Louis, MO). Fresh DSS (MW 36,000-50,000, MP Biomedicals, Solon, OH) was provided daily at 1% (w/v) in the tap water for 4 weeks following AOM injection to facilitate tumorigenesis. Mice were randomly assigned to high fat, lard (HFL) and low fat, control (LFC) treatment diets, with 10 mice/treatment combination, which began 3 days after DSS treatment ended. The diet compositions were based on the AIN-93M formulation (Harlan Teklad, Madison, WI) as previously described with the following modification (24) the high fat diet was composed of 36% lard by weight as the fat source with protein balanced on a caloric basis (**Table 1**). The treatment diets were fed *ad libitum* for 7 ½ weeks at which point the study was terminated due to significant weight loss. Water (with DSS) and LFC and HFL dietary intake were measured daily throughout respective treatment periods. DSS load (mg DSS consumed per g body weight) was calculated for use as a covariate in statistical analysis of colon lesions and inflammatory and insulin resistance endpoints.

*Sample Collection.* Mice were fasted for 6-8 hours, sacrificed by CO<sub>2</sub> asphyxiation and terminal blood was collected by cardiac puncture for blood glucose and serum analysis. Colon and gonadal fat pad samples were removed and weighed. Tissue sections (approximately 2 cm. in length) were taken from the distal end of the colon, flushed with PBS, and preserved in 10% buffered formalin for histological analysis of lesions. Remaining distal and proximal colon sections and fat pads were flash frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

*Lesion Histology.* To estimate the number of pre-neoplastic and neoplastic colonic lesions among mice in different diet and treatment groups, aberrant crypt foci (ACF), mucin

depleted foci, adenomas, intramucosal carcinomas, and invasive carcinomas were counted in histologic sections of colon and the number of each lesion type per mm colon determined. Formalin-fixed colon samples were processed routinely for histopathology, embedded to allow perpendicular sectioning, sectioned at 5 microns, and sections were stained with hematoxylin and eosin. The total length of each sample was determined using morphometric analysis (Image J software, NIH) of digital photomicrographs of histologic sections of colon. Briefly, 20X digital images of each tissue section were collected using a Nikon Eclipse 55i microscope and DSFi-1 digital camera system. Images were opened in the Image J software, the length of the mucosa was recorded using a free-hand line and the length analyzed using the “Measure” function. The sum of each image for the tissue sample was converted to mm by comparison with a micrometer standard. In addition, each tissue section was evaluated histologically by a veterinary pathologist blinded to mouse groups and treatments. Aberrant crypt foci (ACF), mucin depleted foci, adenomas, intramucosal carcinomas, and invasive carcinomas were determined for each sample using standard histologic criteria (25,26). Bifurcated crypts were included as ACF. Mucin depleted foci were identified as crypts absent of goblet cell differentiation. The data is presented as number of ACF, early lesions (ACF plus mucin depleted foci), adenomas, intramucosal carcinomas plus invasive carcinomas (IMC+IC) and total lesions per mm colon.

*Serum Analysis.* Blood glucose was measured using a commercially available glucometer (LifeScan, Milpitas, CA). ELISA or EIA was used to measure the following serum parameters: total adiponectin (R&D Biosystems, Minneapolis, MN), IL-6 (R&D Biosystems), leptin (R&D Biosystems) and insulin (Alpco Diagnostics, Salem, NH). Serum adiponectin was measured in all mice for genotype verification. However, only serum



adiponectin concentrations from Wt mice are reported here as adiponectin concentrations were not detectable in serum from KO mice.

*Quantitative Real Time PCR.* Frozen adipose and colon tissue was ground in liquid nitrogen and RNA was isolated using acid-phenol reagent (TRIzol, Invitrogen, Carlsbad, CA). DNA contamination was removed using DNase-Free (Ambion, Austin, TX) and RNA was visualized on a diagnostic agarose gel to confirm purity. cDNA was synthesized using iScript (Bio-Rad, Hercules, CA). Standard curves were created for all primer pairs by cloning amplified cDNA into pGemT vector (Promega, Madison, WI) and sequenced to confirm the gene target. Primers used are listed in **Table 2**. Concentrations of RNA in samples were quantified on iCycler (Bio-Rad, Hercules, CA) using IQ™ SYBR Green Super Mix kit (Bio-Rad, Hercules, CA). Thermal cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Gene expression in each tissue was normalized to a housekeeper gene ( $\beta$ -Actin or GAPDH) and expressed as log starting quantity.

*Statistical Analysis.* Data were tested for normality and analyzed using general linear models analysis in SAS (Version 9.2; SAS Institute, Cary, NC) with block, genotype, diet, and sex considered fixed effects. Total DSS load and body weight were used as covariates for relevant analyses. Mean separation was performed with differences considered significant at  $P < 0.05$  and tendency at  $P < 0.10$ . Correlation procedure was used to identify significant correlations between lesion types and serum and gene expression parameters. Data are presented as least square means  $\pm$  s.e.m.

## Results

*Physiological response to obesity is genotype- and sex- dependent.* Mice fed the HFL diet gained significantly more weight and had greater adipose mass than mice fed the LFC diet (**Table 3**). Interestingly, KO mice gained significantly less than Wt mice throughout the dietary treatment period in both LFC and HFL diet groups. However, no genotype differences in gonadal fat pad weight were observed when normalized to body weight (**Table 3**), nor were caloric intake ( $P=0.3951$ ), initial body weight ( $P=0.4548$ ) or final body weight ( $P=0.2083$ ) different between genotypes. Although there were no differences in food intake between genotypes, LFC mice consumed more than HFL ( $P<0.0001$ ) and females consumed more than males ( $P=0.0077$ ) on a gram per day basis (data not shown). Because animals were on diet only 7 ½ weeks, serum adiponectin concentrations were not depressed in high fat diet-fed mice. However, there were differences in circulating glucose and insulin concentrations between genotypes, suggesting a role for adiponectin in insulin and glucose regulation (**Table 3**). Both serum glucose and insulin were lower in KO mice than Wt mice, and although the glucose:insulin ratio was not statistically different in Wt compared to KO and in males compared to females, there was a tendency for a positive correlation ( $r=0.3422$ ) between serum adiponectin and glucose:insulin ratio ( $P=0.0595$ ) in Wt mice. It is likely that after 7 ½ weeks of high fat diet feeding, the mice were at an early stage of insulin resistance and were beginning to compensate for loss of insulin sensitivity. Serum leptin concentrations were higher in the HFL diet than LFC and a diet\*sex interaction was observed where males had higher leptin concentrations than females only in mice fed the HFL diet. No significant differences in serum IL-6 concentrations were identified between mice due to low detection levels (data not shown).

*Incidence of AOM/DSS- induced colorectal lesions.* At the time of sacrifice, both early and advanced lesions were detected in colons. Total lesion number per mm colon was significantly higher in Wt than KO mice, and there was a tendency for increased numbers of total lesions in females compared to males and in mice fed LFC diet compared to HFL (**Figure 3**). Interestingly, when analyzed separately, development of early and advanced lesion types was affected differentially by genotype, diet, and sex effects. ACF and early lesions were influenced by genotype and genotype\*diet interaction effects (**Figure 4A, 4B**). Specifically, Wt mice had significantly more ACF and early lesions per mm colon than KO mice. When genotype\*diet interactions were examined, KO mice fed the HFL diet had more early lesions than KO mice fed the LFC diet, but in Wt mice, the LFC fed mice had a higher early lesion incidence than HFL fed mice. Conversely, the advanced lesion type IMC+IC was significantly influenced by sex and diet rather than genotype (**Figure 4D**). Mice fed LFC diet had more IMC+IC than the HFL group and females had more IMC+IC than males. Adenomas are intermediate in severity between early lesions and IMC+IC and were subsequently influenced significantly by genotype, sex, and diet effects with a tendency for genotype\*diet interaction and genotype\*diet\*sex interaction (**Figure 4C**). Thus, as lesions develop and undergo progression to more malignant phenotypes in our adiponectin KO mouse model, the regulation of carcinogenesis switched from adiponectin-dependent to diet- and sex-dependent.

*Colon and adipose tissue respond differentially to high saturated fat-induced obesity.* To evaluate whether the diet effects detected in tumor progression were associated with diet-induced obesity, we measured the expression of genes involved in inflammation, insulin resistance, and adiponectin signaling in colon and adipose tissue. Both Wt and KO mice

responded to a high saturated fat diet with an elevation in pro-inflammatory cytokine gene expression in adipose tissue (**Figure 5A, 5B**). Both IL-6 and TNF $\alpha$  gene expression levels were significantly elevated in mice fed a high fat diet. Although genotype did not have an effect on IL-6 and TNF $\alpha$  expression itself, there was a diet\*genotype interaction whereby Wt mice fed HFL diet had elevated TNF $\alpha$  and IL-6 expression compared to LFC fed mice, but there were no diet related differences in KO mice. Lcn2 was not affected by diet, but a tendency for a sex effect ( $P = 0.0567$ ) was detected with males expressing higher Lcn2 transcript levels than females (data not shown).

Colon tissue did not respond to the dietary treatment with the same inflammatory profile as adipose tissue (**Figure 6**). In colon, there were no diet-induced differences in TNF $\alpha$  ( $P = 0.8159$ ) or TLR4 ( $P = 0.5170$ ) gene expression between treatment groups (data not shown). It is possible that in our model DSS-induced inflammation is negating the effect of diet on colonic inflammation. However, AdipoR1 gene expression was reduced in mice fed the HFL diet (**Figure 6A**). There was a tendency for an effect of a genotype\*sex interaction in AdipoR2 expression whereby expression was highest in male KO mice and female Wt mice (**Figure 6B**). We also detected a tendency for genotype\*diet in insulin receptor, whereby KO mice had higher insulin receptor expression in the group fed LFC diet while Wt mice had higher insulin receptor expression in mice fed the HFL diet (**Figure 6C**). Thus, colonic tissue responds to diet-induced obesity with regulation of adiponectin receptor and insulin receptor expression in the absence of an inflammatory response to diet.

*Specific lesions are correlated with different serum and tissue parameters.* To understand the associations between inflammation, insulin resistance, and tumor progression, we correlated specific colonic epithelial lesion types with serum and tissue parameters

(**Table 4**). Final body weight and fat pad weight are negatively correlated with advanced and total colonic lesions and serum insulin concentrations are negatively correlated with adenoma development. Conversely, blood glucose concentration is positively correlated with early, advanced, and total lesion development. Interestingly, although adiponectin and leptin alone are not significantly correlated to lesion number, a strong positive correlation was observed between serum adiponectin:leptin ratio and numbers of advanced and total colonic lesions. AdipoR1, TLR4, and insulin receptor expression are positively correlated to development of both early and advanced lesion types while AdipoR2 is correlated only to ACF and total early lesions. These correlations demonstrate a possible role for adiponectin and insulin signaling and TLR4 expression in colonic carcinogenesis and progressive development of malignancy.

### **Discussion**

We report herein that adiponectin KO mice have lower incidence of AOM/DSS-induced colorectal lesions than Wt mice, and that this effect was significant in ACF, early lesions, adenomas, and total lesions. This finding is supported by previous literature with respect to DSS-induced colitis, but not AOM-induced carcinogenesis. Fayad et al. determined that, in mice administered 2% DSS for 5 days, mice absent in adiponectin were protected from colonic inflammation (22). The authors showed that adiponectin directly binds basic fibroblast growth factor and heparin binding epidermal growth factor, thereby potentially inhibiting the protective effect of these growth factors against colitis in the epithelium. Conversely, Fujisawa et al. showed that in AOM-induced colorectal carcinogenesis in mice fed a high fat diet for 20 weeks, colon polyps were increased in adiponectin deficient mice compared to wild type mice (27). The authors proposed that

adiponectin was functioning to suppress epithelial proliferation under high dietary fat conditions, thus preventing lesion development. However, it is interesting to note that colonic epithelial proliferation was increased in adiponectin deficient mice compared with wild type mice in both studies. This may indicate a beneficial role for adiponectin deficiency-induced colonic epithelial proliferation in DSS-treated mice and a harmful role for proliferation in AOM-treated mice. In our model, the inflammation induced with DSS following AOM administration seemed to favor the previously reported DSS response with respect to the role of adiponectin. Collectively, these studies indicate that adiponectin appears to have the potential to both promote and prevent colorectal epithelial damage, and the specific role of adiponectin is likely determined by microenvironmental influences, carcinogen, diet, and stage of carcinogenesis.

We found that AdipoR1 is decreased in colon of mice fed HFL diet regardless of genotype. It has been shown that level of adiponectin receptor expression decreases as adiponectin sensitivity declines in obesity (5). Thus, we expected that adiponectin receptors would decline in Wt mice fed HFL diet but that no change would be observed in KO mice. We also found that AdipoR1 is positively correlated to ACF and IMC+IC lesion multiplicity which is consistent with the report by Kim et al. showing up-regulation of AdipoR1 expression in human colon cancer tissue compared with normal colon tissue (28). Because adiponectin is not functioning to regulate AdipoR1 expression in adiponectin KO mice, it is possible that the alterations in AdipoR1 expression we observed in our adiponectin-deficient model were due to the presence of alternative ligands for AdipoR1. Several signaling molecules have been identified that interact with AdipoR1, including endo protein disulphide isomerase (ERp46), APPL1, protein kinase CK2, and receptor for activated C-kinase 1

(RACK1) (29,30,31,32). Of these signaling molecules, CK2 and RACK1 are involved in regulation of pancreatic beta cell signaling (33,34), a signaling cascade highly affected by diet-induced obesity. Moreover, CK2 and RACK1 have potential roles in regulation of colon tumorigenesis through regulation of apoptosis and the cell cycle (35,36). Thus, it is plausible that the presence of these AdipoR1 ligands may be in part responsible for the up-regulation of AdipoR1 expression in mice fed the HFL diet.

To our knowledge, this is the first report of sex differences in development of pre-neoplastic and neoplastic colonic lesions in adiponectin KO mouse model administered AOM/DSS. In this study, female mice developed greater adenoma and IMC+IC multiplicity than male mice. The development of these advanced lesions is likely due to sex-specific differential response to HFL diet. Males had higher insulin concentrations than females and a tendency for lower fat pad weight when normalized to body weight. This is supported by reports of sex differences in insulin regulation, inflammation, and adiposity in response to high fat diet (37,38). This is also supported by epidemiological data which show an association between sex differences and dietary factors in colorectal cancer incidence in humans (39). In transgenic mice with increased circulating adiponectin concentrations, a sex difference was observed with males developing greater tumor multiplicity than females (40). Another explanation for differences between sexes is hormonal disparity. Although estradiol has been implicated as having a role in the initiation stage of colorectal tumorigenesis (41), in the DSS model of colitis, estrogen increased histological scores and disease severity in female mice (42). Our findings are inconsistent with human epidemiological data that show higher incidence of colorectal cancer in males than females (43), and warrant further mechanistic investigation into these sex differences.

Mice of both genotypes responded to high fat diet as expected with an increase in the pro-inflammatory gene expression in adipose tissue of mice fed the HFL vs. LFC diet. However, diet-induced inflammation was not observed in the circulation or colonic tissue. This is likely due to the short duration of dietary treatment, which was sufficient for development of obesity and leptin resistance, but not metabolic complications. Bullen et al. reported that 10 wks of high fat diet feeding are needed to induce a depression in circulating adiponectin concentrations in mice, and that insulin concentrations are lowest at 6 weeks of high fat feeding and become hyperinsulinemic after 6 weeks on a high fat diet (44). Although diet-induced inflammation was not detectable in colon tissue, both LFC mice and females had more advanced lesions than HFL and male groups, respectively. We assert three possible reasons for this association: 1) differences in diet composition and physiological properties such as glycemic index (45) contributed to colorectal tumorigenesis independently or through interaction with genotype or sex-related parameters, 2) tumor formation may be promoted by a low fat diet (46) as observed by Nakamura et al. in human subjects, or 3) the different types and amounts of dietary fat in the LFC vs. HFL diets resulted in IGF-I and IGF-II receptor regulation (47) that contributed to suppression of tumor development in HFL mice. The diets were structured such that protein was balanced calorically. Because the LFC-fed mice consumed more daily feed on a weight basis but less on a caloric basis, the imbalance of both protein and micronutrients consumed may have contributed to diet effects.

Herein, we show that insulin receptor expression is regulated by genotype\*diet interaction and is positively correlated with development of ACF. This increase in insulin receptor expression is due in part to the tendency for increased circulating insulin in the Wt genotype. This finding is consistent with the literature that reports increased insulin receptor



expression in colonic tumors compared with normal mucosa (48). Both insulin and leptin have been shown to independently promote colorectal tumorigenesis (15,49). However, in this study, leptin is increased in mice fed the HFL diet regardless of genotype and has no significant association with colon lesion multiplicity. Interestingly, we observed a strong positive correlation between serum adiponectin:leptin ratio and numbers of adenomas, IMC+ICs, and total lesions that was related to the overall effect of diet on adenomas, IMC+IC, and total lesions. Low adiponectin:leptin ratio has been recognized recently as a prognostic marker related to severity and adverse outcome in colorectal cancer (50) and can also be used as a measure of insulin resistance (51). However, we observed increased severity of colonic lesions in mice with a higher adiponectin:leptin ratio, indicating the importance of adiponectin function in the AOM/DSS model and the previously reported ability of adiponectin to modulate the proliferative effects of leptin in colon epithelial cells (52). Thus, in our model, insulin signaling and serum adiponectin:leptin ratio, but not adiponectin and leptin concentrations alone, are associated with colorectal tumorigenesis.

In summary, we report for the first time that AOM/DSS-induced colorectal carcinogenesis is regulated differentially to adiponectin status, sex, and diet. Early lesion development is determined by genotype, adenomas are determined by genotype, diet, and sex, and advanced colonic neoplasms are determined by diet and sex. Thus, we propose that molecular patterns change throughout development of colorectal cancer such that adiponectin plays an initial role in tumorigenesis that is subsequently regulated by diet and sex as lesions become more advanced. This theory is supported by the publication by Otake et al. that recognized serum adiponectin as a significant risk factor for early cancer and adenomas in patients with colorectal cancer, but found no association between adiponectin and advanced

cancer (53). Based on the literature, it is likely that this sequence of events is specific to AOM/DSS-induced colorectal cancer and not the AOM-only model of carcinogenesis. Our correlations indicate that insulin and glucose regulation, adiponectin signaling, and TLR4 are involved in these events. Future studies are warranted to identify the molecular changes occurring at the transition from early to advanced lesions.

### Author Contributions

RB performed the animal experiment and laboratory work, designed the cell culture experiments, and wrote the manuscript. EW performed histology of colons and contributed to interpretation of results. DB contributed to interpretation of results and helped design the experiment. MS supervised the experiments, edited the manuscript, and contributed to interpretation of results.

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## Tables

Table 1. Diet Composition

<b>Ingredient</b>	<b>LFC<sup>1</sup> (g)</b>	<b>HFL<sup>1</sup> (g)</b>
Casein <sup>2</sup>	140	196
Sucrose <sup>2</sup>	100	100
Corn Starch <sup>2</sup>	456	88
Maltodextrin <sup>2</sup>	155	155
Soybean Oil <sup>2</sup>	50	
Lard <sup>2</sup>		360
Cholesterol <sup>2</sup>		1.5
Cellulose <sup>2</sup>	50	50
Vitamin Mix <sup>2</sup>	10	10
Mineral Mix <sup>2</sup>	35	35
Choline Bitartrate <sup>2</sup>	2.5	2.5
L-Cystine <sup>2</sup>	1.8	1.8
THBQ <sup>2</sup>	0.008	0.008
<b>Total (g)</b>	<b>1000.0</b>	<b>1000.0</b>
% Protein (Total kcal)	14.69	14.69
% Carbohydrate (Total kcal)	73.65	25.4
% Fat (Total kcal)	11.66	59.96
Density (kcal/g)	3.86	5.4

<sup>1</sup>Adapted from AIN-93M Diet

<sup>2</sup>Harlan Teklad, Madison, WI

**Table 2.** *Quantitative Real Time PCR Primers*

<b>Target</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
$\beta$ -Actin	TGAGAGGGAAATCGTGCGTGACAT	ACCGCTCGTTGCCAATAGTGATGA
Gapdh	TCAACAGCAACTCCCCTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA
AdipoR1	TCCTGACTGGCTGAAAGACAACGA	AGATGTTGCCAGTCTCTGTGTGGA
AdipoR2	TGAGCGCTTCTTTCCTGGCAAATG	ATTCCTGCAGGTTTGAGACTCCGT
Insulin Receptor	TTCTTTCCTGCGTGCATTTCCCAC	TTAATCAGGGTGGCCAGTGTGTCT
IL-6	TCCAGTTGCCTTCTTGGGACTGAT	AGCCTCCGACTTGTGAAGTGGTAT
TNF $\alpha$	CCAACGGCATGGATCTCAAAGACA	AGATAGCAAATCGGCTGACGGTGT
Lcn2	TGCCACTCCATCTTTCCTGTT	GGGAGTGCTGGCCAAATAAG
TLR4	CCGCTCTGGCATCATCTTCATTGT	TCCTCCCATTCCAGGTAGGTGTTT



**Table 3. Body Composition and Serum Parameters**

Genotype	Sex	Diet	Weight Gain (g) <sup>a</sup>	Fat Pad Weight (g) <sup>b</sup>	Glucose (mg/dl) <sup>c</sup>	Insulin (ng/ml) <sup>d</sup>	Adiponectin (µg/ml) <sup>e</sup>	Leptin (ng/ml) <sup>f</sup>
KO	F	LFC	3.8 ± 1.4 <sup>a</sup>	1.21 ± 0.10 <sup>c</sup>	267 ± 31 <sup>a</sup>	0.29 ± 0.40 <sup>a</sup>	N/A	14.2 ± 7.7 <sup>a,b</sup>
KO	F	HFL	7.3 ± 1.2 <sup>b,c</sup>	1.24 ± 0.08 <sup>c</sup>	283 ± 28 <sup>a,b</sup>	0.48 ± 0.35 <sup>a,b</sup>	N/A	28.7 ± 5.5 <sup>b,c</sup>
Wt	F	LFC	5.1 ± 1.3 <sup>a,b</sup>	1.12 ± 0.09 <sup>b,c</sup>	325 ± 28 <sup>a,b,c</sup>	0.88 ± 0.32 <sup>a,b,c</sup>	7.88 ± 0.60 <sup>b</sup>	11.1 ± 4.9 <sup>a</sup>
Wt	F	HFL	10.2 ± 1.4 <sup>c,d</sup>	1.22 ± 0.08 <sup>c</sup>	312 ± 31 <sup>a,b,c</sup>	0.64 ± 0.36 <sup>a,b,c</sup>	7.35 ± 0.61 <sup>a,b</sup>	25.6 ± 6.2 <sup>a,b,c</sup>
KO	M	LFC	6.3 ± 1.2 <sup>a,b</sup>	0.89 ± 0.08 <sup>a,b</sup>	293 ± 27 <sup>a,b</sup>	1.40 ± 0.29 <sup>b,c</sup>	N/A	13.3 ± 6.1 <sup>a,b</sup>
KO	M	HFL	10.8 ± 1.3 <sup>c,d</sup>	1.24 ± 0.10 <sup>c</sup>	341 ± 28 <sup>b,c</sup>	1.66 ± 0.33 <sup>c</sup>	N/A	40.5 ± 5.8 <sup>c</sup>
Wt	M	LFC	8.2 ± 1.2 <sup>b,c</sup>	0.85 ± 0.09 <sup>a</sup>	386 ± 27 <sup>c</sup>	1.54 ± 0.31 <sup>c</sup>	6.10 ± 0.65 <sup>a</sup>	14.0 ± 5.6 <sup>a,b</sup>
Wt	M	HFL	12.7 ± 1.4 <sup>d</sup>	1.06 ± 0.11 <sup>a,b,c</sup>	370 ± 27 <sup>c</sup>	2.58 ± 0.32 <sup>d</sup>	6.45 ± 0.63 <sup>a,b</sup>	55.9 ± 5.7 <sup>d</sup>

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Values are least squares means  $\pm$  s.e. Different letters denote statistical significance ( $P < 0.05$ ).

<sup>a</sup> Weight gain is calculated over 7 ½ wk. of experiment diet feeding. Significant effect of diet ( $5.9 \pm 0.6$  vs.  $10.3 \pm 0.6$  for LFC vs. HFL,  $P < 0.0001$ ); Significant effect of genotype ( $7.1 \pm 0.6$  vs.  $9.1 \pm 0.6$  for KO vs. Wt,  $P = 0.0191$ ); Significant effect of sex ( $6.6 \pm 0.8$  vs.  $9.5 \pm 0.8$  for female vs. male,  $P = 0.0376$ )

<sup>b</sup> Fat pad weight normalized to final body weight. Significant effect of diet ( $1.02 \pm 0.05$  vs.  $1.19 \pm 0.05$  for LFC vs. HFL,  $P = 0.0148$ ); Significant sex effect ( $1.20 \pm 0.06$  vs.  $1.01 \pm 0.06$  for female vs. male,  $P = 0.0489$ )

<sup>c</sup> Significant effect of genotype ( $296 \pm 13$  vs.  $348 \pm 14$  for KO vs. Wt,  $P = 0.0074$ ); Tendency for sex effect ( $297 \pm 18$  vs.  $347 \pm 17$  for female vs. male,  $P = 0.0878$ )

<sup>d</sup> Significant effect of sex ( $0.57 \pm 0.22$  vs.  $1.79 \pm 0.18$  for female vs. male,  $P = 0.0004$ ); Tendency for genotype effect ( $0.96 \pm 0.16$  vs.  $1.41 \pm 0.16$  for KO vs. Wt,  $P = 0.0511$ )

<sup>e</sup> Significant effect of sex ( $7.61 \pm 0.43$  vs.  $6.27 \pm 0.47$  for female vs. male,  $P = 0.0366$ )

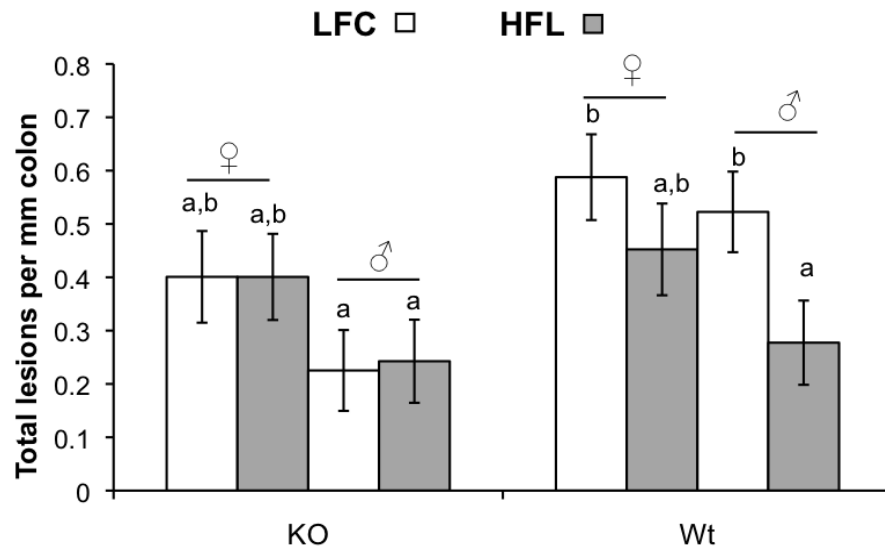
<sup>f</sup> Significant diet effect ( $13.2 \pm 2.6$  vs.  $37.7 \pm 2.6$  for LFC vs. HFL,  $P < 0.0001$ ); Significant diet\*sex interaction ( $P = 0.0112$ )

**Table 4.** *Lesion-Specific Correlations*

	ACF	Early	Adenoma	IMC+IC	Total Lesions
<i>Serum or body composition parameter</i>					
<b>Final Body Weight</b>	-0.18 (0.13)	-0.19 (0.11)	-0.37 (0.0014)	-0.32 (0.0061)	-0.33 (0.0052)
<b>Gonadal Fat</b>					
<b>Weight</b>	-0.16 (0.17)	-0.16 (0.19)	-0.37 (0.0012)	-0.31 (0.0078)	-0.30 (0.0096)
<b>Serum Insulin</b>	-0.07 (0.59)	-0.13 (0.33)	-0.25 (0.05)	-0.14 (0.29)	-0.20 (0.13)
<b>Adiponectin:Leptin</b>	0.50 (0.12)	0.59 (0.054)	0.79 (0.004)	0.69 (0.019)	0.72 (0.013)
<b>Blood Glucose</b>	0.35 (0.0023)	0.34 (0.0038)	0.22 (0.069)	0.38 (0.0009)	0.37 (0.0014)
<i>Colon gene expression parameter (SQ)</i>					
<b>AdipoR1</b>	0.29 (0.050)	0.21 (0.16)	0.11 (0.49)	0.39 (0.0073)	0.32 (0.031)
<b>AdipoR2</b>	0.34 (0.030)	0.37 (0.018)	0.21 (0.19)	0.14 (0.40)	0.27 (0.091)
<b>Insulin Receptor</b>	0.42 (0.0004)	0.34 (0.0055)	0.098 (0.44)	0.39 (0.0015)	0.35 (0.0037)
<b>TLR4</b>	0.50 (<0.0001)	0.42 (0.0004)	0.17 (0.16)	0.50 (<0.0001)	0.41 (0.0005)

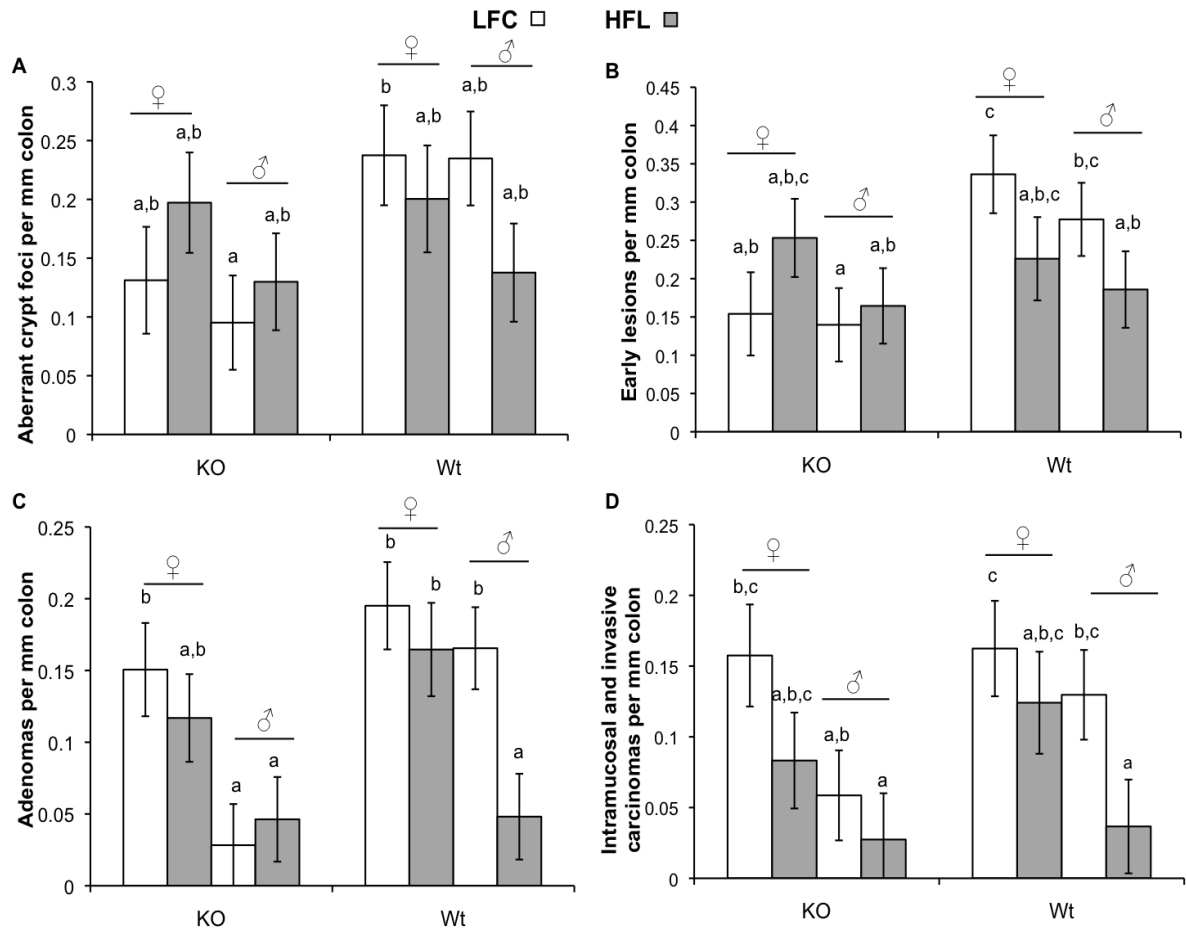
Data represented as correlation R (p-value).

## Figures



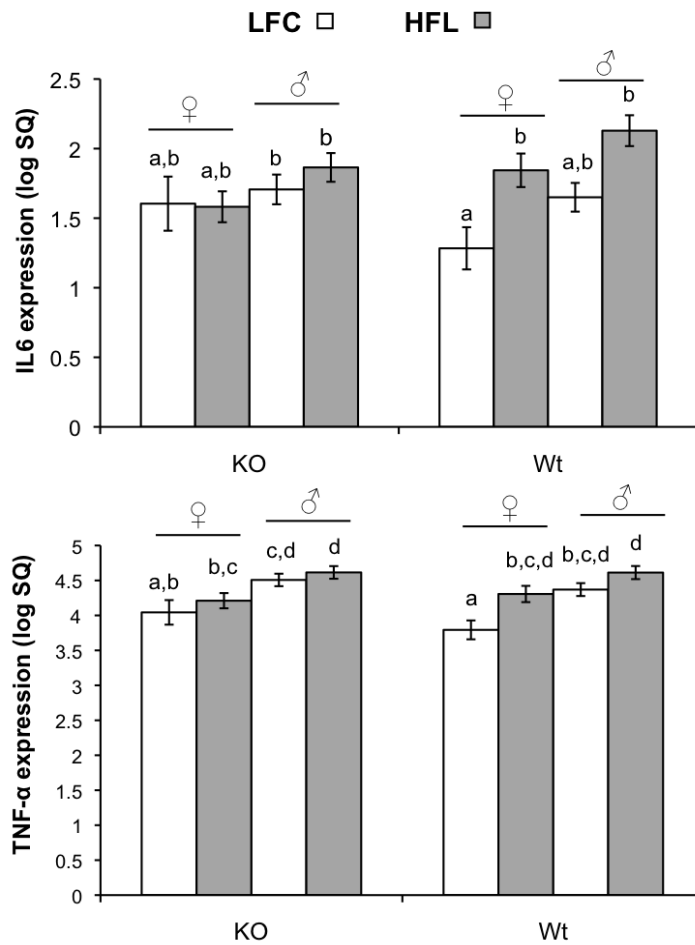
**Figure 3.** Total lesion development is influenced by genotype, diet, and sex.

Total lesions induced by AOM/DSS treatment were measured by histopathology lesion count per mm colon. Significant genotype effect ( $0.32 \pm 0.04$  vs.  $0.46 \pm 0.04$  for KO vs. Wt,  $P = 0.0091$ ); Tendency for diet effect ( $0.43 \pm 0.04$  vs.  $0.34 \pm 0.04$  for LFC vs. HFL,  $P = 0.0808$ ); Tendency for sex effect ( $0.46 \pm 0.05$  vs.  $0.32 \pm 0.05$  for female vs. male,  $P = 0.0819$ ); Tendency for genotype\*diet interaction ( $P = 0.0554$ ).



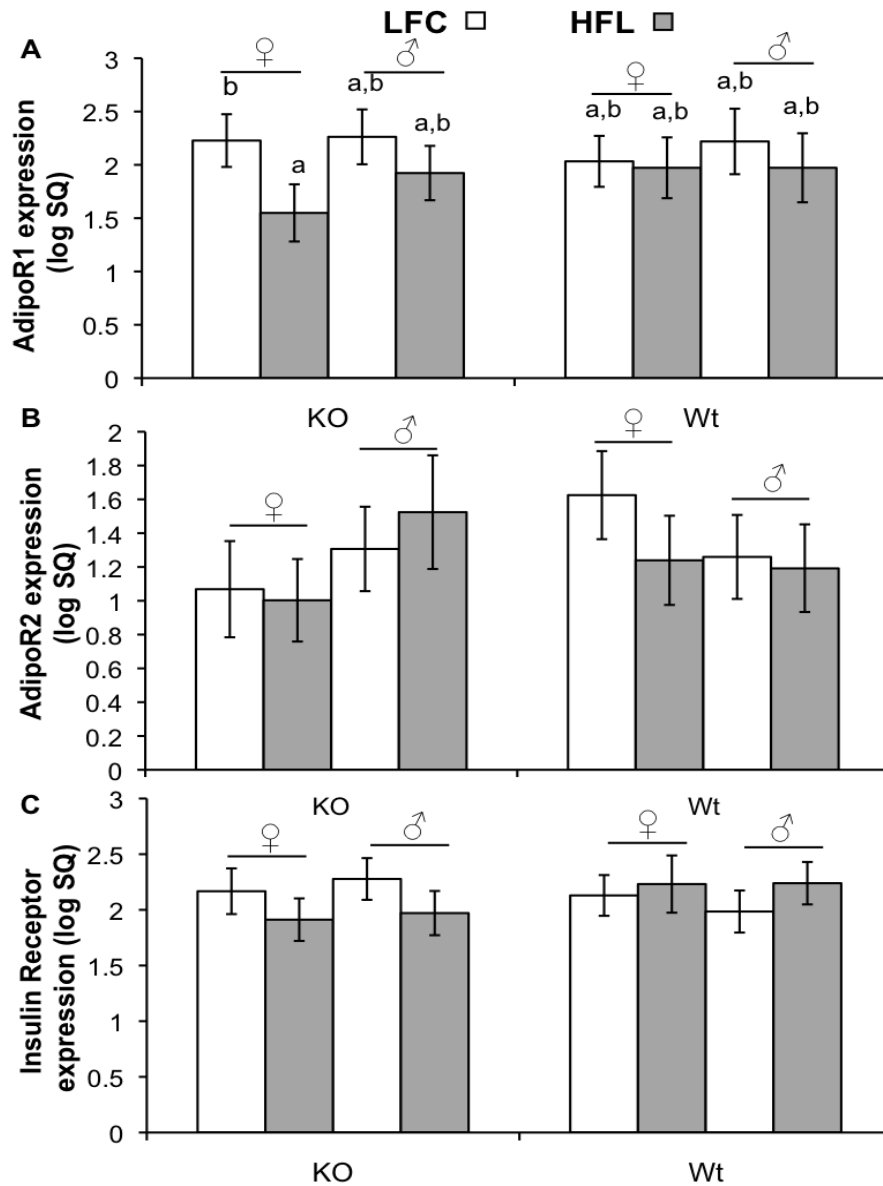
**Figure 4.** Early lesions are influenced by genotype and diet whereas advanced lesions are influenced by sex.

Histological analysis of early and advanced lesion number per mm colon. **(A)** Significant genotype effect ( $0.14 \pm 0.02$  vs.  $0.20 \pm 0.02$ ,  $P = 0.0253$ ); Significant genotype\*diet interaction ( $P = 0.0330$ ). **(B)** Significant genotype effect ( $0.18 \pm 0.02$  vs.  $0.26 \pm 0.02$  for KO vs. Wt,  $P = 0.0222$ ); Significant genotype\*diet interaction ( $P = 0.0140$ ). **(C)** Significant genotype effect ( $0.09 \pm 0.01$  vs.  $0.14 \pm 0.01$  for KO vs. Wt,  $P = 0.0054$ ); Significant diet effect ( $0.13 \pm 0.01$  vs.  $0.09 \pm 0.01$  for LFC vs. HFL,  $P = 0.0388$ ); Significant sex effect ( $0.16 \pm 0.02$  vs.  $0.07 \pm 0.02$  for female vs. male,  $P = 0.0076$ ); Tendency for genotype\*diet interaction ( $P = 0.0911$ ); Tendency for genotype\*diet\*sex interaction ( $P = 0.0900$ ). **(D)** Significant diet effect ( $0.13 \pm 0.02$  vs.  $0.07 \pm 0.02$  for LFC vs. HFL,  $P = 0.0077$ ); Significant sex effect ( $0.13 \pm 0.02$  vs.  $0.06 \pm 0.02$  for female vs. male,  $P = 0.0477$ ).



**Figure 5.** HFL diet induced a pro-inflammatory expression profile in adipose tissue.

Quantitative RT-PCR gene expression analysis of adipose tissue expressed as log SQ. **(A)** Significant effect of diet ( $1.58 \pm 0.07$  vs.  $1.84 \pm 0.06$  for LFC vs. HFL,  $P = 0.0047$ ); Significant effect of genotype\*diet interaction ( $P = 0.0184$ ). **(B)** Significant diet effect ( $4.20 \pm 0.06$  vs.  $4.44 \pm 0.05$  for LFC vs. HFL,  $P = 0.0014$ ); Significant sex effect ( $4.11 \pm 0.08$  vs.  $4.52 \pm 0.05$  for female vs. male,  $P = 0.0008$ ); Tendency for genotype\*diet interaction ( $P = 0.0943$ ).



**Figure 6.** Diet altered *AdipoR1* expression in colonic tissue.

Quantitative RT-PCR gene expression analysis of colon tissue expressed as log SQ. (A) Significant effect of diet ( $2.19 \pm 0.12$  vs.  $1.85 \pm 0.12$  for LFC vs. HFL,  $P = 0.05$ ). (B) Tendency for genotype\*sex interaction effect ( $P = 0.0645$ ). (C) Tendency for genotype\*diet interaction effect ( $P = 0.0685$ ).

## **CHAPTER 4. LOW DOSE DIETARY RESVERATROL HAS DIFFERENTIAL EFFECTS ON COLORECTAL TUMORIGENESIS IN ADIPONECTIN KNOCKOUT AND WILD TYPE MICE**

Submitted to *Nutrition and Cancer: An International Journal*

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### **Abstract**

Obesity is associated with a decrease in the anti-inflammatory hormone, adiponectin, and increases in the circulating concentrations of multiple pro-inflammatory cytokines. These changes contribute to colon tumorigenesis. Resveratrol increases adiponectin production in adipocytes and attenuates the development of colon cancer. Thus, we hypothesized that adiponectin is an integral component of the mechanism by which resveratrol antagonizes colorectal tumorigenesis. To investigate this, we induced tumorigenesis in adiponectin knockout (KO) and wildtype (Wt) C57BL/6 mice through combined azoxymethane and DSS treatment during which mice were fed a high fat, lard-based diet (HFL), or the same diet containing 20 mg/kg resveratrol (HFL+R). After 14 weeks on diet, Wt mice gained more weight, and on a percentage basis, had higher fat mass and lower lean mass than KO mice. Resveratrol tended to attenuate this response in male Wt mice. Resveratrol also tended to reduce aberrant crypt foci development and decrease circulating interleukin 6 and insulin concentrations in male but not female Wt mice. Taken together, resveratrol improved overall health of obese Wt but not KO mice as hypothesized with a differential sex response.

### **Introduction**

Visceral adiposity is highly associated with the development of colorectal cancer and has now been identified as an independent risk factor for colorectal cancer (1,2). Obesity is



associated with changes in adipose tissue that promote colon cancer development including a decrease in the anti-inflammatory hormone, adiponectin, and increases in leptin, insulin, and IL6 (3,4,5). Studies using a co-culture system or conditioned media show that adipocytes can stimulate proliferation of colon cancer cells (5,6). Moreover, culturing colon cancer and intestinal epithelial cells with adiponectin decreases inflammation, activates AMP-activated protein kinase (AMPK), and decrease proliferation (7).

Resveratrol (3,5,4' trihydroxystilbene), an activator of sirtuin 1 (Sirt1) and AMPK, is a compound naturally found in grapes and red wine. Resveratrol has been studied extensively for its anti-inflammatory and anti-carcinogenic properties. Several groups have demonstrated that resveratrol supplementation attenuates colitis and colon tumor multiplicity in mice exposed to dextran sodium sulfate (DSS) and azoxymethane (8,9,10,11). *In vitro* studies to determine the mechanism of resveratrol indicate an inhibitory effect on pro-inflammatory gene expression, including toll like receptor 4 (12) and cyclooxygenase 2 (13), and decreased proliferation of colonocytes (14).

Recently, a role for resveratrol in modulating the adipokine profile has been established in the literature. The addition of resveratrol to a high fat diet increased mitochondrial number and improved insulin sensitivity and the overall metabolic profile in obese mice (15). *In vitro* studies of resveratrol signaling in adipocytes show attenuation of inflammation via inhibition of nuclear factor kappa B (16), and inhibition of adipogenesis (17,18) through a down-regulation in peroxizome proliferator-activated receptor  $\gamma$  and other adipogenic genes. Importantly, resveratrol increases adiponectin expression in human visceral adipocytes (19) and 3T3-L1 adipocytes (20). However, the role of resveratrol in colorectal cancer development and the potential interaction between resveratrol and

adiponectin has not been reported in the context of obesity. We used adiponectin knockout mice, coupled with diet-induced obesity, to test the hypothesis that adiponectin mediates the anti-inflammatory and anti-carcinogenic effects of resveratrol in obesity. This investigation was focused on early lesion development, which we have previously reported to be modulated by low dose dietary resveratrol (21).

### Materials and Methods

*Animals and Diets.* Forty adiponectin knockout (KO) and sixty wildtype (Wt) male and female mice were generated using our previously established colony (22). Complete randomized block design was used with blocks consisting of at least one animal from each diet\*sex\*genotype combination. At 6 weeks of age  $\pm$  1 week, mice were moved to individual housing and acclimated on AIN-93M diet (Harlan Teklad, Madison, WI) for 1 week. Following acclimation, all mice were administered a single injection of 10 mg/kg body weight AOM (Sigma Aldrich, St. Louis, MO) immediately followed by 5 days of 1% DSS (MW 36,000-50,000, MP Biomedicals, Solon, OH) in drinking water. DSS water intake was measured daily, and was used to calculate DSS load (mg DSS consumed per g body weight) for use in statistical analyses. Concurrent with the start of AOM/DSS administration, forty Wt and forty KO mice were randomly assigned one of two diets: high fat lard (HFL) or high fat lard with resveratrol (HFL+R). An additional twenty Wt mice were maintained on the AIN-93M low fat control (LFC) diet for validation of high fat diet-induced obesity. The diet compositions are as previously reported (22) with the addition of 20 mg/kg diet resveratrol (Sigma Aldrich, St. Louis, MO) in the HFL+R diet (**Table 5**) to represent the amount attainable from a low-dose dietary resveratrol aglycone supplements. Experimental diets were fed *ad libitum* for 14 weeks throughout which body weight and dietary intake were

measured daily. Animals were housed in a climate controlled facility with a 12:12 hr light:dark cycle. All experimental protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Iowa State University, Ames, Iowa.

*Sample Collection.* Prior to sacrifice, animals were fasted for 6-8 hrs. Animals were sacrificed by CO<sub>2</sub> asphyxiation followed by terminal cardiac puncture for serum collection. Gonadal fat pad and colon tissues were excised and weighed. Distal colon sections (approximately 2 cm long) were rinsed in PBS and placed in buffered formalin for lesion histology. Gonadal fat pad and carcasses were flash frozen in liquid nitrogen and stored at -80°C.

*Lesion Histology.* Colons were sectioned to allow for histologic analysis of the tissue and the use of remaining colonic tissue for molecular analyses. Aberrant crypt foci (ACF), mucin depleted foci, and adenomas were counted in representative histologic sections of distal colon and the number of each lesion type per mm colon determined. Formalin-fixed colon samples were processed routinely for histopathology, embedded to allow perpendicular sectioning, sectioned at 5 µm, and stained with hematoxylin and eosin. Briefly, 20X digital images of each tissue section were collected using a Nikon Eclipse 55i microscope and DSFi-1 digital camera system. The total length of each sample was determined using morphometric analysis (Image J software, NIH) of digital photomicrographs of histologic sections of colon. The sum of each image for the tissue sample was converted to mm by comparison with a micrometer standard. A veterinary pathologist blinded to mouse treatment groups evaluated each tissue section histologically. Aberrant crypt foci (ACF), mucin depleted foci, and adenomas were determined for each sample using standard histologic criteria (23,24).

Bifurcated crypts were included as ACF. Mucin depleted foci were identified as crypts absent

of goblet cell differentiation. The data is presented as number of ACF and total lesions per mm colon.

*Body Composition.* Frozen mouse carcasses (excluding blood, gonadal adipose tissue, and colon) were thawed to room temperature. Following system calibration, fat mass and lean body mass was determined using an EchoMRI whole body composition analyzer (Houston, TX). Composition analysis was also performed on 10 grams of gonadal fat pad samples from a representative sample of mice fed each diet for use as a correction coefficient. The correction coefficients for respective diets were applied to the weight of removed adipose tissue from each mouse, and the carcass composition data adjusted accordingly to obtain whole body composition. Whole body percent fat and lean were calculated by dividing total weight of fat (g) and lean (g) by whole body weight (g), respectively.

*Adipocyte Size.* Frozen gonadal fat pads samples (n = 4 per diet\*genotype\*sex combination) were fixed in 10% buffered formalin overnight and processed routinely for histologic sections. Five  $\mu\text{m}$  sections were stained with hematoxylin and eosin. To analyze for adipocyte size and number, four fields were captured at 20X magnification for each mouse. Adipocyte area was determined using AxioVision v4.8.2.0 (Carl Zeiss, Germany).

*Serum Analyses.* Blood glucose was measured by commercial glucometer (LifeScan, Milpitas, CA). Serum from terminal blood collection was assayed by ELISA or Multiplex. Total and high molecular weight adiponectin was measured by ELISA (Alpco Diagnostics, Salem, NH). Insulin, MCP1, IL6, TNF $\alpha$ , and leptin were measured using a 5-plex Milliplex Mouse Adipokine kit (Millipore, Billerica, MA) on a multiplex system (Bioplex, BioRad, Hercules, CA).

### *Caco-2 and Stromal Vascular Cell Culture*

*Caco-2 Culture.* Caco-2 cells (ATCC, Manassas, VA) (passage 18) were cultured at 37°C, 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), 100 U/ml penicillin/100 µg/ml streptomycin, and 1% non-essential amino acids (MEM, Gibco, Billings, MT).

*Stromal Vascular Cell Isolation.* Stromal vascular cells (SVCs) were isolated from gonadal fat pads of Wt and KO mice obtained from the adiponectin null breeding colony. Briefly, adipose tissue was excised, placed in cold 1x HBSS buffer, and minced. Tissue was then digested in 50% HBSS/50% LG-DMEM medium with 200 U/ml collagenase shaking for 1 hr. at 37°C. Digested cells were centrifuged 5 min, 1,000  $\times$  g and digestion media was removed. Cells were then incubated at room temperature in red blood cell lysis buffer (0.154 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA) and centrifuged at 1,000  $\times$  g to remove red blood cells. Remaining cells were re-suspended in LG-DMEM growth medium and passed through a 100 µm filter to isolate the SVC fraction. SVCs were grown to passage 2 under 37°C, 5% CO<sub>2</sub> in LG-DMEM growth medium with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mg/ml gentamicin and amphotericin B.

*Experiment 1.* Wt and KO SVCs were plated according to manufacturer's instructions and allowed to adhere for 2 hr. For measure of cell proliferation, media was removed and fresh medium containing 0, 25, or 50 µM resveratrol aglycone (Sigma Aldrich, St. Louis, MO) and BrdU reagent (Chemicon International, Temecula, CA) was added to the cells. BrdU incorporation was measured after 18 hr. The same procedure was followed for Caco-2 cells with 6 hr BrdU reagent incubation before measurement.

*Experiment 2.* Wt and KO SVCs (passage 2) were grown to 80% confluence and differentiated using a previously described protocol (25). At Day 11 post-differentiation, the cells were pre-treated for 18 hr. with fresh medium containing 0 or 25  $\mu$ M resveratrol aglycone (Sigma Aldrich, St. Louis, MO) in DMSO. After 18 hr. pre-treatment, 100 ng/ml LPS was added to the medium for an additional 24 hr. treatment. The culture medium was collected for measurement of mouse IL6 concentration by ELISA (R&D Systems, Minneapolis, MN).

The inflammatory response of Caco-2 cells to resveratrol and adiponectin was measured using conditioned media. The conditioned media was obtained by applying fresh medium (with 1% fetal bovine serum) to fully differentiated Wt and KO adipocytes for 24 hr. Caco-2 cells were incubated for 18 hr. in unconditioned or conditioned medium from Wt or KO adipocytes with DMEM containing 0 or 25  $\mu$ M resveratrol. After pre-treatment, 10  $\mu$ g/ml LPS and 50 ng/ml TNF $\alpha$  (Sigma Aldrich, St. Louis, MO) were added to the culture medium, and cells were incubated for an additional 24 hr. Culture medium was collected for cytokine analysis of human IL6 and IL1 $\beta$  by ELISA (R&D Systems, Minneapolis, MN). All experiments had a minimum of 4 samples per treatment.

*Statistical Analyses.* All residuals were plotted and tested for normality. Data were analyzed using general linear models procedure in SAS (Version 9.2; SAS Institute, Cary, NC). In the mouse experiment, genders were analyzed separately with diet, genotype, and block considered fixed effects with the following exception. Those data containing only Wt mice (serum adiponectin and LFC diet weight, intake, and blood glucose data) were analyzed with gender, diet, and block considered fixed effects. Body weight and DSS intake were used as covariates in relevant analyses. For cell culture studies, genotype of SVCs or conditioned

media and treatment (resveratrol or LPS) were considered fixed effects. Data is reported as least square means  $\pm$  s.e.m. Statistical significance is defined as  $p < 0.05$  and tendency for significance as  $p < 0.1$ .

## Results

*Weight gain and dietary intake.* We first confirmed diet-induced obesity in Wt mice by showing Wt mice gained significantly more weight when fed the HFL diet as compared to the LFC diet (**Figure 7**). Adiponectin genotype altered weight gain in male mice ( $P = 0.0009$ ), but not females, when fed the HFL diet (**Figure 8A**). Interestingly, resveratrol had a tendency to decrease weight gain in the Wt but not KO genotype in both male ( $P = 0.057$ ) and female ( $P = 0.059$ ) mice. These observed differences in weight gain were not due to differences in dietary intake (**Figure 8**).

*Body composition and adipocyte size.* Overall, male Wt mice had significantly higher percent body fat and lower percent lean mass than male KO mice (**Figure 9A,B**). There was a tendency for the HFL+R diet to reduce percent body fat ( $P = 0.077$ ) and increase percent lean mass ( $P = 0.087$ ) in Wt male mice (**Figure 9A,B**). This HFL+R diet effect was not observed in KO male mice, suggesting that resveratrol has a tendency to attenuate high fat diet-induced adiposity only in the presence of adiponectin. In contrast, there were no significant diet or genotype effects on body composition observed in female mice, likely as a result of the greater variability observed within treatments (**Figure 9C,D**).

Analysis of adipocyte size further demonstrated a dimorphic sex response to genotype and experimental diets. In male mice, a genotype effect, but not a dietary effect, was observed in which a greater average adipocyte size was observed in Wt male mice than in KO male mice (**Figure 10A**). Conversely, females exhibited a significant diet effect, but not

a genotypic effect (**Figure 10C**). In female mice, adipocyte area was larger in HFL-fed mice than in HFL+R fed mice. This effect was significant for the Wt genotype with a tendency for significance in the KO genotype.

*ACF and total lesion development.* Aberrant crypt foci (ACF) were the most abundant lesion type observed in the distal colon of all mice. In addition to ACF, mucin depleted foci and adenomas were observed, although primarily in females. Overall, there were no significant differences in lesion number between diet and genotype groups in males or females. However, there was a tendency for a genotype\*diet interaction in ACF ( $P = 0.066$ ) and total lesions ( $P = 0.092$ ) of male mice (**Figure 11A,B**). Specifically, the HFL+R diet tended to decrease ACF and total lesion development ( $P = 0.095$ ,  $P = 0.0773$ , respectively) in Wt male mice but had no effect on KO male mice (**Figure 11A,B**). In female mice, ACF and total lesions tended to be greater in Wt compared to KO genotypes ( $P = 0.062$  and  $P = 0.096$ , respectively), but no interaction between diet and genotype was observed (**Figure 11C,D**).

*Serum Parameters.* Serum insulin was significantly elevated in Wt male mice compared to KO male mice in males (**Tables 12A, 2B**). However, HFL+R diet attenuated the elevation in Wt insulin concentrations with no effect on KO mice. The glucose:insulin ratio, an indicator of insulin sensitivity to glucose, tended to be higher in male KO mice compared to male Wt ( $P = 0.099$ ). Females did not display diet- or genotype-dependent differences in glucose and insulin concentrations or glucose:insulin ratio. Thus, whereas glucose homeostasis had a tendency to be improved by resveratrol in Wt male mice, female mice were not responsive to dietary resveratrol.

There was a tendency for higher serum total adiponectin concentrations in HFL+R fed mice compared to HFL fed mice for both Wt males ( $P = 0.094$ ) and Wt females ( $P =$



0.099) (**Tables 6A, 6B**). However, there were no differences observed between levels of high molecular weight adiponectin in mice of either sex fed either diet or in the ratio of HMW adiponectin:total adiponectin (data not shown). Serum IL6 concentrations in males were significantly affected by both diet ( $P = 0.043$ ) and genotype ( $P = 0.021$ ) with concentrations higher in HFL compared to HFL+R and in Wt mice compared to KO. Interestingly, there was a tendency ( $P=0.061$ ) for a decrease in IL6 concentrations of mice fed HFL+R compared to HFL in Wt but not KO male mice. Serum MCP1 concentrations were similarly affected by both diet and genotype in male mice. However, in contrast to IL6 regulation, MCP1 was significantly decreased by HFL+R diet compared to HFL only in KO male mice. Leptin and TNF $\alpha$  concentrations were not affected by diet or genotype in males or females. Furthermore, female mice did not demonstrate any diet or genotype effects with regard to IL6 or MCP1 serum concentrations.

*Lesion and serum correlations.* A distinct sexual dimorphism exists in the correlation between serum parameters and colon lesions (**Table 7**). In males, ACF number was negatively correlated to total adiponectin ( $P = 0.03$ ) and positively correlated to MCP1 ( $P = 0.02$ ) and TNF $\alpha$  ( $P = 0.098$ ) concentrations. These correlations were reflected in total lesion number. In addition, there was a tendency for a negative correlation between total lesions and serum leptin concentration ( $P = 0.07$ ). In females, ACF and total lesions were not correlated with adiponectin and leptin concentrations but were both strongly positively correlated with serum IL6 ( $P < 0.0001$ ) and TNF $\alpha$  ( $P < 0.0001$ ).

*Stromal vascular cell (SVC) and Caco-2 proliferation and inflammation.* To further investigate the interaction between adiponectin and resveratrol in adipocytes and colon cancer cells, we studied SVCs isolated from gonadal adipose tissue from Wt and KO mice

and Caco-2 colon cancer cells (**Figures 12,13**). Basal SVC proliferation was significantly greater in KO than Wt SVCs (**Figure 12A**). This demonstrated a clear phenotype difference between adiponectin KO and Wt SVCs prior to differentiation and endogenous adiponectin production by Wt cells. Treatment of the cells with resveratrol lowered KO cell proliferation to the level of Wt cells, but had no effect on Wt cell proliferation. We then measured proliferation of Caco-2 colon cancer cells treated with media conditioned from differentiated Wt or KO SVCs. These conditioned medias contained 0.21 ng/ml and 0.02 ng/ml adiponectin, respectively. The Caco-2 cells treated with KO SVC-conditioned media had higher basal proliferation than those treated with Wt SVC-conditioned media, but both treatments caused increased proliferation when compared to the unconditioned media control (**Figure 12B**). Resveratrol treatment reduced proliferation in cells treated with both KO- and Wt-conditioned media to the level of proliferation in unconditioned media. Thus, despite increased proliferation in the absence of adiponectin, the effect of resveratrol on proliferation of SVCs and Caco-2 cells is not dependent on adiponectin.

We then measured the response of adipocytes and colon cancer cells culture in conditioned media to inflammatory stimuli initiated by LPS or LPS/TNF $\alpha$ , respectively. Differentiated KO SVCs had greater basal IL6 production than Wt SVCs. In both cell types, IL6 secretion was significantly elevated with the addition of LPS (**Figure 13A**). Interestingly, resveratrol treatment lowered LPS-induced inflammation as measured by IL-6 concentration in Wt cells but not in KO cells. Conversely, Caco-2 cells treated with KO SVC- or Wt SVC-conditioned media had uniformly low basal IL6 production, but were both responsive to LPS/TNF $\alpha$  treatment (**Figure 13B**). Moreover, resveratrol treatment effectively reduced IL6 production to basal levels in LPS/TNF $\alpha$  stimulated KO SVC- and Wt

SVC-conditioned Caco-2 cells. Therefore, resveratrol effectively attenuated LPS-induced IL6 production in Wt but not KO adipocytes, but was effective in reducing LPS/TNF-induced IL6 production in KO SVC-conditioned and Wt SVC-conditioned Caco-2 cells.

### **Discussion**

Herein we show a tendency for attenuation of early lesion development by resveratrol that is dependent on the presence of adiponectin in male mice. Moreover, male mice had a significant negative correlation between serum adiponectin and ACF. To our knowledge, this is the first report of this interaction in the context of obesity-associated colon carcinogenesis. Interestingly, this interaction is not evident in female mice. This sex disparity is most likely explained by the influence and variability associated with female reproductive hormones. In support of this, two studies have demonstrated an interaction between female reproductive hormones and obesity-related insulin signaling and inflammation in mouse models using established colon cancer lines (26,27). However, the effect of reproductive hormones is not well characterized under tumorigenic conditions.

We also showed that resveratrol-associated improvement in the co-morbidities of obesity were evident primarily in Wt male mice. Specifically, Wt male mice fed HFL+R diet showed a tendency for reduced weight gain, increased percent lean mass, decreased percent body fat, and overall improved serum profile. We propose three possible explanations for this finding: 1) The improvement in the serum profile of Wt male mice may be in part due to the tendency for resveratrol to increase serum adiponectin concentration as we hypothesized. However, resveratrol also tended to increase serum adiponectin in females without a parallel improvement in serum inflammatory and glucose metabolism endpoints. This illustrates a sexual dimorphic response to resveratrol and adiponectin that may be simply due to the more

variable response of female mice to carcinogen and dietary treatments. 2) In addition to actions mediated via adiponectin, it is possible that resveratrol had inhibitory effects on the elevated adipogenesis and inflammation of Wt mice through Sirt1. This role for resveratrol has been previously demonstrated *in vivo* and *in vitro* (16,17,28) 3) Wt mice were more obese and had a more pro-inflammatory profile than KO mice. This discrepancy may explain the more measurable effect of resveratrol on Wt mice.

Although Kineman et al. has previously reported an attenuation of AOM-induced ACF formation with the same resveratrol dose in a low fat diet (21), the lack of a statistically significant response to resveratrol in the present study is likely due to profound DSS- and obesity-induced colonic inflammation. However, differences between ACF assessment methods and colon regions assessed between the studies could also contribute to the lack of significance observed. It is possible that with a greater dietary resveratrol dose, significant results may be obtainable in this obesity model.

In SVC culture, we showed that resveratrol is more effective at attenuating LPS-induced inflammation in Wt compared to KO adipocytes. This supports our hypothesis that the anti-inflammatory effect of resveratrol is enhanced by adiponectin. However, basal IL6 production is higher in KO adipocytes than Wt adipocytes. This is in contrast to our *in vivo* results showing higher circulating IL6 in Wt than KO mice. We also showed that resveratrol was able to reduce proliferation in KO- and Wt-conditioned media treated Caco-2 cells to the same level. The discrepancy between our *in vivo* and *in vitro* experiments is most likely due to differences in resveratrol dose and cancer stage, thereby demonstrating the importance of *in vivo* studies to assess the role of dietary and endogenous signaling molecules on tumorigenesis. The resveratrol doses used in cell culture were chosen to provide a

mechanistic analysis of resveratrol actions on Wt and KO conditions. However, the cell culture doses used would likely not be obtainable in mice fed our low dose resveratrol treatment diets. It is also important to note that the form of resveratrol used in these studies (*trans*-resveratrol aglycone) is not the form present naturally in foods but rather is the form of resveratrol typically used in supplements.

In conclusion, we report a potential effect of resveratrol on obesity-associated colon tumorigenesis in male mice that is not evident in the absence of adiponectin. Moreover, we present a dimorphic gender response to resveratrol and carcinogen treatment. Further studies focusing on resveratrol dosage and this sexual dimorphic response are warranted to improve our understanding of the interaction between resveratrol and adiponectin in colon cancer development.

#### **Author Contributions**

RB performed the animal experiment and laboratory work, designed the cell culture experiments, and wrote the manuscript. EW performed histology of colons and contributed to interpretation of results. JD performed body composition analysis and contributed to interpretation of results. DB contributed to interpretation of results and helped design the experiment. MS supervised the experiments, edited the manuscript, and contributed to interpretation of results.

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## Tables

Table 5. Diet Composition

Ingredient	LFC <sup>1</sup>	HFL <sup>1</sup>	HFL+R <sup>1</sup>
Casein <sup>2</sup>	140	196	196
Sucrose <sup>2</sup>	100	100	100
Corn Starch <sup>2</sup>	456	88	88
Maltodextrin <sup>2</sup>	155	155	155
Soybean Oil <sup>2</sup>	50		
Lard <sup>2</sup>		360	360
Cholesterol <sup>2</sup>		1.5	1.5
Cellulose <sup>2</sup>	50	50	50
Vitamin Mix <sup>2</sup>	10	10	10
Mineral Mix <sup>2</sup>	35	35	35
Choline Bitartrate <sup>2</sup>	2.5	2.5	2.5
L-Cystine <sup>2</sup>	1.8	1.8	1.8
THBQ <sup>2</sup>	0.008	0.008	0.008
Resveratrol Aglycone <sup>3</sup>			0.02
Total (g)	1000.0	1000.0	1000.0
% Protein (Total kcal)	14.69	14.69	14.69
% Carbohydrate (Total kcal)	73.65	25.4	25.4
% Fat (Total kcal)	11.66	59.96	59.96
Density (kcal/g)	3.86	5.4	5.4

<sup>1</sup>Adapted from AIN-93M Diet

<sup>2</sup>Harlan Teklad, Madison, WI

<sup>3</sup>Sigma-Aldrich, St. Louis, MO

**Table 6 (A).** Male Blood and Serum Parameters

	KO		Wt	
	HFL	HRL+R	HFL	HRL+R
Glucose (mg/dl) <sup>a</sup>	336 ± 22	365 ± 22	349 ± 23	320 ± 22
Insulin (pg/ml) <sup>b</sup>	1745.96 ± 320.94 <sup>a</sup>	1874.31 ± 320.94 <sup>a</sup>	2958.22 ± 338.78 <sup>b</sup>	2284.75 ± 320.94 <sup>a,b</sup>
Glucose:Insulin <sup>c</sup>	0.26 ± 0.05	0.26 ± 0.05	0.20 ± 0.05	0.15 ± 0.05
Total AdipoQ (µg/ml) <sup>d</sup>	N/A	N/A	22.72 ± 1.02	25.34 ± 1.02
HMW AdipoQ (µg/ml) <sup>e</sup>	N/A	N/A	5.32 ± 0.64	6.61 ± 0.65
Leptin (ng/ml) <sup>f</sup>	11.64 ± 1.46	11.32 ± 1.39	15.33 ± 1.46	12.46 ± 1.46
IL-6 (pg/ml) <sup>g</sup>	8.86 ± 1.49 <sup>a</sup>	6.78 ± 1.41 <sup>a</sup>	13.69 ± 1.59 <sup>b</sup>	9.32 ± 1.58 <sup>a,b</sup>
MCP-1 (pg/ml) <sup>h</sup>	118.01 ± 13.30 <sup>b</sup>	58.83 ± 20.86 <sup>a</sup>	146.50 ± 17.26 <sup>b</sup>	113.72 ± 14.10 <sup>b</sup>

Values are least squares means ± s.e. Different letters denote statistical significance (P < 0.05).

<sup>a</sup>No significant treatment differences.

<sup>b</sup>Significant genotype effect, P = 0.018.

<sup>c</sup>Tendency for genotype effect, P = 0.099.

<sup>d</sup>Tendency for diet effect, P = 0.094.

<sup>e</sup>No significant treatment differences.

<sup>f</sup>No significant treatment differences.

<sup>g</sup>Significant diet effect, P = 0.043; significant genotype effect, P = 0.021.

<sup>h</sup>Significant diet effect, P = 0.019; significant genotype effect, P = 0.031.

**Table 6 (B).** Female Blood and Serum Parameters

	KO		Wt	
	HFL	HRL+R	HFL	HRL+R
Glucose (mg/dl) <sup>a</sup>	279 ± 22	330 ± 21	304 ± 21	286 ± 22
Insulin (pg/ml) <sup>b</sup>	658.12 ± 135.19	902.49 ± 119.17	852.71 ± 113.55	956.64 ± 127.15
Glucose:Insulin <sup>c</sup>	0.52 ± 0.10	0.49 ± 0.09	0.41 ± 0.09	0.42 ± 0.10
Total AdipoQ (µg/ml) <sup>d</sup>	N/A	N/A	42.54 ± 3.58	51.61 ± 3.70
HMW AdipoQ (µg/ml) <sup>e</sup>	N/A	N/A	11.17 ± 1.17	12.22 ± 1.14
Leptin (ng/ml) <sup>f</sup>	4.60 ± 1.32	4.91 ± 1.19	6.61 ± 1.19	5.83 ± 1.25
IL-6 (pg/ml) <sup>g</sup>	7.09 ± 1.82	7.90 ± 1.56	10.44 ± 1.49	9.69 ± 1.56
MCP-1 (pg/ml) <sup>h</sup>	132.16 ± 31.36	124.81 ± 23.83	157.71 ± 65.00	176.72 ± 36.65

<sup>a</sup>No significant treatment differences.

<sup>b</sup>No significant treatment differences.

<sup>c</sup>No significant treatment differences.

<sup>d</sup>Tendency for diet effect, P = 0.099.

<sup>e</sup>No significant treatment differences.

<sup>f</sup>No significant treatment differences.

<sup>g</sup>No significant treatment differences.

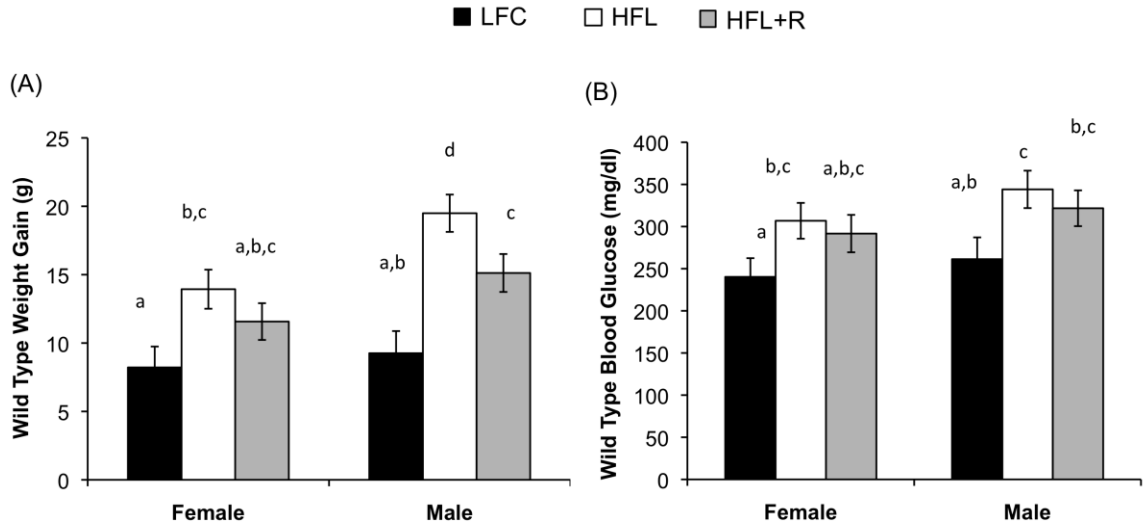
<sup>h</sup>No significant treatment differences.

**Table 7.** Serum parameter and lesion correlations

Serum Parameters	Male		Female	
	ACF	Total Lesions	ACF	Total Lesions
Total Adiponectin	-0.49 (0.03)	-0.40 (0.09)	-0.41 (0.10)	-0.41 (0.10)
Leptin	-0.25 (0.13)	-0.30 (0.07)	0.16 (0.36)	0.18 (0.31)
IL-6	-0.19 (0.26)	-0.15 (0.36)	0.80 (<0.0001)	0.82 (<0.0001)
TNF- $\alpha$	0.38 (0.098)	0.43 (0.06)	0.83 (<0.0001)	0.84 (<0.0001)
MCP-1	0.59 (0.02)	0.45 (0.08)	0.43 (0.096)	0.46 (0.07)

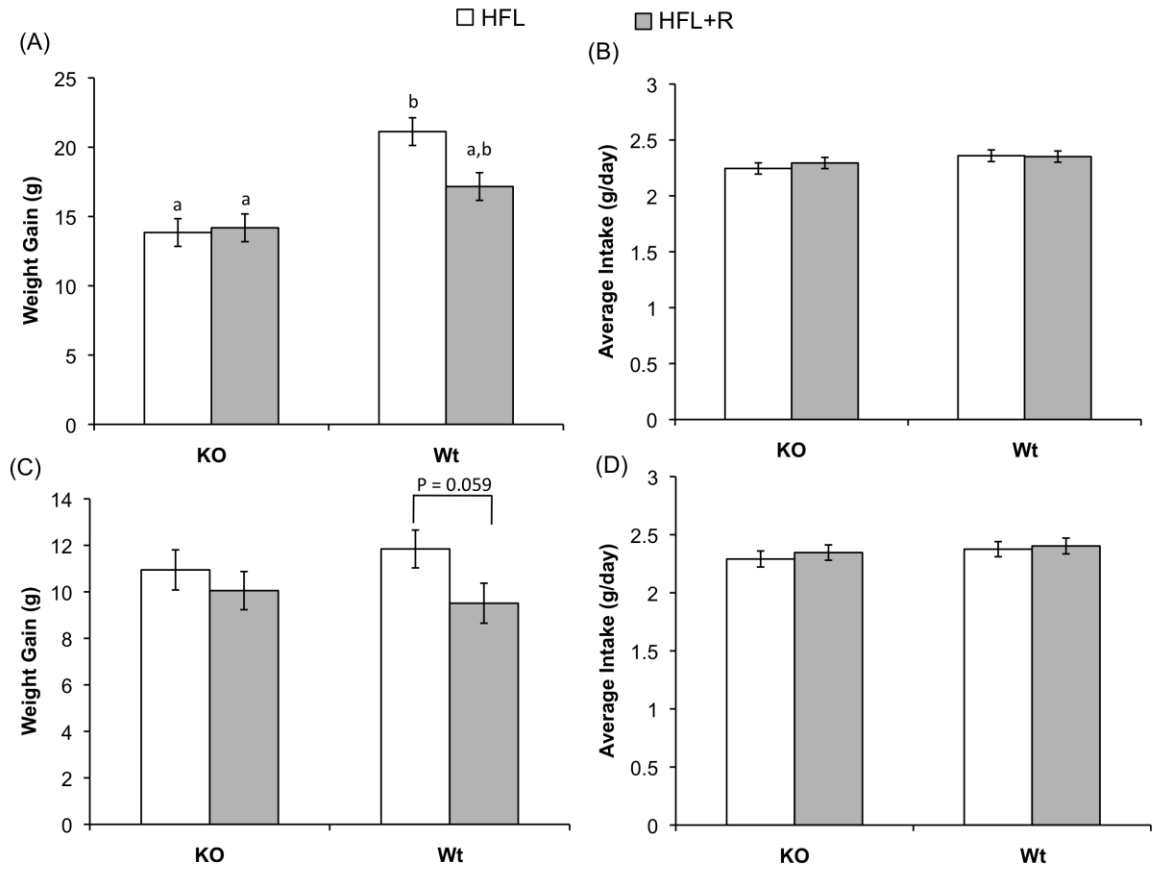
Values are correlation (p-value).

## Figures



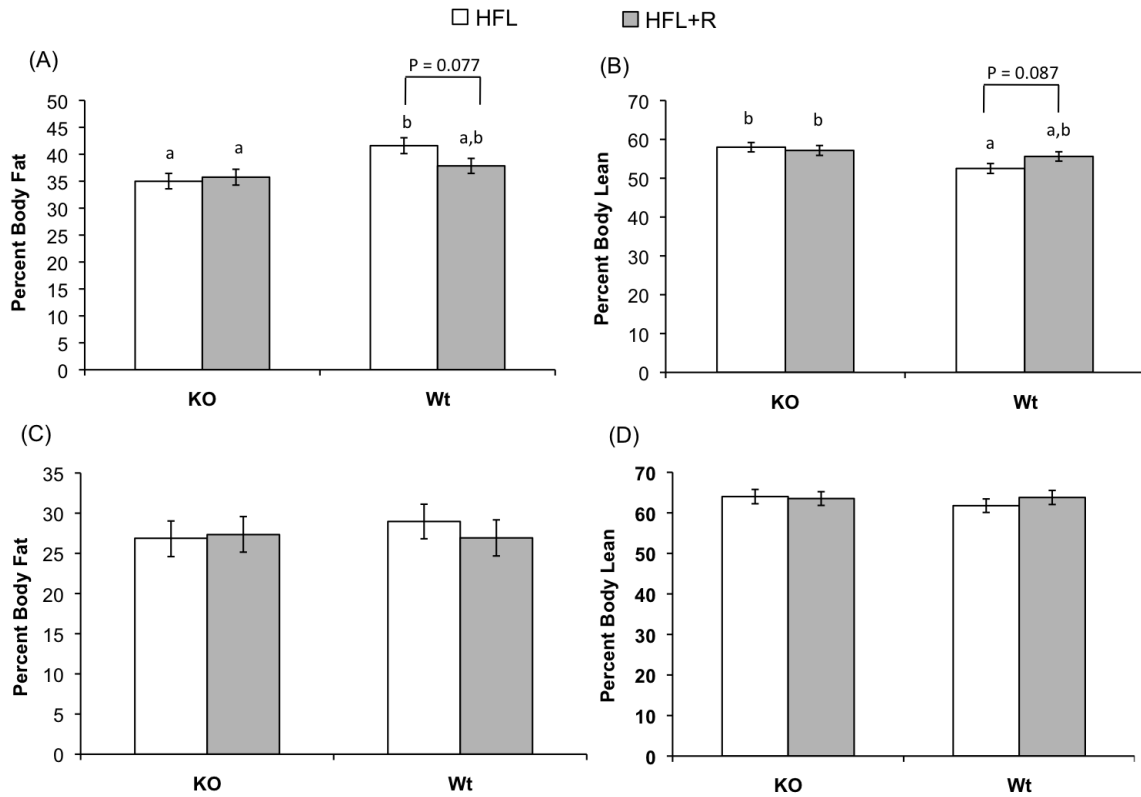
**Figure 7.** HFL causes a significant increase in weight gain and blood glucose concentrations of *Wt* mice, and this is attenuated by RSV.

Wild type weight gain and blood glucose. **(A)** Significant diet effect,  $P < 0.001$ ; tendency for sex effect,  $P = 0.056$ . **(B)** Significant diet effects,  $P = 0.0064$ .



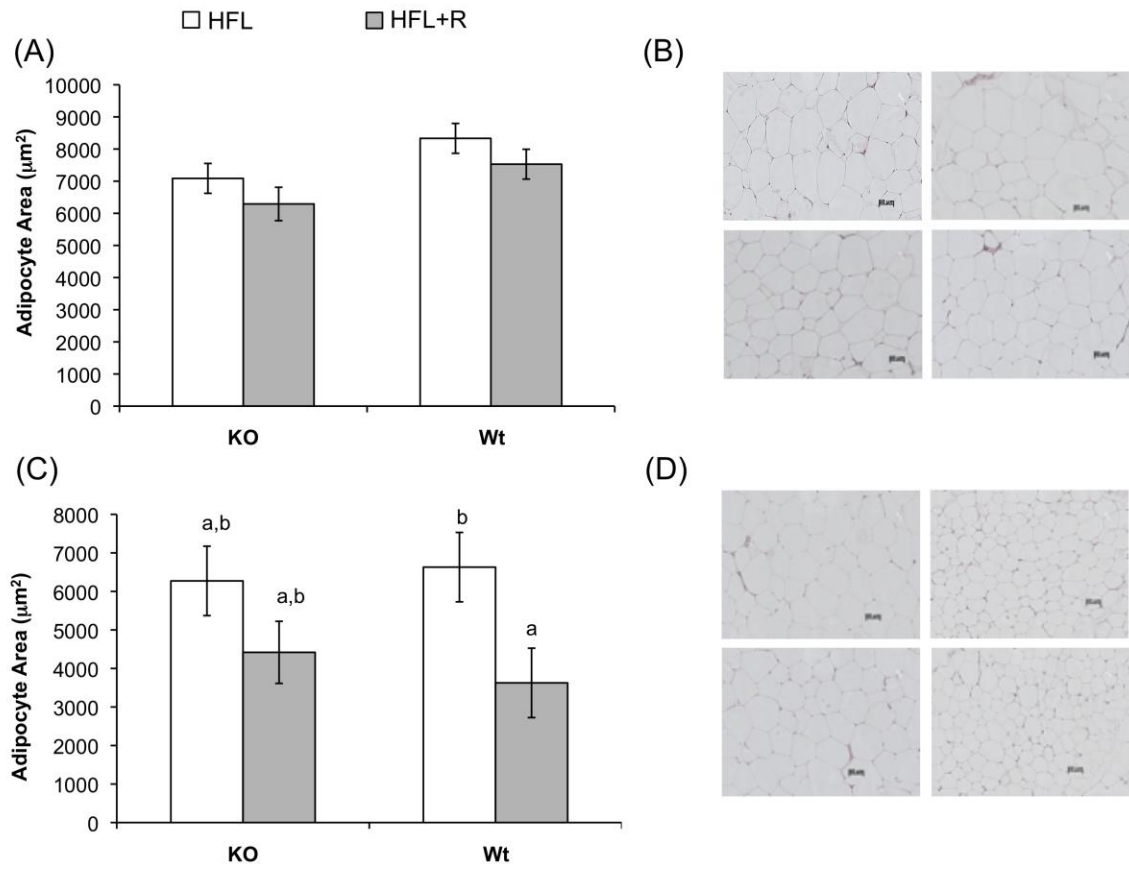
**Figure 8.** RSV had a tendency to attenuate weight gain in Wt but not KO mice without affecting food intake.

Weight gain and average daily intake. Males (A,B); Females (C,D). **(A)** Significant genotype effect,  $P = 0.0009$ . **(C)** Tendency for diet effect,  $P = 0.065$ . **(D)** Tendency for genotype effect,  $P = 0.099$ .



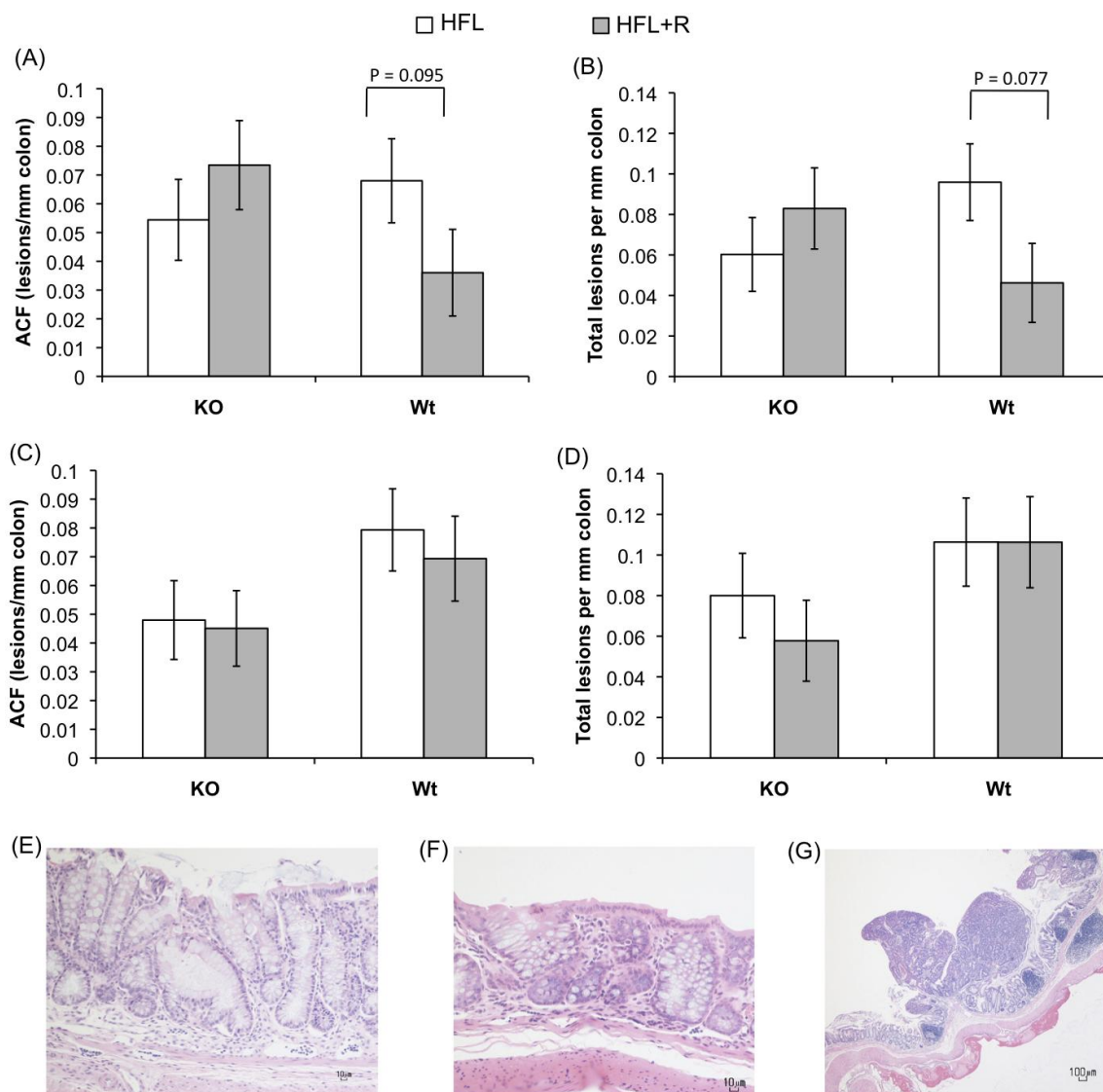
**Figure 9.** RSV had a tendency to increase percent body fat and decrease percent lean mass in male Wt but not KO mice.

Percent total body fat and lean. Males (A,B); Females (C,D). (A) Significant genotype effect,  $P = 0.0045$ . (B) Significant genotype effect,  $P = 0.0077$ .



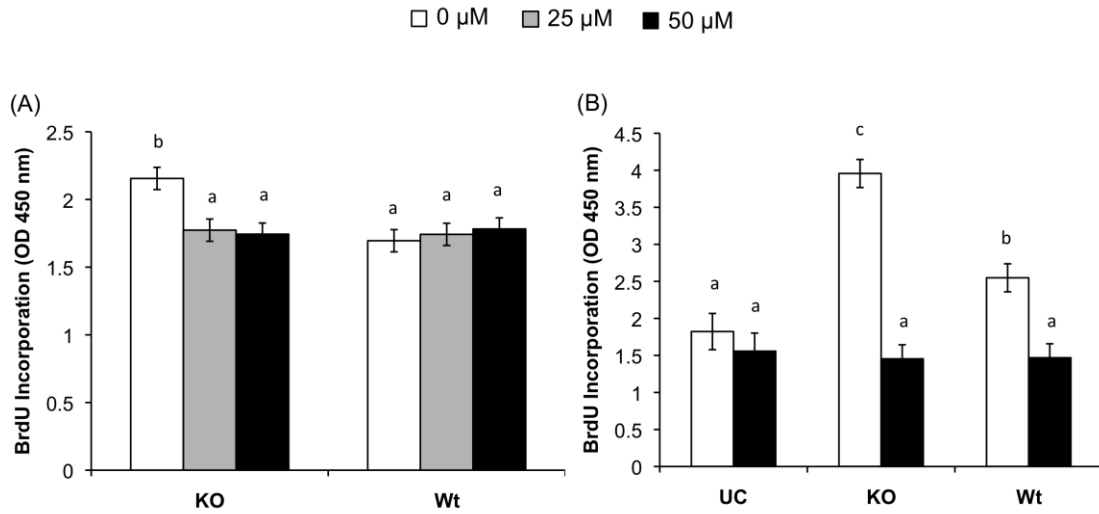
**Figure 10.** RSV significantly decreased adipocyte area in Wt but not KO female mice. Gonadal adipose tissue adipocyte size. Males (A,B); Females (C,D). (A) Tendency for genotype effect,  $P = 0.095$ . (C) Tendency for diet effect,  $P = 0.084$ . B,D: KO, HFL, top left; KO, HFL+R, top right; Wt, HFL, bottom left; Wt, HFL+R, bottom right.



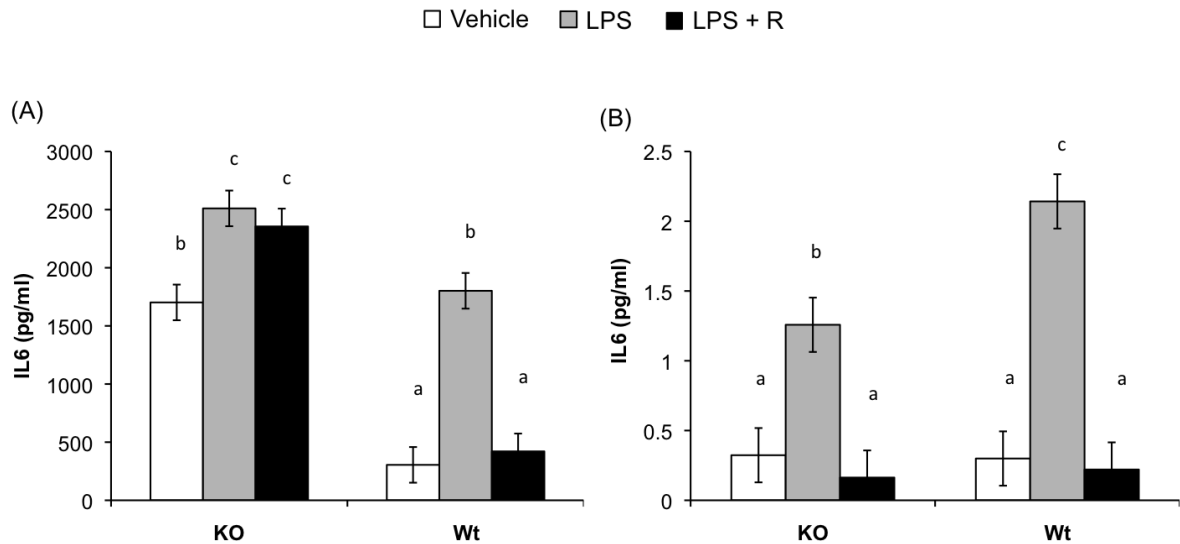


**Figure 11.** RSV has a tendency to decrease AOM-induced early and total lesion number in male Wt but not KO mice.

Colonic aberrant crypt foci and total lesions represented as lesion number per mm distal colon. Average colon section length was 39 mm, and an average of 4 lesions were counted per section. Males (A,B); Females (C,D). (A) Tendency for diet\*genotype interaction,  $P = 0.092$ . (B) Tendency for diet\*genotype interaction,  $P = 0.066$ . (C) Tendency for genotype effect,  $P = 0.062$ . (D) Tendency for genotype effect,  $P = 0.096$ . (E) Representative ACF. (F) Representative MDF. (G) Representative adenoma.



**Figure 12.** Proliferation is attenuated in SVCs and Caco-2 cells by RSV. SVC and Caco-2 cell proliferation. **(A)** SVC BrdU incorporation. Significant effect of SVC genotype,  $P = 0.0338$ . Tendency for effect of RSV concentration,  $P = 0.0886$ . Significant SVC\*RSV interaction,  $P = 0.0116$ . **(B)** Caco-2 BrdU incorporation. Significant effect of SVC genotype,  $P = 0.0003$ . Significant effect of RSV concentration,  $P < 0.0001$ . Significant SVC\*RSV interaction,  $P = 0.0001$ .



**Figure 13.** Adiponectin interacts with RSV to attenuate LPS-induced inflammation in adipocytes, but not Caco-2 cells. SVC and Caco-2 inflammation. **(A)** SVC culture medium IL-6. Significant effect of SVC genotype,  $P < 0.0001$ . Significant effect of RSV concentration,  $P < 0.0001$ . Significant SVC\*RSV interaction,  $P = 0.002$ . **(B)** Caco-2 culture medium IL-6. Tendency for effect of SVC genotype,  $P = 0.07$ . Significant effect of RSV concentration,  $P < 0.0001$ . Tendency for SVC\*RSV interaction,  $P = 0.06$ .

## CHAPTER 5. A MULTI-CELL COMPARISON OF EFFECTS OF RESVERATROL ON LPS-INDUCED INFLAMMATION IN CELL TYPES INVOLVED IN OBESITY-ASSOCIATED COLON CANCER

Submitted to *BMC Cancer*

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### Abstract

Resveratrol (RSV) has been shown to inhibit the metabolic effects of obesity as well as colon tumorigenesis and has recently been shown to attenuate obesity-associated colon tumorigenesis. Moreover, RSV is a known activator of the mediators of inflammation, sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK). However, the roles of Sirt1 and AMPK in the anti-inflammatory function of RSV are not fully elucidated. The aim of this study was to compare the roles of Sirt1 and AMPK in RSV's attenuation of LPS-induced inflammation among human adipocytes, HT29 colon cancer cells, and U937 monocytes. This was investigated through the use of EX 527, a Sirt1 inhibitor, and Compound C, an AMPK inhibitor. In adipocytes, RSV attenuated LPS-induced MCP-1 production in part through activation of Sirt1 but not AMPK. Conversely, Compound C reversed RSV's attenuation of LPS-induced lipolysis suggesting a role for AMPK in the metabolic effects of RSV. In HT29 cells, Sirt1 mediated RSV's effect on LPS-induced reactive oxygen species production and proliferation. However, RSV and EX 527 had no effect on IL-8 production. U937 cells exhibited LPS-induced inflammation that was attenuated by RSV with antagonistic roles of AMPK and Sirt1. Taken together, our data suggest that RSV elicits different molecular responses of AMPK and Sirt1 in the response to LPS stimulation among adipocytes, colon cancer cells, and monocytes. Further research is needed to elucidate how these cell and tissue types interact under RSV supplementation to prevent obesity-associated colon cancer.

## Introduction

Obesity is associated with elevated risk, reoccurrence, and mortality from colorectal cancer (1,2). The development of obesity-associated colon cancer requires the involvement of adipocytes, macrophages, and colon epithelial cell types as inflammatory mediators. The chronic, systemic inflammation characteristic of obesity is a result of increased adipose-resident macrophages, adipose- and diet-derived saturated fatty acids, and increased colonic endotoxin transport and toll-like receptor 4 (TLR4) expression (3). Stimulation of TLR4 by circulating lipopolysaccharide (LPS) and saturated free fatty acids increases the activity of the pro-survival and pro-inflammatory transcription factors, nuclear factor- $\kappa$  B (NF- $\kappa$ B) and activator protein 1 (AP-1) in adipocytes and macrophages (4). Subsequently, adipose- and macrophage-derived cytokines and reactive oxygen species directly contribute to colon tumorigenesis through promotion of survival and proliferation of damaged cells (5,6,7).

Resveratrol (RSV, trans-3,5,4' trihydroxystilbene), a polyphenol found naturally in grapes and red wine, has been demonstrated to have anti-tumorigenic and anti-obesity effects in human models studied *in vivo* and *in vitro* (8,9,10). Moreover, we recently demonstrated a tendency for RSV to attenuate obesity-associated colorectal cancer in a mouse model (11). Inhibition of both NF- $\kappa$ B and AP-1 pathways have been implicated in the action of RSV. In human myeloid, epithelial, and lymphoid cells, RSV was shown to prevent LPS-induced activation of NF- $\kappa$ B and AP-1 (12). Furthermore, treatment of 3T3-L1 murine adipocytes with LPS-stimulated RAW264.7-conditioned media resulted in ERK and NF- $\kappa$ B activation that was attenuated with RSV (13). Similarly, RSV or similar pterostilbene analogues have been reported to down-regulate NF- $\kappa$ B and p38 MAPK in HT-29 human colon cancer cells (14,15).

RSV is an activator of sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK), and it is thought that Sirt1 and AMPK mediate the anti-inflammatory effects of RSV. Although there have been reports of the roles of Sirt1 (16,17,18,19) and AMPK (16,20) in the regulation of NF- $\kappa$ B and MAPK pathways in various cell types, the independent roles of RSV-induced Sirt1 and AMPK in attenuation of inflammation in obesity-associated colon cancer has not been fully elucidated. Moreover, we have previously shown that murine adipocytes and Caco-2 colon cancer cells respond differentially to RSV with respect to presence of adiponectin, an AMPK activator (11,21). Thus, the purpose of the experiments described herein was to mechanistically compare the response of cell types involved in obesity-associated colon cancer to RSV treatment under LPS stimulation.

### **Materials and Methods**

*Materials.* Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone (Logan, UT). RPMI 1640 medium was purchased from American Type Culture Collection (ATCC, Manassas, VA). The NF- $\kappa$ B p65 inhibitory peptide was purchased from Imgenex (IMG-2003, San Diego, CA). SB 203580, Cardamonin, EX 527, and Compound C were purchased from Tocris Bioscience (Ellisville, MO). All reagents not specified were purchased from Sigma (St. Louis, MO).

*Culturing of human adipocytes.* Human subcutaneous pre-adipocytes pooled from 6 non-diabetic, non-obese female donors were purchased from Zen Bio (Research Triangle Park, NC). Pre-adipocytes were cultured in low glucose DMEM with 10% FBS and 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. For differentiation to adipocytes, pre-adipocytes were grown to confluence and incubated in differentiation medium containing 100 nM human insulin, 33  $\mu$ M biotin, 17  $\mu$ M pantothenic acid, 0.1% ITS (insulin transferrin sodium

selenite) supplement, 5  $\mu\text{M}$  Troglitazone, 1  $\mu\text{M}$  dexamethasone, and 0.5 mM IBMX for 6 days. Differentiation medium was removed at Day 6, and fresh medium containing 100 nM insulin, 33  $\mu\text{M}$  biotin, 17  $\mu\text{M}$  panthothenic acid, and 0.25  $\mu\text{M}$  dexamethasone was applied every 2 days until experimental treatments were applied between Days 12-14.

*Culturing of HT29 human colon cancer and U937 human macrophages.* HT29 and U937 cells were purchased from ATCC (Manassas, VA). HT29 cells were cultured in high glucose DMEM medium with 10% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. U937 monocytes were cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. HT29 and U937 cells were plated at  $1 \times 10^6$  cell/ml for experimental treatments.

*Cell culture experiments.* Differentiated human adipocytes (Day 12-14), HT29 colon cancer cells, and U937 monocytes were plated and cultured as described above. Treatment medium consisted of regular growth medium for each cell type with or without the addition of vehicle (0.1-0.5% DMSO), 1  $\mu\text{g}/\text{ml}$  LPS, and 20  $\mu\text{M}$  RSV. The following inhibitors were added concurrently with LPS and/or RSV treatment: 20  $\mu\text{M}$  cardamonin (inhibitor of I $\kappa$ B degradation), 10  $\mu\text{M}$  p65 inhibitory peptide, 20  $\mu\text{M}$  SP 600125 (JNK inhibitor), 20  $\mu\text{M}$  SB 203580 (p38 inhibitor), 20  $\mu\text{M}$  EX 527 (Sirt1 Inhibitor), and 10  $\mu\text{M}$  Compound C (AMPK Inhibitor). All treatments were applied for 24 hours.

*Proliferation.* BrdU reagent (Chemicon International, Temecula, CA) was applied to treated HT29 cells according to the manufacturers instructions and incubated for 8 hours. BrdU incorporation was detected by measuring absorbance at 450/550 nm.

*Reactive oxygen species production.* ROS production was measured in confluent HT29 cells and adipocytes following the 24 hour incubation in treatment medium as

previously described (4). Briefly, treatment medium was removed and cells were rinsed in warm 1x phosphate buffered saline (PBS). The cells were incubated for 45 minutes at 37°C in 10  $\mu$ M OxyBURST Green H<sub>2</sub>DCFDA (Invitrogen, Carlsbad, CA) in warm 1x PBS buffer. The detection agent was removed, and cells were rinsed 3 times with warm 1x PBS. Florescence was measured at absorbance and emission wavelengths of 495 nm and 527 nm, respectively. Data was normalized to protein concentration in each well as determined by bicinchoninic acid assay (BCA) (Pierce, Rockford, IL).

*Quantitative Real-Time PCR.* RNA was isolated using acid-phenol reagent (TRIzol, Invitrogen, Carlsbad, CA), and possible DNA contamination was removed using DNase-Free (Ambion, Austin, TX). cDNA was synthesized using iScript (Bio-Rad, Hercules, CA). Standard curves were created for all primer pairs by cloning amplified cDNA into pGemT vector (Promega, Madison, WI) and sequenced to confirm the gene target. The primer sequences used are listed in **Table 8**. mRNA transcript abundance was quantified on an iCycler (Bio-Rad, Hercules, CA) with IQ™ SYBR Green Super Mix kit (Bio-Rad, Hercules, CA). Thermal cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Gene expression in each tissue was normalized to a housekeeper gene ( $\beta$ -Actin) and expressed as log starting quantity.

*Glycerol release.* Glycerol release into treatment medium was measured in differentiated adipocytes. Free Glycerol Reagent was added to samples and standards as described by the manufacturer (Sigma, St. Louis, MO). Absorbance was measured at 540 nm.

*Cytokine and chemokine quantification.* Secreted cytokines and chemokines were measured by Multiplex or ELISA. For Multiplex analysis, treatment medium was analyzed using the Human Cytokine/Chemokine Pre-Mixed 14 Plex Milliplex kit (Millipore, Billerica,



MA) on a Bioplex system (BioRad, Hercules, CA). ELISAs were used to measure human MCP-1 and IL-8 production in the culture medium (R&D Biosystems, Minneapolis, MN).

*Transcription factor activation.* Nuclear extracts were isolated using the Cayman Nuclear Extraction Kit (Cayman Chemical, Ann Arbor, MI). NF- $\kappa$ B p65 transcription factor activation was measured by ELISA (Cayman Chemical, Ann Arbor, MI). AP-1 c-Jun transcription factor activation was measured by ELISA (TransAM AP-1 c-Jun, Active Motif, Carlsbad, CA). Data was normalized to protein concentration in each sample as determined by BCA (Pierce, Rockford, IL).

*Statistical analysis.* Data was tested for normality by residual analysis. Data was analyzed using the general linear model procedure in SAS (Version 9.2; SAS Institute, Cary, NC) with culture treatment as the fixed effect. Quantitative PCR data was analyzed with RSV, LPS, and RSV\*LPS interaction as fixed effects. Total protein concentrations or housekeeper mRNA transcript abundance were used as covariates for normalization of relevant analyses. Data is reported as least square means  $\pm$  s.e.m. Statistical significance is defined as  $p < 0.05$  and tendency for significance as  $p \leq 0.1$ .

## Results

We first compared the activation of the RSV downstream target pathways, MAPK and NF- $\kappa$ B, by LPS in monocytes, adipocytes, and colon cancer cells. A panel of 14 cytokines and chemokines were measured in each cell type. Each cell type expressed a unique cytokine and chemokine profile represented in **Figure 14**. In U937 monocytes, each inhibitor significantly attenuated LPS-induced IL-8 or MCP-1 production with the most robust reduction from the JNK inhibitor, SP 600125, and the non-specific NF- $\kappa$ B inhibitor, Cardamonin (**Figure 14A,B**). Similarly, LPS-induced IL-8 and TNF- $\alpha$  production were

significantly reduced in HT29 cells in response to all inhibitors (**Figure 14C,D**). Adipocytes showed a more variable response to the inhibitors, with a significant inhibition of IL-6 in response to all inhibitors and an inhibition of MCP-1 production in response to Cardamonin only (**Figure 14E,F**). Taken together, this data demonstrates that both NF- $\kappa$ B and MAPK pathways are important mediators of LPS-induced inflammatory cytokine and chemokine production in these cell types.

To determine the potential roles of AMPK and Sirt1 in the regulation of LPS response by RSV, the AMPK and Sirt1 inhibitors, Compound C and EX 527, were utilized. In U937 cells, AMPK or Sirt1 inhibitors in concert with RSV and LPS treatment reduced IL-8 production greater than the combined treatment of RSV and LPS alone (**Figure 15A**). In HT29 cells, LPS increased IL-8 production, but the combined treatment of LPS and RSV was unable to significantly reduce LPS-induced inflammation (**Figure 15B**). Sirt1 inhibition resulted in no change in IL-8 production from that of the LPS and RSV treatment. Interestingly, AMPK inhibition of the LPS and RSV treatment significantly reduced both IL-8 production and proliferation (**Figure 15B, 16A**). RSV decreased proliferation, and Sirt1 inhibitor reversed the effects of RSV on ROS production and proliferation of HT29 cells (**Figures 16A, 16B**). In adipocytes, the Sirt1 but not AMPK, inhibitor reversed RSV's inhibition of LPS-induced MCP-1 and ROS production. (**Figures 15C, 16D**). However, only the AMPK inhibitor significantly increased glycerol when added to the combined treatment of LPS and RSV in adipocytes. Thus, Sirt1 appears to be important in mediating the anti-inflammatory effect of RSV in adipocytes and colon cancer cells while AMPK may be important in mediating metabolic effects. The metabolic effects of AMPK may be driven by RSV-induced increase in the insulin-sensitizing hormone, adiponectin. To this end, we

showed that RSV significantly increased adiponectin mRNA transcript abundance under vehicle conditions, but had no effect under LPS stimulation (**Figure 17**).

To connect the pro-inflammatory endpoints to the downstream molecular targets of RSV, we identified the roles of AMPK and Sirt1 in transcription factor activation in the MAPK and NF- $\kappa$ B pathways. NF- $\kappa$ B p65 and c-Jun, a major component of the AP-1 heterodimer, were chosen based on the successful attenuation of LPS-induced cytokine and chemokine production in the presence of p65 and JNK pathway inhibitors demonstrated in **Figure 14**. In U937 monocytes under LPS stimulation, RSV had a tendency ( $P = 0.09$ ) to decrease NF- $\kappa$ B p65 activation that was further decreased by Sirt1 inhibitor (**Figure 18A**). The opposite was observed for c-Jun activity whereby the AMPK inhibitor further decreased c-Jun activity compared to RSV's effect on LPS (**Figure 19A**). In adipocytes, RSV significantly inhibited LPS-induced p65 activation, and RSV-induced inhibition was reversed to the level of LPS activation in the presence of both inhibitors (**Figure 18C**). The activation of c-Jun in adipocytes was less consistent. Interestingly, RSV significantly increased c-Jun activity under vehicle conditions ( $P = 0.001$ ) and had a tendency to increase c-Jun activity under LPS stimulation ( $P = 0.1$ ) (**Figure 19C**). Our data suggests this effect of RSV was independent of AMPK or Sirt1. There was no observed effect of RSV or Sirt1 and AMPK inhibition on HT29 transcription factor activation (**Figures 18B, 19B**), suggesting that transcriptional regulation may be precluded by 24 hr. post-LPS stimulation in this cell type.

Because Sirt1 was most effective in the anti-inflammatory action of RSV, we measured the transcriptional regulation of Sirt1 by RSV and LPS (**Figure 20**). In U937 and HT29 cells, Sirt1 transcription was elevated significantly by LPS treatment (**Figures 20A, 20B**). There was also a tendency for up-regulation of Sirt1 transcript by RSV under Vehicle

conditions in U937 cells ( $P = 0.1$ ) and under LPS conditions in HT29 cells (0.07). In adipocytes, there were no significant treatment effects of RSV or LPS (**Figure 20C**).

However, Sirt1 transcript had a tendency for up-regulation by RSV under vehicle conditions ( $P = 0.06$ ) but not LPS.

### Discussion

*Adipocytes.* We demonstrated herein that cell types involved in obesity-associated colon cancer responded to RSV by different mechanisms 24 hours after LPS stimulation. Adipocytes responded to LPS stimulation with elevated production of IL-6 and MCP-1, which was significantly attenuated by RSV. As evidenced by MCP-1 production in response to Sirt1 and AMPK inhibitors, Sirt1 but not AMPK in part mediated the anti-inflammatory effect of RSV. Similarly, Sirt1 but not AMPK reversed RSV's inhibition of LPS-induced ROS generation. It is likely that the anti-inflammatory effect of RSV in adipocytes is mediated in part by inhibition of p65 activity. To this end, our data suggests that both AMPK and Sirt1 mediate RSV's attenuation of p65 activity. Interestingly, c-Jun was increased by RSV independent of Sirt1 and AMPK in adipocytes. This finding is supported by a report by Deck et al. that showed RSV increases AP-1 activity in Hek293 cells (22). In contrast to the anti-inflammatory function of RSV in adipocytes, AMPK appeared to be an important regulator of RSV's attenuation of LPS-induced lipolysis. Furthermore, RSV increased expression of the AMPK activator, adiponectin, in adipocytes. It is possible that in an *in vivo* obese scenario, this function of AMPK may indirectly impact inflammation through decreasing free fatty acid-induced inflammation (23).

*HT29 colon cancer cells.* HT29 cells responded differentially to RSV compared to adipocytes. While LPS induced IL-8 and TNF $\alpha$  production in HT29 cells, RSV did not

significantly attenuate IL-8 production. In support of this, RSV has previously shown to have no effect on IL-8 gene expression in Caco-2 murine colon cancer cells (24). Interestingly, the combined treatment of AMPK inhibitor and RSV decreased IL-8 production, suggesting a RSV-independent role for AMPK under LPS stimulation. Sirt1 inhibition suggested a role for Sirt1 in mediating the attenuation of proliferation and ROS generation by RSV in HT29 cells. However, we did not detect an effect of RSV on p65 or c-Jun activity. This may be due to lack of continued LPS and RSV stimulation throughout the 24 hour treatment period or involvement of alternate NF- $\kappa$ B and AP-1 heterodimer components.

*U937 monocytes.* Monocytes, although responsive to LPS and RSV, demonstrated opposite roles for AMPK and Sirt1. RSV attenuated LPS-induced IL-8 production, and both AMPK and Sirt1 inhibition amplified rather than reversed the RSV response. Similarly, Sirt1 inhibition maximized RSV's attenuation of p65 activity, and both AMPK and Sirt1 maximized RSV's attenuation of c-Jun activity. Interestingly, Sirt1 expression was elevated by LPS in monocytes as in HT29 cells. This may be explained by the increased Sirt1 expression and nuclear accumulation but not activity shown by Liu et al. in development of LPS tolerance by a monocytic cell line (25). Conversely, RSV increased Sirt1 expression in HT29 but not U937 cells, suggesting that Sirt1 is differentially regulated by RSV under LPS stimulation between these cell types.

Taken together, these results demonstrate that adipocytes, colon cancer cells, and monocytes respond to LPS stimulation with different cytokine and chemokine profiles, and RSV acts differentially on these inflammatory pathways among cell types. However, these cell types are not independent from one another *in vivo*. In obesity-associated colon tumorigenesis, monocytes infiltrate both adipose and colonic tissue, and locally interact with

these tissues to stimulate inflammation. Moreover, we have previously shown that the adipokine profile influences colon cancer cell proliferation and inflammation both *in vitro* and *in vivo* (11). Future *in vivo* studies aimed at investigating tissue-specific RSV signaling in obesity-associated colon tumorigenesis are warranted.

There are limitations to this use of inhibitors that should be considered in the interpretation of these results. First, the use of one inhibitor per target does not allow us to identify any bias or lack of specificity for that inhibitor. For most meaningful data, multiple inhibitors should be used. Second, we have not demonstrated in the cell lines used, that the target pathways were down-regulated by the inhibitors. Finally, the intended specificity of the inhibitors for specific pathway components may not have an overall effect on the pathway.

#### **Author Contributions**

RB designed and performed the experiments and wrote the manuscript. MS supervised the experiments, edited the manuscript, and contributed to interpretation of results.

#### **Acknowledgements**

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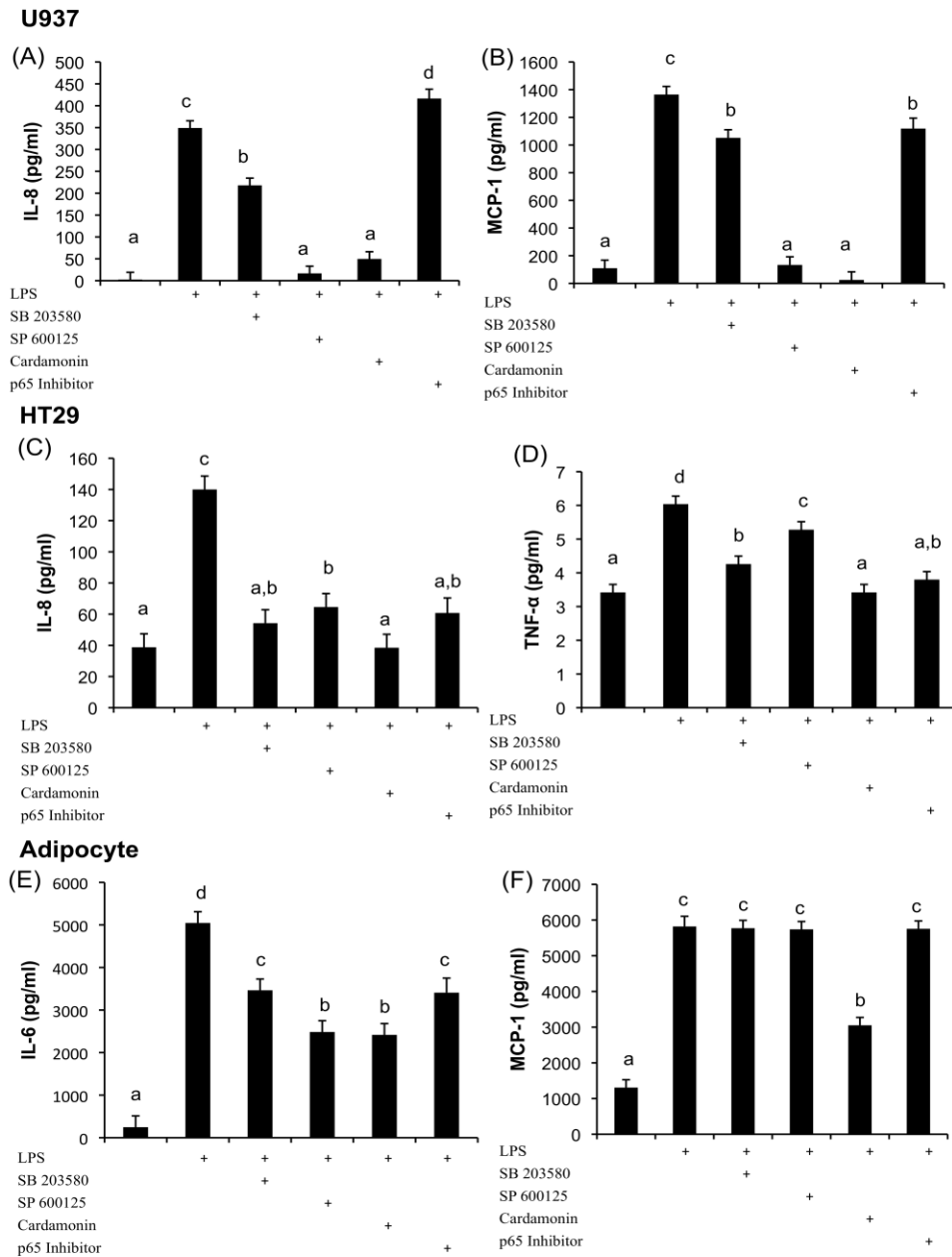


## Tables

**Table 8.** *Quantitative Real Time PCR Primers*

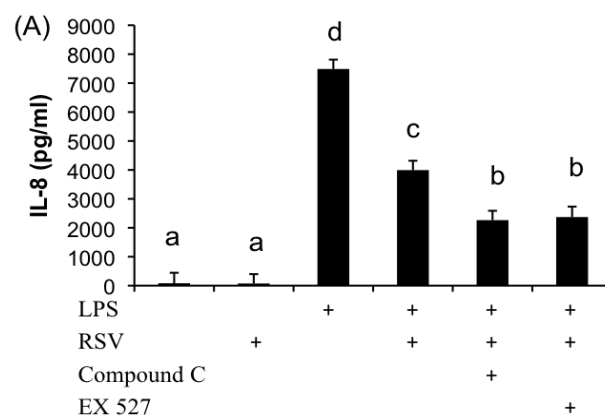
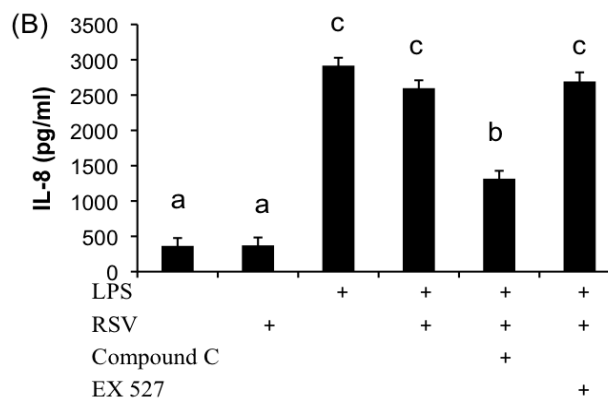
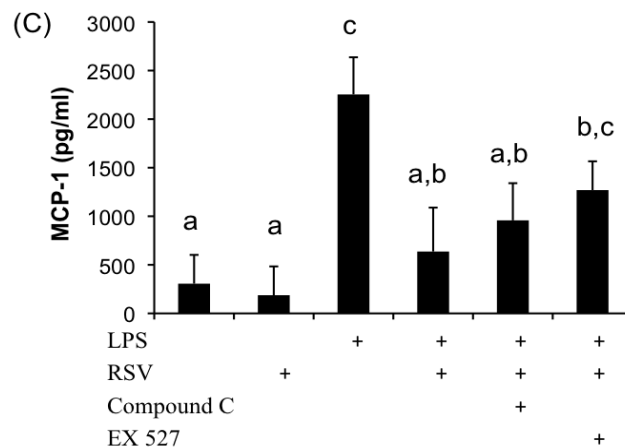
<b>Target</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
$\beta$ -Actin	CAGCCATGTACGTTGCTATCCAGG	AGGTCCAGACGCAGGATGGCATG
Adiponectin	ATCCAAGGCAGGAAAGGAGAACCT	TGGTAAAGCGAATGGGCATGTTGG
Sirt1	TCCTGGACAATTCCAGCCATCTCT	TTCCAGCGTGTCTATGTTCTGGGT

## Figures



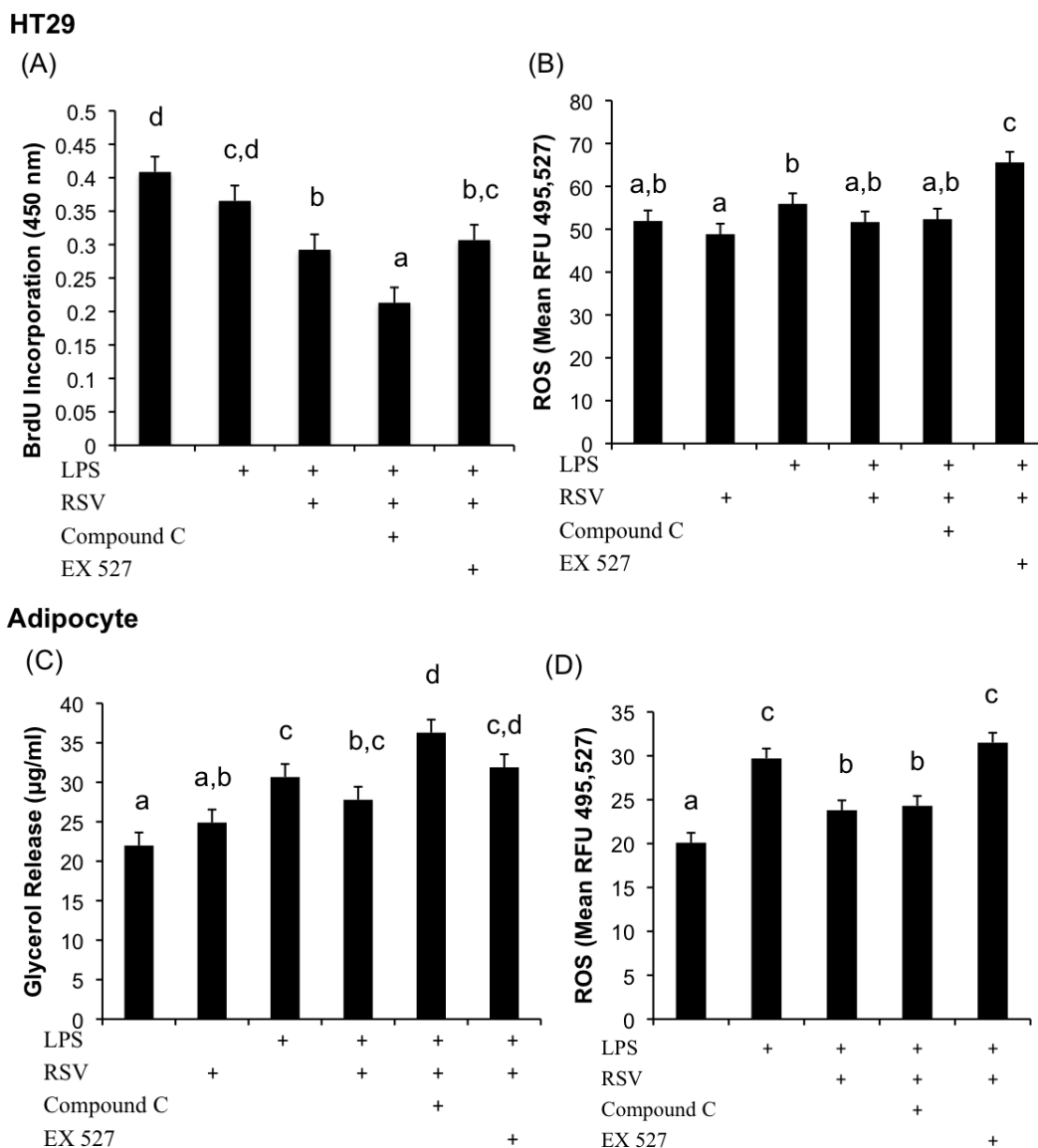
**Figure 14.** Comparison of the roles of MAPK and NF- $\kappa$ B pathways in cytokine and chemokine response to LPS in the presence of specific inhibitors.

SB 203580 (p38 Inhibitor), SP 600125 (JNK Inhibitor), Cardamonin (I $\kappa$ B degradation Inhibitor). (A,B) U937 monocytes, A: P < 0.0001, B: P < 0.0001. (C,D) HT29 colon cancer cells. C: P < 0.0001, D: P < 0.0001. (E,F) Adipocytes, E: P < 0.0001, F: P = < 0.0001. n = 8 per treatment.

**U937****HT29****Adipocyte**

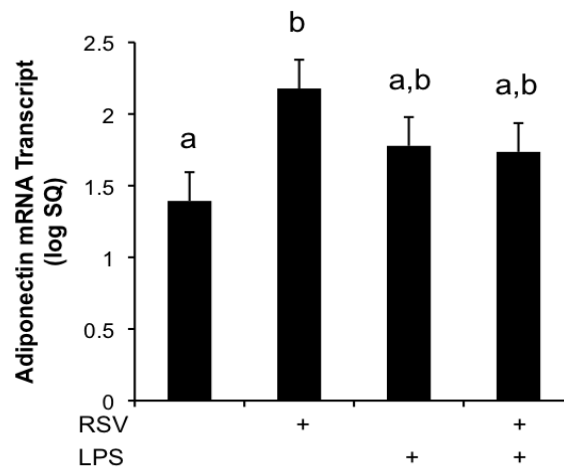
**Figure 15.** *Sirt1* mediates the anti-inflammatory action of RSV.

Compound C (AMPK Inhibitor), EX 527 (Sirt1 Inhibitor). (A) U937 monocytes,  $P < 0.0001$ . (B) HT29 colon cancer cells,  $P = < 0.0001$ . (C) Adipocytes,  $P = 0.0006$ .  $n = 6$  per treatment.



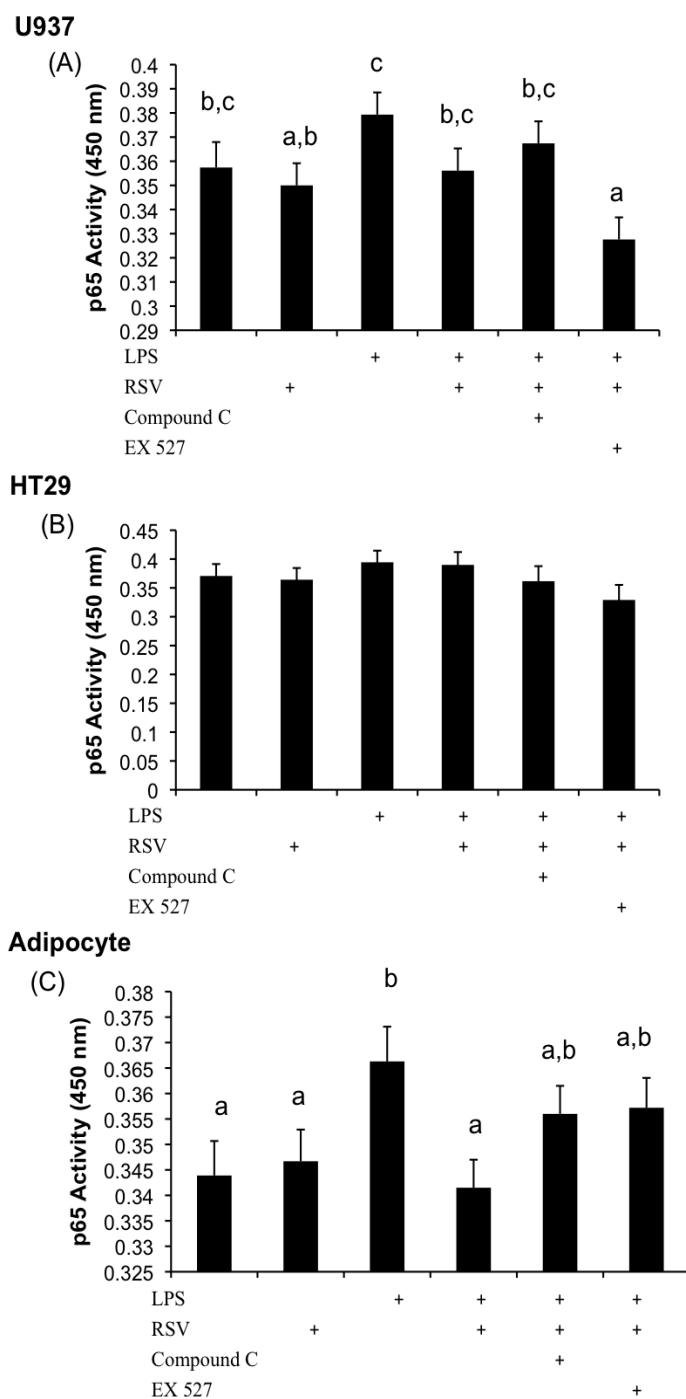
**Figure 16.** Roles of AMPK and Sirt1 in ROS production, proliferation, and lipolysis.

Compound C (AMPK Inhibitor), EX 527 (Sirt1 Inhibitor). (A) HT29 proliferation,  $P < 0.0001$ . (B) HT29 ROS generation,  $P = 0.0004$ . (C) Adipocyte glycerol release,  $P < 0.0001$ . (D) Adipocyte ROS generation,  $P < 0.0001$ .  $n = 6 - 8$  per treatment.



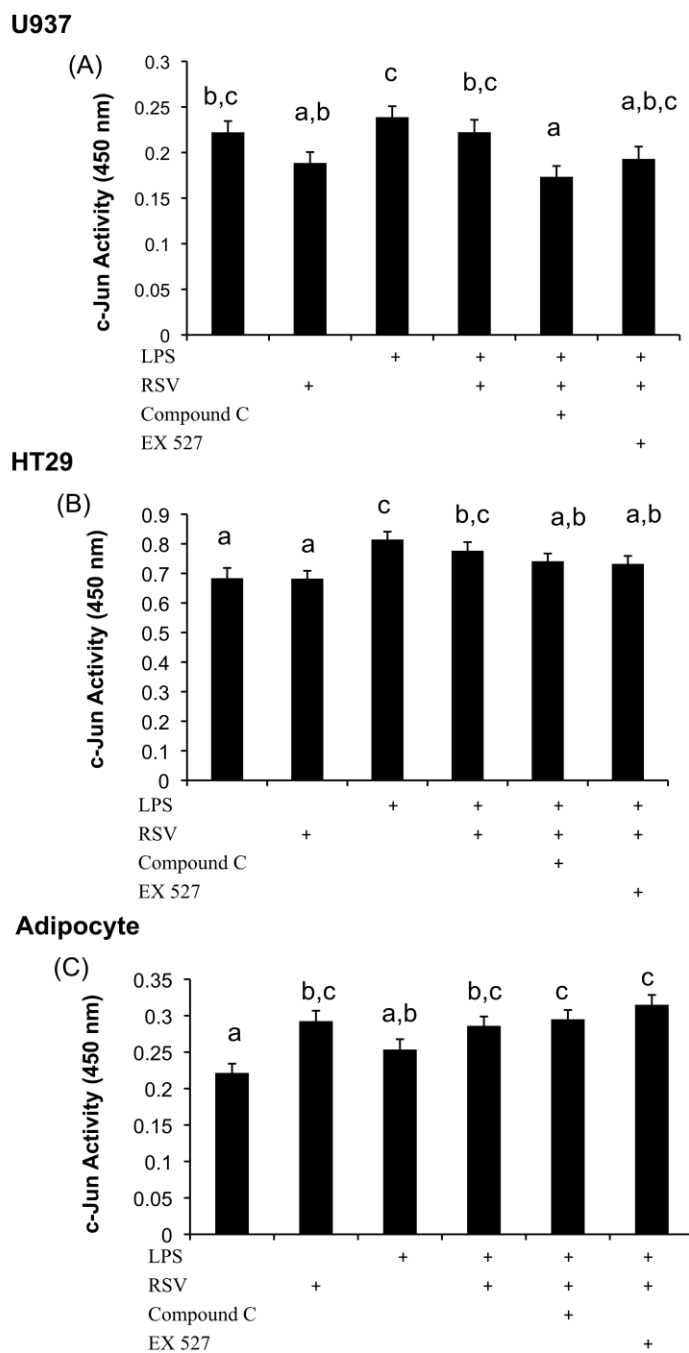
**Figure 17.** Effect of RSV and LPS on adiponectin expression in adipocytes.

RSV P = 0.082, LPS P = 0.89, RSV\*LPS P = 0.06. n = 5 per treatment.



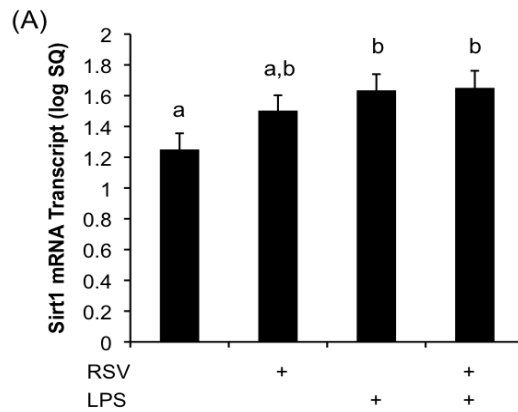
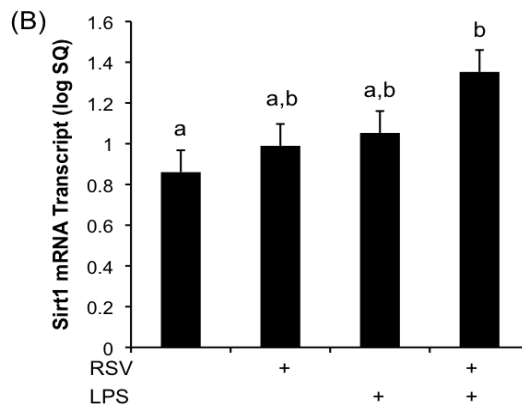
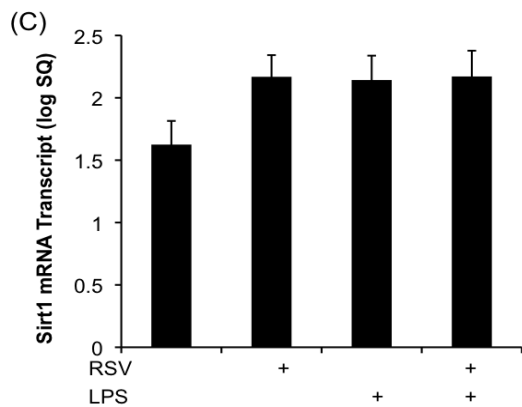
**Figure 18.** *NF- $\kappa$ B* p65 activity is differentially mediated by AMPK and Sirt1 in monocytes, colon cancer cells, and adipocytes.

Compound C (AMPK Inhibitor), EX 527 (Sirt1 Inhibitor). (A) U937 monocytes,  $P = 0.016$ . (B) HT29 colon cancer cells,  $P = 0.33$ . (C) Adipocytes,  $P = 0.07$ ,  $n = 6$  per treatment.



**Figure 19.** AP-1 c-Jun activity is differentially mediated by AMPK and Sirt1 in monocytes, colon cancer cells, and adipocytes.

Compound C (AMPK Inhibitor), EX 527 (Sirt1 Inhibitor). (A) U937 monocytes,  $P = 0.008$ . (B) HT29 colon cancer cells,  $P = 0.03$ . (C) Adipocytes,  $P = 0.0009$ ,  $n = 6$  per treatment.

**U937****HT29****Adipocyte**

**Figure 20.** *Sirt1* mRNA transcript abundance is regulated by RSV and LPS.

Compound C (AMPK Inhibitor), EX 527 (Sirt1 Inhibitor). (A) U937, RSV P = 0.21, LPS P = 0.03, RSV\*LPS P = 0.29. (B) HT29, RSV P = 0.06, LPS P = 0.02, RSV\*LPS = 0.44 (C) Adipocyte, RSV P = 0.20, LPS P = 0.17, RSV\*LPS P = 0.20. n = 5 per treatment.



## CHAPTER 6. GENERAL CONCLUSIONS

The incidence of obesity has recently risen dramatically in developed countries, resulting in increased risk of obesity-related morbidities such as colon cancer (1,2). Development of obesity-linked colon cancer is accompanied by a shift to an overall pro-inflammatory adipokine environment with decreased circulating concentrations of the anti-inflammatory adipokine, adiponectin. The role of adiponectin in colon cancer development has been investigated, yielding controversial results asserting that adiponectin both protects and promotes colon cancer in response to different dietary and carcinogenesis models (3,4). RSV, a dietary polyphenol, is proposed to have anti-obesity and anti-tumorigenic effects, which have been attributed in part to activation of SIRT1 and AMPK activation (5). However, the role of RSV in obesity-associated colon cancer has not been previously reported.

We first sought to investigate the role of adiponectin in obesity-associated colon cancer through the use of an adiponectin knockout mouse model (Chapter 3). To this end, we treated adiponectin KO and Wt mice with a combined AOM/DSS treatment followed by 7 ½ weeks of high fat or low fat diet feeding. In this study, KO mice developed fewer lesions than Wt mice. This finding was in contrast to the proposed role of adiponectin in AOM models of colon cancer (4), but consistent with its reported role in the inflammatory DSS model (3). Moreover, we showed that lesion formation was differentially regulated by genotype, diet, and sex. Specifically, early lesions were regulated by diet whereas advanced lesions were regulated by diet and sex. The intermediate lesion type, adenoma, was regulated by diet, genotype, and sex. This was the first report of a disparity between lesion stage and the effect of adiponectin genotype, sex, and dietary treatment.

Interestingly, the high fat diet promoted pro-inflammatory gene expression in adipose tissue but not colon. This is likely due to the robust colonic inflammatory response initiated by the DSS treatment. Because the DSS treatment preceded the dietary treatments, all mice had substantial colonic immune cell infiltration independent of dietary treatment. The primary difference between the DSS and AOM models is initiation of inflammation. It has been suggested that in the AOM model, adiponectin functions to attenuate NF- $\kappa$ B signaling, resulting in decreased proto-oncogene activation and related tumorigenesis (4). Conversely, in the DSS model, adiponectin may be preventing NF- $\kappa$ B transcribed growth factors from repairing damaged epithelium, thus promoting colonic damage and tumorigenesis. This theory is supported by the findings of Fayad et al. that showed under DSS-induced pro-inflammatory conditions, adiponectin binds growth factors preventing the repair of damaged epithelium (3). Thus, it is becoming evident that adiponectin may be effective at attenuating colonic tumorigenesis under non-inflammatory but not pro-inflammatory conditions. These dual roles of adiponectin require further investigation and likely explain the highly variable nature of data relating adiponectin to colon cancer in the human population.

Because we showed that the adiponectin genotype primarily influenced early lesion development, we used our knockout mouse model at the ACF stage to evaluate the interaction between RSV and adiponectin in obesity-associated tumorigenesis (Chapter 4). In this study, RSV had a tendency to attenuate ACF formation in Wt but not KO mice. This was the first report of the tendency for an interaction between RSV and adiponectin in colon tumorigenesis. In addition to its effects on ACF, RSV also interacted with the adiponectin genotype to attenuate weight gain, adipocyte size, and insulin and IL-6 concentrations. With the exception of adipocyte size, this interaction was observed in male but not female mice.

This sex difference is most likely explained by the influence of reproductive hormones. In support of this, two studies have demonstrated an interaction between female reproductive hormones and obesity-related insulin signaling and inflammation in mouse models using established colon cancer cell lines (6,7). However, the effect of reproductive hormones is not well characterized under tumorigenic conditions.

The absence of significant dietary effects is likely due to the use of low-dose RSV in combination with a high saturated fat diet and DSS treatment. We hypothesize that the dose of RSV used (20 mg/kg diet) was insufficient to overcome these inflammatory insults both at the tissue and circulation levels. The lack of a low fat control baseline for gene analysis may have limited the detection of attenuation of inflammatory gene expression by RSV in adipose tissue. Moreover, the Birt laboratory has previously demonstrated that this dose of RSV is effective at attenuating ACF formation in a low fat control AOM rodent model that lacked induction of inflammation (8).

To further investigate the interaction between RSV and adiponectin at the cellular level, we isolated gonadal adipose-derived stromal vascular cells from adiponectin KO and Wt mice for treatment with RSV. We also generated conditioned media from the differentiated adipose-derived SVCs for treatment of Caco-2 colon cancer cells. These experiments showed that in adipocytes, RSV's action was dependent on the presence of adiponectin to attenuate LPS-induced inflammation. Conversely, in colon cancer cells treated with adipocyte-conditioned media, RSV attenuated inflammation and cell proliferation in the presence and absence of adiponectin. This suggested differential mechanisms of RSV action between cell types.

Finally, we sought to compare the mechanistic role of RSV in LPS-induced inflammation across cell types involved in obesity-associated colon tumorigenesis (Chapter 5). For this set of experiments, we utilized human colon cancer cells, monocytes, and adipocytes in which we compared the roles of AMPK and Sirt1 in RSV stimulated attenuation of LPS-induced inflammation and ROS production through use of an AMPK and a Sirt1 inhibitor. Our results suggest that RSV functions differentially between these cell types. In adipocytes, RSV attenuated LPS-induced MCP-1 production and lipolysis, and Sirt1 and AMPK inhibitors reversed this, respectively. The action of RSV was mediated in part by downstream attenuation of p65 activation. In HT29 colon cancer cells, RSV failed to significantly inhibit IL-8 production, but did inhibit ROS generation and proliferation. The addition of the Sirt1 and AMPK inhibitors suggested that this was in part through Sirt1 but not AMPK signaling. U937 monocytes, in contrast to adipocytes and colon cancer cells, did not demonstrate a role for Sirt1 or AMPK in RSV's attenuation of IL-8 production. It is important to note that we did not confirm Sirt1 or AMPK inhibition by the inhibitors used, and the data should therefore be interpreted with this in mind. Moreover, because only one inhibitor was tested for each Sirt1 and AMPK, the specificity and bias of each is unknown in these cell lines.

These findings led us to propose a model for RSV function in prevention of obesity-associated colon cancer. In this model, we hypothesize that RSV acts directly on adipocytes in adipose tissue to inhibit inflammation and metabolic dysregulation associated with obesity through activation of AMPK and Sirt1. In colon epithelial cells, we hypothesize that RSV attenuates proliferation and ROS production in part via Sirt1 activation. Moreover, we hypothesize that RSV-induced reduction of inflammation in adipose tissue will further

attenuate inflammation and proliferation in the colon. The anti-inflammatory effects of RSV on both adipose and colon tissues then inhibits monocyte infiltration and the propagation of inflammation in these tissues. Therefore, we propose that RSV attenuates obesity-associated colon cancer through its direct action on adipose and colon tissues.

Overall, we have presented evidence that adiponectin, diet, and gender interact in the development of obesity-associated colon tumorigenesis under pro-inflammatory conditions. We further demonstrated that RSV is a promising therapeutic strategy for prevention of obesity-associated colon cancer through its interaction with adiponectin. Finally, we showed that RSV signals differentially between cells types involved in obesity-associated colon cancer to inhibit inflammation and ROS generation. Obesity-associated colon cancer is a multi-factorial disease, and effective preventative therapies should target both adipose and colon tissue.

The continuation of this research can proceed in several directions. First, studies can be aimed at resolving the seemingly opposing roles of adiponectin in colon tumorigenesis. This would require the use of a variety of dietary and inflammatory conditions both *in vivo* and *in vitro*. Additionally, the observed differences between male and female responses to diet and carcinogen could be investigated. Because comparisons between sexes are rarely made in animal studies, this research could have a large impact on both the obesity and cancer research fields. To further study therapeutic strategies aimed at obesity-associated colon cancer, there is ample opportunity for testing and identification of novel compounds that target RSV signaling pathways and demonstrate similar or greater bioactivity than RSV itself. Furthermore, our mechanistic analysis of RSV action can be expanded to additional human cell lines and subsequently to an *in vivo* tumorigenesis model. Finally, the expression

profile of specific colonic lesion types throughout development from healthy tissue to carcinoma and the effect of RSV on each lesion type can be investigated. These findings would help identify the ideal window of time in the progression colon tumorigenesis for RSV treatment.

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