INVESTIGATING THE MULTI-TARGETED ANTI-CANCER AND
CHEMOPREVENTIVE POTENTIAL OF GSE IN PRE-CLINICAL MODELS OF
COLORECTAL CANCER

by

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Date  07/02/13
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Investigating the Multi-targeted Anti-cancer and Chemopreventive Potential of GSE in Pre-Clinical Models of Colorectal Cancer

Thesis directed by Professor Rajesh Agarwal

Colorectal cancer (CRC) is the third leading cause of cancer-associated deaths in the world; in this regard, there is an interest in cancer prevention and treatment strategies. Colon carcinogenesis involves a number of etiological factors, and thus to create effective preventive and treatment strategies, molecular targets need to be identified and targeted prior to disease progression.

Non-toxic chemoprevention strategies need to be developed; Grape seed extract (GSE) is one such agent, whose beneficial effects have been well documented in multiple cancer models. The present studies examine the chemopreventive and anti-cancer efficacy of GSE treatment through *in-vitro* and *in-vivo* pre-clinical models of CRC. *In-vitro* studies elute GSE efficacy and mechanisms of apoptotic death in human CRC cell lines. *In-vivo* studies assessed GSE efficacy as a chemoprevention agent against azoxymethane (AOM)-induced colon tumorigenesis in the A/J mouse model. Furthermore, these studies characterized the metastasis of azoxymethane-induced colon tumors to lung mouse model and evaluated grape seed extract (GSE) efficacy against this pre-clinical metastatic CRC model.

GSE is a complex mixture of polyphenolic compounds, including catechin and epicatechin; we aimed to identify potential targets of this extract utilizing Drug Affinity
Response Target Stability (DARTS). This DARTS technique involves separation of proteins from GSE-treated and control-treated human CRC cells. Altered protein bands that are enriched due to GSE treatment were isolated and analyzed via LC/MS; the resulting peptides were then identified via MASCOT. These results revealed an overall downregulation of proteins involved in the ER stress response.

Overall the completed studies in this thesis indicated that GSE is a non toxic pleiotropic agent that targets multiple protein pathways involved in the development of colon carcinogenesis; these pathways included: ER stress pathway; intrinsic and extrinsic apoptotic pathways; proliferation pathways and inflammation. The pre-clinical efficacy studies have indicated that GSE would be a safe, effective, long-term treatment for the prevention and treatment of human CRC and should be further investigated in Phase I clinical trials.

The form and content of this abstract are approved. I recommend its publication.

Approved: Rajesh Agarwal
ACKNOWLEDGEMENTS

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<td>Acetaldehyde</td>
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<td>Arachidonic acid</td>
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<td>ACS</td>
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<td>ADH</td>
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<td>MMPs</td>
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MRI  Magnetic Resonance Imaging
MS   Mass Spectrometry
mTOR Mammalian target of Rapamycin
MUC1 Mucin 1, cell surface associated
NAC  N-acetyl Cysteine
nACHRs Nicotinic Acetylcholine Receptors
NFκB Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NKX2.1 NK2 homeobox 1
NNK Nicotine-derived Nitrosamine Ketone
NO   Nitric Oxide
NOC  Nitroso compounds
Nrf2 Nuclear Transcription Factor Erythroid 2p45
NSAIDs Non-steroidal Anti-inflammatory Drugs
NUCL Nucleolin
NuRD Nucleosome Remodeling and Histone Deacetylase
OGG1 8-Oxoguanine glycosylase
PAHs Polycyclic Aromatic Hydrocarbons
PAR Proline and acidic acid-rich
PARP Poly ADP Ribose Polymerase
PCA Protocatechuic acid
PCD Programmed Cell Death
PCNA Proliferating Cell Nuclear Antigen
PCR Polymerase Chain Reaction
<table>
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<td>PDIA3</td>
<td>Protein Disulfide Isomerase Family A</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic Polypeptide Peroxisome</td>
</tr>
<tr>
<td>PPARα</td>
<td>Proliferator-activated receptor-α</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RAS</td>
<td>RAit Sarcoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time-PCR</td>
</tr>
<tr>
<td>SAM</td>
<td>S-Adenosylmethionine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
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<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
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<tr>
<td>SP-D</td>
<td>Surfactant D</td>
</tr>
<tr>
<td>SSB</td>
<td>Single Strand Breaks</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor Associated Antigen</td>
</tr>
<tr>
<td>TCF-4</td>
<td>Transcription factor 4</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor Necrosis Factor-related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>TRF2</td>
<td>Transferrin Receptor 2</td>
</tr>
<tr>
<td>TTF-1</td>
<td>Thyroid Transcription Factor-1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>UGTs</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>VC</td>
<td>Vector Control</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>XIAP</td>
<td>X-linked Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray Repair Cross-Complementing Protein-1</td>
</tr>
<tr>
<td>YY</td>
<td>YY Peptide</td>
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CHAPTER I

IDENTIFYING MOLECULAR TARGETS OF LIFESTYLE MODIFICATIONS IN COLON CANCER PREVENTION

Introduction

It is projected that by 2030 the number of new cancer cases will increase by 70% worldwide due to demographic changes alone; the significant rise is attributed to the adoption of western lifestyle habits (Franceschi and Wild, 2013). Globally, colorectal cancer (CRC) is the 2nd most common cancer in women and the 3rd most common in men; according to the American cancer society (ACS), in the United States alone, there are estimated to be approximately 50,830 deaths associated with CRC in 2013. CRC diagnostic screening strategies are available, however, the compliance is low; further conventional treatments result in severe toxicity and do not decrease disease incidence (Diaz et al., 2006; Bretthauer, 2010). With this in mind, based on existing knowledge about risk factors and natural CRC disease progression, prevention strategies need to be developed and implemented within the community. There is extensive research examining the risk factors for CRC, including high red meat consumption, high fat low fiber diet, alcohol and tobacco consumption, obesity, lack of physical activity and sleep deprivation (Huxley et al., 2009; Wei et al., 2009; Basterfield and Mathers, 2010; Thompson et al., 2011). These lifestyle factors are the major causes of disease

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burden globally, and combined with physician’s tendency to prescribe medication rather than prescribing a healthy lifestyle, have resulted in a global health crisis (Yousefi et al., 2012; Franceschi and Wild, 2013). However, advancing knowledge investigating the molecular basis of carcinogenesis has created a remarkable opportunity to develop effective prevention strategies through lifestyle modification. Identifying molecular targets of lifestyle modification allows researchers to develop effective non-toxic interventions, thus further allowing physicians to design personalized lifestyle prescriptions based on patient history, resulting in improved patient health and well being.

**Identifying Molecular Pathways Involved in CRC Etiology**

CRC is a complex multi-factorial disease; prior to diagnosis, there are decades of complex genetic and environmental interactions that ultimately lead to disease initiation, promotion, and progression (Nambiar et al., 2010). The cellular environment plays a large role in the disease, and as early as 1863, it was hypothesized that the origin of cancer was at sites of chronic inflammation (Balkwill and Mantovani, 2001). During the normal inflammatory response, anti-inflammatory cytokine production follows the production of pro-inflammatory cytokines, creating a balanced environment. Chronic inflammation can be the result of persistent initiating factors in the surrounding environment, or faulty repair mechanism resulting in unrepaired damage, ultimately leading to neoplasia (Coussens and Werb, 2002).

During chronic inflammation, imbalances between the production of reactive oxygen species (ROS) and the detoxification of these reactive species result in oxidative
stress with in the target tissue; this stress can result in DNA damage and further reduce DNA repair (Coussens and Werb, 2002). On the other hand, cells can inherit changes in phenotype, independent of alterations in the DNA; termed epigenetics, more specifically chromatin based events that regulate the DNA template (Dawson and Kouzarides, 2012). Further alterations in gene methylation and acetylation status result in altered chromatin regulators and gene expression (Dawson and Kouzarides, 2012). These epigenetic changes have been associated with oncogene modification or abnormal expression patterns that can lead to the induction and maintenance of various cancers (Dawson and Kouzarides, 2012). At the same time, the tumor microenvironment can also alter the DNA template resulting in modified protein translation, function and transport. (Xu et al., 2005).

Once the cells are transformed, the extent to which inflammatory cell populations infiltrate the tissue depends on the balance of cytokines released from the tumor microenvironment. Inflammatory cells are able to produce a wide array of cellular signals including ROS, proteases [i.e. MMPs (Matrix metalloproteinase)], soluble cell death ligands [i.e. TNF-α (tumor necrosis factor alpha)], interleukins (ILs) (i.e. IL-6) and interferons (IFNs) (Coussens and Werb, 2002). NFkB is a central mediator of the immune response that further regulates inducible-nitric oxide synthase (iNOS) which is an enzyme that catalyzes the formation of nitric oxide (NO), leading to inflammatory and hormone signaling modulation (Fujimoto et al., 2005). Additionally, signaling lipid mediators, specifically prostaglandins (PGs), are formed during oxidation of arachidonic acid (AA) via cyclooxygenases (COX-1 and COX-2) which are involved in numerous processes including inflammation, hormone regulation, and cell growth (Vane et al.,
Inflammatory mediators are also involved in the regulation of various growth factors and hormones, including VEGF (Vascular endothelial growth factor) and IGF-1 (Insulin-like growth factor-1), which are involved in tumor cell proliferation and metastasis (Akagi et al., 1998).

The cells’ response to the above stimuli depends on its ability to adapt and continue on the path of cell survival, allowing cells to repair DNA damage and continue through the cell-cycle, or direct towards the path of programmed cell death (PCD). Hallmarks of colon carcinogenesis are uncontrolled cellular proliferation and resistance to cell death; therefore, strategies that focus on these processes would be of clinical significance. Proliferative signaling pathways often begin with activation of a receptor tyrosine kinase (RTK) (e.g. EGFR, epidermal growth factor receptor) by a growth factor and subsequent downstream protein activation (i.e. MAPK, mitogen-activated protein kinase) that can lead to activation of multiple pathways involved not only in proliferation but also cell-cycle regulation and cellular metabolism (Fritz and Fajas, 2010). Proliferation is controlled by cell cycle regulatory pathways and involves DNA replication and cellular division; this process is controlled by a cascade of protein phosphorylation events and a set of protein checkpoints that can arrest the cell at specific stages of the cell cycle (Collins et al., 1997). PCD is an intracellular process that refers to apoptosis, autophagy and programmed necrosis. The process of apoptosis is highly complex involving two main pathways, the extrinsic or death receptor pathway (i.e. DR4/DR5) and the intrinsic or mitochondrial-derived pathway (Elmore, 2007). Autophagy is a lysosomal-dependent pathway involving degradation and recycling of cellular components; controlled via an autophagy-related gene network (Liu et al., 1998).
Programmed necroptosis is a result of the initiation of different receptor-sensor complexes via various stimuli, including TNF-α and ROS species (Chan et al., 2011). Colorectal carcinogenesis involves initiating events resulting in genomic alteration, which leads to neoplastic growth, resulting in the alteration of microenvironment and further tumor promotion from adenoma to adenocarcinoma, ultimately resulting in disease progression or metastasis, via internal and external cellular stimuli. A wide variety of cellular pathways are involved throughout the colorectal carcinogenesis process, thus, identifying molecular targets that are altered in response to lifestyle choices is key in understanding how to effectively prevent CRC.

**Biological responses of meat consumption in relation to CRC risk**

Humans over a long period of time have adapted to consuming large amounts of lean red meat; for many adults in the US, red meat is part of a habitual diet with average consumption of red meat per day being 128g (Mann, 2000; Daniel et al., 2011). However, numerous epidemiological studies suggest an association between red and processed meat consumption and the risk of CRC (Chan et al., 2011). Red meat is considered the intake of mutton, lamb, veal, pork and beef, and processed meat is defined as the total intake of cured or preserved meats, sausages, ham, and bacon (Chan et al., 2011). To develop prevention strategies that will reduce CRC risk, we need to further understand the role of these factors in colorectal carcinogenesis.

Consumption of red and processed meats may induce several biological responses that could be responsible for the increased risk of CRC development as shown in Table 1-1 and Figure 1-1. White meat is not associated with an increased risk of CRC, so what
dietary factor/s in red meat increases the risk of cancer development? One important dietary factor could be heme, an iron porphyrin pigment of red meat, present 10-fold higher in red meat compared to white meat (Schwartz and Ellefson, 1985; Ishikawa et al., 2010). A free ferrous iron is released when heme oxygenase-1 (HO-1) resolves dietary heme in the intestinal mucosa (Ishikawa et al., 2010). Iron itself has been linked to the increased production of ROS, specifically H$_2$O$_2$, which can further induce genetic mutations, inflammatory mediators and other cytotoxic effects (Klaunig and Kamendulis, 2004; Knobel et al., 2007). H$_2$O$_2$, more specifically, induces IL-8, IL-6, IL-1β and TNF-α cytokine production, and can further activate NFκB and AP-1 transcription factors promoting a pro-inflammatory signal (Morcillo et al., 1999; Haddad et al., 2001; Yamamoto et al., 2003).

Another dietary factor that could contribute to CRC risk is malondialdehyde (MDA), a known mutagen, which is formed during lipid peroxidation and found at high

### Table 1-1: Molecular targets of lifestyle modifications for CRC prevention

<table>
<thead>
<tr>
<th>Red Meats</th>
<th>Detox Pathways</th>
<th>Oxidative Stress</th>
<th>Inflammation</th>
<th>DNA Repair</th>
<th>Epigenetic</th>
<th>Oncogene Alteration</th>
<th>Hormone Signaling</th>
<th>Proliferation</th>
<th>Apoptosis</th>
<th>Cell Cycle</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>CYPs, Catalase</td>
<td>NO, HIF-1α, NADPH oxidase</td>
<td>TNF-α, IL-6</td>
<td>p53</td>
<td>SAM, p16</td>
<td>p53</td>
<td>EGFR</td>
<td>AP-1, PI3K/Akt, ERK 1/2</td>
<td>P53, PI3K/Akt</td>
<td>AP-1, p16</td>
<td>VEGF, MMP-2, MMP-9</td>
</tr>
<tr>
<td>Tobacco</td>
<td>CYPs, GSTs, UGTs</td>
<td>ROS</td>
<td>NFκB, COX-2</td>
<td>OGG1, APEX1/APE1, XRCC1</td>
<td>KXAS/BRAP</td>
<td>C-myc</td>
<td>EGFR</td>
<td>MAPK, AP-1, EGFR</td>
<td>Bcl-2, Bad, MAPK</td>
<td>AP-1</td>
<td>MMP-2, VEGF</td>
</tr>
<tr>
<td>PA/Obesity</td>
<td>SOD, Nrf2, GSTs</td>
<td>ROS, Nrf2</td>
<td>TNF-α, IL-6, iNOS, NFκB, COX-2</td>
<td>C-myc, β-catenin</td>
<td>YY, GLP-1, PP, Leptin, Insulin, IGFBP-3, PPARα, GSK3β</td>
<td>Leptin, MAPK, PI3K/Akt, PCNA, β-catenin, GSK3β</td>
<td>Leptin, MAPK, PI3K/Akt</td>
<td>Cyclin D</td>
<td>VEGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circadian Clock</td>
<td>CYPs, HIF, E4BP4</td>
<td>TRβ, ROS</td>
<td>NFκB</td>
<td>p53</td>
<td>C-myc, β-catenin</td>
<td>p53, p21</td>
<td>p53, p21, Bcl-2, Bim, cc3, cc9</td>
<td>p21, Wnt1, Cyclin D1E, p53, p21</td>
<td>VEGF</td>
<td></td>
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</tr>
</tbody>
</table>
levels in the plasma when beef is consumed compared to chicken (Basu and Marnett, 1983; Toden et al., 2010). MDA exposure results in colonic DNA damage, and studies have shown that there is a dose-dependent positive association between MDA plasma levels and the extent of DNA signal-strand breaks (SSB) and double-strand breaks (DSB) (Toden et al., 2010). Additionally, red meat consumption leads to the production of N-nitroso compounds (NOC) in the large bowel, which can directly result in DNA damage and further through the binding of telomere stabilizing proteins (i.e. TRF2) can lead to DNA adduct formation (O'Callaghan et al., 2012). Moreover, red meat consumption initiates epigenetic changes in host DNA; genotoxic exposure results in modification of NuRD histone deacetylase complexes that control transcriptional repression, including histone deacetylase-2 (HDAC-2) (Feng and Zhang, 2003; Hebels et al., 2012). Furthermore, animal-fat oxidation occurs when red meat is consumed, resulting in formation of oxysterols and aldehydes that can alter hormone signals such as TGF-β; these external stimuli can result in uncontrolled proliferation (Biasi et al., 2008). Apart from these changes, genes that are involved in cancer metastasis are also modified in response to red meat exposure; specifically, the Notch signaling pathway, which is abnormally activated in CRC and plays a key role in epithelial-mesenchymal transition (EMT) (Bolos et al., 2007; Wang et al., 2010; Hebels et al., 2012).

**Identifying molecular targets of alcohol-induced CRC**

Alcohol (ethanol), by virtue of its oral consumption, not only reaches the gastrointestinal tract but also reaches every cell of the body, and is a major risk factor for
Figure 1-1 Summarizing the effects of red meat consumption on colon carcinogenesis pathways.
Figure 1-1 Summarizing the effects of red meat consumption on colon carcinogenesis pathways. Consumption of red meat has been shown to induce epigenetic changes in host DNA: These changes occur specifically through altering the levels of histone deacetylase-2 (HDAC-2). Red and processed meat further contains iron, heme and nitrosyl heme, all of which at high levels may increase the risk of CRC development. Both heme and nitrosyl heme undergo catalysis resulting in the formation of N-Nitroso compounds (NOC); these NOCs can either result in DNA damage or DNA adduct formation. Red meat consumptions specifically leads to mutations in p53 and KRAS genes, further leading to the initiation and progression of colon cancer. Alternatively heme catalysis can also lead to lipid peroxidation end products, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), oxysterols, and aldehydes. MDA exposure can result in DNA adduct formation, leading to DNA mutations and aberrant proliferation, further contributing to the initiation of colorectal cancer. 4-HNE is cytotoxic and genotoxic compound, that targets colon cells that carry a wild-type APC gene; this selective toxicity results in enhancement of colon cells that carry a mutated APC gene, resulting in CRC promotion and progression. Additionally lipid peroxidation results in the formation of oxysterols and aldehydes, which further alter hormone signaling, specifically TGF-β, ultimately resulting in uncontrolled proliferation that contributes to the promotion and progression of CRC. Another major component of red meat is iron, which is absorbed mainly through dietary intact; ferric iron (Fe\(^{3+}\)) binds to transferrin, resulting in receptor activation and endocytosis. Ferric iron is further converted to ferrous iron (Fe\(^{2+}\)) via divalent metal transporter 1 (DMT1), which then contributes to the cell’s overall iron pool. Iron has been linked to the production of ROS species, specifically H\(_2\)O\(_2\); these reactive species can then upregulated inflammatory mediators, such as NFκB, IL-6, IL-8, IL-1β, and TNF-α, leading to the promotion and progression of CRC. Furthermore IL-1β signaling upregulates NFκB, which then activates DMT1 iron transporters, resulting in increased levels of ferrous iron with in the cell, representing a feed-back loop in iron regulation. All the photographs of lifestyle interventions were provided by http://www.freedigitalphotos.net/.
the development as well as progression of various forms of cancer including CRC 
(Boffetta and Hashibe, 2006;Haas et al., 2012). Alcohol is acknowledged as a carcinogen 
by the World Health Organization (WHO) and the International agency for Research on 
Cancer (IARC), and its intake is categorized into less than 12g of alcohol, moderate 
alcohol intake (12-35g/day), and high alcohol intake (>35g/day). One unit of alcohol is 
one 12oz beer, 4oz of wine, or 1.5oz of liquor, which is equivalent to approximately 7.9 
grams of alcohol (Kontou et al., 2012). During metabolism, alcohol dehydrogenase 
(ADH), catalase, and cytochrome P450 subenzyme 2E1 (CYP2E1) catalyze the oxidation 
of alcohol to acetaldehyde (AA), a group I carcinogen (Haas et al., 2012;Mikko, 2012). The mechanisms involved in alcohol-induced cancer progression are difficult to resolve 
due to the limitations involved in determining individual risks, because in most cases 
smoking and chronic inflammation are additional risk factors common in patients that 
over consume alcohol (Haas et al., 2012). Developing effective prevention strategies 
requires knowledge of the existing etiologic factors that contribute to alcohol-induced 
carcinogenesis.

The numerous molecular pathways that are involved in alcohol exposure and 
CRC etiology are reviewed in Table 1-1 and Figure 1-2. Chronic alcohol consumption 
results in nutritional deficiencies, specifically decreasing the absorption of folic acid and 
vitamins B1, B2 and B12, which make the cells to become vulnerable to the increase in 
ROS resulting in oxidative stress; this results in a variety of pathway alterations within
Figure 1-2 Summarizing the effects of chronic alcohol consumption on the development of CRC.
Figure 1-2 Summarizing the effects of chronic alcohol consumption on the development of CRC. The chronic consumption of alcohol leads to dietary deficiencies in the following vitamins and minerals, vitamins A, B1, B2, B12, and folic acid. These deficiencies can further lead to alterations in epigenetic regulators: Folate deficiency specifically leads to altered S-adenosylmethionine (SAM) synthesis, resulting in altered p16 gene expression. These epigenetic modifications are involved in the initiation and promotion of CRC. Beer consumption can lead to increased ROS species; specifically maleic and succinyllic acids that are found in beer can resulted in increase gastric secretion. This acidic environment can then lead to alteration in the microflora present in the gut, ultimately resulting in increase ROS production. Consumed ethanol is metabolized via oxidation to acetaldehyde (AA); this metabolism is mediated via alcohol dehydrogenase (ADH), cytochrome P450 subenzyme 2E1 (CYP2E1), and catalase. Additional metabolic byproducts of ethanol metabolism include ROS, which are released during the CYP2E1 oxidation reaction; these reactive intermediates enhance lipid peroxidation leading to DNA adduct formation. Adduct formation can then result in DNA mutations, specifically in the p53 gene, which contributes to the initiation and promotion of CRC. AA alone is a mutagenic compound known to form adducts with DNA and proteins, as well as, induce DNA mutations; furthermore protein adduct formation has been shown to induce inflammatory responses, via TNF-α and IL-6. These inflammatory mediators further regulate the expression of matrix metalloproteinase MMP-2, -7, and –9, which are involved in the promotion and progression of CRC. Additionally AA can be oxidized by aldehydes dehydrogenase 2 (ALDH2) resulting in acetate and ROS formation; these ROS species have been indicated in the activation of NADPH oxidase. NADPH oxidase is an enzyme complex that further modulates downstream effectors proteins, such as ERK1/2 and HIF-1α signaling; this downstream protein modification results in alteration of proliferative and metastatic signaling pathways, which are involved in CRC promotion and progression. All the photographs of lifestyle interventions were provided by http://www.freedigitalphotos.net/.
the cell (Testino, 2011). Furthermore, fermented alcoholic beverages, such as beer and wine, contain compounds that contribute to ROS generation; specifically, maleic and succinyllic acids that stimulate gastrin-mediated secretion potentially altering the colon microflora resulting in increased nitric oxide (NO) production (Haas et al., 2012). Additionally, Vitamin A synthesis is mediated via the CYP2E1 enzyme, which also metabolizes alcohol; this substrate competition induced from chronic alcohol consumption results in decreased Vitamin A levels (Testino, 2011). Decreased vitamin A levels result in decreased expression of the AP1 gene, which is involved in cell cycle regulation and inflammation (Testino, 2011). Likewise, alcohol consumption results in increased inflammation, as demonstrated by increased secretion of inflammatory mediators, such TNF-α and IL-6, when CRC cells are exposed to alcohol (Zhao et al., 2004;Amin et al., 2009). Moreover, as a result of long-term alcohol consumption, folate levels are decreased, further altering the synthesis of S-adenosylmethionine (SAM), a key methyl donor involved in epigenetic alterations leading to modification of gene expression (Sauer et al., 2010). Similarly, alcohol exposure has also been shown to alter the histone methylation pattern of the p16 gene promoter, resulting in decreased p16 protein levels and uncontrolled cell-cycle regulation (Sauer et al., 2010). In addition, accumulation of intracellular ROS leads to the induction of NADPH oxidase and downstream pathways such as hypoxia-inducible factor-1α (HIF-1α) signaling; leading to up regulation of PI3K/AKT and VEGF signaling, which are involved in apoptotic and metastatic signaling (Morgensztern and McLeod, 2005;Wang et al., 2012a). Likewise, additional proliferative and metastatic pathways are modulated by alcohol consumption; these include MMP-2, MMP-7, MMP-9, EGFR and ERK 1/2, which in turn promote
proliferative and EMT pathways (Forsyth et al., 2010).

**Molecular mechanisms of tobacco-induced colorectal carcinogenesis**

Everyday humans are exposed to a variety of toxic and carcinogenic compounds due to life style habits including smoking tobacco. It has been estimated that tobacco has killed more than 5 million people in 2008 and will be responsible for the death of more than 8 million by 2030 (Lodovici and Bigagli, 2009). A wide variety of malignancies are associated with tobacco consumption, with the strongest associations seen not only in the respiratory tract, but the gastrointestinal and urogenital systems; it is estimated that approximately 20% of CRC cases can be attributed to tobacco exposure (Giovannucci and Martinez, 1996; Tsoi et al., 2009). The major classes of carcinogenic compounds in tobacco smoke are polycyclic aromatic hydrocarbons (PAHs), aromatic amines, nitrosamines, and heterocyclic amines (HCAs); these carcinogenic compounds can enter the alimentary tract or the circulatory systems. (Fischer et al., 1990; Kasahara et al., 2008). With in the body, these compounds are then metabolized by CYPs (CYP1A1, CYP1A2, CYP2E1, CYP2A6), leading to DNA-adduct formation, or by glutathione S-transferases (GSTs) (GSTM1, GSTT1, GSTP1) leading to excretion (Barrowman et al., 1989; Guengerich and Shimada, 1991; Alexandrov et al., 1996; Koh et al., 2011).

Identifying the molecular mechanisms involved in tobacco detoxification allows the development of effective lifestyle modification strategies to prevent CRC development in tobacco smokers.

There are multiple molecular pathways involved in tobacco exposure and CRC etiology, which are reviewed in Table 1-1 and Figure 1-3. Nicotine exposure results
the activation of nicotinic acetylcholine receptors (nAChRs) and may contribute to cancer progression; coupled with the fact that, as tumors progress, there is an increased expression of nAChRs, further demonstrating the involvement of this pathway during carcinogenesis (Russo et al., 2012). Nitrosamines, such as nicotine-derived nitrosamine ketone (NNK), are high affinity ligands for the nAChR signaling and have been shown to increase intracellular ROS levels in human CRC cells (Ye et al., 2004). This oxidative stress triggers activation of inflammatory pathways including that of NFκB, which further acts as a positive regulator of COX-2 expression (Kosaka et al., 1994). There are additional biomarkers that indicate tobacco exposure; these biomarkers include oxidative DNA base modification or DNA-adduct formation, both of which have been shown to be elevated in colon tissue of smokers (Alexandrov et al., 1996; Kasahara et al., 2008; Lodovici and Bigagli, 2009). These events trigger DNA repair, specifically base excision repair (BER) pathway, which is one of the four major DNA repair pathways, involving multiple proteins such as OGG1, APEX1/APE1, and XRCC1 (Kasahara et al., 2008). Another potential mechanism involved in the initiation of tobacco-induced colorectal carcinogenesis could be epigenetic changes, resulting in KRAS and BRAF gene silencing that have been observed in the majority of the diagnosed CRC cases (Samowitz et al., 2006; Rosenberg et al., 2007).

Another class of receptors involved in nicotine signaling includes β-adrenoceptors, which can initiate a number of physiological responses, including metabolic and immunomodulatory responses (Civantos Calzada and Aleixandre de Artinano, 2001; Oberbeck, 2006).
Figure 1-3  Summary of the effects of cigarette smoke on the etiology of CRC.
Cigarette smoke contains nicotine as well as numerous carcinogenic compounds that effect the initiation, promotion, and progression of CRC. These carcinogenic compounds include polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HCAs), and aromatic amines; these compounds have the ability to induce epigenetic changes in \textit{KRAS} and \textit{BRAF} genes, both of which are imported in preventing the initiation of CRC. Furthermore these compounds can be bioactivated via cytochrome P450 subenzyme (CYPs); this activation can result in DNA adduct formation, potentially resulting in DNA mutations in \textit{KRAS}, \textit{BRAF}, and \textit{MYC} if the DNA damage is not repaired. DNA repair pathways such as the base excision repair (BER) pathway can reverse the damage induced by these carcinogens, through upregulation of various repair proteins such as, OGG1, APEX1/APE1, and XRCC1. Alternatively these carcinogenic compounds can be excreted from the body, via glutathione S-transferases (GSTs) and/or UDP-glucuronosyltransferases (UGTs). Specific carcinogenic compounds, such as nicotine-derived nitrosamine ketone (NNK) have been shown to induce the production of ROS; these reactive intermediates can then activated numerous molecular pathways including MAPK and NFκB. The MAPK signaling cascade has numerous potential protein targets, including NFκB, AP-1, and C-myc; the activation of these pathways can further result in increased inflammatory markers such as COX-2, leading to the initiation and promotion of CRC. Additionally NNK can activate nicotinic acetylcholine receptors (nAChRs), resulting in β-adrenaline upregulation; β-adrenaline can upregulate COX-2 levels, further leading to VEGF upregulation. Alternatively β-adrenaline can bind and activate the β-adrenaline receptor leading to cascade activation; receptor stimulation triggers ERK1/2 activation leading to upregulation of downstream targets such as, Bcl-2, Bad, and AP-1. The downstream targets of the β-adrenaline receptor lead to the induction apoptosis and further activation of metastatic proteins such as MMPs; both of which are important pathways involved in the development and progression of CRC. Additionally cigarette smoke contains nicotine which can activated multiple cell membrane receptors including, nAChRs, β-adrenaline, and EGFR. Activation of these various membrane receptors leads to the upregulation of inflammatory, apoptotic, and metastatic proteins, such as COX-2, Bad, Bcl-2, AP-1 and VEGF. All the photographs of lifestyle interventions were provided by http://www.freedigitalphotos.net/.
Once activated, these receptors increase inflammatory signals and metastatic mediators, such as COX-2 and MMP-2 (Schuller et al., 1999; Hori et al., 2011). Together with nAChR signaling, β-adrenoceptors have been linked to metastatic growth factors, such as VEGF (Lutgendorf et al., 2003; Wong et al., 2007). Chronic nAChRs and β-adrenoceptors activation due to tobacco smoke exposure has further been linked to apoptotic signaling; specifically via phosphorylation of pro-apoptotic proteins, BCL-2 and BAD (Heusch and Maneckjee, 1998; Jin et al., 2004). In addition, smoke inhalation initiates and promotes proliferative pathways, specifically MAPK signaling, which is activated in response to NNK exposure (Ye et al., 2004).

**Physiological responses to physical activity and obesity-induced CRC**

According to the Center for Disease Control (CDC) and the WHO, obesity and cancer are two major epidemics in the United States, and prevalence has dramatically increased in the last few decades. High consumption of processed foods, animal fat, and a high calorie diet are risk factors for CRC development. Overweight is defined as those who have a body mass index (BMI) ≥ 25 kg/m², obese individuals have a BMI ≥ 30 kg/m²; currently 66% of adults are overweight and 33% are obese (Flegal et al., 2012; Vucenik and Stains, 2012). In 2008, there were over 500 million people that were in the obese category, as a result of a chronic positive energy balance. This lack of equilibrium leads to the systemic secretion of various factors, such as TNF-α, IL-6, insulin, insulin-like growth factor-1 (IGF-1), adiponectin and leptin, which play an important role in carcinogenesis including CRC (Harvey et al., 2012; Vucenik and Stains, 2012). Over the last 20 years, there has been a growing interest in the benefits of physical activity and cancer etiology; current
physical activity guidelines recommend healthy adults to perform a minimum of 60-min moderate-intensity or 30-min of vigorous-intensity exercise daily to promote health, according to the American Institute of Cancer Research (AICR) (Wiseman, 2008). There are a number of observational studies examining prevention strategies with convincing evidence that physical activity reduces CRC risk, when comparing the most active with the least active individuals (Winzer et al., 2011). Repeated aerobic exercise has shown to decrease blood pressure, fasting glucose, insulin, and atherogenic lipids (Pedersen and Fischer, 2007). Currently, there are numerous pathways that are modified with physical activity that could target colon carcinogenesis; specifically, hormonal, inflammatory, angiogenic, apoptotic and proliferative pathways (Winzer et al., 2011). Identifying biomarkers and further examining their mechanistic role in CRC initiation, promotion and progression will, thus, further the development of physical activity interventions for the prevention of CRC.

Additionally, Table 1-1 and Figure 1-4 are a review of the numerous cellular pathways that are affected by physical activity and obesity, which are further involved in CRC etiology. During exercise, a 10- to 40-fold increase in oxygen uptake occurs relative to the resting state which can cause an increased ROS formation (Na and Oliynyk, 2011). It has been reported that vigorous exhausting exercise increases DNA damage, while moderate exercise does not increase, but instead, alleviates the oxidative stress and DNA damage (Poulsen et al., 1999; Sato et al., 2003).
Figure 1-4 Summarizing the effects of physical activity and obesity on the development of CRC.
Vigorous physical activity had been shown to induce the production of ROS leading to DNA adduct formation, potentially affecting the initiation of CRC. Alternatively moderate physical exercise has been shown to induce an innate immune response through IL-6 receptor activation and downstream protein upregulation; this adaptive immune response is thought to protect the organisms against chronic inflammatory conditions, which have been shown to increase the risk of CRC development. Conversely moderate physical activity has been shown to inhibit the expression of other inflammatory mediators, such as iNOS, which may play a role in chronic inflammatory conditions. Moderate physical activity has been shown to alter specific hormones that are related to appetite and satiety signals; these hormones include YY, GLP-1, and PP all of which are involved in glycogen secretion. Practicing moderate physical activity also results in the upregulation of superoxide dismutase (SOD); this enzyme further activates detoxification pathways via upregulation of the Nrf2 proteins and GSTs enzymes. Additional SOD inhibits PPAR-α leading to the reduced expression of VEGF, which is involved in the promotion and progression of CRC. Obesity is a result of an energy imbalance that results in aberrant activation various receptor proteins that are involved in inflammation, proliferation, and hormone regulation. This abnormal activation creates an environment that is chronically inflamed and can result in the initiation, promotion and progression of CRC. One of the major inflammatory mediators is TNF-α, which is activated at the cellular membrane resulting in protein cascade activation; TNF-α activation can result in induction NFκB and COX-2 signaling which initiates an inflammatory response. Additionally the TNF-α receptor pathways can trigger activation of the GSK3β enzyme; this enzyme regulates numerous proteins, including β-catenin and NFκB. β-catenin further regulates multiple proteins that are involved CRC development; these proteins include C-myc, cyclin D, and VEGF, which are involved in oncogene signaling, cell cycle regulation, and metastatic development. Adiponectin is another factor that is highly expressed in obese individuals, which has been shown to increase glucose uptake and fatty acid oxidation; once the adiponectin receptor is activated it results in PKA kinase upregulation, which further inhibits NFκB in adipocytes. This inhibition ultimately leads to downregulation of cell adhesion molecules that are important regulators of CRC promotion and progression. Insulin signaling is another important pathway that is chronically upregulated in obese individuals; binding of insulin to its receptor triggers a conformational change that results in the activation of downstream kinase targets such as, PI3K. PI3K has the ability to trigger PKB kinase activation, which ultimately results in increased glycogen secretion and decreased gluconeogenesis; alternatively PI3K can activate the JAK protein kinase, which plays a role in immune function and inflammatory responses. Another factor that has been shown to activate the JAK protein is leptin; this factor is secreted from adipose tissue and in addition to activating JAK it has also been shown to activate the MAPK signaling cascade. Furthermore both the JAK and MAPK signaling pathways involve the regulation of proliferative and metastatic signals, such as PCNA and VEGF; both of these pathways are important regulators of CRC promotion and progression. All the photographs of lifestyle interventions were provided by http://www.freedigitalphotos.net/.
Examining detoxification pathways during physical activity revealed that superoxide dismutase (SOD) was upregulated, as well as, downstream protein players, such as nuclear transcription factor erythroid 2p45 (NF-E2)-related factor (Nrf2) (Asghar et al., 2007). Nrf2 then translocates to the nucleus to initiate transcription of additional detoxifying enzymes, such as GSTs (Asghar et al., 2007). Physical activity has the ability to increase energy expenditure and decrease energy intake through hormone modulation; acute exercise results in increased levels of certain satiety hormones, specifically polypeptide YY, GLP-1 and PP, all of which are involved in glycogen secretion, the major energy source in the body (Martins et al., 2008). Leptin is another example of the ability of physical activity to modulate hormones-leptin levels decrease with moderate exercise; this hormone is synthesized by adipocytes and is important in regulating food intake (Ahima et al., 1996). Furthermore, leptin is involved in proliferative and apoptotic signaling, specifically activation of MAPK and PI3K/AKT pathways (Cirillo et al., 2008). Correspondingly, proliferative marker such as proliferating cell nuclear antigen (PCNA) is downregulated during moderate physical activity (Demarzo et al., 2008).

The increase of adipose tissue observed in obese individuals results in systemic low-grade inflammation, via up regulation of TNF-α; this sustained up regulation can lead to chronic inflammatory conditions that elevate the risk of CRC development (Drevon, 2005). However, with regular exercise, the anti-inflammatory effects of acute bouts of exercise protect against chronic systemic low-grade inflammation. Physical activity has been shown to increase inflammatory mediators such as IL-6 (Kim et al., 2009; Brandt and Pedersen, 2010). It is speculated that this increased IL-6 production during exercise may work in an endocrine-type fashion and increase hepatic glucose production or
lipolysis in adipose tissue (Pedersen and Febbraio, 2008). It is hypothesized that induction of the adaptive immunity through moderate exercise can ultimately prevent chronic inflammatory conditions. Furthermore, physical activity was able to decrease the expression of iNOS and TNF-α in the plasma and colon mucosa, supporting an overall anti-inflammatory role of moderate exercise (Na and Oliynyk, 2011). Physical activity was also shown to significantly decrease the IGF-1/Insulin-like growth factor-binding protein 3 (IGFBP-3) ratios and aberrant β-catenin signaling (Ju et al., 2008). An inappropriate stabilization, translocation and activation of β-catenin are hallmarks of sporadic and familial CRC. Thus, physical activity modulation of β-catenin results in downstream modulation of oncogenic target genes such as c-myc and VEGF (Zhang et al., 2001; Barker and Clevers, 2006; Saif and Chu, 2010). Moreover, peroxisome proliferator-activated receptor-α (PPARα) is a metabolic regulator that regulates glucose and lipid homeostasis; physical activity targets PPARα, decreasing it’s expression, further resulting in decreased levels of VEGF, a key regulator of metastasis (Wang et al., 2006; Peters et al., 2011).

**Circadian rhythm alteration and increased risk of CRC**

A convincing body of evidence suggests that there are severe repercussions of circadian disruption resulting in physiological and patho-physiological consequences. This is not surprising, considering that in last 2.5 billion years during which life has evolved on the planet, there has been a predictable rhythm between the rotating surfaces of the earth and the sun. The cells of the most organisms reveal circadian rhythms known as the circadian clock; 24-hour timekeeping in the central clock of the brain and the
peripheral tissues. This circadian clock modulates transcription-translation feed-back loops that are generated by core circadian clock genes, including \textit{PER-1, 2, and 3}, and \textit{CLOCK} (Ko and Takahashi, 2006). Suppression of these genes occurs during CRC promotion and progression; coupled with the fact that \textit{Per-2} knock out mice are associated with a tumor prone-phenotype, indicates the important role this 24-hour clock plays during carcinogenesis (Fu et al., 2002; Sjoblom et al., 2006). Correspondingly, each organism has evolved the mechanisms to adjust to the solar day and deal with cycles of energy overload during the day and cycles of dearth at night; protecting essential machinery from excess solar, chemical and biochemical energy. In addition, biological timing of DNA damage repair, cellular proliferation, and apoptosis are under the control of the circadian rhythm (Hrushesky et al., 1998; Granda et al., 2005; Kang et al., 2009). Consistent with these facts, women and men that are chronically exposed to night shift work, have a 50% increased risk of developing CRC compared to day shift workers; understanding this circadian time structure and the biological targets provides unique opportunities to prevent colon carcinogenesis (Hrushesky et al., 2009). Identifying molecular targets whose expression is controlled by the circadian clock is key when designing prevention strategies for CRC. A review of the circadian rhythm pathways that are involved in CRC etiology is shown in Table 1-1 and Figure 1-5. The circadian clock also controls pathways involved in detoxification enzyme expression, such as CYPs; furthermore, it has been shown that these detoxification genes are expressed in a rhythmic pattern throughout the GI tract (Hoogerwerf et al., 2007; Sladek et al., 2007).
Figure 1-5  Summarizing the effects of circadian rhythm on CRC development.
Figure 1-5 Summarizing the effects of circadian rhythm on CRC development. The mammalian circadian clock takes 24hr to complete and is a self-sustaining feedback loop of core clock genes. This group of clock genes regulated various cellular processes including: detoxification; DNA repair; proliferation; cell cycle regulation; apoptosis; metastatic signaling and inflammation. Furthermore these cellular processes are then regulated in a time specific manner; disruption of this circadian rhythm results in abnormalities in these processes, which contribute to CRC development. The major genes regulating this 24hr timekeeping are PER1 and PER2; these genes modulate proliferative, apoptotic, inflammatory, and metastatic signaling. These genes have been shown to alter the expression of β-catenin; this protein is involved in proliferation and cell cycle regulation, specifically cyclin protein regulation. Additionally β-catenin can modulated oncogenic proteins such as C-myc, which have been shown to regulate iron metabolism; as discussed in Figure 1-2, iron metabolism results in ROS formation, further triggering an inflammatory response and NFκB activation. Moreover β-catenin can modify growth and metastatic signals mediate via VEGF protein upregulation. Another protein that is under the control of PER1/PER2 genes is ATM, which is upregulated in response to cellular stress; this stress regulator protein can further modify downstream targets, such as p53. The p53 protein, known as the guardian of the genome, can upregulate p21Cip1 protein levels resulting in cell cycle arrest; the arrest allows the cell to repair the DNA damage that has occurred due to cellular stress. The PER1/PER2 gene complex further inhibits apoptotic signaling; specifically through Bim protein inhibition, which results in downregulation of cleaved-caspase-3 (cc3) and PARP. Alternatively a group of binding proteins are regulated in a circadian manner, these include HLF and E4BP4; these binding proteins further modulate detoxification enzymes, such as cytochrome P450 subenzyme (CYPs). In addition HLF/E4BP4 binding proteins inhibit the apoptotic process through inhibition of the pro-apoptotic protein Bcl-xL; Bcl-xL inhibition results in down regulation to cleaved-caspase-9 (cc9). Additional genetic regulators of the circadian process are p21Cip1 and WEE1; these genes are involved in numerous cellular processes including proliferation, cell cycle regulation, and oncogene signaling. Both WEE1 and p21Cip1 regulate Cyclin D and Cyclin E activity; these cyclin proteins are involved in cell cycle regulation, which is abnormally regulated during CRC development. Additionally p21Cip1 genetic regulation can lead to the induction of cellular apoptotic signals, via cc3 induction; p21Cip1 can further regulate oncogenic proteins such as C-myc, which is an important protein in the development and progression of CRC. All the photographs of lifestyle interventions were provided by http://www.freedigitalphotos.net/.
Proline and acidic amino acid-rich (PAR) basic leucine zipper (bZip) transcription factors are also circadian clock-controlled, and form a complex with hepatic leukemia factor (HLF) or E4 promoter binding protein-(E4BP4); this group of transcription factors are responsible for xenobiotic detoxification (Murakami et al., 2008). Moreover, the rhythmic regulation of p21Cip1/Wee1 gene controls numerous physiological processes, which gates other growth and cell cycle regulators including DNA replication and mitosis (Bjarnason et al., 2001; Matsuo et al., 2003). Additionally, elimination of the Per-2 gene in mice resulted in lack of p53 protein activity, which is involved in DNA repair, cell-cycle, and cell death pathways (Fu et al., 2002). Cancer cells also express higher levels of PER-2 compared to normal cells; correspondingly, PER-2 circadian expression has been shown to modulate proliferative, cell-cycle and apoptotic pathways, specifically c-myc, cyclin D, β-catenin and VEGF (Zhang et al., 2001; Fu et al., 2002; Wood et al., 2008). Iron is an important mineral involved in metabolism, respiration and DNA synthesis; regulated through transferrin receptor 1 (TfR1) which binds and internalizes transferrin and also exhibits a 24-hour rhythm (Sorokin et al., 1989). Furthermore, iron metabolism and immunity are closely related; TNF-α and IL-6 inflammatory cytokines directly stimulate iron storage proteins (Brock and Mainou-Fowler, 1986; Torti et al., 1988; Rogers, 1996).

**Biological targets of nutraceuticals for CRC prevention**

A healthy diet is important in maintaining basic physiological functions within the body. With that in mind, the ACS has recommended an intake of ≥5 serving of fruits and vegetables and further limiting the consumption of refined grains, sugars, fats and red
meat. Fruits and vegetables are part of a large plant kingdom that is a vast source of phytochemicals, and a number of these compounds have been investigated for their anti-cancer and chemoprevention efficacy (Ramos, 2008). Furthermore, probiotic therapy has been shown to exert health benefits and these beneficial organisms further increase the production of various vitamins such as folic acid. Lifestyle interventions based on natural sources not only have the potential to prevent carcinogenesis, but also are beneficial in maintaining good health because they are natural sources of vitamins, minerals and fiber. Furthermore, these nutraceuticals make ideal chemopreventive agents due to their multi-targeted effects and selective toxicity towards cancer cells. These nutraceuticals are also efficacious against various forms of cancer, can be administered by oral route, are accepted by the general public, and are produced at a low cost (Galati and O'Brien, 2004). Furthermore, nutraceutical administration can target numerous cellular processes, all of which are important regulators during the development of CRC as outlined in Figure 1-6.

Probiotic consumption: Residing in the GI tract are a variety of microorganisms comprising of bacteria, archaea, viruses, and unicellular eukaryotes, which are know to augment a variety of immunological and metabolic pathways (Licciardi et al., 2010; Vipperla and O'Keefe, 2012). Collectively, there are over 800 species and 7000 strains that form over 100-trillion super colonies, which are influenced by several factors including diet, environment, stress and disease (Burk et al., 2003; Vipperla and O'Keefe, 2012).
Summarizing the molecular targets of dietary agents. There are a number of dietary agents that prevent CRC development, including probiotics, fiber, folic acid, grape seed extract (GSE), Silibinin (Sb), curcumin (CUR), and (-)-Epigallocatechin-3-gallate (EGCG). Further there are a multiple pathways modified due to dietary interventions that are involved in colon carcinogenesis these include: detoxification; oxidative stress; DNA repair; oncogene; epigenetic; inflammation; hormone signaling; proliferation; apoptosis; cell cycle; and metastatic pathways. All the photographs of dietary agents were provided by http://www.freedigitalphotos.net/.
Intestinal microbial composition has become an emerging factor in CRC susceptibility, since these organisms impact multiple physiological functions that are related to cancer progression, cell proliferation, differentiation, metabolism of essential nutrients, and stimulation of intestinal immunity; similarly, probiotics have been shown to prevent carcinogenesis through a variety of mechanisms (Zhu et al., 2011; Kumar et al., 2013). The fields of nutrition, microbiology, and genomics are converging rapidly, providing insight into the molecular mechanisms involved in the chemopreventive efficacy of probiotics; further fostering the development of inexpensive preventive therapies for CRC. The development of probiotic-based CRC prevention therapy has great therapeutic implications, due to its ability to target multiple pathways. Probiotic multi-targeted approach results in increased DNA repair and detoxification enzymes; furthermore, probiotic consumption inhibits multiple processes including DNA mutation, DNA adduct formation, growth signaling, insulin signaling and inflammation as shown in Figure 1-6.

_Saccharomyces boulardii_ is a non-pathogenic yeast species that has demonstrated anti-oxidant, metabolic, anti-toxin, and anti-inflammatory effects (Czerucka et al., 1994; Castagliuolo et al., 1996; Dalmasso et al., 2006). Compounds produced by the intestinal microbiota, specifically polyamines, have been shown to reduce oxidative stress (Noack et al., 1998; Rhee et al., 2007). Several mechanisms by which probiotics may suppress CRC development have been suggested, including induction of the adaptive immune response through increased production of inflammatory cytokines, such as TNF-α and IL-6; further modulating downstream detoxification pathways (CYPs, GSTs, COX-2) and reducing the uptake of carcinogenic compounds (Rafter, 2004). Mechanistic studies indicate that _Saccharomyces boulardii_ modulates the host-pathogen interaction;
specifically through ERK1/2 MAPK pathway, which is down regulated in both in vitro and in vivo studies (Dahan et al., 2003; Chen et al., 2006b). Modulation of the MAPK pathway can lead to altered hormone, inflammatory, apoptotic, proliferative, and metastatic signals. *Bifidobacterium animalis* also increases polyamine concentrations and has been shown to alter IGF-1 expression (Matsumoto et al., 2011). Similarly, *Bacillus polyfermenticus*, also known to possess chemopreventive efficacy, has been shown to modulate inflammatory and proliferative signals, such as those associated with EGFR pathway (Kim et al., 2006; Lee et al., 2007; Park et al., 2007). The ErbB receptor family, including EGFR, is important in cancer development and these family members are over expressed in various cancers including CRC (Holbro et al., 2003; Kamath and Buolamwini, 2006). ErbBs also regulate the cell cycle effecting cyclin D1 levels and further downstream oncogenic targets, such as c-myc (Slansky and Farnham, 1996; Rafter, 2004).

**Dietary fiber consumption**: Epidemiological studies indicate that diets rich in fruits, vegetables and legumes are of great importance when considering CRC prevention strategies; these foodstuffs are rich in soluble dietary fiber (DF) and insoluble fibers, starches, oligosaccharides, and phenolic compounds (Michels, 2005; Vergara-Castaneda et al., 2010). These DFs are fermented via microorganisms in the colon resulting in several physiological effects: production of short chained fatty acids (SCFA), butyrate, propionate, and acetate; furthermore, these compounds have been shown to induce apoptosis in human CRC cells (Chen et al., 2006a; Campos-Vega et al., 2009). Potential mechanisms, associated with fiber consumption and reduced CRC risk, include: dilution of carcinogens, reduction of transit time, production of SCFAs, and reduction of tumor-
promoting substances. Further identifying these molecular mechanisms, induced after fiber consumption, would play an important role in CRC prevention.

DF was shown to decrease levels of deoxycholic acid (DCA), which is a bile acid produced via the dehydroyxylolation of primary bile acids and is known to be a direct as well as in-direct tumor promoter in the colon (Zampa et al., 2004; Stein et al., 2012). Similarly, butyrate induces GSTs in human CRC cells, which results in carcinogen detoxification (Pool-Zobel et al., 2005). Furthermore, DF acts as an anti-inflammatory agent decreasing the levels of IL-6 and TNF-α; additional studies have also shown the ability of DF to inhibit COX-2 and iNOS gene expression (Reddy et al., 2000; Kaczmarczyk et al., 2012). Additionally, DF intake was associated with the increased expression of DNA repair genes, specifically Apex, Xrcc4 and Xrcc5, which bind to DNA ligase initiating the repair of DSB (Vergara-Castaneda et al., 2010). Similarly, butyrate induces apoptotic signaling in CRC cells through up regulation of BAK, cleaved caspase-3 and cleaved PARP, and loss of mitochondrial function (Ruemmele et al., 1999). Furthermore, other SCFAs found in legumes altered the expression of β-catenin, p53, p21Cip1, Bax, and casp3 genes in a carcinogen induced CRC model; all of which are important modulators of cell cycle, proliferation, and cell death (Feregrino-Perez et al., 2008). Butyrate also alters epigenetic pathways through histone deacetylase (HDAC) inhibition in CRC cells (Waldecker et al., 2008). This alteration leads to hyperacetylation of histone residues altering gene transcription and expression; for example HDAC inhibition via SCFAs results in upregulation of p21Cip1, which is involved in cell cycle, proliferation, differentiation and apoptosis (Chen et al., 2004).
**Folic acid supplementation:** Folates are water-soluble B vitamins that are important cofactors in DNA synthesis and methylation pathways. Humans need to consume folic acid from exogenous sources to support these essential functions; good sources of folates include leafy greens, vegetables, yeast extracts, and citrus fruits (Lucock, 2000). Studies indicate a 40-60% reduction in the risk of CRC in individuals with high folate intake (Kim, 1999; 2007). One of the potential mechanisms in CRC development and progression is folate deficiency (FD); low levels lead to DNA strand breaks, impaired DNA methylation and repair (Kennedy et al., 2011). CRC patients frequently have decreased folate levels; this deficiency is recognized as one of the metabolic stressors of colorectal carcinogenesis (Martinez et al., 2004). The mechanisms and protein targets by which folate conveys protection against CRC development need to be investigated to develop strategies that effectively target and prevent colon carcinogenesis. Furthermore, supplementation with folic acid has clinical potential; this is due to its ability to target multiple pathways that are involved in CRC initiation, promotion, and progression. Folic acid supplementation results in increased DNA repair proteins, as well as, inhibits multiple processes including DNA mutation, epigenetic silencing, DNA adduct formation, growth signaling, metastatic signaling and inflammatory processes as shown in Figures 1-2-1-4 and Figure 1-6.

FD may play a role in chronic inflammation through activation of NF-κB and TNF-α, perpetually creating inflammatory signals (Wang et al., 2012b). Furthermore, it has been reported that DNA repair genes are up regulated in folate-depleted cells, specifically Xrcc5, Apex and PCNA, supporting folic acids’ role in DNA repair and synthesis (Duthie et al., 2008; Sadik and Shaker, 2012). Additionally, PCNA plays a role
in proliferative pathways, as well as, in DNA repair, indicating an additional mechanism
of action in which folic acid is protective against CRC development. Folate
supplementation studies reveal its ability to increase the stability of p53, which is
involved in DNA repair, proliferation, and cell death pathways (Sadik and Shaker, 2012).
Studies have also elucidated FD role in epigenetic modifications; its deficiency was
associated with decreased global DNA methylation. Furthermore, different folate species
are used for methylation versus DNA synthesis; these species are distributed
differentially in normal colon tissue compared to cancer tissues (Liu et al., 2012a). In
addition, FD has been shown to enhance EMT signaling in HCT116 colon cancer cells,
through activation of MMPs, up regulation of \textit{Snail} and suppression of \textit{E-cadherin}
(Wang et al., 2012b).

\textit{Nutritional supplementation with grape seed extract}: Another supplement that
has shown promising chemopreventive and anti-cancer potential in numerous types of
cancer, including skin, prostate, breast, lung and CRC, is grape seed extract (GSE) (Kaur
et al., 2009a). GSE supplementation has vast chemopreventive potential; this is due to its
ability to target multiple proteins that are involved in the development of CRC, as
elaborated in Chapter II. GSE supplementation results in increased cancer cell apoptosis,
as well as, it inhibits multiple processes such as signaling related to epigenetics, growth,
proliferation, oncogenes, insulin, metastasis and inflammation as shown in Figures 1.1-1.6.

GSE is a complex mixture of polyphenols, specifically proanthocyanidins. \textit{In-vivo}
studies with GSE have shown its anti-inflammatory effects reducing the expression of
iNOS and COX-2 (Velmurugan et al., 2010b). Additionally, GSE has also been shown to
decrease global methylation, and it also decreases DNA-methyltransferase (DNMT) activity and protein levels, indicating GSE’s role during epigenetic gene modification (Vaid et al., 2012). Furthermore, GSE inhibited aberrant β-catenin expression and downstream proteins, cyclin D1 and c-myc (Velmurugan et al., 2010b). Moreover, mechanistic studies of GSE, at various stages of CRC development, identified apoptosis induction as the major factor in the chemopreventive efficacy of GSE against CRC; specifically it activates caspase-3, -8, -9 resulting in the cleavage of PARP, DNA fragmentation, and programmed cell death (Derry et al., 2012). The apoptotic effect was specific to CRC cells, with minimal effect on normal colon epithelial cells; furthermore, this effect was attenuated with antioxidant treatment, indicating ROS as a potential upstream stimulus in GSE-induced CRC cell death (Derry et al., 2012). In addition, GSE has been shown to modulate p21Cip1 levels; resulting in decreased proliferation, leading to cell-cycle arrest, and further downstream pathway activation, including that of ERK1/2 (Kaur et al., 2011b).

*Herbal supplementation with silibinin:* Silibinin is a flavonolignan constituent of silymarin, which is extracted from the milk thistle plant (*Silybum marianum*). It is non-toxic and has been used traditionally as well as clinically to treat various liver diseases and liver complications (Post-White et al., 2007). In past two-decades, however, Silibinin has been investigated for its cancer chemopreventive properties; this natural non-toxic agent is found efficacious in *in-vivo* preclinical studies against skin, lung, prostate, bladder, and CRC cancer models (Kumarasamy and Agarwal., 2008; Rajamanickam and Agarwal., 2008). Several *in vitro* and *in vivo* studies have shown strong preventive and/or therapeutic efficacy of silibinin against CRC growth and progression (Rajamanickam et
al., 2009; Rajamanickam et al., 2010; Ravichandran et al., 2010; Raina et al., 2013). Silibinin herbal supplementation already has potential against CRC; this is due to its ability to target multiple proteins that are involved in the initiation, promotion, and progression of CRC. Silibinin supplementation results in increased levels of detoxification enzymes; it also inhibits multiple processes in carcinogenesis, such as those involved in DNA mutation, growth/ proliferation, metastatic signaling and inflammation, as shown in Figures 1-6.

Mechanistic investigations have revealed that the anti-CRC effects of Silibinin were mainly due to its anti-proliferative and pro-apoptotic effects; furthermore, Silibinin strongly inhibits the Wnt/β-catenin pathway, which is known to play an essential role in the development and progression of CRC (Kaur et al., 2009b; Kaur et al., 2010; Rajamanickam and Agarwal, 2008; Rajamanickam et al., 2009). Towards this effect, Silibinin strongly inhibited β-catenin-dependent transcriptional activity of Tcf-4 in CRC cells, which was followed with a decrease in the expression of its transcriptional targets, namely cyclin D1 and c-myc (Kaur et al., 2010). Furthermore, the pro-apoptotic effects of Silibinin are via the down regulation of the anti-apoptotic proteins BCL-2, MCL-1, XIAP and survivin and up regulation of pro-apoptotic proteins such as Bax (Velmurugan et al., 2010a; Kauntz et al., 2012a). Additional studies have shown that Silibinin up regulates death receptors, DR4/DR5, at the transcriptional level; these receptors are involved in the extrinsic apoptotic pathway, which further supports the ability of Silibinin to induced apoptotic death as an additional mechanism involved in its chemopreventive efficacy (Kauntz et al., 2012b). Studies have also revealed that Silibinin strongly inhibits PI-3K-AKT–mTOR but activates MEK1/2-ERK1/2 pathways for its biological effects.
manifested in terms of induction of autophagic type PCD in CRC cells (Raina et al., 2013a). Silibinin is also known to cause endoplasmic reticulum stress in CRC cells together with glucose uptake inhibition as well as energy restriction; due to interference in essential cellular processes such as mitochondrial metabolism, phospholipid and protein synthesis; the cellular damage to CRC cells by Silibinin is severe and irreparable (Raina et al., 2013a). Importantly, silibinin is non-toxic to normal colon cells (Rajamanickam et al., 2009; Rajamanickam et al., 2010). Raina et al have also shown that Silibinin has the potential to strongly inhibit TNFα-induced NF-κB activation in human CRC cells (Raina et al., 2013b). These observations were also corroborated by in vivo studies, which showed that anti-inflammatory mechanisms of Silibinin are associated with decreased expression of COX2, and iNOS levels, together with inhibition of NF-κB transcriptional activity (Rajamanickam et al., 2009; Rajamanickam et al., 2010; Ravichandran et al., 2010; Raina et al., 2013b). Moreover, other studies have shown that Silibinin reduced inflammatory mediators, such as TNF-α and IL-1β; further modifying downstream target proteins, such as MMP-7 (Kauntz et al., 2012a). Likewise, Silibinin alters numerous pathways that are involved in cell invasion and metastasis via alteration of the JNK pathway and down regulation of AP-1, resulting in decreased MMP-2 levels (Lin et al., 2012). Silibinin has been shown to alter xenobiotic metabolizing enzymes; specifically, reducing CYP2E1 activity which is involved in metabolic activation of carcinogens, and in contrast, Silibinin upregulated Phase II enzymes including, GSTs, which are important in metabolic detoxification (Sangeetha et al., 2012). Importantly, Hoh et al in a completed pilot study with Silibinin in CRC patients with colon adenocarcinoma have shown high bioavailability of Silibinin in
colonic tissue of CRC patients (Hoh et al., 2006). Thus, the ongoing research on the usefulness of Silibinin has strong translational implications in the prevention and management of CRC.

**Curcumin dietary supplementation:** Curcumin (CUR) is the active phenolic compound extracted from the spice turmeric, derived from the rhizome *Curcuma longa*. CUR has been shown to possess anti-cancer and chemopreventive efficacy, and in clinical trials, CUR is effective in reducing CRC burden (Hatcher et al., 2008). CUR dietary supplementation has the potential to target multiple signaling pathways that are involved in the development of CRC. CUR consumption results in increased cancer cell apoptosis, and levels of detoxification enzymes; it also inhibits multiple processes including that of DNA mutation and signaling related to growth, metastasis, and inflammation, as shown in Figures 1-1,1-6.

Numerous studies have examined pharmacological effects of CUR in CRC such as induction of apoptosis, and inhibition of proliferation, oxidative stress and angiogenesis (Shehzad et al., 2010). CUR has been shown to up regulate GST enzymes in multiple studies; furthermore, CUR has been shown to induce ROS in cancer cells, resulting in p21Cip1 up regulation and G2/M cell-cycle arrest (Ye et al., 2007; Odenthal et al., 2012; Yogosawa et al., 2012). CUR has also been shown to alter MAPK pathway resulting in decreased expression of TNF-α and COX-2 expression in colon mucosa, resulting in an overall decrease in inflammation (Camacho-Barquero et al., 2007). Additionally, CUR has been shown to down regulate NF-κB and IL-6 secretion, which are also key regulators of the chronic inflammatory response (Tu et al., 2012). Concentrated analogs of CUR have been also shown to inhibit NF-κB translocation, thus
resulting in a down regulation of gene targets, such as c-myc, cyclin D1, and BCL-2 (Chen et al., 2011). CUR also induces mitochondrial stress and apoptotic cell death through up regulation of Bax, Bim, Bak, Puma and Noxa, and down regulation of anti-apoptotic proteins such as BCL-2 and BCL-xl (Basile et al., 2013). This induced mitochondrial dysfunction may be due to the ability of CUR to induce ER-stress resulting in accumulation of ubiquitinated proteins; this protein accumulation could also induce autophagy (Basile et al., 2013). Furthermore, CUR analogs were able to decrease VEGF and MMP-9 expression, indicating the ability of CUR to prevent metastatic disease (Chen et al., 2011).

**Dietary intervention with EGCG:** Tea is one of the worlds’ most consumed beverages made from the leaves of *Camellia sinensis*; (-)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol. Tea has been cultivated and used medicinally for thousands of years due to the beneficial health effects. Furthermore, it has been extensively studied for its chemopreventive efficacy (Bode and Dong, 2009; Yang et al., 2009). Dietary intervention with EGCG has great anti-CRC potential; this is due to its ability to target multiple proteins that are involved in the development of CRC, as shown in Figures 1.1-1.6.

A number of potential mechanisms have been proposed for the chemopreventive efficacy of EGCG including: enhancement of antioxidant activity, alteration of hormone and growth factor signaling, and induction of cell cycle arrest. Due to the polyphenolic structure of EGCG, tea polyphenols are strong antioxidants and thus prevent the formation of ROS. Similarly, EGCG has been shown to initiate transcription factors in CRC cells, such as Nrf2, which further induces phase II enzymes such as UDP-
glucuronosyltransferases (UGTs) (Zhang et al., 2009). Furthermore, EGCG decreases growth and hormone signals specifically EGF, IGF-1, and VEGF indicating its role in proliferation, insulin and metastatic signaling (Khan and Mukhtar, 2008). EGCG inhibits additional proliferative and cellular division pathways in CRC cells, via alteration of MAPK/ERK1/2 and p21Cip1 pathways, ultimately resulting in cell cycle arrest (Lin et al., 1999; Larsen and Dashwood, 2010). Moreover, EGCG has the ability to induce apoptotic death of cancer cells via p53, p21Cip1 and PUMA up regulation (Thakur et al., 2010). EGCG can also modify epigenetic expression patterns in CRC cells via alteration of DNMT activity, resulting in gene silencing (Fang et al., 2003). EGCG was also observed to inhibit β-catenin translocation and VEGF expression, which further supports its chemopreventive efficacy (Kondo et al., 2002)

The role of vaccination for CRC prevention

Chronic inflammatory conditions have been shown to increase the incidence of CRC (Terzic et al., 2010). The question still remains: does inflammation precede cancer or does cancer precede inflammation. During chronic inflammation, the tissue environment contains a diverse population of leukocytes that secrete and express factors that affect cell proliferation and genomic instability (Balkwill et al., 2005; Lin and Karin, 2007). Antigen presenting cells such as dendritic cells (DCs) have the ability to acquire antigens and migrate to lymph nodes, which can lead to T-cell stimulation, specifically CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTLs). These cells can be hijacked for anti-tumor activity; furthermore, vaccines have been designed to present antigens against cancer cells, causing immune cell infiltration in the tumor
microenvironment and resulting in decreased tumor growth. This immune recognition is mediated by toll-like receptors (TLRs) which bridge the innate and adaptive immunity (Iwasaki and Medzhitov, 2004). Specific cancer antigens including carcinoembryonic antigen (CEA), are utilized clinically to monitor CRC disease progression and is one of the most promising tumor associated antigens (TAAs) (Thompson et al., 1991). The theory behind cancer vaccination is that priming of the innate immunity to neoantigens may clear the tissue site and prevent chronic inflammation, or with tumor specific neoantigens, vaccination could prevent cancer progression. There have been several clinical trials investigating vaccination for CRC, DC based vaccines in particular, that have shown to induce a specific immune response (Mosolits et al., 2005). Furthermore, vaccination has great clinical potential with treatment resistant patient sub-types; this is due to its ability to target specific proteins that are involved in the progression of CRC. Vaccination inhibits multiple processes that are involved in the development of metastatic CRC including growth and oncogenic signaling, and inflammatory processes, as shown in Figures 1.1-1.2 and Figures 1.4-1.5.

Tumor vaccines have been designed to target oncogenes, specifically c-myc, which is over expressed in 80% of CRC; vaccination resulted in generation of CD4+ and CD8+ T cells that infiltrated the tumor site (Williams et al., 2008). Additionally, they have been designed to target other mediators, such as TGF-β, which plays a role in inflammatory, proliferative, and apoptotic pathways; vaccination resulted in inhibition of tumor growth (Roberts, 2002). Furthermore, CRC vaccines have been designed to target epithelial specific proteins such as MUC1, which is over expressed in chronic inflammatory diseases, such as inflammatory bowel disease, resulting in an increased
innate immune response and decreased chronic inflammation (Beatty et al., 2007; Furr et al., 2010). Consequently, the anti-tumor efficacy of MUC1 vaccine was mediated by CD4+ T cells, FasL and TNF-α signaling, resulting in induction of the innate immunity and apoptosis (Sugiura et al., 2008). Clinically, anti-VEGF (Bevacizumab) vaccines are currently utilized, however, adverse effects have been observed, and therefore, different vaccination formulations are being designed against VEGF with anti-metastatic potential and no toxicity, supporting a role for vaccination in CRC control and management (Rad et al., 2007).

**Lifestyle recommendations**

Consumption of red meat is standard in the normal American diet; however, due to the numerous associations between red meat consumption and CRC risk, it is recommended to limit the consumption of red meat to 50g/day, which is equivalent to 2oz, about ½ the size of your palm. Additionally, consuming other foodstuffs along with red meat could be beneficial in decreasing the detrimental effects of red meat consumption. For example, probiotic consumption alters heme metabolism and reduces inflammation. Probiotics, fiber, Silibinin and CUR have the ability to increase DNA repair enzymes; again, neutralizing the detrimental effects of high red meat consumption. Furthermore, nutraceuticals such as GSE, Silibinin and CUR have all been shown to possess anti-inflammatory effects, indicating that these agents would be beneficial in inhibiting red meat-induced inflammation.

Alcohol is considered a carcinogen, and therefore, to prevent CRC, daily intake of alcohol should be kept to 20g/day or below to be considered as safe exposure, which is
equivalent to approximately 2 glasses of wine or beer (Haas et al., 2012). Additionally, other measures can be taken to limit toxicity. Probiotics not only can increase DNA repair enzymes, but these organisms also produce folic acid, which is a chronic nutritional deficiency in individuals that consume alcohol on a regular basis. In addition, folic acid supplementation has been shown to decrease the incidence of DNA mutations that may result following alcohol exposure. Furthermore, alcohol consumption has been shown to increase proliferation; in contrast fiber, GSE, Silibinin, CUR and EGCG have been shown to alter β-catenin signaling resulting in decreased proliferation rates. Downstream targets of β-catenin signaling include MMP-7, which is involved in CRC metastatic disease progression. Nutraceuticals such as Silibinin and CUR have been shown to decrease MMPs expression, specifically MMP-2 and MMP-7, indicating the ability of these supplements to decrease the detrimental effects that result from chronic alcohol exposure.

Tobacco smoke contains nicotine and numerous carcinogenic compounds that result in colon carcinogenesis, and there are no safe exposure guidelines for tobacco smoke. Therefore, for preventing CRC, individuals should not smoke tobacco, and additional lifestyle modifications should be considered. Specifically, foodstuffs that target carcinogen detoxification through up regulation of GSTs and DNA repair proteins would be extremely beneficial; these include probiotics, folic acid, fiber, and phytochemicals such as, Silibinin and CUR. Additionally, tobacco smoke exposure results in epigenetic modifications of KRAS and BRAF, and consumption of fiber, GSE or EGCG has the ability to inhibit these epigenetic changes. Similarly, the epigenetic modifications resulting from tobacco exposure can initiate alterations in proliferative and
apoptotic signaling cascades, resulting in uncontrolled proliferation and apoptosis resistance. To counteract this, it would be beneficial to consume EGCG, which has been shown to inhibit aberrant β-catenin signaling, inhibit proliferation and inflammatory signaling. Furthermore, nutraceuticals, such as GSE, Silibinin, fiber, EGCG and CUR have been shown to induce apoptotic cancer cell death. In addition, probiotics, GSE, Silibinin, CUR, and EGCG all produce anti-inflammatory effects, which would be beneficial in inhibiting the inflammatory mediators that are up regulated in response to tobacco smoke and contribute to CRC disease promotion and progression. Coupled with the fact that Silibinin has also been shown to inhibit VEGF levels, these dietary factors have enormous potential for CRC prevention as a result of tobacco exposure.

Obesity has become a major epidemic in the US, and obesity-related conditions such as cancer are on the rise as well. The most significant lifestyle intervention that would prevent CRC development and be further beneficial to overweight and obese individuals would be increased physical activity. This change in lifestyle would result in decreased adipose tissue and thus decrease angiogenic and inflammatory mediators that are released from the fat tissue. In addition, physical activity has the ability to prevent chronic inflammation, through inhibition of TNFα and induction of the innate immune response. Moreover, physical activity also increases the release of hormones that regulate satiety signals, ultimately restoring the balance between energy intake and expenditure. However, there are a number of diet alterations that can also modify obesity-related signals, specifically, GSE, Silibinin, probiotics and EGCG have all been shown to beneficially modify insulin signaling. Like physical activity, additional nutraceuticals can alter GST activation; these include probiotics, fiber, Silibinin and CUR, all of which
result in increased detoxification. Obesity-induced CRC is also related to uncontrolled proliferation rates, which also could be modified through fiber, GSE, and EGCG supplementation. Furthermore, consumption of Silibinin or CUR could decrease VEGF levels, which are elevated in obese individuals, further inhibiting CRC disease progression and metastasis.

Maintaining a regular daily schedule that coincides with the circadian rhythm is also important in preventing cancer development including CRC. Furthermore, as a proof of principle, these clock genes are suppressed during CRC promotion and progression. It would be recommended to work day shifts; however, that may not be an option for everyone. If night-shift work is necessary, supplementing with compounds that decrease inflammation and control the cell-cycle could alleviate the damaging effects of circadian rhythm disruption. Specifically, probiotics, fiber, GSE, Silibinin, CUR, EGCG and specific vaccinations all decrease inflammation. Moreover, fiber, GSE, Silibinin, CUR and EGCG modulate p21Cip1 protein, which causes cancer cell-cycle arrest as well as inhibition of proliferation.

Overall, the above lifestyle choices can result in damaging or beneficial effects, the key is to find a balance. Furthermore, increasing the protective lifestyle factors is associated with decreased rates and incidence of CRC. The current knowledge highlights the multiple pathways and molecular targets involved in lifestyle choices that can result in CRC progression or prevention. This comprehensive review sheds light on the etiology and pathogenesis of CRC, and provides additional evidence that lifestyle modifications are important for the prevention of CRC.
CHAPTER II

GRAPE SEED EXTRACT (GSE) SUPPLEMENTATION

Introduction

Grape seed extract (GSE) is a complex mixture of polyphenolic compounds; these are naturally occurring compounds that are widely found in fruit, vegetables, nuts, seeds, tea, wine, flowers, and bark (Nandakumar et al., 2008). Specific natural food sources of these beneficial compounds include onions, kale, broccoli, apples, cherries, fennel, sorrel, celery, parsley, thyme, ted pepper, citrus, cocoa, grapes, berries and legumes (Kaur et al., 2009a; Romagnolo and Selmin, 2012). The anti-carcinogenic and chemopreventive efficacy of these polyphenolic compounds has been well documented. *In-vitro* and *in-vivo* studies by our group have demonstrated GSE anti-cancer and chemopreventive efficacy in various pre-clinical models of epithelial cancers including colorectal cancer (CRC); further clinical studies indicate that GSE treatment is well tolerated and bioavailable (Kaur et al., 2006a; Raina et al., 2007; Kaur et al., 2008; Velmurugan et al., 2010b; Velmurugan et al., 2010c; Eich N., 2012). Phase I clinical trials are ongoing aimed at investigating GSE’ anti-cancer efficacy in breast cancer; GSE has already been shown to protect against ultraviolet damage, and also reduce pain (Bagchi et al., 2000; Banerjee and Bagchi, 2001; Eich N., 2012). The polyphenolic compounds in GSE target key colon carcinogenesis pathways including proliferation, inflammation, angiogenesis, invasion, and metastasis pathways (Romagnolo and Selmin, 2012). With the above knowledge,
GSE supplementation would make an excellent nutraceutical intervention in the clinical setting for the long-term anti-cancer treatment and CRC prevention.

**Grape Seed Extract**

*GSE composition:* Nutritional supplements, such as GSE, are considered safe for long-term human consumption. Currently, the FDA categorizes GSE as generally recognized as safe (GRAS), this category is reserved for natural products that have been consumed over thousands of years, with limited toxicity; under this category, GSE supplements are not governmentally regulated. Additionally, many factors can influence the chemical profile of any given GSE supplement; however, the major constituents of this natural extract are polyphenolic flavonoids. These flavonoid compounds are concentrated on the skin and seed of the grape; the phenolic content of GSE depends on grape variety, geographical and climatic factors, cultural practices, stage of ripeness, and the vegetative vigor of the plant (Obreque-Slier et al., 2010). The flavonoid compounds in GSE depend on the evolution of the phenolic compounds during grape growth; additionally the concentration of the phenolic compounds decreases as the grape matures and ripens (Obreque-Slier et al., 2010). Furthermore, how the extraction procedure has been done also affect GSE composition; typical extraction methods include ethanol and water, and ethyl acetate.

Additionally, the flavonoid compounds found in GSE are part of a heterogeneous group that can form oligomers or polymers; this heterogeneous group of flavonoid compounds can be further subdivided into six classes, namely flavonols (quercetin), flavones (apigenin), flavanones (hesperidins), flavan-3-ols (catechin), anthocyanidins
(cyanidin), and isoflavones (genistein) (Nandakumar et al., 2008; Romagnolo and Selmin, 2012). Flavan-3-olic compounds in GSE, specifically catechin and epicatechin, form oligomers or polymers; GSE polymers or proanthocyanidins are mainly dimers, trimers, and polymerized monmeric catechins; these proanthocyanidins are synonymous with condensed tannins (Nandakumar et al., 2008). Catechin contains 2 benzene rings (A-B ring) and a dihydropyran heterocyclin (C-ring); the cis configuration is epicatechin (Fig. 2-1). The A-ring of the catechin molecule is similar to resorcinol moiety, a dihydroxy benzene; the B-ring is a catechol moiety, a 1,2-dihydroxybenzene; and the C-ring of catechin contains an additional hydroxyl group located on carbon 3 (Fig. 2-1).

![Catechin and Epicatechin](image)

**Figure 2-1 Chemical structure of the main polyphenolic compounds in GSE.** Specifically, catechin and epicatechin that form oligomers or polymers; GSE polymers or proanthocyanidins are mainly dimers, trimers, and polymerized monmeric catechins.

**GSE metabolism and bioavailability:** There are several unique biochemical and physio-chemical conditions within the gastrointestinal (GI) tract that need to be considered when discussing GSE metabolism; these conditions include digestive enzymes, buffers and temperature, and pH alteration (Wang et al., 2013). Catechins are absorbed through intestinal cells and metabolized extensively, due to the fact no native catechins are present in bioavailability analyses. There are six alternative routes of
chemical exposure to the intestinal mucosa that include transcellular passive diffusion, paracellular diffusion, transcellular diffusion by endocytosis, transcellular diffusion using lipid particles, paracellular passive diffusion modulation tight junctions; and carrier-mediated diffusion and active transport. Further, intestinal transport of catechins occurs via monocarboxylic acid transporters.

The oxidation of catechin increases as the pH of the surrounding environment increases; furthermore, the auto oxidation of these compounds is initiated via transition metals in the environment, resulting in the formation of superoxide (\(\bullet \text{O}_2^-\)) and hydroperoxyl radicals (\(\bullet \text{HO}_2^-\)), which further reduces to hydrogen peroxide (H\(_2\)O\(_2\)). Additionally, catechin is oxidized to protocatechuic acid (PCA) via *Acinetobacter calcoaceticis*; a bacteria species present in the gut responsible for glucose metabolism. Catechins can also be oxidized into PCA via *Bradyrhizobin japonicum*, a legume nitrogen-fixing bacterial species (Wang et al., 2013). Bacterial degradation of catechins leads to circulatory absorption after 4h of consumption; these microbial metabolites are substrates for catechol-o-methyltransferases (COMT), resulting in metabolites present within the plasma and urine of animals supplemented with proanthocyanidins (Choy et al., 2013). Moreover, phase II glucuronide and methylated metabolites of these phenolic compounds within GSE have been detected *in-vivo* in the plasma as well as kidney and liver tissues; additionally, sulfated metabolites were detected in the urine of animals (Wang et al., 2013). It is unclear if catechin undergoes glucuronidation with in the intestines or within the liver.

The biological activity of polyphenolic compounds such as proanthocyanidins, largely depends on the compounds bioavailability. Many polyphenols are poorly
absorbed when taken orally; this limited uptake is a significant obstacle in designing effective clinical therapy. However, bioavailability studies in humans indicated that following 1 g of GSE given orally, plasma concentration of epicatechin peaks 1-2hr after administration; plasma epicatechin levels are 172ng/ml during peak metabolism; moreover, studies have indicated that repeated exposure to proanthocyanidins increases the bioavailability of these phenolic compounds (Eich N., 2012). Persistent exposure of the gut environment to increased concentrations of catechins and PCA allows these compounds to exert local beneficial effects as well as systemic effects. Additionally, time course studies have revealed that the absorption of proanthocyanidin dimers, specifically proanthocyanidin B1 and proanthocyanidin B2, is ~100 fold greater than the monomeric flavanols (Nandakumar et al., 2008). However, monomeric flavanols have shown a direct protective effect on the intestinal mucosa; GSE flavanols protect the intestinal tissue against damaging oxidative stress and the action of carcinogens (Nandakumar et al., 2008).

**GSE nutraceutical properties:** GSE supplementation has shown promising chemopreventive and anti-cancer potential in numerous cancer types, including skin, prostate, breast, lung and CRC (Kaur et al., 2009a). GSE supplementation has multi-factorial chemopreventive potential; this is due to GSE ability to target multiple protein pathways that are involved in colon carcinogenesis. Overall, GSE supplementation in CRC pre-clinical models results in increased cancer cell apoptosis, as well as, inhibition of multiple processes such as epigenetic signaling, cellular growth, proliferation, oncogene modification, insulin signaling, metastatic development and inflammation (Derry et al., 2013b).
**GSE preclinical in-vitro efficacy:** Numerous studies have shown that GSE treatment of human colon cancer cells results in dramatic induction of apoptotic cell death and down-regulation of proliferation; regarding clinical significance, was the observation that GSE treatment was more effective as CRC progressed (Derry et al., 2012; Dinicola et al., 2012). Detailed mechanistic studies of GSE efficacy, at various stages of CRC development, identified apoptosis induction as the major result of GSE treatment; specifically it activates Caspase-3, -8, -9, resulting in the cleavage of PARP, DNA fragmentation, and programmed cell death (Derry et al., 2012). Furthermore, GSE apoptotic inducing effect was attenuated with antioxidant pre-treatment, indicating oxidative stress as a potential upstream stimulus in GSE-induced CRC cell death (Derry et al., 2012); this is covered in more detail in Chapter III. Similarly, in human CRC cells, GSE treatment triggers cancer cell apoptosis through ROS species generation and an increase in intra-cellular calcium. Furthermore, detailed molecular studies revealed increased ROS and intracellular Ca\(^{2+}\) levels further activated the ERK signaling pathway (Dinicola et al., 2013). Additionally, GSE has been shown to modulate p21Cip1 levels, a key protein involved in cell-cycle and apoptotic regulation; p21Cip1 altered protein expression results in decreased proliferation, leading to cell-cycle arrest, and further downstream pathway activation, including that of ERK1/2 pathway (Kaur et al., 2011a). Furthermore, to determine the specificity of GSE supplementation, viability assay were preformed on normal colon epithelial cells; GSE specifically targeted human CRC cells, with minimal effect on normal colon epithelial cells (Derry et al., 2012).

GSE treatment of head and neck squamous cell carcinoma (HNSCC) cells resulted in decreased expression of proliferative markers such as EGFR, as well as GSE
treatment exhibited anti-inflammatory effects through inhibition of NFκB activation (Sun et al., 2012b). Additionally, GSE proanthocyanidins have been shown to affect human pancreatic carcinoma cell lines; GSE inhibited proliferation and invasive potential via down-regulation of matrix metalloproteinase-2 or -9 (MMP-2, MMP-9) in these cancer cells (Chung et al., 2012). Moreover, in endothelial cells, GSE supplementation inhibited the secretion of MMP-2 and -9; Additionally GSE down-regulated proliferative and angiogenic proteins, such as, EGFR and VEGF (Huang et al., 2012). GSE proanthocyanidins are also shown to inhibit skin cancer cell growth through alteration of epigenetic pathways, ultimately resulting in decreased global DNA methylation, DNMT activity and histone deacetylase activity (Vaid et al., 2012).

Furthermore, GSE proanthocyanidin treatment altered miRNA signaling in rodent hepatocytes; the altered miRNA are involved in lipogenesis, specifically miR-33 and miR-122 (Baselga-Escudero et al., 2012). GSE protects against the deleterious effects of oxidative damage on plasma proteins; this protection exhibits GSE antioxidant potential (Bijak et al., 2012). Moreover, GSE had shown remineralizing potential and thus prevent tooth decay (Benjamin et al., 2012). Additionally, GSE exhibits anti-bacterial properties against the following specific food borne pathogens: *Escherichia coli; Listeria monocytogenes; Salmonella enterica* and *staphylococcus* (Friedman et al., 2013).

**GSE preclinical in-vivo efficacy:** Numerous pre-clinical models of CRC examined GSE efficacy; these studies revealed numerous effects of GSE including anti-inflammatory, anti-proliferative and pro-apoptotic effects (Velmurugan et al., 2010b; Velmurugan et al., 2010c; Derry et al., 2013a). Studies in genetic and sporadic pre-clinical models of CRC revealed GSE anti-inflammatory effects, specifically through
down-regulation of iNOS and COX-2 protein expression (Velmurugan et al., 2010b). Furthermore, in pre-clinical models of CRC, GSE supplementation inhibited aberrant β-catenin expression and downstream proteins, cyclin D1 and c-myc; these altered protein expression profiles led to cell-cycle arrest and decreased proliferation (Velmurugan et al., 2010b). Recent studies in pre-clinical models of sporadic CRC, investigating GSE efficacy, reveal GSE ability to decrease tumor multiplicity, decrease proliferation, induce apoptosis, alter cytokine expression, decrease inflammation, modify miRNA expression profiles, decrease oncogene expression and decrease angiogenic signals (Derry et al., 2013a), which is all covered in more detail in Chapter IV.

GSE proanthocyanidins also inhibit UVB-induced skin phototoxicity; GSE exerted anti-inflammatory properties, specifically through iNOS and NFκB signaling inhibition. Additionally, detailed molecular studies revealed activation of caspases and ERK 1/2 signaling as the major apoptotic and proliferative signals involved in GSE anticancer properties (Filip et al., 2013). Alternatively, GSE treatment in a xenograft model of human head and neck carcinoma (HNSCC) has indicted the generation of reactive oxygen species (ROS) as the upstream mechanism of GSE-mediated cancer cell death; mechanistic studies supplementing with GSE revealed these reactive species induce DNA-damage, which leads to cancer cell-cycle arrest and apoptotic cell death (Shrotriya et al., 2012). GSE polyphenolic compounds induce apoptosis in non-small cell lung cancer (NSCLC); this apoptotic response is initiated via loss of mitochondrial membrane potential, followed by caspase cascade activation (Singh et al., 2011).

GSE proanthocyanidins have been shown to alleviate symptoms associated with allergic inflammation in animal models of asthma; mechanistic studies reveal that GSE
anti-inflammatory mechanism of action specifically includes cytokine down-regulation and up-regulation of antioxidant ratios (Lee et al., 2012). GSE proanthocyanidins have been shown to exhibit immunomodulatory effects and inhibit the growth of sarcoma tumor cells; the GSE phenolic compounds increased the thymus and spleen weight, further increasing TNF-α secretion in serum (Tong et al., 2011). Additionally, GSE has been shown to reduce colitis disease severity; ulcerative colitis is induced by dextran sulphated sodium (DSS) mimicking the human colon inflammatory disorder (Cheah et al., 2013). GSE proanthocyanidins have shown to protect liver tissue against ischemia reperfusion injury, in diet-induced obese animals; the major mechanism of GSE efficacy in this study was attributed to increased activity of ROS scavengers, and decreased cytokines levels, which indicates decreased inflammation (Song et al., 2012). GSE treatment also inhibits chronic autoimmune inflammation that occurs during rheumatoid arthritis (RA) progression; GSE specifically inhibits inflammation through down-regulation of NKκB signaling (Park et al., 2012).

GSE supplementation has been shown to alter glucose homeostasis. Mechanistic studies indicate GSE consumption decreases dipeptidyl-peptidase 4 (DPP4) levels in human colon cancer cells; in-vivo studies in diet-induced obese mice show improvement in glycemic regulation upon GSE supplementation (Gonzalez-Abuin et al., 2012). GSE supplementation has also been shown to alter insulin signaling in-vivo, resulting in fewer incidences of insulinemia; GSE specifically target β-cells reducing triglyceride content (Castell-Auvi et al., 2013). GSE supplementation in insulin resistant rats improved insulin and adiponectin signaling; GSE specifically upregulated Akt and GLUT4 insulin signaling pathways, improving insulin resistance (Meeprom et al., 2011). At the
molecular levels, consumption of GSE proanthocyanidins has been shown to improve mitochondrial function and energy metabolism; 5 h after GSE administration, plasma triglycerides levels had dramatically decreased, as well as the levels of free fatty acids and glycerol levels (Pajuelo et al., 2011). Furthermore, GSE treatment inhibits lipid peroxidation through increased GSH antioxidant activation (Abbas and Sakr, 2013).

GSE has also been shown to cross the blood-brain barrier and exert neuroprotective effects; pre-clinical animal models indicated that GSE reduces methylmercury-induced neurotoxicity in rats (Yang et al., 2012). GSE has been further shown to inhibit the arsenic-induced liver toxicity in rats, via suppression of TGF-β/Smad signaling and suppression of NADPH oxidase (Pan et al., 2011). GSE proanthocyanidins have also shown a protective role against nephropathy; GSE treatment decreased oxidative stress in kidney tissue (Ozkan et al., 2012).

**GSE clinical efficacy:** Presently, there is limited data available in regards to clinical trial evaluating the efficacy of GSE proanthocyanidins. However, GSE proanthocyanidin supplementation has been proven in various pre-clinical as well as clinical models of cardiovascular disease; GSE provides a potent anti-oxidant resulting in improved cardiac function, reduced myocardial infarct, decreased ROS and MDA formation (Bagchi et al., 2003). Clinical studies were conducted evaluating GSE supplementation on hypercholesterolemic patients; the studies reveal GSE ability to reduce oxidized LDL, a known biomarker of cardiovascular disease (Bagchi et al., 2003). Additionally, a meta-analysis of randomized controlled trial investigating GSE efficacy against cardiovascular disease revealed GSE activity in at least one endpoint of the
following: systolic or diastolic blood pressure, heart rate, total cholesterol, low-density or high-density lipoprotein cholesterol, and triglycerides (Feringa et al., 2011).

GSE supplementation had also been shown clinically to treat various neurological disorders including supranuclear palsy; GSE treatment inhibits the abnormal protein folding that results in neurodegenerative diseases (Pasinetti et al., 2010). Additionally, on-going phase I clinical trials are investigating GSE ability to inhibit aromatase, an enzyme that converts androgen to estrogen, and is normally over expressed in breast cancer (Kijima et al., 2006).

**Therapeutic impact of GSE for CRC treatment and prevention:** Given the above information, there is significant experimental date supporting the beneficial effects of GSE proanthocyanidins; numerous *in-vivo* and *in-vitro* studies have indicated GSE supplementation as an effective anti-cancer and chemopreventive agent. Considering that current conventional CRC therapies such as chemotherapy, radiation, and surgery, result in severe patient toxicity and ultimately tumor therapy resistance, there is an urgent need for effective non-toxic long-term treatments (Diaz et al., 2006; Shekhar, 2011).

Accordingly, additional strategies like the use of natural products such as GSE, that targets multiple pathways, are one such approach to prevent colon carcinogenesis and further reduce disease incidence.

Furthermore, detailed *in-vitro* mechanistic studies have provided insight into GSE’ potential anti-cancer and chemopreventive mechanisms of action; fully understanding GSE mechanisms of action will allow the identification of patient populations that will benefit from GSE supplementation. Some of the potential mechanisms of GSE chemopreventive and anti-cancer efficacy include anti-proliferative,
cell-cycle arrest, pro-apoptotic, anti-inflammatory and anti-angiogenic as summarized in Figure 2-2. GSE has additionally shown anti-bacterial, anti-viral, anti-oxidant, and cardio protective effects (Kaur et al., 2009a).

Figure 2-2 Molecular pathways involved in the biologically activity of GSE proanthocyanidins. Specifically, GSE constituents alter Oxidative stress; Metastasis; Oncogene signaling; Epigenetic signaling; Inflammation; Hormone signaling; and Proliferative, cell-cycle and apoptotic pathways.

Cancer is continuing to become a global health crisis; furthermore, current therapeutic interventions have proven ineffective at reducing CRC disease incidence, emphasizing the need to focus on prophylactic CRC disease interventions, including lifestyle modifications and nutraceutical administration. Prevention or the treatment of cancer via nutraceutical administration has vast implications for human health; these naturally occurring agents can reduce cancer incidence, reduce socioeconomic burden, and are cost-effective clinical strategies. However, these GSE proanthocyanidins need to
be further investigated in clinical trials, further validating the extensive pre-clinical studies. Once such information is available, GSE supplementation would make an excellent adjuvant to conventional chemotherapy; reducing toxicity without sacrificing efficacy. In conclusion, GSE proanthocyanidins might be a more safe and effective chemopreventive and chemotherapeutic agent than the current standard of care against cancer of most organs, including CRC.
CHAPTER III

DIFFERENTIAL EFFECTS OF GRAPE SEED EXTRACT AGAINST HUMAN COLORECTAL CANCER CELL LINES: THE INTRICATE ROLE OF DEATH RECEPTORS AND MITOCHONDRIA

Introduction

Colorectal cancer (CRC) is the third leading (both genders combined) cause of cancer-related deaths in the United States [American Cancer Society: Cancer Facts and Figures 2012.]. The incidence of CRC is also increasing world-wide, with an over-all lifetime risk of 1 in 19, due to adoption of western lifestyle habits; high fat diet, alcohol consumption, and sedentary lifestyle (Bretthauer, 2010). Compliance with screening recommendations is low; as such, 65% of the new CRC cases are diagnosed at a stage where they have already progressed to advanced disease (Bretthauer, 2010). Conventional therapies for CRC, e.g. chemotherapy, radiation, and surgery, result in severe toxicity and associated therapy resistance (Diaz et al., 2006; Shekhar, 2011). Such limitations have resulted in a shift towards additional strategies involving the use of natural dietary/non-dietary products, which target multiple pathways in cancer cells and are associated with limited or no toxicity (Ramasamy and Agarwal, 2008; Kaur et al., 2009a).

In this regard, studies have indicated that high consumption of fruits and vegetables or their bio-active components can decrease CRC incidence by ~ 40%

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(Marshall, 2008; Ramasamy and Agarwal, 2008). Grape Seed Extract (GSE) is one such widely consumed dietary supplement (with 95% standardized proanthocyanidins) that has been shown to possess anti-cancer, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral effects (Bagchi et al., 1997; Mayer et al., 2008; Kaur et al., 2009a; Hogan et al., 2011). The United States Food and Drug Administration classifies GSE in GRAS ‘generally regarded as safe’ category. Pre-clinical studies have established strong GSE efficacy against prostate, colon, lung, breast, skin, and other cancers (Kaur et al., 2009a). Clinical studies with GSE have indicated that its active components are bioavailable and well tolerated (Ferruzzi et al., 2009; Prasain et al., 2009; Eich N., 2012). However, till date, the specificity of GSE to target CRC cells and the associated-mechanisms involved in GSE-induced CRC cell death are not well studied. Therefore, in the present study, we investigated the molecular mechanisms involved in GSE-induced CRC cell death at various stages of the malignancy. Though it has been previously shown by a number of research groups, including ours, that GSE has the ability to induce CRC cell death in both in vitro and in vivo CRC models, one limitation of all the in vitro studies investigating GSE efficacy in CRC was that the in-vitro experimental design failed to take into consideration the different stages of this deadly malignancy as well as normal colon epithelial cells (Kaur et al., 2006b). Our present study is unique in that aspect, as we chose multiple CRC cell lines, which differ in their metastatic potential; this strategy helped in elucidating the differential effects of GSE against human CRC cell lines, where this dietary agent was more effective as the CRC malignancy stage worsens with minimal, if any, effect on normal colon cells.
Materials and methods

Reagents: Standardized preparation of GSE was a gift from Kikkoman Corp. (Nado City, Japan). The composition of the GSE preparation is listed as: 89.3% procyanidins, 6.6% monomeric flavonols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash. Dimethyl Sulfoxide (DMSO) and N-acetyl cysteine (NAC) were from Sigma Chemical Co. (St. Louis, MO); Trypan blue 0.4% was from Invitrogen (Carlsbad, CA). Primary antibodies used were anti-cleaved caspase-9, anti-cleaved caspase -8, anti-cleaved caspase -3, anti-cleaved PARP, anti-COX IV, anti-AIF, anti-Bak, anti-Bik, anti-p53, anti-p21Cip1, and anti-Puma (Cell Signaling Technology, Beverly, MA); anti-DR4, anti-DR5, and anti-Mcl-1s (Santa Cruz Biotechnology, Santa Cruz, CA); anti-cytochrome-c, and anti-GADPH (BD Biosciences, San Jose, CA); and anti-β-actin (Sigma, St. Louis, MO). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were purchased from Invitrogen and Cell Signaling Technology, respectively. Z-VAD-Fmk, DR4/Fc, and DR5/Fc were from R&D (Minneapolis, MN).

Cell lines: SW480 and HCT116 cells were purchased from American Type Culture Collection (Manassas, VA). SW620 cells were a gift from Dr. Pamela Rice, University of Colorado, Denver. NCM460 cells were from InCell Corporation (San Antonio, TX). HCT116 vector control and HCT116 p53 double knockout cells were a kind gift from Dr. Bert Vogelstein (The Johns Hopkins Kimmel Cancer Center). Cells were maintained at 37°C in a humidified 5% CO2 atmosphere in DMEM (HCT116), Leibovitz-L15 (SW480), and RMPI (SW620) media supplemented with 10% FBS and 1% penicillin/streptomycin. NCM 460 cells were maintained under similar conditions in M3:10 media (InCell Corporation).
Assessment of cell viability and apoptosis: Cells were plated at a density of 5,000/cm² in 60-mm culture plates under standard conditions and treated subsequently either with DMSO alone or with varying concentrations of GSE (0-100 µg/mL) in DMSO. At the end of desired treatment times (12-48 h), cells were harvested by brief trypsinization and counted using a hemocytometer. Trypan blue dye exclusion assay was used to differentiate between live and dead cells. The final concentration of DMSO in the culture medium during different treatments did not exceed 0.1% (v/v). To quantify GSE-induced apoptotic death, Annexin V and propidium iodide staining was done using Vybrant Apoptosis Assay kit 2 as per the manufacturer's protocol and the stained cells were analyzed by FACS analysis, utilizing the core service of the University of Colorado Cancer Center (Aurora, CO), in order to quantify the apoptotic cells. In inhibitor studies, CRC cells were pre-treated for 2h with all-caspases inhibitor z-VAD-fmk (50 µmol/mL), DR4/Fc (100 ng/mL), DR5/Fc (100 ng/mL) or a combination of both DR4Fc and DR5/Fc prior to GSE exposure. In the experiments with NAC, CRC cells were pretreated for 1h with NAC (10 mM), in pH-7.5 adjusted media, followed by exposure to GSE (20 or 50 µg/ml) for 12h. Mitochondrial and cytosolic fractions of CRC cells were prepared as described previously (Agarwal et al., 2003).

Western immunoblotting: At the end of each treatment, cell lysates were prepared in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonlfy fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin]. Immunoblot analysis using equal amount of protein lysate per sample was done. Membranes were probed with desired primary antibodies followed by peroxidase-
conjugated appropriate secondary antibody and visualized by enhanced chemiluminescence detection system. Some blots were multiplexed or stripped and reprobed with different antibodies including those for loading control.

**Determination of mitochondrial membrane potential ($\Delta \Psi_m$):** Changes in $\Delta \Psi_m$ were assessed using the cationic dye, DiOC$_6$ (3) that accumulates in mitochondria with active membrane potential. Emission increases due to dye stacking. The stain intensity decreases when agents disrupt the $\Delta \Psi_m$. Following 3-24h GSE treatment (20 and 50µg/ml), cells were exposed to 40nM DiOC6(3) for 20 min at 37°C and then cells were washed with fresh medium and fluorescence visualized at 488 nm using the live imaging equipment: Operetta High-Content Imaging and Analysis System. The median values of green fluorescence, from 9 different focal points within the well, were determined with Harmony software. This live-imaging method allows for the quantification of cells with depolarized mitochondria.

**Statistical analysis:** All the data shown are mean ± SE, representative of at least three independent experiments. Statistical significance of differences between control and GSE-treated samples were calculated by one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). $P$ values of ≤0.05 were considered significant.

**Results**

**GSE treatment causes growth inhibition and induces death in human CRC cells:**

First, we evaluated the efficacy of GSE against a panel of human CRC cell lines, based on phenotypic and genetic variations, so as to cover different clinical stages of CRC, viz., SW480 (stage II CRC with mutant $p53$), SW620 (stage III CRC with mutant $p53$),
HCT116 (stage IV CRC with wt p53) cells. Trypan blue exclusion assays were performed to differentiate between live and dead cells. In the results, we saw that GSE at 25, 50 and 100 µg/ml doses resulted in a dose-dependent growth inhibition of SW480 cells after 12, 24 and 48 h treatment; concomitantly, an increase in SW480 dead cell population [17-37% (P<0.05-0.001), 19-38% (P<0.01-0.001) and 15-60% (P<0.01-0.001)] was also observed under the same concentrations and treatment conditions (Fig. 3-1A). In case of SW620 and HCT116 cells (Fig. 3-1B & C), GSE showed significant effect on growth inhibition at comparatively lower doses (10-30 µg/ml) to those effective (25-100 µg/ml) in SW480 cells. In SW620 and HCT116 cells, these GSE doses were also effective in causing significant cell death [50-60% (P<0.01-0.001] in as early as 12 h, though the dead cell numbers did not increase further at later time points utilizing the LD50 dose of GSE. Overall, GSE (10-100 µg/ml) treatment for 12-48 h significantly suppressed cell growth and induced cell death in a dose-dependent manner in the cell lines studied.

Notably, since these CRC cells represent different stages of CRC, our findings showing stronger GSE inhibitory effects in cell lines that are more aggressive in nature have significant translational implications in CRC management. In addition to the viability studies, we also examined GSE effect on cell-cycle distribution of CRC cells. GSE arrested SW480 cells in G2M phase, SW620 in the G1 phase, and HCT116, partly, in G1 and G2 phases (Appendix A). However, since the predominant effect of GSE in these CRC cells was cell death, we chose to focus the mechanistic studies only on this aspect of GSE effect.
Figure 3-1 GSE treatment results in growth inhibition and death of human CRC cells. The biological activity of Kikkoman GSE was assessed in terms of its effect on cellular proliferation and viability of human CRC cells, measured as total live cell number and percent dead population after GSE exposure in A) SW480, B) SW620, and C) HCT116 CRC cells. GSE doses ranged from 10-100 µg/mL, and effect was observed for 12-48 hrs. The data shown here is representative of the mean of 3 independent values; bars, S.E.M; * P≤0.05; # P≤0.01; $ P≤0.001; control (DMSO). S.E.M, standard error mean.
GSE treatment activates both the intrinsic and extrinsic apoptotic pathways in human CRC cells: Next, to identify the nature of GSE-mediated CRC cell death, quantitative apoptosis assay with AnnexinV/ Propidium iodide (PI) followed by FACS analysis was performed. GSE treatment for 12h induced strong apoptotic death, causing a 1.5-3-fold ($P<0.05$-$P<0.001$) increase in apoptotic SW480 cells at 25-100µg/mL doses (Fig. 3-2A). Unlike SW480 cells, a much lower GSE concentration was effective in inducing apoptosis in the more metastatic cells lines, SW620 and HCT116, based on their cell death effects with GSE in Fig 1. In these cell lines, significant apoptotic death (~11-fold, $P<0.001$; Fig. 3-2A) was observed which remained consistent across different doses. Based on these results, showing strong apoptotic effect of GSE in a panel of human CRC cell lines, we next assessed the involvement of both extrinsic and intrinsic pathways in GSE-caused apoptotic death of these CRC cell lines. In all cell lines, GSE treatment induced cleavage of caspases 8, -9, -3 and PARP, suggesting the involvement of different executioner pathways in GSE induced apoptotic death (Fig. 3-2B). A time course assessment of cytochrome c (cyto c) release in the cytoplasm of GSE treated CRC cells indicated that cyto c was released within 9h in SW480 and SW620 cells while in HCT116 cells the release was seen to a lesser extent after 12h (Fig. 3-2C).

Since reduction of $\Delta \Psi_m$ is believed to be an early event during apoptosis (Decaudin et al., 1997;Elmore, 2007), to further solidify above findings, we examined the kinetics of the $\Delta \Psi_m$ in GSE-treated CRC cells. Live fluorescence (Operetta imaging) using the Cationic dye DiOC$_6$(3) as an indicator of dissipation of $\Delta \Psi_m$ revealed a time-dependent decay (Fig. 3-3) of DiOC$_6$(3)-fluorescence (indicating a decline in $\Delta \Psi_m$).
Figure 3-2 GSE treatment induces apoptotic death in human CRC cells. A) GSE causes apoptotic death of SW480, SW620, and HCT116 cells. Cells were treated with DMSO (control) or different concentrations of GSE (10-100µg/mL) for 12 hours. At the end of treatment, cells were collected and stained with Annexin V-propidium iodide and analyzed by flow cytometry. % apoptotic death of CRC cell is represented by columns (mean values of three independent samples for each treatment ± S.E.M) * P≤0.05; $ P≤0.001. B) Apoptotic effect of GSE involves cleavage of caspases-9, -8, -3, and PARP. Both adherent and non-adherent cells were harvested and cell lysates were prepared in nondenaturing lysis buffer and immunoblotting was performed as detailed in Materials and Methods section. C) Apoptotic effect of GSE is accompanied with the release of cytochrome c (cyto c) in the cytoplasm of CRC cells. Membranes were also stripped and reprobed with anti-GADPH antibody to confirm equal protein loading. Probing with COX IV antibody revealed that the cytosolic fractions were non-contaminated with mitochondrial fractions. S.E.M, standard error mean.
Figure 3-3 GSE treatment causes loss of mitochondrial membrane potential in human CRC cells. Cells were exposed to 40nM DiOC6(3) for 20 min at 37°C and then cells were washed with fresh medium and fluorescence visualized at 488 nm using the live imaging equipment: Operetta High-Content Imaging and Analysis System. A) The median values of green fluorescence, from 9 different focal points within the well, were determined with Harmony software and are represented as columns ± S.E.M; * P≤0.05; # P≤0.01; $ P≤0.001. B) Representative photographs (20x) of 9 different focal points per well are shown. S.E.M, standard error mean.
In SW80 and SW620 cells, the decline in DiOC₆(3)-fluorescence was seen as early as 3h, which became more significant by 12h. While in HCT116 cells, the decline in \( \Delta \Psi_m \) was not significant by 3h but became strongly significant by 9h.

**GSE treatment also induces caspase-independent intrinsic apoptotic pathway in human CRC cells and causes differential modulation of apoptotic proteins:** Mutations within the caspase protease family are commonly observed in malignancies (Devarajan et al., 2002; Kim et al., 2003; Soung et al., 2005; Soung et al., 2006; Cox et al., 2007). Several reports indicate caspase-8 mutations in breast and gastric cancers, while silent mutations of caspase-9 have been reported in CRC (Devarajan et al., 2002; Kim et al., 2003; Soung et al., 2005; Soung et al., 2006; Cox et al., 2007). Therefore, for an effective clinical CRC therapy, the treatment agents also need to circumvent such genetic variations/limitations and display potential to induce caspase-independent apoptotic death in the cancer cells, as well. In this context, we next examined whether GSE also had the potential to induce caspase-independent apoptotic death in the CRC cell lines. CRC cells were pre-treated with Z-VAD-FMK, an irreversible pan-caspase inhibitor, prior to 12h GSE treatment (20-50µg/mL) and apoptotic death was assessed (Fig. 3-4A). Importantly, we observed that the apoptosis induced by GSE in all three CRC cell lines was either unaffected or marginally decreased in presence of pan-caspase inhibitors. Thus, regardless of caspase inactivation, GSE was able to induce significant apoptotic death in all CRC cell lines.
Figure 3-4 GSE treatment causes caspase-dependent and caspase independent apoptotic cell death in CRC cells. A) Effect of pre-treatment (2 h) with all-caspase inhibitor Z-VAD-Fmk (50µM) on GSE (20-50µg/mL, 12h) induced apoptotic death in CRC cells. % apoptotic death of CRC cell is represented by columns (mean values of three independent samples for each treatment ± S.E.M). # P≤0.01. B) Apoptotic effect of GSE is accompanied with the release of AIF in the cytoplasm of CRC cells. Membranes were also stripped and re-probed with anti-GADPH antibody to confirm equal protein loading. C) Apoptotic effect of GSE involves differential up regulation of Death receptors and modulation of the expression of pro- and anti-apoptotic proteins. S.E.M, standard error mean.
Since a group of pro-apoptotic proteins such as AIF and Endonuclease G (Endo G), involved in caspase-independent apoptotic cell death, are also released from mitochondria, and on translocation to the nucleus initiate DNA fragmentation and chromatin condensation (Elmore, 2007), we next examined their involvement in GSE-caused apoptotic death. Subsequent to GSE treatments, time-course evaluation of cytosolic fractions of CRC cells showed that in SW620 and HCT116 cells, AIF was released within 12h, while it was only released by 24 h in SW480 cells, indicating that indeed AIF was also involved in apoptotic induction by GSE (Fig. 3-4B). In other study, while expression of Endo G showed a marginal increase in SW480 cells after GSE exposure, its involvement in GSE-caused apoptosis of SW620 and HCT116 cells was not significant (Appendix B).

Next, we analyzed the ability of GSE to modulate a group of additional pro- and anti-apoptotic proteins that play an essential role in the extrinsic and intrinsic apoptotic pathways (Fig. 3-4C). Results showed that GSE treatment caused an up regulation of Death receptors, DR4 and DR5 in SW480 and SW620 cells, while only DR5 was specifically up regulated in HCT116 cells. Furthermore, the expression of pro-apoptotic proteins Mcl-1s and Bik was increased by GSE in all three CRC cell lines (Fig. 3-4C); however, there was down regulation of anti-apoptotic proteins Bcl-2 and XIAP by GSE only in SW480 cells. Due to genetic variations within different CRC cell lines, SW480 and SW620 cells harbor mutant but HCT116 with wild-type p53, we also assessed GSE effect on p53 and its transcriptionally activated targets, which could play an important role in both cell cycle progression and apoptosis. Importantly, GSE caused an up regulation of p53 expression together with an increase in the levels of p21Cip1, Puma
and Bak in HCT116 cells (Fig. 3-4C), and only an increase in p21Cip1 in SW620 cells (Fig. 3-4C).

*Apoptotic death induced by GSE in human CRC cells is mediated by Death receptors and oxidative stress:* To further examine the involvement of Death receptors in the GSE-induced apoptotic cell death in CRC cell lines, cells were pretreated with DR4/Fc and/or DR5/Fc chimeric proteins so as to inhibit the extrinsic apoptotic signals prior to GSE exposure (20-50µg/mL for 12h) and then assessed for apoptotic death (Fig. 3-5A). In SW480 cells, pre-treatment with a single chimera protein DR4/Fc or DR5/Fc or a combination of both chimeras resulted in a marginal to significant attenuation of GSE-induced apoptotic death; the combination showing significantly more decrease (∼ 50%) in apoptotic cells than the single chimeras alone (Fig. 3-5A) suggesting an overlapping involvement of DR4 and DR5 in GSE-induced apoptotic death of SW480 cells. In SW620 cell line, however, only DR5/Fc chimera was able to marginally decrease the GSE-induced apoptotic death; the combination of both chimeras together not being more potent than the single DR5/Fc chimera alone (Fig. 3-5A). In HCT116 cells, DR5/Fc chimera alone was effective in significantly decreasing GSE-induced apoptotic death; the combination of both chimeras failed to enhance the protective anti-apoptotic effect of DR5/Fc, suggesting the involvement of only DR5 in GSE-induced apoptotic death of HCT116 cells (Fig. 3-5A).
Figure 3-5 GSE-mediated apoptotic death involves oxidative stress mediated up regulation of Death receptors DR4 and DR5. A) Effect of inhibition of Death receptor mediated signaling on the apoptotic effect induced by GSE in CRC cells. CRC cells were either pre-treated for 2hr. with DR4/Fc (100ng/mL) or DR5/Fc (100ng/mL) chimeric proteins or a combination of both, prior to GSE exposure (20-50µg/mL, for 12h) and then % apoptotic death was evaluated. Effect of pre-treatment of CRC cells with anti-oxidant, 10mM NAC, for 1hr prior to GSE exposure on, B) Apoptotic effect induced by GSE, C) modulation of Death receptors by GSE. * P≤0.05; #P≤0.01; $ P≤0.001. S.E.M, standard error mean.
Considering the dependence of GSE-mediated cell death on the Death receptor pathway, we next focused on the upstream receptor stimulus. Several reports have indicated that proteasomal inhibition can lead to Death receptor up regulation and protein accumulation of pro-apoptotic molecules namely Bik, Bim and Mcl-1 (Nencioni et al., 2005; Nikrad et al., 2005). To examine this possibility, we determined the efficacy of GSE to inhibit proteasomal activity directly in a cell free system (co-incubation with 20S proteasomal subunit) as well as in the cellular system (exposure of cells to GSE). Interestingly, GSE was able to inhibit the proteasomal activity of 20S subunit in a cell free system more significantly than epoxomicin, a known proteasomal inhibitor. However, GSE displayed no such effect in presence of cellular environment, suggesting that other triggers were involved in causing the increase in the expression of the molecules involved in apoptosis (Appendix C).

We next focused on an alternative upstream stimulus, oxidative stress, which has been implicated in Death receptor activation, mitochondrial DNA damage, and apoptosis cascade initiation (Kaur et al., 2006b; Zhang et al., 2006; Ricci et al., 2008), for its possible role in GSE-mediated apoptosis. For this, CRC cells were pre-incubated with the anti-oxidant NAC prior to GSE (20-50µg/mL for 12h) exposure (Fig. 3-5B). Interestingly, NAC pretreatment resulted in 25% ($P<0.01$), 65% ($P<0.001$), and 37% ($P<0.001$) attenuation in GSE-caused apoptotic cell death in SW480, SW620, and HCT116 cells, respectively (Fig. 3-5B). Following these results, we also performed immunoblotting to visualize the changes in the protein expression of these Death receptor molecules resulting from NAC pre-treatment (Fig. 3-5C). The results obtained complimented the earlier observations using the chimera proteins in the receptor
inhibition studies, i.e., in SW480 cells, NAC pre-treatment resulted in down-regulation of both DR4 and DR5 receptors, while in SW620 cells no attenuation in the levels of death receptors was observed, in fact the expressions were slightly more in the NAC+GSE groups (Fig. 3-5C). Furthermore, we also observed down-regulation of DR5 protein expression levels with NAC pre-treatment in HCT116 cells, while there was no effect on DR4 levels. Overall, these results indicated that GSE-induced apoptotic death in SW480 and HCT116 cells is mediated via the extrinsic pathway with oxidative stress as a possible upstream stimulus working through Death receptors. However, in SW620 cells, oxidative stress was a stimulus for GSE-caused CRC cell death independent of Death receptors involvement.

**GSE-mediated death is specific to CRC cells and is independent of their p53 status:** To investigate whether the GSE-induced cell death effects were specific to CRC cells and that it had no effect on normal colon cells, NCM 460 (normal colon epithelial cell line) cells were exposed to different doses of GSE (25-100µg/mL) for 12-48h (Fig. 3-6A) and cellular viability was assessed by Trypan blue exclusion assay. Importantly, no growth inhibition was observed in these normal colon cells due to GSE treatment. There was also no effect on cellular viability till 24h of GSE exposure, however, a marginal increase (~8%) in dead cell population was observed after 48h exposure to the highest dose (100µg/mL) of GSE (Fig. 3-6A).

In other studies, based on the fact that there was a strong increase in p53 and its transcriptionally activated targets such as p21Cip1, Puma and Bak in HCT116 cells after GSE treatment, we further explored the role of p53 in GSE-induced CRC cell death.
Figure 3-6 GSE-mediated cellular death is independent of p53 status of human CRC cells. The cytotoxic effect of Kikkoman GSE on, A) normal colon epithelial cells, NCM460; B) HCT116 p53 KO, and C) vector control, HCT116 VC cells. Effect on cellular proliferation and viability of cells was measured by assessing total live cell number and percent dead population after GSE treatment GSE doses ranged from 10-100µg/mL, and effect was observed for 12-48 hrs. The data shown here is representative of the mean of 3 independent values; bars, SD; * P≤0.05; # P≤0.01; $ P≤0.001; control (DMSO). D) Effect of GSE exposure on cleavage of caspases-9, -8, -3 and PARP in HCT116 p53 KO and HCT116 VC cells. S.E.M, standard error mean.
HCT116 p53 double knockout cells (HCT116 p53 KO) and vector control cells (HCT116 VC) were treated with GSE (10-30µg/mL) for 24h; viability assays (Fig. 3-6B & C) showed almost comparable GSE responses in both p53 KO and VC cells. Furthermore, in both cell lines, we observed an up regulation of cleaved caspase-3, -9, -8, and cleaved PARP expression levels with GSE treatment, indicating that the apoptotic response induced by GSE was independent of p53 status (Fig. 3-6D).

Discussion

Mechanisms that contribute to multi-drug tumor cell resistance are known to involve mutations that result in uncontrolled cellular proliferation and apoptosis resistance; therefore, strategies that focus on inhibiting these processes, at any stage of malignancy, would be of clinical significance (Kelloff et al., 1996). The process of apoptosis is highly complex involving a cascade of molecular events; the two main pathways involved in this process are: extrinsic or death receptor pathway and the intrinsic or mitochondrial-driven pathway (Elmore, 2007). There is evidence of cross-talk between these pathways and both pathways converge into the same executioner pathway (Igney and Krammer, 2002). The extrinsic pathway involves transmembrane receptor-mediated protein interactions. These death receptors are part of the tumor necrosis factor (TNF) receptor gene super family, and include DR4 and DR5 (Elmore, 2007). Ligand binding, for example Tumor Necrosis factor-related apoptosis-inducing ligand (TRAIL), and adaptor protein recruitment, such as Fas associated death domain (FADD), allow for the formation of the death-inducing signaling complex (DISC), which results in autocatalytic cleavage/activation of procaspase-8 and executioner pathway activation.
(Kischkel et al., 1995). The intrinsic pathway, on the other hand, involves non-receptor mediated protein interactions and mitochondrial initiated events (Elmore, 2007). The stimuli that initiate the intrinsic pathway can be either positive or negative; negative signals involve the absence of certain growth hormones and cytokines, and positive signals include radiation, hypoxia, and presence of free radicals (Elmore, 2007). These stimuli ultimately alter $\Delta \Psi_m$ resulting in the opening of the mitochondrial permeability transition pore. Loss of this membrane potential results in the release of pro-apoptotic proteins into the cytosol and executioner pathway activation (Elmore, 2007). Regulation of these mitochondrial apoptotic events occurs via members of the Bcl-2 family of proteins; these proteins can have either a pro-apoptotic or anti-apoptotic effect: anti-apoptotic molecules include: Bcl-2, Mcl-1L and Bcl-xL; and pro-apoptotic proteins include: Bik, Bim, Bak, Puma and Mcl-1s (Marsden et al., 2002). Another major player in apoptosis is the p53 tumor suppressor molecule; p53-dependent cell death is executed by transcriptional activation of pro-apoptotic family members such as p21Cip1, Puma, Noxa, and Bak, which can translocate to the mitochondria and induce cytochrome c release and result in executioner pathway activation (Schuler and Green, 2001; Shen and White, 2001).

Clinically, a number of cancer types have been shown to be sensitive to TRAIL gene therapy resulting in the activation of the extrinsic pathway and ultimately cancer cell death (Eggert et al., 2000; Hinz et al., 2000; Fulda et al., 2001). However, resistance to TRAIL-mediated apoptosis in cancer cells has become a challenging issue in the clinic, with a number of cancer types, including CRC, exhibiting no response to therapy (Griffith et al., 1998; Kim et al., 2000; Zhang et al., 2000; Zhang and Fang, 2005).
Furthermore, mutations within the family of caspase proteases have also been reported in CRC, suggesting that effective clinical therapies are needed to circumvent these obstacles (Kim et al., 2003; Soung et al., 2006).

In this regard, the results of the present study are highly significant as they identify GSE as a potential anti-cancer therapeutic agent that has the capacity to induce both extrinsic and intrinsic apoptotic pathways selectively in CRC, but not normal colon epithelial cells. Previous studies have indicated caspase-dependent and caspase-independent pathways in response to GSE treatment, however this effect was GSE preparation specific, only examined a single non-metastatic colon cancer cell line, and did not utilize caspase pathway inhibition to determine its dependence on GSE-induced apoptotic death (Dinicola et al., 2010). In this regard, our study was designed to determine GSE dependence on caspase activation in various stages of CRC through irreversible pathway inhibition, and our results indicated that indeed GSE-induced apoptotic cell death is not entirely caspase-dependent but also involves the activation of caspase-independent pathways, which is mediated in part through the release of AIF. In addition, the GSE-mediated cell death was also dependent, in part, on the activation of the extrinsic pathway via death receptor up regulation in SW480 and HCT116 cells; SW620 cells failed to show any involvement of death receptors in GSE caused cell death. Interestingly, oxidative stress generated by GSE exposure was also identified to play an important role in the cell death of all CRC cell lines, as an upstream stimulus, causing either up regulation of death receptors (SW480 and HCT 116 cells) or generating cytotoxic stress (SW620 cells) affecting the mitochondrial membrane permeability. The upstream stimulus of oxidative stress not being able to cause significant death receptor up
regulation, yet causing a significant apoptotic response in SW620 cells, can be explained by the fact that in this cell line we observed p21Cip1 up regulation independent of p53 protein activation; previous work has linked GSE generated oxidative stress to p21Cip1 expression which leads to increased apoptotic response. Furthermore, our results indicated that loss of ΔΨm occurs prior to the release of cyto c followed by activation of intrinsic and extrinsic pathways, suggesting that the intrinsic pathway may be the initiating pathway in GSE-mediated apoptotic cell death. The GSE mediated apoptotic events were also found to be associated with differential modulation of pro- and anti-apoptotic proteins. In addition, the cytotoxic effect exhibited by GSE against CRC cells was found to be independent of p53 status of the CRC cell lines, though, it did induce the p53-dependent apoptotic pathway in HCT116, through up regulation of p53, Bak, p21Cip1, and Puma. This observation is highly significant, given the fact that one of the common mutations in CRC is missense mutations in the TP53 gene, which encodes for the p53 protein (Ahnen et al., 1998).

Importantly, GSE-mediated cell death efficacy was found to be specific against CRC cells as it exhibited no toxicity in normal colon epithelial cells. The fact that severe toxicity can result from conventional CRC therapies, for example, it took a long time to establish an optimal 5-FU regime that had less severe toxicity, higher response rate, and overall improved long-term survival (Braun and Seymour, 2011), further reaffirms the benefits of GSE use. Another most significant finding of the present study is that GSE showed cytotoxic efficacy against different CRC cell lines, which differed in their metastatic potential; interestingly, the anti-cancer efficacy of GSE increased as the metastatic potential of CRC cells increased. Based on these observations, we can
speculate that GSE intervention may serve as an effective, multi-target, non-toxic CRC therapy for the control of the disease at any stage of the malignancy.
CHAPTER IV

GRAPE SEED EXTRACT EFFICACY AGAINST
AZOXYMETHANE-INDUCED COLON TUMORIGENESIS IN A/J MICE:
INTERLINKING miRNA WITH CYTOKINE SIGNALING AND INFLAMMATION

Introduction

Colorectal cancer (CRC) is the second leading (both genders combined) cause of cancer-related deaths, and the third most frequently diagnosed malignancy in the United States. Adoption of western and sedentary lifestyles increase CRC incidence world-wide, with most (~75%) of CRC developing sporadically (Bretthauer, 2010). Despite efforts, compliance with screening recommendations is low and not enough to halt cancer growth/progression and prevent associated mortality (Bretthauer, 2010). Traditional CRC chemopreventive agents, such as NSAIDS, exert gastrointestinal side effects, suggesting that additional approaches are needed to manage CRC (Baron, 2009; Lanas et al., 2009; Lanza et al., 2009). Use of natural products with limited or no toxicity is one such approach to prevent, suppress or reverse the carcinogenesis process (Rajamanickam and Agarwal, 2008). In this regard, several studies have indicated that one-third of all cancer deaths in US could be prevented through diet modification and that high consumption of fruits and vegetables or their bioactive components could significantly decrease CRC.

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Grape seed extract (GSE) is one such natural agent, rich in proanthocyanidins, which has shown cancer chemopreventive and anti-cancer efficacy in various pre-clinical cancer models including CRC (Kaur et al., 2009a; Velmurugan et al., 2010b; Velmurugan et al., 2010c).

Importantly, clinical studies have shown that active components of GSE are bioavailable and well tolerated in humans (Eich N., 2012). However, to our knowledge, GSE has not been evaluated for its chemopreventive efficacy in mouse model of sporadic CRC. Considering that the sporadic CRC patient population represents the largest clinical population of diagnosed CRC, we aimed to evaluate GSE efficacy against a chemical carcinogen azoxymethane (AOM)-induced colon tumorigenesis in A/J mouse model that mimics generation of sporadic neoplastic lesions as observed in the human colon (Rosenberg et al., 2009). Furthermore, we also conducted mechanistic studies interlinking miRNA expression with cytokine signaling and inflammation in overall efficacy of GSE against colon tumorigenesis.

**Materials and methods**

*Reagents:* GSE-standardized preparation was a gift from Kikkoman Corp. (Nado City, Japan). As reported earlier, the analyzed composition of GSE is 89.3% proanthocyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash (Veluri et al., 2006). AOM was purchased from Sigma (St. Louis, MO) and dissolved in saline. Primary antibodies include, anti-PCNA from Dako; anti-iNOS, anti-VEGF, anti-Ago2 from Abcam (Cambridge, MA); anti-COX-2, anti-C-myc, anti-β-catenin from Santa Cruz Biotechnology (Dallas, TX); anti-phospho-ERK1/2 and anti-
NFκB/p65 from Cell Signaling (Beverly, MA); and anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies from Invitrogen (Grand Island, NY) and Cell Signaling, respectively. Mouse cytokine antibody array was from RayBiotech (Norcross, GA). miRNA was isolated using Qiagen miRNeasy Kit, amplified by Qiagen RT² miRNA qPCR kit and quantified using miRNA Cancer PCR array (Qiagen, Valencia, CA).

*Animals and treatments:* Animal experiment was done under institutional guidelines using approved IACUC protocol. Animals were maintained under standard conditions with free access to water and food. GSE was mixed in AIN-76A powder diet (Dyets Inc.) at 0.25% and 0.5% (w/w). 5 week old male A/J mice (from Jackson Laboratory) were fed AIN-76A powder diet for two weeks (to acclimatize them to new diet) and then divided into 5 groups: [1] negative control group (n=20) on AIN-76 diet; [2] positive control group (AOM, n=35), injected with 5 mg/kg AOM i.p. once a week for 6 weeks, and on AIN-76A diet; [3] AOM+0.25% GSE group (n=35), GSE supplemented diet was started 2 weeks post last AOM injection and continued for 18 weeks (n=25) or 28 weeks (n=10); [4] AOM+0.5% GSE group (n=35), GSE supplemented diet was started 2 weeks post last AOM injection and continued for 18 weeks (n=25) or 28 weeks (n=10); and [5] 0.5% GSE group, GSE supplemented diet was started at 5 weeks of mice age and continued for remainder of study. Body weight and diet consumption were recorded weekly. At the end of the study, at 33 and 43 weeks of age, mice were sacrificed, entire colon excised starting from ileocecal junction to anal verge and cut open longitudinally along main axis and gently flushed with ice-cold PBS, divided in to three equal sections (proximal, medial and distal), tumors counted, and
tumor diameters measured with digital calipers under dissecting microscope. Colon tissues and/or tumors were either fixed flat in formalin and embedded in paraffin, snap-frozen in liquid nitrogen, or stored in Qiagen RNAlater (Valencia, CA).

**Magnetic Resonance Imaging (MRI):** Gadolinium enhanced T1-weighted MRI was also employed to non-invasively assess colon tumor progression in mice. Bruker multi slice multi echo (MSME) T1-scans were performed at a Bruker 4.7 Tesla PharmaScan (Bruker Medical, Billerica, MA) following a bolus injection of 0.1 mmol/kg MultiHance via a tail catheter on anesthetized mice (2% isoflurane). A mouse volume transmitter/receiver coil (36 mm diameter) was used for all MRI studies using flowing parameters: FOV=4cm, slice thickness 1 mm, number of slices 16 (coronal) and 40 (axial), TR/TE=725/11 ms, number of averages 2, matrix size 256x256, flip angle 180. Total acquisition time was 6.5 min for each plane. All imaging acquisition and analysis was performed using Bruker ParaVision software (at the Animal Imaging Shared Resources, University of Colorado Anschutz Medical Campus).

**Mouse cytokine expression:** Tissue lysates of colonic mucosa with tumors from randomly selected animals in different groups were applied to Mouse Cytokine Antibody Array. Expression of various cytokine molecules was analyzed in duplicate on the membranes, which were scanned and quantified by ImageJ, and densitometric data analyzed using antibody array analysis tool.

**Mouse miRNA expression:** miRNA isolation was done utilizing Qiagen miRNeasy Kit, starting with 20mg of mouse colonic mucosa with tumors. Isolated miRNA was used for miRNA arrays using Qiagen RT² miRNA qPCR kit utilizing Applied Biosciences Fast-Real time PCR 7500. Replicates were performed and the
average ΔCt value of each miRNA assay was calculated for each group. The ΔΔCt was calculated for each miRNA between = ΔCt (AOM+0.5% GSE) - ΔCt (AOM), then the fold-change was calculated for each miRNA.

Immunohistochemistry (IHC) and statistical analyses: Paraffin-embedded sections (5 µm) of distal colon were subjected to standard IHC procedures described previously (Velmurugan et al., 2010b). Dilutions of primary anti-bodies used were anti- PCNA (1:250), anti-iNOS (1:100), anti-COX-2 (1:50), anti-phosho-ERK1/2 (1:100), anti-Ago2 (1:100), anti-β-catenin (1:50), anti-NFkB/p65 (1:50), anti-VEGF (1:100) and anti-c-myc (1:25). Negative staining controls were used for each protein. Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system (Promega) following manufacturer’s protocol (Velmurugan et al., 2010b). Microscopic analyses were performed using Zeiss Axioscope 2 microscope; photomicrographs captured by Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. Quantification of IHC data includes counts from both colonic mucosa and tumor staining and is shown as mean ± standard error mean (SEM) from 10 fields/section in each group. Statistically significant difference between AOM and AOM+GSE groups was analyzed for tumor multiplicity, tumor size and protein expression using one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). P values of ≤0.05 were considered significant.
Chemopreventive efficacy of GSE against AOM-induced colon tumorigenesis in A/J mice. The details of the experimental protocol are outlined in Methods section. (A) Real-time non-invasive assessment of tumor numbers and sizes formed after GSE feeding in AOM exposed A/J mice using non-invasive gadolinium-enhanced T1-MRI. Representative coronal and axial T1-weighted MRI images are presented for untreated negative control (left panel, coronal image), AOM untreated (middle, coronal) and AOM + 0.5% GSE-fed mice (right, axial). Effect of GSE feeding on (B) tumor multiplicity in colon and small intestine and (C) tumor size in colon, in AOM-induced colon tumorigenesis in A/J mice. Columns represent mean ± SEM (error bars) in each group; * P≤0.05; # P≤0.01; $ P≤0.001. NC (negative control)
Results

*Dietary GSE feeding suppresses AOM-induced colon and small intestine tumorigenesis visualized via MRI*: GSE feeding did not show considerable difference in diet consumption and body weight gain profiles between AOM and AOM+GSE-fed mice during the entire study (Appendix D). Real time assessment of colon tumor formation was performed in negative control, AOM and AOM+0.5% GSE mice at 32 weeks of age using MRI (Fig 4-1A). We did not detect any tumors, other lesions or abnormalities in negative control mice, which were further confirmed upon necropsy (performed one week post-MRI at 33 weeks of mice age) (Fig. 4-1A, left panel).

*Dietary GSE feeding decreases proliferation but induces apoptosis in its efficacy against AOM-induced colon tumorigenesis*: CRC growth and progression to advanced stages is associated with enhanced cell proliferation and evasion of apoptosis (Rosenberg et al., 2009), and therefore, the agents which could inhibit proliferation and induce apoptosis could play an important role in controlling CRC growth and progression (Rajamanickam and Agarwal, 2008). Since we found strong efficacy of GSE in preventing AOM-induced colon tumorigenesis, tissue samples were next analyzed for proliferation and apoptotic indices. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in AOM+GSE-fed groups compared to AOM alone (Fig. 4-2A). Quantification of PCNA staining showed 17±2% PCNA-positive cells in AOM+0.25% and AOM+0.5% GSE-fed groups of mice compared to 47±5% in AOM alone (Fig. 4-2A), accounting for 65% (P<0.001) decrease in proliferation.
Fig. 4-2 Dietary GSE feeding decreases proliferation but induces apoptosis in its efficacy against AOM-induced colon tumorigenesis. (A) Anti-proliferative, and (B) pro-apoptotic effects of GSE feeding on colon/tumor tissue from AOM-induced colon tumorigenesis in A/J mice. Immunohistochemical staining for PCNA was based on DAB staining as detailed in “Materials and Methods”. Proliferation index was calculated as the number of positive cells × 100 / total number of cells counted under ×400 magnifications in 10 randomly selected areas in each sample, and shown as mean ± SEM (error bars) for each group. Apoptosis was analyzed by TUNEL staining in colon/tumor tissues as detailed in “Materials and Methods”. Apoptotic index was calculated as the number of TUNEL-positive cells × 100 / total number of cells counted under ×400 magnifications in 10 randomly selected areas in each sample, and shown as mean ± SEM (error bars) for each group. $ P \leq 0.001$. Representative pictographs are depicted at ×400. NC (negative control)
Regarding in vivo apoptotic response of GSE feeding on AOM-induced colon tumorigenesis, qualitative microscopic examination showed an increased number of TUNEL-positive cells in AOM+GSE-fed groups compared to AOM alone (Fig. 4-2B). The number of TUNEL-positive apoptotic cells were ∼5% (Fig. 2B, P<0.001) in both AOM+GSE-fed groups compared to 1.7±0.43% in AOM alone, accounting for a 3-fold (P<0.001) increase (Fig. 4-2B).

Dietary GSE feeding alters cytokine and miRNA expression profiles as well as miRNA processing machinery in its efficacy against AOM-induced colon tumorigenesis:

The mixture of host-derived cytokines produced in the tumor microenvironment plays an important role in cancer progression where cancer cells exploit host-derived cytokines to promote growth, attenuate apoptosis, and stimulate invasion and metastasis (Dranoff, 2004). Thus, manipulation of the cytokine balance could be exploited for both prevention and treatment of various malignancies including CRC (Dranoff, 2004). To examine whether GSE efficacy against AOM-induced colon tumorigenesis involves altered expression of various cytokines, we used inflammatory cytokine antibody array analysis on colonic mucosa with tumors from AOM and AOM+0.5% GSE groups (Fig. 4-3A). Our results indicated that GSE feeding causes an alteration in the expression of various cytokines/interleukins involved in innate immunity, proliferation and apoptotic cell death. Specifically, GSE up regulated the levels of B-lymphocyte chemo attractant (BLC ~8-fold), granulocyte macrophage-colony stimulating factor (GM-CSF ~10-fold), chemokine CCL1 (I-309 ~9-fold), interleukin (IL)-1α (~10-fold), IL-23 (~20-fold) and M-CSF (~9-fold), but down regulated IL-1ra (~26-fold), IL-2 (~9-fold) and IL-27 (~8-fold) levels compared to AOM alone (Fig. 4-3A).
Fig. 4-3 Dietary GSE feeding alters cytokine and miRNA expression profiles as well as miRNA processing machinery in its efficacy against AOM-induced colon tumorigenesis. Alteration in (A) cytokine and (B) miRNA expression profiles, and (C) Ago2 expression in colonic mucosa with tumors from AOM-induced colon tumorigenesis in A/J mice. The arrays were done as detailed in “Materials and Methods. Immunohistochemical staining for Ago2 was based on DAB staining as detailed in “Materials and Methods”. Representative pictographs are depicted at ×400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate), +3 (strong) and +4 (very strong). Columns represent mean ± SEM (error bars) in each group. $ P≤0.001. NC (negative control)
miRNAs are small non-coding RNAs which are implicated in cell cycle regulation, cell growth and differentiation, stress responses, and apoptosis (Croce, 2009). In addition, miRNAs also play an essential role during carcinogenesis and their expression patterns have been now correlated with disease prognosis in cancer patients (Croce, 2009). Furthermore, knock down or re-expression of specific miRNAs is shown to induce drug sensitivity, inhibit proliferation, and suppress invasion of cancer cells (Croce, 2009). In view of above summarized critical roles of miRNAs, next we assessed whether their altered expression also contributes to GSE efficacy against AOM-induced colon tumorigenesis. A comparison of miRNA expression profiles of the tissue from AOM and AOM+0.5% GSE groups showed that GSE feeding causes an up regulation of Snord 68 (~6500-fold), miR-19a (~2400-fold), miR-20a (~1000-fold) and miR-let7a (~1200-fold) (Fig. 4-3B), but down regulates miR-205 (~400-fold), miR-135b (~100-fold), miR-196a (~24-fold), miR-21 (~25-fold), miR-148a (~21-fold) and miR-103 (~9-fold) (Fig. 4-3B). Furthermore, IHC analysis revealed a ~6.5 fold induction in the level of Ago2, which is involved in miRNA processing, in AOM+0.5% GSE group compared to AOM alone, indicating an increased RNA-induced gene silencing in response to GSE feeding (Fig. 4-3C) (Taliaferro et al., 2013).

Based on an extensive literature search for the possible role of above identified various cytokines/interleukins and miRNAs with altered expression following GSE treatment and the associated cross-talks among them we were able to identify a pattern in their modulation (Dranoff, 2004; Slaby et al., 2009; Schetter et al., 2010). Specifically, we found that GSE mediates its efficacy against AOM-induced colon tumorigenesis by possibly modulating NF-κB and its downstream targets related to inflammatory signaling,
β-catenin signaling, and MAPK pathway. Accordingly, next we investigated their possible roles in GSE efficacy against AOM-induced colon tumorigenesis.

**Dietary GSE feeding inhibits NF-κB activation and decreases expression of associated molecules in its efficacy against AOM-induced colon tumorigenesis:**

Transcription factor NF-κB mediates the transcription of various genes which are associated with tumor initiation, promotion, progression, metastasis as well as inflammation, including those for COX-2, iNOS and VEGF (Greten et al., 2004). Inflammation is implicated in the pathogenesis of CRC development, and chronic inflammation increases CRC risk (Dyson and Rutter, 2012). In addition, experimental and epidemiological evidences indicate an up regulation of COX-2, iNOS and VEGF (downstream target genes of NF-κB) in various malignancies, including human CRC (Ohta et al., 2006). Together, based on this information and our array data analyses suggesting the involvement of NF-κB and its downstream inflammatory molecules in GSE efficacy, IHC analyses were done to examine their expression. Our results showed that GSE feeding indeed interferes with NF-κB activity, as observed by a significant decrease in nuclear expression of NF-κB/p65 in the distal colon/tumor tissues by both GSE doses (Fig. 4-4A). Next we examined the protein levels of COX-2 and iNOS (Fig. 4-4B & C). AOM alone treated mice showed a significant increase in COX-2 and iNOS immunoreactivity scores (~10 folds (P<0.001) and ~2.5 folds (P<0.001) respectively) in the distal colon/tumor tissues, compared to untreated negative controls.
Fig. 4-4 Dietary GSE feeding inhibits NF-κB activation and decreases associated molecules in its efficacy against AOM-induced colon tumorigenesis.

Immunohistochemical staining for (A) NF-κB/p65 (B) COX-2, (C) iNOS, and (D) VEGF was based on DAB staining as detailed in “Materials and Methods”. Representative pictographs are depicted at ×400. Percent positive cells were calculated as number of brown nuclei × 100 / total number of cells counted under ×400 magnifications in 10 randomly selected areas in each sample, and shown as mean ± SEM (error bars) for each group. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate), +3 (strong) and +4 (very strong). Columns represent mean ± SEM (error bars) in each group. $ P ≤ 0.001. NC (negative control)

Importantly, mice in the AOM+GSE-fed groups exhibited a significant down regulation of COX-2 and iNOS protein levels in the distal colon/tumor tissues (Fig. 4-4B & C).

Similarly, a decrease in VEGF immunoreactivity scores (Fig. 4-4D) was also observed in the distal colon/tumor tissues from the mice in AOM+GSE-fed groups compared to AOM alone.
Dietary GSE feeding modulates β-catenin and MAPK pathways in its efficacy against AOM-induced colon tumorigenesis: Our array data analyses also suggested a possible involvement of β-catenin and MAPK signaling in GSE efficacy against AOM-induced colon tumorigenesis, and accordingly, next we focused on these two pathways. β-catenin is important in the development of CRC, and an inappropriate stabilization, translocation and activation of β-catenin occur in sporadic and familial CRC resulting in downstream modulation of oncogenic target genes such as c-myc and VEGF (Barker and Clevers, 2006). IHC results for β-catenin staining revealed a significant dose-dependent reduction (P<0.001) in the nuclear expression of β-catenin in the distal colon/tumor tissues of AOM+GSE-fed groups of mice compared to AOM alone (Fig. 4-5A).

Following the above results and to further examining the downstream β-catenin pathway targets, we assessed the expression of C-myc (Barker and Clevers, 2006). The quantification of IHC staining for C-myc in distal colon/tumor tissues revealed a significant (P<0.001) dose-dependent reduction in AOM+GSE groups compared to AOM alone (Fig. 4-5B).

The RAS-RAF-MEK-MAPK signaling cascade plays an important role in tumorigenesis including CRC (Santarpia et al., 2012), and accordingly we also investigated the role of this pathway in GSE efficacy against AOM-induced colon tumorigenesis by assessing the expression of extracellular signal-regulated kinase 1/2 (ERK1/2), which is phosphorylated in response to RAS activation (Santarpia et al., 2012). Microscopic examination of distal colon/tumor tissue sections from all groups revealed a significant reduction (94-97%, P<0.001) in the phosphorylation of ERK1/2 (Fig. 4-5C) in AOM+GSE–fed groups compared to AOM alone.
Fig. 4-5 Dietary GSE feeding modulates β-catenin and MAPK pathways in its efficacy against AOM-induced colon tumorigenesis. Immunohistochemical staining for (A) β-catenin, (B) C-myc, and (C) phospho ERK 1/2 was based on DAB staining as detailed in “Materials and Methods”. Representative DAB-stained tissue specimens from AOM control and AOM+ 0.5% GSE-fed groups showing brown-colored positive cells or cytosolic staining are depicted at ×400 magnifications. Percent positive cells were calculated as number of cells with brown cytoplasmic or nuclei staining × 100 / total number of cells counted under ×400 magnifications in 10 randomly selected areas in each sample, and shown as mean ± SEM (error bars) for each group. Immunoreactivity (represented by intensity of brown staining) was scored as 0 (no staining), +1 (weak), +2 (moderate), +3 (strong) and +4 (very strong). Error bars indicate ± SEM, $ P≤0.001. NC (negative control).
Discussion

This is the first study reporting that long-term dietary feeding of GSE (0.25% and 0.5% w/w in AIN-76A diet) for 18 and 28 weeks following exposure to AOM results in a significant decrease in AOM-induced colon tumor multiplicity. More importantly, GSE feeding caused a significant reduction in colon tumor size in a dose-dependent manner. Furthermore, GSE feeding did not show any effect on food consumption and body weight-gain profiles, which is consistent with previous anti-cancer efficacy studies with GSE in other cancer models (Kaur et al., 2009a). In the present study, we also established the feasibility of non-invasive real-time examination of colon lesions by gadolinium-enhanced MRI, which allowed the visualization of colon tumors in AOM mouse model with 75–81% sensitivity. This, somewhat low, sensitivity of MRI (as compared to necropsy results) can be attributed to non-optimal experimental conditions during tumor visualization (such as small size lesions and bowel movements), which could possibly be improved in future by altering image contrast as reported for other studies (Young et al., 2009). Biomarker analyses in colonic mucosa with tumors indicated that GSE feeding decreases proliferation but induces apoptosis in its chemopreventive efficacy. We also focused our efforts on identifying the targets and pathways modulated by GSE in its colon cancer chemopreventive efficacy by utilizing cytokine and miRNA arrays. Our results and their analyses showed that a wide number of cytokines/ILs and miRNAs were significantly modulated by GSE which were associated with alterations in NF-κB, β-catenin and MAPK signaling, suggesting that inflammation, proliferation and apoptosis are targeted by GSE in its chemopreventive efficacy.
Further analysis of array data showed that GSE decreases IL-2, which is an important finding because activation of NF-κB, a transcription factor associated with inflammation, leads to IL-2 production (Arima et al., 1992). GSE also significantly downregulated the levels of IL-27 and IL-1α, which are known to modify NF-κB signaling (Hartupee et al., 2008; Guzzo et al., 2012). Similarly, miRNA array analysis showed that GSE upregulates miR-19a, which is linked to NFκB regulation (Gantier et al., 2012). miR-20a is known to inhibit HIF-1α pathway, which plays a major role in the survival of cancer cells in tumor microenvironment; importantly, GSE upregulated miR-20a suggesting its activity in inhibiting HIF-1α pathway and its downstream target VEGF (Semenza, 2002; Kang et al., 2012). Similarly, miRNA array results showed that GSE causes a dramatic down regulation of miR-205, which targets VEGF (Wu et al., 2009) that is also transcriptionally regulated by NF-κB. Consistent with these observations, our results showed that GSE decreases the expression of nuclear NF-κB p65 subunit as well as those of COX-2, iNOS and VEGF. These results are important as over expression of pro-inflammatory molecules, such as iNOS and COX-2, is observed in AOM-induced CRC and selective COX-2 inhibitors have been shown to prevent disease progression (Rao et al., 2002; van der Woude et al., 2004). In addition, decreased expression of the pro-angiogenic factor VEGF suggests that GSE might also interference in tumor angiogenesis; a strong dose-dependent decrease in colon tumor size by GSE supports this notion. Together, these results suggested that GSE exerts its efficacy against colon tumorigenesis at least in part by modulating NF-κB signaling and its downstream transcriptional targets COX-2, iNOS and VEGF, which are implicated in inflammation,
tumor promotion, progression and metastasis of CRC (Greten et al., 2004; Pikarsky et al., 2004; Karin, 2006).

β-catenin signaling is extensively studied for its critical role in human CRC growth and progression (Barker and Clevers, 2006), suggesting that the agents targeting this pathway would be useful in CRC control. In our study, we found that GSE causes an increase in M-CSF, which is linked to β-catenin signaling (Fujita and Janz, 2007). In addition, GSE also significantly decreased miR-135b, which is also linked to β-catenin pathway (Huang et al., 2010). Moreover, GSE up regulated let-7a, which is implicated in inhibiting the expression of c-myc oncogene, a downstream target of β-catenin (Sampson et al., 2007). Consistent with these results, GSE significantly down regulated β-catenin protein expression, which was also associated with the decreased expression of its downstream target C-myc (Barker and Clevers, 2006), suggesting their possible role in GSE efficacy against colon tumorigenesis.

Cytokine/IL array findings also showed up regulation of BLC, CCL1/I-309 and IL-1α, but down regulation of IL-1rα and IL-27 by GSE; all these molecules are involved in regulating the MAPK pathway (Foey et al., 1998; Saklatvala et al., 1999; Louahed et al., 2003; Burkle et al., 2007). Furthermore, in miRNA array analysis, GSE showed an up regulation of let-7a, which is known to inhibit MAPK pathway (Johnson et al., 2005). We also found that GSE decreases miR-205, which is up regulated in some malignancies and known to interact with both MAPK and NOTCH pathways (Xie et al., 2012). Notably, MAPK signaling cascade is known to play an important role in tumorigenesis by modulating cell growth, differentiation, proliferation, apoptosis, and migration (Santarpia et al., 2012). GSE also decreased miR-103, which is involved in oncogenic KRAS
signaling and is up regulated in CRC cells (Ota et al., 2012). Our IHC results clearly showed that GSE causes a strong decrease in MAPK/ERK1/2 phosphorylation, which is consistent with our cytokine/IL and miRNA array profiles. Together, these results suggested that GSE exerts its efficacy against colon tumorigenesis at least in part by also modulating KRAS-MAPK signaling.

In addition to the miRNAs described above and their association with signaling pathways, GSE also modulated the expression of several additional miRNAs, which are implicated in CRC. For example, GSE down regulated miR-135b that is up regulated in CRC tissue compared to the normal surrounding tissue and has been specifically shown to target the APC gene involved in CRC (Nagel et al., 2008; Xu et al., 2012). Similarly, GSE decreased miR-196a, the high-levels of this miRNA exert a pro-oncogenic effect in CRC cells (Schimanski et al., 2009). Likewise, the ability of GSE to decrease miR-21 is of clinical significance, because its increased levels correlate with increased stage of clinical CRC (Kjaer-Frifeldt et al., 2012; Xu et al., 2012). Importantly, in this study, we also observed predominate global up regulation of miRNAs by GSE, coupled with down regulation of oncogenic miRNAs and up-regulation of tumors suppressive miRNAs. Accordingly, we also investigated a key protein involved in miRNA homeostasis, namely Ago2, which binds to the mRNA and represses translation Colon tissue analysis revealed significant up regulation of Ago2 protein in colons of AOM+GSE-fed groups, which further supports our miRNA array results (Taliaferro et al., 2013). Together, this is the first study showing alteration in miRNAs expression and cytokine signaling by GSE in its chemopreventive efficacy against AOM-induced colon tumorigenesis in A/J mouse sporadic CRC model.
CHAPTER V

CHARACTERIZATION OF THE COLON TUMORS METASTASIS TO LUNG
IN A MOUSE MODEL RELEVANT TO HUMAN SPORADIC COLORECTAL
CANCER AND EVALUATION OF GSE EFFICACY

Introduction

In most industrialized countries, cancer remains to be the major cause of disease deaths; the majority of these deaths are a result of cancer metastatic growth (Taketo, 2011). Furthermore, colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States, and is expected to result in 50,830 deaths during 2013 alone; most (~75%) of these CRC cases developing sporadically. At the time of diagnosis, clinical and pathological staging of CRC is closely related to survival rate. For example, according to the U.S. Department of Health & Human Services (HHS), the 5-year survival rate of CRC, confined to the bowel wall, is 90%; however, 65% of the patients exhibit advanced disease at the time of diagnosis, and a further progression to the lymph nodes decreases the 5-year survival rate between 35-60%. If a distant metastasis occurs, the survival rate decreases to 10%; common organ sites of clinical CRC metastasis include liver, lung, peritoneum, and ovaries, according to U.S. Department of Health & Human Services (HHS). The tissue specific markers have been important tools utilized by diagnostic pathologists to determine metastatic tumor origin. For example, caudal type homeobox 2 (CDX2) is a highly sensitive marker of primary and metastatic colorectal adenocarcinomas, exhibiting 100% accuracy of intestinal tumors (Werling et
al., 2003). Similarly, the biomarkers such as thyroid transcription factor-1 (TTF-1) are specifically expressed with in the lung, thyroid, and brain tissues (Boggaram, 2009).

Traditional treatment for CRC includes adjuvant chemotherapeutic agents and surgery depending on location of metastatic growth; however the current chemotherapeutic agents induce patient toxicity, which includes the risk of death (Ohe, 2002). Moreover current chemotherapeutic agents are not capable of increasing long-term patient survival or possess the ability to cure metastatic disease. With the above information in mind, it is evident that there is the clinical need to develop non-toxic, effective, long-term treatment regimes. An alternative approach to the current chemotherapeutic agents would include natural products; these natural agents have limited or no toxicity and can be administered long-term to prevent, suppress or reverse colon carcinogenesis (Rajamanickam and Agarwal, 2008). Correspondingly, there are multiple reports indicating that one-third of all cancer deaths in the US could be prevented through high consumption of fruits and vegetables or their bioactive components (Kelloff et al., 1996; Rajamanickam and Agarwal, 2008; Kaur et al., 2009a). Natural agents such as grape seed extract (GSE) have been shown to be bioavailable, safe and efficacious during clinical trials against multiple cancer types; additionally GSE exhibits significant efficacy against pre-clinical models of CRC (Kaur et al., 2009a; Velmurugan et al., 2010b; Velmurugan et al., 2010c; Eich N., 2012).

Scientific progression in the field of cancer molecular biology and genetics has fostered the development of effective patient therapies, which are attributed to the development of pre-clinical animal models that mimic the human disease condition. However, the pre-clinical research field has not yet developed an efficient and effective
model of carcinogen-induced colon tumorigenesis that metastasizes to lung; this metastatic tumor formation accounts for 20% of the diagnosed patient population. Additionally, this lung metastasis patient population may not have the option of surgery, further decreasing the probability of patient survival. With this in mind, the aim of this study was to characterize lung nodules found in A/J mice following azoxymethane (AOM) exposure and establish their origin as colon tumors. Rodent experimental models of colon carcinogenesis have been utilized for over 80 years; the use of rodent models has a number of advantages to study the pathogenesis of CRC, including model reproducibility, access to different genetic backgrounds, and that these models recapitulate human cancer development. To mimic human sporadic CRC development, there are a variety of carcinogens that induce colon tumors; however, AOM-induced CRC takes advantage of the organotropism of the carcinogen where AOM specifically induces tumors in the colon and small intestine with highest incidence in the distal region of the colon (Rosenberg et al., 2009). Notably, AOM-induced colon tumorigenesis model is the most commonly used pre-clinical animal model of human sporadic CRC (Rosenberg et al., 2009); however, previous studies have not reported colon tumors metastasis to lung in this model. Additionally, investigating GSE efficacy in an in situ pre-clinical animal model of CRC metastasis is highly desirable, which combined with the vast pre-clinical GSE efficacy studies in various models of colon carcinogenesis, would help provide much needed evidence to initiate clinical trials investigating GSE efficacy against human CRC growth, progression and metastasis to distal organs including lung.
Material and methods

Reagents: GSE-standardized preparation was a gift from Kikkoman Corp. (Nado City, Japan). The preparation composition is as follows: 89.3% procyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash. Purchased antibodies include anti-PCNA (1:250 dilutions, Dako), anti-CDX2 (1:100 dilutions, Abcam), anti-CK20 (1:100 dilutions, Abcam), anti-Surfactant D (1:100 dilutions, Abcam), anti-TTF-1 (1:100 dilutions, Abcam). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were purchased from Invitrogen (Carlsbad, CA) and Cell Signaling Technology (Beverly, MA). RNA was isolated via the Qiagen RNeasy Kit, amplified via the Qiagen RT² RNA qPCR kit. Additionally RNA transcript was quantified via specific RNA TagMan primers for CDX2, Surfactant D, and TTF-1 from Life technologies (Grand Island, NY). Primer for mouse CK20 was purchased from Invitrogen (Carlsbad, CA).

Animals and treatments: Male A/J mice were purchased from Jackson Laboratory, and experiments were done with an approved protocol by IACUC. AOM was purchased from Sigma (St. Louis, MO) and dissolved in saline. GSE was mixed in AIN-76 powder diet at 0.25% (w/w). Animals, maintained under standard conditions with free access to water and food (AIN-76 diet), were divided in to 3 groups, and treated as: (1) control group (n=20), (2) positive group (n=35), injected with 5 mg/kg dose of AOM i.p. once a week for 6 weeks, (3) AOM+0.25% GSE (n=35) initiated 2 weeks post AOM and continued for 18 weeks (n=25) and 28 weeks (n=10) and continued for remainder of study. Body weight and diet consumption were recorded weekly. At 33 and 43 weeks of age mice were sacrificed, entire lung was excised, gently flushed with ice-cold PBS,
tumors counted and fixed flat in formalin, and embedded in paraffin for immunohistochemical (IHC) studies, frozen in liquid nitrogen for protein isolation, or stored in Qiagen RNA later (Valencia, CA). Additionally, colon and small intestine tumors were also documented and stored as detailed in Chapter IV.

**Immunohistochemistry (IHC):** Paraffin-embedded sections (5 µm) of pulmonary tissue were subjected to standard IHC procedures described previously (Velmurugan et al., 2010b). Dilutions of primary anti-bodies used were anti-PCNA (1:250), anti-CDX2 (1:100), anti-CK20 (1:100), anti-Surfactant D (1:100), anti-TTF-1 (1:100). Negative staining controls were used for each protein. Microscopic analyses were performed using Zeiss Axioskope 2 microscope; photomicrographs captured by Carl Zeiss AxioCam MrC5 camera with Axiovision Rel 4.5 software. Quantification of IHC data includes staining counts from control pulmonary tissue, AOM exposed pulmonary tumor tissue and the AOM exposed pulmonary tissue surrounding the tumor tissue and is shown as mean ± standard error mean (SEM) from 10 fields/section from four animals in each group. Statistically significant difference between control, AOM and AOM+GSE groups was analyzed using one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). *P* values of ≤0.05 were considered significant.

**RNA isolation and Real Time-PCR (RT-PCR):** RNA isolation: was completed following manufactures protocol utilizing the Qiagen RNeasy Kit, starting with 20mg of mouse colon mucosal tissue and 20mg of mouse lung tissue, from two treatment groups. RNA concentration was determined with a NanoDrop 2000 (Thermo Scientific) and used for the following studies.

Reverse Transcription-PCR and cDNA synthesis: Qiagen RT² RNA qPCR kit was
used following manufactures protocol and the First Strand cDNA Synthesis Reaction was stored at -20°C.

RNA primers and Real Time-PCR: isolated RNA was used for transcript expression following the manufactures protocol. The commercially available and pre-validated TaqMan primer/probe set used was: CDX2 (CDX2; Mm01212280_m1). The primer set for cytokeratin 20 was designed 5’-GCGTTTATGGGGGTGCTGGGAG-3’ (F) and 5’-AAGGCTTGGGCGGTGCGTCTC-3’ (R). Utilizing Applied Biosciences Fast-Real time PCR 7500 the samples were placed in the real-time thermal cycler and entered the program: 1 cycle of 10 min at 95°C, followed by 40 cycles of: 15 sec. at 95°C, then, 30 to 40 seconds at 60°C, then 30 seconds at 72°C. SYBR Green fluorescence was detected and the threshold cycle (Ct) was calculated for each well. The resulting threshold cycle values were exported, for data analysis all Ct values reported as greater than 35 or as N/A (not detected) change to 35. Replicates were performed and the average ΔCt value of each RNA assay was calculated for each treatment group. The ΔΔCt was calculated for each RNA between = ΔCt (AOM) - ΔCt (control), then the fold-change was calculated for each RNA transcript from control and AOM treated. RNA levels were further normalized to ribosomal RNA levels to determine overall transcriptional modification.

Statistical analysis: All data shown are mean ± SE when indicated and representative of at least 4 different animals. Statistical significance between colon and lung tissue from control and AOM-treated sample groups compared for analyses of protein expression and were calculated by one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA) P values of ≤0.05 were considered significant.
Results

*AOM-induced colon carcinogenesis results in lung metastatic tumors formation, and dietary GSE feeding inhibits them*: A previous group had shown 29% and 35% incidences of lung and liver tumors, respectively in ICR mouse strain after 30 weeks of AOM injection (Fukutake et al., 1998). However, neither of the authors characterized the origin of these tumors in lung and liver, nor did they evaluate whether they are colon tumors metastasized to lung and liver. During our AOM dose standardization study for colon tumorigenesis in A/J mice, we observed upon necropsy that, in addition to colon and small intestine tumors, 4 out of 6 mice also exhibited lung tumors after 30 weeks (43 weeks of mice age) post-last dose of AOM treatment. Based on this unexpected finding, as detailed in Methods, we designed a long-term protocol to identify and characterize the origin of these lung tumors and to evaluate GSE efficacy towards them. At the end of the study, upon necropsy at 33 and 43 weeks of mice age, lung tumors were visible to the naked eye; in total 15 lung tumors were observed in the AOM alone group of mice, however, only 5 tumors were observed in AOM + 0.25% GSE group, accounting for a 67% decrease in total lung tumor number (Fig. 5-1A-C). Also importantly, the tumor size in AOM + GSE group was <1mm in all cases compared to AOM alone where 9 out of 15 tumors were in 1-2 or 2-3 mm range (Fig. 5-1B).
Figure 5-1 AOM-induced colon carcinogenesis results in lung metastatic tumor growth. The details of the experimental protocol are outlined in the Methods section. (A) Gross examination of isolated lung tissue revealed the formation of lung tumors in animals that were injected with AOM, no lung tumors were observed in the control animals. These lung tumors formed on the exterior portion of the lung lobes. (B) Lung tumor quantification revealed 15 total lung tumors in the AOM alone group. However, in animals injected with AOM and supplemented with 0.25% GSE, only 5 lung tumors were observed in this treatment group. Furthermore, GSE supplementation reduced the overall lung tumor size, no lung tumors larger than 1mm were observed with GSE treatment. (C) Summarizing the above data per experimental group including: lung tumor incidence rates; total lung tumor number, and number of tumors that were formed in tumor bearing mice. GSE supplementation reduced lung tumor incidence by 53%, reduced total tumor number by 66%, and no animals were observed with multiple lung tumors upon GSE supplementation.
In terms of incidence, 11 out of 35 mice developed lung tumors in AOM alone group, accounting for a 32% incidence rate; only 5 out of 34 animals displayed these lung tumors in AOM + GSE-fed group that was a 15% incidence rate, and thus GSE feeding was also able to inhibit lung tumor incidence by 53% (Fig. 5-1C). All the mice were also monitored throughout the study on a regular basis to investigate if dietary GSE had any negative impact; our primary measure of toxicity was body weight gain profile that was recorded weekly and did not differ significantly among the controls and GSE-fed groups (Appendix D).

*Histological examination of lung tumors:* Upon AOM-injection, pulmonary tumor formation occurred most commonly on the right superior portion and the left inferior regions of the lung lobes. Pathological examination of control and AOM-treated lung tissue stained via H&E exhibited characteristics of adenocarcinoma formation, which originated from mucin cellular origin (Fig. 5-2A). Pulmonary tissue was further stained for PCNA as an indicator of the rate of cellular proliferation; AOM-injection resulted in increased nuclear expression of PCNA. Quantification of IHC results indicated that AOM injection resulted in a proliferative index of 29% and with in the lung tumor tissue this increased minimally to 33% (Fig. 5-2B). Furthermore GSE supplementation decreased proliferation rates significantly within these lung tumors, by 48% compared to the surrounding lung tissue (Fig. 5-2B).
Figure 5-2  Histological examination of the AOM-induced CRC lung metastatic tumors. (A) H&E staining of control and AOM treated lung tissue revealed distinct histological differences between the two groups. Examination of control animals revealed normal lung structure; however upon AOM injection, pathological examination revealed the formation of adenocarcinoma of mucin origin. (B) Immunohistochemical staining for PCNA was preformed, as detailed in Methods section, to determine proliferation rates within the lung tissue of control, AOM-injected, and AOM-injected + 0.25% GSE animals. Proliferation index was calculated as the number of positive cells × 100 / total number of cells counted under ×400 magnifications, in four different animals, 10 randomly selected areas for each animal, graphs shown as mean ± SEM (error bars) for each group. P≤0.01 was considered significant. Representative pictographs are depicted at x100 and ×400.
Identification of colon tissue biomarkers within the AOM-exposed pulmonary tissue: Utilizing biomarkers that are specifically expressed in the colon tissue, we aimed to identify and characterize the origin of the lung tumors that form upon AOM injection in A/J mice. Control and AOM-injected colon and pulmonary tissue were analyzed via IHC for the expression of a colonic specific transcription factor, CDX2. As a positive control for CDX2 expression, we utilized our control colon tissue samples. As anticipated, there was a high level of CDX2 expression within the mucosal region of the control colon tissue (Fig. 5-3A). Examining control lung tissue revealed minimal CDX2 staining via IHC; however, upon AOM exposure, lung tissue exhibited an increased expression of this colon specific biomarker (Fig. 5-3A). To quantify these biomarkers, we separated the AOM-injected lung tissue into two groups, tumor tissue and the tissue surrounding the tumor. Quantification of CDX2 levels revealed that AOM-injection resulted in a 73% increase in protein expression with in the lung tumors compared to the surrounding AOM exposed lung tissue (Fig. 5-3A); similar tissue samples from AOM + GSE group of mice showed comparable findings (Fig. 5-3A). Additionally, RNA was extracted from control and AOM-injected colon and pulmonary tissues and analyzed via RT-PCR to detect the transcript levels of CDX2 in these tissues. As anticipated, CDX2 expression was high with in the colon mucosal tissue of control and AOM-treated animals; average CDX2 mRNA levels ranged from 320 pg/ng 18s in control tissue to 395pg/ng 18s in AOM colon tissue (Fig. 5-3B). Isolated RNA from lung tissue revealed no expression of CDX2 mRNA within control lung tissue, via RT-PCR; additionally there was minimal expression of CDX2 in the lung tissue surrounding the tumors (Fig. 5-3B). However, examining the mRNA levels from the lung tumor tissue revealed a 15-
fold upregulation in CDX2 expression; these results indicated the presence of colon biomarkers with in this metastatic lung tumor (Fig. 5-3B).

Figure 5-3 Examining the colon specific biomarker CDX2 in AOM-induced CRC lung metastatic tumors. Immunohistochemical staining for (A) CDX2 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at X100 and x400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as +1 (no staining), +2 (weak), +3 (moderate) and +4 (strong). Quantification was done in four separate animals, examining 10 randomly selected areas in each animal. Columns represent mean and ± SEM (error bars) in each group. P≤0.001 was considered significant. (B) RT-PCR was preformed to evaluate transcriptional expression of Cdx2 in colon tissue from control and AOM-exposed animals. In addition transcriptional Cdx2 expression was evaluated in lung tissue from control, AOM-exposed tissue surrounding the lung tumors, and within the AOM-lung tumors. Columns represent the mean transcript level (normalized to ribosomal RNA) of four animals, ± SEM (error bars) in each group.
To further confirm this AOM-induced lung metastatic CRC mouse model we examined an additional colon biomarker, CK20; CK20 is a cytokeratin that is expressed in epithelia cells, and is expressed in virtually all cases of CRC carcinomas (Chu et al., 2000). Staining revealed increased CK20 expression upon AOM injection and further IHC quantification revealed that AOM-injection resulted in a 90% increase of protein expression compared to control lung tissue (Fig. 5-4A). Furthermore, CK20 expression was increased within the lung tumors by 63% compared to the surrounding lung tissue (Fig. 5-4A). Dietary GSE feeding resulted in a decreased expression of CK20 within the lung tumors compared to AOM-exposed lung tissue; staining quantification revealed a 65% decrease in CK20 protein expression (Fig. 5-4A). Additionally, RNA was extracted from control and AOM-injected colon and lung tissues and analyzed via RT-PCR to detect the transcript levels of CK20 in these tissues. As anticipated, CK20 expression was high within the colon mucosal tissue of control and AOM-treated animals; average CK20 mRNA levels ranged from 469 pg/ng 18s in control tissue to 569 pg/ng 18s in AOM colon tissue (Fig. 5-4B). Isolated RNA from lung tissue revealed minimal expression of CK20 mRNA within control lung tissue; however, there was 20-fold induction of CK20 expression in the lung tissue surrounding the tumors (Fig. 5-4B). Notably, mRNA levels from the lung tumor tissue revealed a dramatic 40-fold induction of CK20 expression; these results further confirm the presence of colon biomarkers within this metastatic lung tumor originating from primary colon tumors (Fig. 5-4B).
Figure 5-4 Examining the colon specific biomarker CK20 in AOM-induced CRC lung metastatic tumors. Immunohistochemical staining for (A) CK20 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at X100 and ×400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as +1 (no staining), +2 (weak), +3 (moderate) and +4 (strong). Quantification was done in four separate animals, examining 10 randomly selected areas in each animal. Columns represent mean and ± SEM (error bars) in each group. P≤0.001-P≤0.05 was considered significant. (B) RT-PCR was preformed to evaluate transcriptional expression of CK20 in colon tissue from control and AOM-exposed animals. In addition transcriptional CK20 expression was evaluated in lung tissue from control, AOM-exposed tissue surrounding the lung tumors, and within the AOM-lung tumors. Columns represent the mean transcript level (normalized to ribosomal RNA) of four animals, ± SEM (error bars) in each group. P≤0.001 was considered significant.
Investigating lung tissue biomarkers in AOM exposed lung tissue: Additionally we wanted to investigate lung tissue origin markers in the AOM exposed lung tissues, utilizing biomarkers that are specifically expressed in the lung tissue, to further confirm the origin of the observed lung tumors. Surfactant D (SP-D) is expressed in type II cells with in the lung tissue and it has been shown to be expressed at elevated levels within spontaneous adenocarcinoma (Zhang et al., 2003). Control and AOM-injected colon and lung tissues were analyzed via IHC for the expression of the lung specific protein, SP-D. As a negative control for SP-D expression, we utilized our control colon tissue samples. As anticipated, there was a minimal expression of SP-D within the mucosal region of the AOM exposed colon tissue (Fig. 5-5A). Examining control lung tissue revealed minimal SP-D staining via IHC; however upon AOM exposure lung tissue exhibited an increased expression of this lung specific biomarker (Fig. 5-5A). To further quantify this biomarker, we separated the AOM-injected lung tissue into two groups, tumor tissue and the surrounding tumor tissue. SP-D quantification revealed that AOM-injection resulted in a 58% increase in protein expression in the surrounding lung tissue compared to control lung tissue; however, in the lung tumors, IHC quantification of SP-D expression revealed an 89% decrease in protein expression (Fig. 5-5A). Also noteworthy, GSE treatment downregulated SP-D expression with in the lung tumors compared to surrounding lung tissue; staining quantification revealed an 82% decrease in SP-D protein expression (Fig. 5-5A).
Figure 5-5  Examining the lung specific biomarkers Surfactant D and TTF-1 in AOM-induced CRC lung metastatic tumors. Immunohistochemical staining for (A) Surfactant D was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at X100 and ×400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as +1 (no staining), +2 (weak), +3 (moderate) and +4 (strong). Quantification was done in four separate animals, examining 10 randomly selected areas in each animal. Columns represent mean and ± SEM (error bars) in each group. P≤0.001 was considered significant. (B) TTF-1 was based on DAB staining as detailed in Methods section. Representative pictographs are depicted at X100 and ×400. Immunoreactivity (represented by cytosolic intensity of brown staining) was scored as +1 (no staining), +2 (weak), +3 (moderate) and +4 (strong). Quantification was done in four separate animals, examining 10 randomly selected areas in each animal. Columns represent mean and ± SEM (error bars) in each group. P≤0.001 was considered significant.
With the above results in mind and further considering the fact that SP-D protein is important against host–defense, and therefore, could be upregulated in the AOM tumor tissue due to increased inflammation, we aimed to investigate an additional lung origin biomarker to further confirm this AOM-induced lung metastatic CRC mouse model, specifically TTF-1 (Zhang et al., 2003). TTF-1, also known as Nkx2.1, is a thyroid transcription factor that is essential to lung tissue formation and has been shown to be upregulated in lung adenocarcinoma (Kimura et al., 1996; Kaufmann and Dietel, 2000). Staining revealed TTF-1 expression in the control lung tissue and AOM-injection tissue surrounding the lung tumors; however, TTF-1 expression was decreased with in the lung tumors of AOM-injected animals and IHC quantification revealed a 47% decrease in TTF-1 expression (Fig. 5-5B). Furthermore, dietary GSE feeding resulted in a decreased expression of TTF-1 with in these lung tumors of AOM treated animals; staining quantification revealed a 77% decrease in TTF-1 protein expression (Fig. 5-5B)

**Discussion**

The present study aimed to identify and characterize lung metastasis of AOM-induced colon tumors in mouse model; this model is unique in that lung metastatic tumor formation occurs in-situ originating from primary colon tumors upon AOM-injection. Currently, there are no pre-clinical models of carcinogen-induced lung metastatic tumor growth originating from primary tumor growth in the colon; typical CRC metastasis animal models involve complicated surgical techniques utilizing immune compromised animals and tissue transplantation to study advanced CRC disease progression. For example, a model of CRC lymphatic metastasis involved nude mice that were injected subcutaneously with human HT-29 CRC cells on the side flanks of the animal; the
resulting tumor was then removed and further surgically implanted into the cecum of the second generation of mice, which resulted in CRC adenocarcinoma formation and lymphatic metastasis (Sun et al., 2012a). The hepatic metastatic CRC animal models utilize severe immune compromised mice and surgical splenic injection of human SW620 CRC cells; this initial surgery is then followed by a splenectomy (Xu et al., 2010). Another orthotopic hepatic model involves laparotomy to inject SW480 human CRC cells into the cecum of the animal that results in liver tumor formation after 8 weeks (Ding et al., 2011). Furthermore, additional orthotopic liver and lung metastasis models also include surgery and injection or implantation of human tissue into immune compromised animals at various tissue sites including colon and rectum, portal vein, intraperitoneal cavity, and the tail vein (Naomoto et al., 1987; Sirovich et al., 1999; Sturm et al., 2003; Donigan et al., 2010; Ishihara et al., 2010). With the above information in mind, all the current pre-clinical models of metastatic CRC progression are costly, technically challenged, time-consuming, and do not represent an in situ model of human CRC metastasis. The model described in our study involves simple technical procedures and results in primary colon adenocarcinoma and lung metastatic tumor formation; recapitulating the human disease progression in a fully immune competent A/J mice.

First evidence that supported this model as an AOM-induce model of lung metastatic CRC development was based on the histological properties of the lung tumors. The AOM-induced metastatic lung tumors formed on the exterior portion of the lung lobes; the location of these tumors suggested a potential lymphangitic spread, indicating that AOM-induced colon tumors had progression to lymphatic infiltration. However, if the metastatic spread had been arterial, we would have also observed tumor formation
around the pulmonary arteries toward the center of the lung lobes. Moreover, we did not observe any spontaneous lung tumor formation in the control animals; this observation suggests the observed lung tumors were a result of the AOM-induced colon tumorigenesis. Furthermore, the animal that were sacrifice at the later time point of 43 weeks of age had a higher incidence rate of lung tumor formation; indicating this is a progression pre-clinical CRC metastatic model. We do realize the possibility that due to AOM exposure, we potentially could have induced metaplasia within the pulmonary tissue; metaplasia is the transformation of one tissue type to an alternative cell phenotype. Clinically, it is defined as the formation of intestinal tissue from the esophageal epithelium, known as Barrett’s esophagus (Slack, 2004). More specifically, metaplasia is the process that involves the transformation of epithelia cells to goblet cells; however, histological examination of lung tumors in our study revealed intestinal gland-like structure instead of granule goblet cell type. Furthermore, pathologically, metaplasia would result in heterogeneous abnormal lung structure, which is composed of large air spaces lined with highly proliferative cuboidal epithelium (Okubo and Hogan, 2004); however, such metaplastic phenotype was not observed in our histological examination of the tissues.

To further characterize our pre-clinical AOM-induced lung metastasis CRC mouse model, we utilized specific cellular origin markers; these biomarkers are currently utilized in the clinic to identify the primary site of adenocarcinoma presenting at metastatic sites. The CDX2 protein plays an important role in proliferation and differentiation of intestinal tissue; it is normally expressed in the nucleus of epithelia cells within the colon, from the proximal to the distal regions; however, cytosolic expression
of CDX2 is indicative of intestinal differentiation and adenocarcinoma formation (Werling et al., 2003; Wani et al., 2009). Furthermore, clinically, the expression of CDX2 has been shown to identify colonic origin in resected primary and metastatic adenocarcinomas from the lung; CDX2 is considered the gold standard biomarker due to its 100% percent specificity and 100% sensitivity (Werling et al., 2003). High levels of this protein has been found in clinical CRC adenocarcinoma specimens (Werling et al., 2003). To further confirm the origin of lung tumors in our pre-clinical AOM-induced lung metastasis CRC model, we examined an additional colonic origin marker namely CK20, which is a cytokeratin typically expressed in epithelial cells; positive expression of CK20 has been seen in all cases of CRC carcinomas; however, the levels did not coordinate with clinical stage (Chu et al., 2000; Samija et al., 2013). Taken together the above information and the fact that we observed high expression of both CDX2 and CK20, known colonic biomarkers, at mRNA and protein levels in lung tumors convincingly suggests that these lung tumors originated from primary colon tumors, in this AOM-induced CRC lung metastasis mouse model. Our IHC results for SP-D and TTF-1 further supported this conclusion.

Furthermore investigating GSE efficacy in this pre-clinical azoxymethane-induced lung metastasis CRC mouse model revealed significant inhibition of colon tumor metastasis to lung tumor formation. Additionally, examination of individual animal colon tumor multiplicity data revealed that the animals with the highest tumor multiplicity did not exhibit secondary metastatic lung tumor formation upon GSE supplementation. Further examining the tumor multiplicity data in the AOM-group alone revealed the formation of lung metastatic tumors regardless of primary colon tumor size;
indicating in this model that secondary lung tumor growth does not depend on large primary colon tumor growth. These findings indicate GSE’s ability to decrease the angiogenic process and thus further inhibiting the metastatic spread. In this regard, our group and others have shown that GSE modulates a number of factors involved in the metastatic process; specifically, GSE treatment decreases VEGF, TNF-α, MMP-1,-3,-7,-8,-9 and MMP-13 protein expression (Kaur et al., 2009a; La et al., 2009; Chao et al., 2011). In conclusion, the development of this preclinical AOM-induced lung metastasis CRC mouse model is a significant scientific contribution; this in-situ sporadic CRC metastatic lung model will aid in the development of effective clinical agents that inhibit CRC disease progression. Furthermore, agents such as GSE, that targets multiple pathways, which are involved in angiogenesis and distant metastatic tumor growth, could be ideal candidates for their clinical potential in controlling CRC growth, progression as well as metastasis to distal organs such as lung.
CHAPTER VI

PROFILING OF PROTEIN TARGETS
AFTER GSE TREATMENT IN IN-VITRO MODELS OF CRC

Introduction

Natural products have been used exclusively for thousands of years; these natural products were the ultimate source of small molecule drugs exhibiting numerous beneficial effects that have been used since 2737 B.C. (Lomenick et al., 2011). There are millions of species that contain compounds with valuable pharmacological properties; only in the last half a century has the scientific community begun to tap into the vast source of scientific knowledge within the environment. Many of these small-molecule drugs comprise the majority of today’s medicines; however, specific protein targets of these small molecules remain a key challenge in creating effective clinical therapies (Lomenick et al., 2009; Kumar, 2012a; Kumar, 2012b; Villalba and Alcain, 2012).

Examining drug-protein interactions, prior to pre-clinical efficacy studies, allows scientist to effectively screen for the best small molecule candidates and further predict any associated toxicity with the drug administration. Taken the above information, in this study, we aimed to profile potential protein targets of grape seed extract (GSE) in human CRC cells.

Grape seed extract (GSE) is a complex mixture of proanthocyanidins; these proanthocyanidins are widely distributed throughout the plant kingdom and are present in high quantities within the seeds of the grapes. As GSE is composed of highly
polymerized proanthocyanidins, their characterization remains to be very challenging due to the variability of the composition of flavanols units, linkage positions and molecular size (Hayasaka et al., 2003; Shi et al., 2003; Nassiri-Asl and Hosseinzadeh, 2009).

Additionally, when considering a complete cellular system, which is composed of numerous chemical compounds and various proteins, there needs to be sensitive affinity-based techniques to identify and quantify these agents-protein interactions. Current affinity-based techniques that are utilized to characterize complex chemical protein mixtures are limited by the need to modify the small molecule. An alternative approach is an indirect non-affinity technique; however, these techniques depend on the ability of the small molecule to induce the specific cellular or biochemical readout (Lomenick et al., 2009; Lomenick et al., 2011). To overcome this obstacle, there has been the recent development of a simple approach that analyzes direct drug binding to targets; this technique is a universal applicable target identification approach. The theory behind the drug affinity responsive target stability (DARTS) technique is that a given protein may become less susceptible to proteolysis, when it is drug-bound, versus drug-free protein (Lomenick et al., 2009; Lomenick et al., 2011). In the current study, we aimed to identify potential protein targets of chemical components of GSE, via the DARTS technique, in human colorectal cancer (CRC) cells.

Identifying specific protein targets will aid in development of effective, long-term treatments and prevention approaches for CRC, which is the third most deadly malignancy in the US according to the American Cancer Society; over 50,800 deaths in 2013 would occur as a result of advanced CRC progression. Current therapeutic agents can induce severe patient toxicity upon administration; these current limitations
emphasize the need to develop safe, effective, long-term chemoprevention agents. GSE is one such agent that is a heterogeneous mixture of polyphenolic compounds; identifying the molecular targets of GSE will further solidify GSE clinical application for CRC treatment and prevention. Additionally, identification of GSE molecular targets potentially could further identify other disease conditions that would benefit from GSE treatment.

Materials and methods

Reagents: Standardized preparation of GSE was a gift from Kikkoman Corp. (Nado City, Japan). The composition of the GSE preparation is listed as: 89.3% procyanidins, 6.6% monomeric flavanols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash. Dimethyl Sulfoxide (DMSO) was from Sigma Chemical Co. (St. Louis, MO). Primary antibodies used were anti-GRP78, anti-calnexin, anti-IRE1α, anti-ATF6α, anti-eIF2α, and anti-integrin β1 (Cell Signaling Technology, Beverly, MA); anti-CK-1, and anti-DHE3 (Abcam, Cambridge, MA); and anti-β-actin (Sigma, St. Louis, MO). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were purchased from Invitrogen and Cell Signaling Technology, respectively.

Cell lines and treatments: SW480 and HCT116 cells were purchased from American Type Culture Collection (Manassas, VA). SW620 cells were a gift from Dr. Pamela Rice, University of Colorado, Denver. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere in DMEM (HCT116), Leibovitz-L15 (SW480), and RPMI SW620) media supplemented with 10% FBS and 1% penicillin/streptomycin.
For western immunoblotting protein analysis, human CRC cells were plated at a density of 5,000/cm² in 100-mm culture plates under standard conditions and treated subsequently either with DMSO alone or with varying concentrations of GSE (20-50 µg/mL) in DMSO. At the end of desired treatment times (12h), cells were harvested by brief trypsinization.

For the DARTS experiments, human CRC cells were plated at a density of 5,000/cm² in 60-mm culture plates under standard conditions and treated subsequently either with DMSO alone or with varying concentrations of GSE (60-100 µg/mL) in DMSO. At the end of desired treatment times (3h), cells were harvested by brief trypsinization. The cell lysates were then utilized in the following DARTS protocol.

Drug affinity response target stability (DARTS) with complex protein mixtures:
The protocol was followed as previously published; briefly cell lysates were prepared in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin] (Lomenick et al., 2009). Bradford analysis was preformed to ensure equal amount of protein lysate per sample. All steps were preformed on ice to prevent premature protein degradation; each sample was then quickly warmed to room temperature and proteolysed with 1:25, 1:50, 1:100 ratio of Pronase versus cell lysates protein concentration for 5 min. To stop proteolysis, 0.5 M EDTA (pH 8.0) was added to each sample at a 1:10 ratio, mixed well, and placed on ice.

SDS-PAGE and Comassie blue staining: Polyacrylamide gels were prepared according to the standard protocol, samples were loaded, gels were run @ 25 mA (2 gels
run @ 50 mA) in 1x SDS Running Buffer. At the end, the separated proteins were either transferred to a membrane (see Western Immunoblotting) or gels were stained with Coomassie. Briefly, gels were placed into fixing solution (50:10:40/methanol:acetic acid:H$_2$O) for 25 - 30 mins, and then washed three times with water, shaking for 5 mins each. Gels were then stained in coomassie working solution (500 mL Methanol+ 30 mL Coomassie concentrated stain solution + 400ml milli-Q water + 100 ml acetic acid, mixed and filtered using a 0.22 micron pre-sterilized filter) for about 25-40 minutes, gently rocking at room temperature. Gels were then destained with destaining solution [45:10:45 (methanol: acetic acid: H$_2$O) until no background staining (shaking about 2-3 hours and changing solution 3 to 4 times). Gels were finally stored in glass containers in 5% acetic acid solution at 4°C until in-gel digestion was performed.

In gel-digest and LC/MS analysis: Gel bands were cut out and prepared for mass spectrometry analysis with trypsin digestion. Briefly, gel pieces were placed on a clean plate, excised each band with a clean new scalpel; the larger pieces were cut to 1x1mm pieces. The gel pieces were washed twice with destaining buffer [25mM NH$_4$HCO$_3$ / 50% ethanol (EtOH)] and incubated for 20min @ 25°C, followed by dehydration of the gel pieces in 100% AcN for 10min at 25°C, with repeating the process until gel pieces were dry. Gel pieces were further dried in a speed-vac for 5 min and then re-hydrate in reduction buffer [10mM dithiothreitol (DTT) in 50mM ABC, solution is further diluted 1M DTT] and incubate for 60min at 56°C. Alkylation buffer was then added [55mM iodoacetamide in 50mM ABC] and samples were incubate in the dark for 45min at 25°C; followed by a gel wash with digestion buffer for 20min at 25°C. Additionally, these gel pieces were dehydrate again with 100% AcN for 10min at 25°C; followed by drying
again for 5min in the speed-vac. Gel pieces were then again rehydrated in trypsin at 37°C ~20min and then further incubated for digestion with trypsin overnight at 37°C. The peptides were the extracted from the gel matrix by incubating gel pieces with extraction buffer [3% trifluoroacetic acid (TFA)/ 30% AcN], followed by gel dehydration and removal of the supernatant.

The digested proteins were loaded onto a Magic AQ C18 reverse phase nano column (Bruker) using a nano-Advance autosampler and nano flow UPLC (Bruker). Mobile phases consisted of H₂O + 0.1% formic acid (A) and 89.95% acetonitrile, 9.95% H₂O, 0.1% formic acid (B). Peptides were chromatographically separated at a flow rate of 500nl/min using a gradient of 5-45% B over 30 minutes followed by a column wash at 95% B for 5 minutes. An Amazon Speed ion trap equipped with a Captive Spray source (Bruker) was used for MS/MS analysis of the eluting peptides. Proteinscape software (Bruker) was used to submit the data to Mascot v.2.4 for database searching and the Percolator algorithm rescored peptide and protein matches.

*Western immunoblotting:* At the end of each treatment, cell lysates were prepared in nondenaturating lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin]. Immunoblot analysis using equal amount of protein lysate per sample was done. Membranes were probed with desired primary antibodies, concentrations are as follows: anti-GRP78 (1:300); anti-calnexin (1:10000); anti-IRE1α (1:200); anti-ATF6α (1:100); anti-eIF2α (1:500); anti-integrin β1 (1:500); anti-CK-1 (1:1000), and anti-DHE3 (1:1000) followed by peroxidase-conjugated appropriate secondary antibody and visualized by
enhanced chemiluminescence detection system. Some blots were stripped and reprobed with different antibodies including those for loading control.

**Statistical analysis:** All the data shown are mean ± SE, representative of at least three independent experiments. Statistical significance of differences between control and GSE-treated samples were calculated by one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). *P* values of ≤0.05 were considered significant.

**Results**

*Utilizing DARTS, enriched protein bands were identified after GSE treatment in human CRC cells:* To identify potential protein targets of GSE we utilized DARTS; the basic strategy of DARTS is shown in Fig. 6-1A. GSE is a complex mixture of proanthocyanidins; specific monomeric phenolic compounds include catechin and epicatechin (Fig. 6-1B). GSE treatment was done at 60-100 µg/mL doses in a panel of human CRC cell lines; these cell lines were chosen based on phenotypic and genetic variations, so as to cover different clinical stages of CRC, viz., SW480 (stage II CRC), SW620 (stage III CRC), HCT116 (stage IV CRC) cells. After GSE treatment, cell lysates were isolated and followed by proteolysis at varying concentrations of Pronase (1:25, 1:50, 1:100). The proteins were separated and stained via SDS-PAGE with Coomassie blue; this experiment revealed multiple enriched protein bands due to GSE treatment. For example, in SW480 human CRC cells, GSE treatment (100 µg/mL) resulted in enrichment of 3 main peptide bands; enriched bands occurred around 75KDa, 53KDa, and 52KDa molecular weights (Fig. 6-1C, left).
Figure 6.1 DARTS enriched protein bands were identified after GSE treatment in human CRC cells. A) Summarizes the DARTS theory behind target stability through drug-protein interaction. Treatment of human CRC cells with GSE (60-100 µg/mL) for 3h resulted in binding of GSE components to target proteins; these proteins were isolated, undergo proteolysis, further separated and finally identified via LC/MS. B) The two major constituents of GSE are catechin and epicatechin, these flavanoids are able to exist as monomers, but can further polymerize to form large dimers and trimers, also known as GSE proanthocyanidins. C) After the proteolysis of the control and GSE treated samples, they were separated via SDS-PAGE, followed by Commassie staining. Additionally, varying concentrations of Pronase were used to digest the protein samples, 1:25, 1:50, and 1:100. D) A sample LC/MS chromatograph from human GSE treated CRC cells demonstrates the complex protein/peptide mixture.
However, the DARTS technique in SW620 human CRC cells revealed that GSE treatment (60 µg/mL) resulted in enrichment of 6 main peptide bands; enriched bands occurred around the molecular weights of 75KDa, 74KDa, 53KDa, 52KDa, 40KDa, and 20KDa (Fig. 6-1C, middle). Moreover, DARTS in HCT116 human CRC cells revealed that GSE treatment (60 µg/mL) resulted in enrichment of 5 main peptide bands at the molecular weights of 75KDa, 74KDa, 53KDa, 52KDa, and 40KDa (Fig. 6-1C, right). Observing the resulting LC/MS chromatogram demonstrated the presence of complex mixture of peptides in these bands; a representative chromatogram profile is shown in Fig. 6-1D.

*MASCOT protein identification:* To further interpret LC/MS information, peptide mass fingerprints were imported into the MASCOT search engine; via this database, statistically, the most probable proteins that were targeted by GSE treatment were identified (Table 6-1).

<table>
<thead>
<tr>
<th>Proteins enriched with GSE treatment</th>
<th>SW480</th>
<th>SW620</th>
<th>HCT116</th>
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<tbody>
<tr>
<td>GRP78</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>PDIA3</td>
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<tr>
<td>HSP7c</td>
<td>X</td>
<td>X</td>
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<td>NUCL</td>
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<tr>
<td>DHE3</td>
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<td>GRP75</td>
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</tr>
<tr>
<td>K2Cl</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>ALDOA</td>
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</table>
In SW480 CRC cells, GSE treatment potentially upregulated the expression of GRP78, PDIA3, HSP7c, DHE3 and K2C1; in SW620 CRC cells, GSE treatment enriched peptide bands specific for GRP78, PDIA3, HSP7c, NUCL, DHE3 and GRP75 (Table 6-1); and in HCT116 human CRC cells, GSE treatment enriched specific peptide mass fingerprints for GRP78, PDIA3, GRP75, K2C1 and ALDOA (Table 6-1).

MASCOT results also revealed the number of peptides identified and further calculated the sequence coverage for the identified peptide mass fingerprints. As an example, examining the sequence coverage for GRP78 in all three cell lines revealed a high percent of sequence coverage; specifically, the GRP78 protein peptide sequence for the three cell lines was: SW480-49%; SW620-31% and HCT116-40% (Table 6-2).

Examining the identified proteins, which could be possible direct targets of components of GSE, revealed a potential global protein modification following GSE treatment. Furthermore, examining the spectral output by LC/MS and the MASCOT data revealed that GSE exposure resulted in numerous oxidative protein modifications, specifically on methionine amino acids residues (Table 6-2). These oxidative methionine residue modifications occurred in the following identified proteins: GRP78, PDIA3, HSP7c, DHE3, GRP75, K2C1 and ALDOA (Table 6-2).

Glucose related protein-78 (GRP78) is a member of the HSP family of molecular chaperones and is required for ER integrity and stress induced autophagy (Schonthal, 2012). Protein disulfide-isomerase A3 (PDIA3) is also localized to the ER and involved in protein folding; additionally, nucleolin (NUCL) is involved with ribosome maturation and assembly (Srivastava et al., 1990; Dunn et al., 2001).
Glutamate dehydrogenase 1 (DHE3) is a mitochondrial matrix enzyme; the
glucose related protein-75 (GRP75) protects against oxidative stress and localized to the
mitochondria (Wadhwa et al., 2002; Lehmann and Ratajczak, 2008). Additionally, GSE
may interact with HSP7c, which acts as a transcriptional repressor; this protein is further
activated upon cellular stress. Keratin type II cytoskeletal 1 (K2C1) is involved in kinase regulation and responses to oxidative stress stimuli (Collard et al., 2001). Finally, fructose- bisphosphate aldolase (ALDOA), a key enzyme involved in glycolysis is a potential target of GSE (Gamblin et al., 1991). The above potentially identified proteins that are modified by GSE treatment and their cellular localization are summarizing in Figure 6-2.

Figure 6-2 Summary of potential protein targets of GSE treatment. Multiple proteins were potentially identified via LC/MS analysis coupled with MASCOT protein identification. The above figure summarizes the potentially proteins targets of GSE that presented the greatest sequence coverage in our MASCOT analysis; further the figure additionally displays the cellular localization of the GSE targeted proteins. The protein targets are as follows: GRP78; PDIA3; HSP7c; NUCL; DHE3; GRP75; K2C1 and ALDOA.
**Validation of GSE protein targets in human CRC cells:** The DARTS technique, combined with LC-MS analysis and MASCOT, identified potential protein targets of the GSE components. A number of the fore mentioned proteins and summarized in Figure 6-2, are involved in protein folding and ER integrity. To investigate the potential effect of GSE treatment on the ER stress pathways, we treated human CRC cells with this agent. GSE treatment (20-50 µg/mL) of SW480, SW620, and HCT116 CRC cells resulted in dramatic down-regulation of GRP78; this effect is due to oxidation of this protein ultimately resulting in protein degradation (Fig. 6-3A). Calnexin protein levels were unchanged after 12h of GSE treatment in all three cell lines; however, IRE1α protein levels decreased in all three cell lines (Fig. 6-3A). Additionally, GSE treatment of SW480 and SW620 CRC cells resulted in a down-regulation of ATF6α; however, no ATF6α protein alteration was observed in HCT116 cells (Fig. 6-3A). Furthermore, eIF2α protein levels were increased in SW480 CRC cells; in contrast, eIF2α levels were decrease in SW620 and HCT116 CRC cells (Fig. 6-3A). We also examined DHE3 levels in SW480 and SW620 CRC cells; results indicated an increased expression of DHE3 in SW480 CRC cells, however, no change in protein levels was observed in SW620 cells (Fig. 6-3B). Examining the potential extracellular matrix as targets of GSE indicated no direct effect on protein levels of K2C1 and a known binding partner Integrin β1 in HCT116 CRC cells (Fig. 6-3)
Figure 6-3 Validation of GSE protein targets in human CRC cells. A) GSE was administered to SW480, SW620, and HCT116 human CRC cells. Cells were treated with DMSO (control) or different concentrations of GSE (20-50µg/mL) for 12 hours. At the end of treatment, cells were collected and both adherent and non-adherent cells were harvested and cell lysates were prepared in nondenaturing lysis buffer and immunoblotting was performed as detailed in Materials and Methods section. GSE treatment resulted in downregulation of GRP78, IRE1α, ATF6α, eIF2α. No change in protein expression was observed for calnexin. B) Examined GSE treatments effects on mitochondrial and cytoskeleton proteins with in SW480, SW620 and HCT116 human CRC cells. GSE treatment did increase levels of DHE3, but no effects were observed in SW620 cells. In HCT116 cells no alteration in K2C1 and Integrin β1 levels were observed. Membranes were also stripped and reprobed with anti-β-actin antibody to confirm equal protein loading.
Discussion

One in four deaths in the United States is cancer-related and CRC is the second leading cause of cancer-associated deaths, according to the American Cancer Society (ACS). Screening strategies are utilized but have not reduced disease incidence or mortality. In this regard, there is an interest in preventive strategies focusing on natural small-molecule drugs or nutraceuticals that can be administer on a daily basis for the treatment and prevention of CRC. Furthermore, Americans have spent billions of dollars on over-the-counter dietary supplements with limited scientific evidences for their beneficial effects. GSE is one such nutraceutical, a complex mixture of flavanoids, specifically proanthocyanidins, which are the most abundant phenolic compounds in grapes; furthermore the grape seeds contain the highest percentage of proanthocyanidins. However, due to GSE composition variability, it is difficult to characterize these complex mixtures. Alternatively, the fact that GSE is a complex mixture of chemical components, allows this supplement to target multiple protein pathways in its efficacy against colon carcinogenesis. Furthermore, identifying GSE target proteins will aid in development of effective clinical treatment and prevention strategies for CRC; GSE target protein identification is a key indicator of clinical success. Through this knowledge, researchers can predict in advance any additional off target effects of GSE; this allows for effective screening of natural compounds in general, prior to pre-clinical development.

However, current methods to identify protein targets of complex polyphenolic mixtures, require alteration in the chemical compounds, to allow for detection; these affinity-based methods include: Matrix-based affinity detection; Genetic yeast three-hybrid and phage cloning (Lomenick et al., 2009). Alteration of the chemical compounds
is not desirable, due to the potential structure alterations, thus altering potential protein binding; The DARTS technique is a new method that like affinity methods relies on the affinity of the small molecule to bind to the target protein. This target affinity allows identification of direct GSE target proteins; however, the key advantage of DARTS over current affinity based techniques, is that it doesn’t require chemical alteration of the components of GSE. DARTS allows for identification of potential target proteins that can then be further validated through molecular and biochemical techniques. These experiments verify DARTS as an efficient test and screen to verify drug-protein interaction; DARTS is a useful discovery tool, applicable to complex protein mixtures such as human cancer cells.

The results of the current study elucidated the upstream stimulus responsible for the chemopreventive and anti-cancer efficacy of GSE in CRC model. These results indicated the initial mechanism of action of GSE, which was responsible for its anti-cancer efficacy; specifically how GSE targets ER stress response proteins, resulting in overall downregulation of proteins involved in translation. Furthermore examining the spectral output by LC/MS and the MASCOT data revealed that GSE exposure resulted in oxidative protein modifications, specifically on methionine amino acids residues (Table 6-2). Other studies have shown that GSE proanthocyanidins result in oxidative modification of SOD and catalase, specifically on cysteine and methionine residues, resulting in increased SOD and catalase activity (Chis et al., 2009). In the above study, we observed oxidative protein modification and an altered ER stress response protein expression, in human CRC cells, as a result of GSE treatment.
The endoplasmic reticulum (ER) is an essential organelle, responsible for protein folding and secretion, lipid biosynthesis, and calcium homeostasis (Tsai and Weissman, 2010). The ER is a major organelle for sensing oxygen and nutrients; the stresses that disrupt ER function, from the surrounding cellular microenvironment, result in the accumulation of unfolded proteins (Tsai and Weissman, 2010). The ER stress response can be triggered following imbalances in cellular homeostasis; this cellular response can adopt a pro-survival effort or a pro-apoptotic effort (Schonthal, 2012). GRP78/BiP is a major component of the ER stress response system; notably, GRP78 is found to be frequently upregulated in tumor cells (Schonthal, 2012), and importantly, it is an effective specific target the GSE modifies. Oxidative modification of GRP78 was detected at 3h of GSE treatment in all three human CRC cell lines; this protein modification further resulted in downregulation of GRP78 proteins levels after 12hs of GSE treatment (Table 6-2, Fig. 6-3A), which might be a selective GSE effect in inducing apoptotic death in CRC cell lines but not normal colon epithelial cells as reported by us recently (Derry et al., 2012).

Furthermore, GSE treatment also resulted in the downregulation of downstream targets of GRP78, namely IRE1α, ATF6α and eIF2α. IRE1α is triggered, by GRP78, in response to the accumulation of unfolded proteins within the ER lumen (Zhang et al., 2005). Regarding ATF6α, its levels were decreased in response to GSE treatment in SW480 and SW620 cells; ATF6α senses ER protein mis-folding stress and is further involved in protein secretion and degradation within the golgi apparatus (Wu et al., 2007). Furthermore, GSE treatment in SW480 cells resulted in upregulation of eIF2α; while in the other 2 cell lines, SW620 and HCT116, GSE treatment resulted in a
downregulation of eIF2α. The eIF2α protein is another sensor that is typically upregulated due to ER stress; this eIF2α pathway is also activated via ERK signaling (Back et al., 2009). In SW480 CRC cells, GSE treatment has been shown to cause upregulation of ERK phosphorylation and activation; however, this effect has not been found in SW620 and HCT116 CRC cells. Together, these results coincide with the altered eIF2α expression pattern in these three CRC cell lines by GSE treatment; importantly, the ability of GSE to downregulate eIF2α indicates that GSE induces oxidative stress, leading to the accumulation of un-translated and unfolded proteins. Furthermore, GSE’ ability to decrease pan-signaling protein GRP78, resulting in downregulation of downstream protein pathways namely IRE1α, ATF6α and eIF2α, possibly also resulted in the inhibition of the un-folded protein response; this inhibition results in damage to the ER membrane causing a loss of membrane potential. Indeed, GSE has been previously shown to decrease mitochondrial, as well as, ER membrane potential in these CRC cells; further similar effects were observed in prostate cancer cells with GSE treatment (Agarwal et al., 2002; Derry et al., 2012). In addition, the loss of ER membrane potential could also alter intra-cellular Ca²⁺ levels; altered Ca²⁺ levels have been shown to further result in IRE1α protein alteration. The intra-cellular Ca²⁺ levels can also result in mitochondrial membrane permeability further leading to the activation of caspase-8 and caspase-9; recent mechanistic studies investigating GSE efficacy in these CRC cell lines have revealed the activation of caspase-8 and -9 as a result of GSE treatment, leading to cancer cell apoptotic death (Derry et al., 2012).

Of central importance to metabolic pathways are the amino acids glutamine and glutamate; the key enzymes involved in their synthesis are localized almost exclusively to
the mitochondria (Kovacevic and McGivan, 1983; Prickett and Samuels, 2012; Teh and Chen, 2012). Specifically, glutamate dehydrogenase (DHE3) is localized to the inner surface of the mitochondria membrane and is involved in glutamate synthesis (Kovacevic and McGivan, 1983). Alteration of glutamine metabolism occurs in many forms of cancer; this is due to the fact that glutamine is a precursor to purine and pyrimidine synthesis but it also is the major substrate for tumor energy metabolism (Kovacevic and McGivan, 1983; Prickett and Samuels, 2012; Teh and Chen, 2012). Additional investigation of GSE potential protein targets revealed that in SW480 cells GSE protein oxidation resulted in upregulation of DHE3 expression; however this effect was not observed in SW620 and HCT116 CRC cell lines. Further activity studies would be ideal to identity and validate DHE3 activity post GSE treatment in these CRC cell lines.

Cancer is a disease in which a number of biological processes are lost or perturbed; this includes the alteration of cellular morphology leading to tissue disruption and invasive characteristics (Hall, 2009; Sipos et al., 2012). The cellular cytoskeletons are key players in organizing cytoplasmic organelles, defining cell polarity, and generating pushing and contractile forces (Hall, 2009). To further investigate GSE ability to alter cellular structural proteins, we examined the protein expression of K2C1 and its known binding partner Integrin β1; results revealed no alteration of K2C1 or Integrin β1 expression levels in response to GSE treatment. However, these findings do not rule out the possible that oxidation of K2C1 could result in, alteration of kinase activity, activation of an oxidative stress response and further alteration in cellular morphology. Further examination of PKC and SRC kinase activity may reveal the role of K2C1 oxidation in response to GSE treatment.
Overall, this study indicates that DARTS is a useful affinity technique that allows researchers to identify potential protein targets of small molecules within complex protein mixtures. Specifically, DARTS identified eight overall proteins that could be potential targets of GSE treatment in human CRC cells; these potential targets are: GRP78, PDIA3, HSP7c, NUCL, DHE3, GRP75, K2C1, and ALDOA. Furthermore, the LC/MS data collected in this study promotes further investigation into the other potential protein targets of GSE; additional protein were identified via MASCOT but we did not focus on them in the present study due to low sequence coverage. In summary, identifying the potential targets of GSE treatment involved in its anti-cancer and chemopreventive efficacy against CRC, allows the further developed of this natural supplement in the clinical setting. This information, combined with the vast pre-clinical GSE efficacy studies, would further solidify GSE as a safe, effective, multi-targeted anti-cancer and chemopreventive agent for CRC.
CHAPTER VII

SIGNIFICANCE

Cancer is a global health problem and incidence rates are not currently decreasing; according to the ACS, in the United States, it is estimated that over 1,660,000 new diagnosed cancer cases will occur in 2013 alone. Furthermore, it is projected, that by 2030, the number of new cancer cases will increase 70%, due to demographic changes alone; from this estimation in 2030, one could estimate 2,822,000 newly diagnosed cancer patients (Franceschi and Wild, 2013). Globally, Colorectal Cancer (CRC) is the 2nd most common cancer in women, and the 3rd most common cancer in men; additionally according to the ACS, CRC will result in over 50,830 deaths in 2013, from a public health standpoint, these numbers are unacceptable. Additionally, in 2010 alone, the US spent an estimated $125 billion on cancer care; furthermore, during this same year, the US spent an estimated $62,000-$94,000 per cancer patient, this is a considerable public health and economic cost (Yabroff et al., 2011). The current treatment regime for a CRC patient includes surgery, radiation, and/or traditional chemotherapeutic agents, depending on stage of diagnosis; these conventional therapeutic treatments, however, pose the risk of severe toxicity upon administration. Furthermore, considering that the current conventional therapies have been unable to decrease CRC incidence, the question remains whether the money being spent is worth the cost.

Considering the above information, which emphasizes the clinical need to focus on prevention strategies, as well as, designing non-toxic, affordable, effective anti-cancer
treatments. CRC is a multi-factorial disease that results from decades of complex genetic and environmental interactions that can ultimately lead to CRC initiation, promotion, and progression. Various epidemiological studies have indicated lifestyle choices as a major factor that contributes to CRC disease risk. These deleterious lifestyle factors are generally referred to as Western lifestyle habits and include: high-fat diet; low-fiber diet; tobacco exposure; alcohol consumption and sedentary lifestyle. These lifestyle habits are modifiable parameters that can be altered to decrease CRC incidence. To limit CRC risk, people should decrease: exposure to tobacco; consumption of red-meat and alcohol. Additionally, to further decrease their risk of developing CRC, people need to consider increasing: physical activity, consumption of fruits and vegetables, and nutraceutical administration.

The standard American diet includes the consumption of red meat; however, due to the numerous associations between red meat consumption and CRC risk, it is recommended to limit the consumption of red meat to 2oz/day. Moreover, consuming other foodstuffs along with red meat would be a beneficial strategy to combat the deleterious effects of red meat consumption; specifically, the foodstuffs that counter-act the red meat effect could include: probiotics; silibinin; dietary fiber; GSE and curcumin. These nutraceuticals possess anti-inflammatory properties, as well as, the ability to increase DNA repair enzymes; these beneficial qualities would decrease the carcinogenic properties of red meat.

Another risk factor for CRC development is alcohol consumption; further considering that alcohol is a carcinogen, to prevent CRC, daily intake of alcohol should be kept to 2 glasses of wine or beer/day to be considered as safe exposure (Haas et al.,
2012). However, to counteract the deleterious effects of alcohol, one could take additional measures to limit toxicity. Some beneficial foodstuffs that can be administered prior to alcohol consumption include: probiotics; folic acid supplements; dietary fiber; GSE; silibinin; curcumin and EGCG. These beneficial nutraceuticals aid in alcohol detoxification, increase DNA damage repair enzymes and decrease aberrant cellular proliferation and angiogenic signals, and thus decrease the detrimental effects resulting from chronic alcohol exposure.

Additionally, tobacco exposure increases the risk for CRC development; tobacco smoke contains numerous carcinogenic compounds that result in colon carcinogenesis. With this in mind, there are no safe exposure guidelines for tobacco smoke; therefore, for CRC prevention, individuals should not smoke tobacco. Nicotine, however, is highly addictive, so additional lifestyle modifications should be considered; specific foodstuffs include: Probiotics; Folic acid; Dietary fiber; Silibinin; EGCG and curcumin. These nutraceuticals target carcinogen detoxification and epigenetic pathways; these natural agents further decrease abnormal cellular proliferation and inflammation. These beneficial multi-targeted dietary factors have enormous potential for CRC prevention as a result of tobacco exposure.

Another major risk factor for CRC development is obesity; obesity has become a major epidemic in the US, 1 out of 3 Americans are overweight. The most significant lifestyle interventions that would prevent CRC development would be diet modification and increased physical activity; these lifestyle changes would dramatically decrease the incidence of obesity and related-diseases, including CRC. Physical activity results in increased release of satiety hormones, and decreased inflammation and adipose tissue.
However, there are additional diet alterations that can also modify obesity-related signals, thus preventing development of CRC; these include: GSE; Silibinin; Probiotics; EGCG; Silibinin and curcumin. The majority of these beneficial nutraceuticals modify insulin signaling, increase detoxification enzymes, and decrease uncontrolled proliferation and angiogenic signals. These nutraceuticals have the ability to inhibit CRC disease progression and metastasis; they are pleiotropic agents that down regulate numerous abnormal cellular pathways that have become elevated in obese individuals.

Increasing the protective lifestyle factors and limiting the deleterious choices will decrease a person’s incidence of developing CRC in his/her lifetime; however, the key is to find a balance. Ultimately, the above lifestyle choices can result in damaging or beneficial effects. The above knowledge highlights the multiple lifestyle choices that can be modified to prevent CRC; further, elucidating specific nutraceuticals that would be beneficial for CRC prevention based on patient lifestyle choices. These nutraceuticals are pleiotropic agents that target multiple cellular pathways involved in colon carcinogenesis; these pathways include proliferative, inflammatory, apoptotic, epigenetic, DNA repair and angiogenic pathways.

Additional strategies, like the use of nutraceuticals, such as grape seed extract (GSE), that targets multiple pathways, is one such approach to prevent colon carcinogenesis and further reduce CRC disease incidence. GSE is a complex mixture of naturally occurring polyphenolic compounds, and there is significant experimental date supporting the beneficial effects of GSE proanthocyanidins, specifically its anti-cancer and chemopreventive efficacy. The supporting studies include numerous clinical, in-vivo, and in-vitro studies that have demonstrated GSE is non-toxic, pleiotropic, and
efficacious nutraceutical. Furthermore, numerous detailed *in-vitro* mechanistic studies, including the work in this thesis, have revealed GSE’ mechanism of action, that is responsible for its anti-cancer and chemopreventive efficacy. Some of the potential mechanisms responsible for the chemopreventive and anti-cancer efficacy of GSE include: anti-proliferative; induces cell-cycle arrest; pro-apoptotic; anti-inflammatory and anti-angiogenic. Moreover, GSE has additional benefits that include: anti-bacterial; anti-viral; anti-oxidant, and cardio protective effects.

Further investigating GSE efficacy in human CRC cells that vary in their metastatic potential, provide mechanistic details into GSE’ ability to induce cancer cell death at any stage of CRC disease progression. Hallmarks of colon carcinogenesis involve mutations that result in uncontrolled cellular proliferation and apoptosis resistance; additionally, these same mutations contribute to multi-drug tumor cell resistance. Focusing on clinical strategies that target these uncontrolled processes would be of clinical significance. Apoptosis and cellular proliferation are highly complex processes involving a cascade of molecular events. The two main apoptotic pathways include: the extrinsic or death receptor pathway and the intrinsic or mitochondrial-derived pathways; furthermore, there is evidence that these pathways participate in cross-talk and converge into the same executioner pathway (Igney and Krammer, 2002). The extrinsic pathway involves death receptor activation, specifically DR4 and DR5, which results in autocatalytic cleavage/activation of procaspase-8 and the executioner pathway activation (Kischkel et al., 1995; Elmore, 2007). The intrinsic pathway, on the other hand, involves non-receptor mediated protein interactions and mitochondrial initiated events;
once stimulated, it results in the release of pro-apoptotic proteins and executioner pathway activation.

Clinically, a number of cancer types have been shown to be sensitive to TRAIL gene therapy that results in the activation of the extrinsic apoptotic pathway; however, resistance to TRAIL-mediated apoptosis in cancer cells has become a clinically challenging issue for CRC treatment, with patients exhibiting no response to therapy (Griffith et al., 1998; Kim et al., 2000; Zhang et al., 2000; Zhang and Fang, 2005). Additionally, mutations in proteins involved in apoptosis have also been reported in CRC; suggesting that, to design effective clinical therapies for CRC, these obstacles need to be overcome (Kim et al., 2003; Soung et al., 2006). In this regard, the studies in present thesis are highly significant in that they identify GSE as a potential anti-cancer therapeutic agent, which has the capacity to induce both extrinsic and intrinsic apoptotic pathways selectively in CRC cells. Unique to our study is the experimental design to determine GSE caspase-dependence in various stages of CRC; results indicated that indeed GSE induced apoptotic cell death is caspase-dependent and caspase-independent pathways. Interestingly, the upstream stimulus to the GSE-induced cell death was oxidative stress in all three CRC cell lines; furthermore, our mechanistic studies revealed the loss of mitochondrial ($\Delta\Psi_m$) and ER membrane potentially, followed by activation of intrinsic and extrinsic apoptotic pathways. The GSE-mediated apoptotic events were also found to be associated with differential modulation of pro- and anti-apoptotic proteins. Importantly, GSE-mediated cell death was specific to human CRC cells, exhibiting no toxicity in normal colon epithelial cells. Another significant finding of our study was that
the anti-cancer efficacy of GSE increased as the metastatic potential of CRC cells increased.

Additionally, the study in present thesis is the first detailed *in-vivo* study to report that long-term feeding of GSE (0.25% and 0.5%) after AOM carcinogen exposure results in decreased colon tumor multiplicity. Furthermore, GSE supplementation significantly reduced colon tumor size. In this study, we also established a non-invasive real-time imaging technique, MRI, to examine GSE efficacy in real-time. To reveal GSE mechanisms of action in the AOM-induced CRC model, we further examined biomarkers in colonic mucosa with tumors; these studies revealed that GSE supplementation decreased proliferation, but induced apoptosis in its chemopreventive efficacy. Additionally, this study revealed a number of altered cytokines/ILs and miRNAs in response to GSE treatment, which were associated with alterations in NF-κB, β-catenin and MAPK signaling; this information suggests that inflammation, proliferation and apoptosis are targeted by GSE in its chemopreventive efficacy against CRC. In addition to the miRNAs association with signaling pathways, GSE also modulated the expression of several additional miRNAs, which are implicated in CRC. For example, GSE down regulated miR-135b, miR-196a, miR-21, all of which show increased expression in CRC tissue compared to the normal surrounding tissue (Nagel et al., 2008; Schimanski et al., 2009; Yabroff et al., 2011; Kjaer-Frifeldt et al., 2012; Xu et al., 2012). Together, this is the first study to show the ability of GSE to beneficially modulate miRNA and cytokine signaling in its chemopreventive efficacy against AOM-induced colon tumorigenesis model.
Once CRC progresses to an advanced stage, primary tumor cells migrate to distant sites; the main sites of metastatic CRC tumor growth are the liver and the lung. Additionally, the majority (~65%) of CRC patients already present advanced disease at the time of diagnosis; current conventional clinical strategies are not effective in inhibiting disease progression or metastasis. This lack of clinical efficacy could be a reflection on the lack of effective models of CRC metastasis available in the pre-clinical setting; without effective models that recapitulate the human disease condition, it is difficult to design effective CRC therapies for metastatic disease. The studies in present thesis also aimed to identify and characterize the AOM-induced lung metastasis CRC mouse model. In this model of CRC metastasis, upon AOM-injection, primary colon tumors progress to advance disease, and further result in in-situ lung tumor formation. Currently, the pre-clinical models of metastatic CRC progression do not effectively mimic human CRC metastasis, are costly, technically advanced, and time-consuming. The model described in our work utilizes immune competent animals, involves simple technical procedures, results in primary colon adenocarcinoma, and lung metastatic tumor formation; this pre-clinical animal model recapitulates the human CRC disease progression. Moreover, to further characterize this pre-clinical AOM-induced lung metastasis CRC mouse model, we utilized cellular origin markers; these biomarkers are currently utilized in the clinic to identify the primary site of adenocarcinoma present at metastatic sites, specifically CDX2 and CK20. Colon biomarkers included CDX2 and CK20; clinically CDX2 is considered the gold standard biomarker due to its 100% percent specificity and 100% sensitivity (Werling et al., 2003). Due to the originality of this pre-clinical AOM-induced lung metastasis CRC model, we further wanted to
examine additional colonic origin markers. CK20 is a cytokeratin typically expressed in epithelial cells; positive expression of CK20 was seen in all cases of CRC carcinomas (Chu et al., 2000; Samija et al., 2013). Considering the above information and the fact that we observed expression of both CDX2 and CK20, known colonic biomarkers, our finding suggests that the pulmonary tumors observed in our study, originated from primary colon tumors in this AOM-induced CRC lung metastasis mouse model. Furthermore, examining the transcript levels of these proteins verified the upregulation of these colonic markers within the lung tumor tissue in the AOM-induced CRC mouse model.

Additional evidence that supports that the observed tumors were a result of the AOM-induced CRC includes the observation that the AOM-induced metastatic lung tumors formed on the exterior portion of the lung lobes indicating a potential lymphangitic spread. Moreover, we did not observe any spontaneous lung tumor formation in the control animals. Furthermore, investigating GSE efficacy in this pre-clinical AOM-induced lung metastasis CRC mouse model revealed significant inhibition of primary colon tumor growth as well as secondary metastatic lung tumor formation. The development of this preclinical AOM-induced lung metastasis CRC mouse model is a significant scientific contribution; this model will aid in the development of effective clinical agents that inhibit CRC disease progression. Furthermore, agents such as GSE, that have shown efficacy in metastatic CRC tumor models and target multiple pathways, have vast clinical potential.

Further identifying the specific protein targets of GSE treatment will solidify GSE as a safe and effective chemopreventive and anti-cancer agent; GSE target protein identification allows for prediction of any potential off-target effects, thus increasing the
clinical success of GSE. However, current methods to identify protein targets of complex polyphenolic mixtures, requires alteration of the chemical compounds, to allow for detection (Lomenick et al., 2009). A new affinity method is the DARTS technique that, like affinity methods, relies on the affinity of the small molecule to bind to the target protein, which allows identification of potential direct GSE target proteins. Our other studies in this thesis verified DARTS as an efficient test to screen for drug-protein interaction. Additionally, the results of our study elucidated the upstream stimulus responsible for the chemopreventive and anti-cancer efficacy of GSE in a pre-clinical model of CRC. Specifically, how GSE targets ER stress response proteins; additional analysis revealed that GSE exposure resulted in oxidative protein modifications, specifically on methionine amino acids residues. We also observed oxidative protein modification and altered ER stress response protein expression, in human CRC cells, as a result of GSE treatment. GRP78 is a major component of the ER stress response system, shown to be upregulated in tumor cells (Schonthal, 2012). GSE treatment resulted in downregulation of GRP78 proteins levels; additionally, GSE treatment resulted in downregulation of downstream targets of GRP78, namely IRE1α, ATF6α, and eIF2α.

The above information supports GSE ability to induce oxidative stress, leading to the accumulation of un-translated and unfolded proteins. Additionally, and of central importance to metabolic pathways, is glutamate dehydrogenase (DHE3), which is a key enzyme involved in glutamate synthesis that is localized to the mitochondria membrane (Kovacevic and McGivan, 1983). Alteration of glutamine metabolism occurs in many forms of cancer; additional investigation of GSE potential protein targets revealed that GSE protein oxidation resulted in upregulation of DHE3 expression (Kovacevic and
McGivan, 1983). However, further activity studies are necessary to validate DHE3 activity post GSE treatment in these human CRC cells. Overall, this study indicated that DARTS specifically identified eight proteins that could be potential targets of GSE treatment in human CRC cells; these potential targets are: GRP78; PDIA3; HSP7c; NUCL; DHE3; GRP7; K2C1, and ALDOA. Further mechanistic studies revealed that oxidation of the GRP78 protein, by GSE, resulted in protein downregulation; this down-regulation triggered a global downregulation of the ER stress pathways. Identifying the potential targets of GSE treatment involved in its anti-cancer and chemopreventive efficacy against CRC, allows the further developed of this natural supplement in the clinical setting.

Although the experiments contained with in this dissertation provide detailed *in-vitro* and *iv-vivo* mechanistic studies investigating GSE’ chemopreventive and anti-cancer efficacy, further studies would be useful to fully elucidate GSE’ mechanism of action. Specifically, considering the *in-vitro* studies, it would be beneficial to determine the connection between the GSE-induced ER stress and the robust GSE-induced apoptotic cell death observed in these same CRC cell lines. I would hypothesis that the connection resides within the alteration of Ca$^{2+}$ levels within the cellular environment; specifically, GSE treatment results in loss of ER membrane integrity and thus releases Ca$^{2+}$ into the intramembrane space, resulting in induction of apoptotic cell death. Numerous fluorescent dyes are available to detect Ca$^{2+}$ levels; with this in mind, it would be easy to design a time-course study investigating GSE effect on intracellular Ca$^{2+}$ levels as a function of time. Further examining GSE effect on calreticulin may also shed some light on the role of Ca$^{2+}$ in the GSE-mediated ER stress and apoptotic cell death.
Moreover, to further confirm GSE protein targets, additional protein analysis could be preformed for other DARTS identified GSE target proteins, including: GRP75; HSP7c; NUCL, and ALDOA. Additional DARTS assays could also be preformed utilizing signal components of GSE, specifically catechin or epicatechin, to determine what specific components of GSE target which proteins.

Furthermore, *in-vivo* studies could be preformed to further solidify GSE chemopreventive and anti-cancer efficacy; specifically, determination of GSE bioavailability in the efficacy studies in animals would be of clinical significance. Compounds within GSE could be isolated and quantified within the plasma and urine of these animals. Furthermore, considering miRNA expression is becoming a key clinical indicator of treatment success, we could also investigate GSE ability to alter miRNA expression within the plasma samples of these animals. To further validate GSE ability to decrease the formation of CRC lung metastatic lung tumors, we could examine the expression of other angiogenic markers, besides VEGF expression, such as: Integrins or MMPs. This metastatic CRC lung tumor model could be further utilized to test varying concentrations of GSE; specifically examining if there is a dose-response relationship between GSE feeding and metastatic lung tumors development. Additionally to examine GSE potential as an adjuvant chemotherapeutic agent, *in-vivo* studies could be preformed combining GSE supplementation with conventional chemotherapeutic agents, such as 5-FU. These additional studies, combine with the above information, and with the vast pre-clinical GSE efficacy studies, solidify GSE as a safe, effective, multi-targeted, anti-cancer and chemopreventive agent for CRC. These findings emphasizing the need to further evaluate GSE efficacy in human Phase I clinical trials for the treatment of CRC.
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APPENDIX A

GSE TREATMENT RESULTED IN CELL-CYCLE ARREST OF HUMAN CRC CELLS IN CHAPTER III

A  SW480

B  SW620

C  HCT116

187
APPENDIX B

GSE TREATMENT RESULTED IN ALTERATION OF CASPASE-INDEPENDENT CELL DEATH PATHWAYS IN CHAPTER III
APPENDIX C

THE EFFECTS OF GSE TREATMENT ON PROTEASOMAL ACTIVITY IN CHAPTER III

A  CT Proteasomal Activity

B  SW480  SW620
APPENDIX D

THE EFFECTS OF GSE TREATMENT ON ANIMAL BODY WEIGHT IN CHAPTER IV AND CHAPTER V

![Graph showing the effects of GSE treatment on animal body weight.](image-url)
APPENDIX E

GSE TREATMENT INHIBITED TUMOR MULTIPLICITY IN ALL REGIONS OF THE COLON IN CHAPTER IV

![Graph showing tumor multiplicity in different regions of the colon at 33 and 43 weeks.](image-url)