EMERGING EPigenetics, MetaboloMics AND OXidative Stress IN CANCER CHEmoprevention: The Potential Of DEITARY PHYtoCHEMICALS

By

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Cancer chemoprevention is defined as the strategy to block or slow the onset of premalignant tumors using relatively nontoxic chemical substance. Recently, accumulating experimental evidence has suggested that epigenetic alterations are involved in cancer development. The scope of epigenetics lies on the molecular interface between genetics and environmental factors; external factors switch genes on and off by influencing how cells read the genes, therefore, to regulate the transcriptomic profiles of organisms. DNA methylation, histone covalent modification and remodeling as well as miRNA-mediated gene silencing represents the major mechanisms that play important roles in epigenetic control of gene expression. Increasing evidence supporting redox imbalance and aberrant reactive oxygen species (ROS) are closely linked to the oncogenesis of various cancers. ROS-induced oxidative stress regulates multiple redox signaling
pathways that ultimately impact on cellular metabolic rewiring. Interestingly, epigenetic modifications such as DNA methylation and histone acetylation are sensitive to cellular metabolic status. Strong molecular link between metabolic reprogramming and epigenetic modifications through key metabolic intermediates, such as nicotinamide adenine dinucleotide (NAD), α-ketoglutarate (aKG), S-adenosyl methionine (SAM), and Acetyl-CoA (AcCoA), which are cofactors for the epigenetic enzymes and work as hubs between epigenetic processes and oxidative stress responses has been reported.

This thesis focused on elucidating the underlying intricate biological connectivity between metabolomic, epigenomic and transcriptomic regulation in blocking pro-tumorigenic signaling and elicit cancer-protective effects by dietary phytochemicals including Fucoxanthin (FX), Butyrate (B), Ursolic acid (UA) and Curcumin. The Nuclear Factor Erythroid-2 like 2 (NRF2)-ARE (antioxidant response element) signaling axis plays a critical role in many phytochemical-mediated cellular defense against oxidative and chemical stresses via induction of cellular defense and antioxidant enzymes. Normal healthy cells often maintain a low basal Nrf2 expression/activation, while cancer cells hold high intrinsic Nrf2 activity. Activation of Nrf2 protects normal cells from oxidative stress and cell damage, while activation of Nrf2 in cancer cells enhances drug resistance and cancer cell survival. Inhibition of Nrf2, on the other hand, sensitizes cancer cells to chemotherapeutic agents. Specifically, studies have shown that the higher the presence of the phase II detoxifying/antioxidant enzymes including heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO-1), NADPH, and
superoxide dismutase (SOD) in the normal healthy tissue/cells, the less susceptible to cancers. NRF2 and its interaction with AREs increase the transcription of these enzymes in normal condition. Previous in vivo studies have shown that NRF2 knockout (KO) mice exhibit significantly lower levels of cellular defense in various tissues, with an increased risk of developing carcinogen-induced cancers. Oppositely, cancerous and otherwise diseased cells often exhibit dysfunctional NRF2 regulation and overexpression of NRF2 in the nucleus of cells. This overabundance of NRF2 in the nucleus leads to increased expression of antioxidant response and cell survival genes often leading to drug insensitivity or resistance. This has become a common area of research for many groups and several small molecule inhibitors of NRF2 have been developed and implicated in overcoming multidrug resistance in cancer.

Our group utilized multi-omics approaches to evaluate the role of Nrf2 and the impact of FX on tumor promoter 12-O-tetradecanolyphorbal-13-acetate (TPA)-induced normal skin cell JB6 transformation. FX blocked TPA-induced ROS and oxidized glutathione (GSSG)/reduced glutathione (GSH), an oxidative stress index, in Nrf2 wild-type (WT) but not Nrf2-Knockdown (KD) cells. Both Nrf2 KD and TPA altered cellular metabolisms and metabolites which are tightly coupled to epigenetic machinery. The suppressive effects of FX on TPA-enhanced SAM (S-adenosyl methionine)/SAH (S-adenosylhomocysteine), a biosensor of the cellular metabolic state to influence the activity of methyltransferase enzymes, was abrogated by Nrf2 KD indicating Nrf2 plays a critical role in FX-mediated metabolic rewiring and its potential consequences on epigenetic reprogramming.
FX/Nrf2’s redox signaling drives metabolic rewiring causing epigenetic and transcriptomic reprogramming potentially contributing to the protection of TPA-induced JB6 cellular transformation skin cancer model. Besides the FX/Nrf2-mediated cancer protective effects in normal healthy cells, we also found that butyrate (B), a short-chain fatty acid produced from dietary fiber, increased NRF2 negative regulator Kelch-like ECH-associated protein 1 (KEAP1) expression (KEAP1-NRF2 signaling pathway) through inhibiting it’s promoter CpG methylation which further result in the NRF2 inhibition in colorectal cancer (CRC) HCT116 cells. Associative analysis of DEGs (differentially expressed genes) from RNA-seq and DMRs (differentially methylated regions) from CpG methyl-seq identified the tumor suppressor gene ATP binding cassette A1 (ABCA1) and tumor promote gene Early growth response protein 3 (EGR3) were correlated with their promoters’ CpG methylation indicating B regulates cancer markers through modulating their promoter methylation. B activated the mitochondrial tricarboxylic acid (TCA) cycle while inhibited the methionine metabolism which are both tightly coupled to the epigenetic machinery. B also regulated the epigenetic enzymes/genes including DNMT1, HAT1, KDM1A, KDM1B and TET1. Altogether, B’s regulation of metabolites coupled to the epigenetic enzymes illustrates the underlying biological connectivity between metabolomics and epigenomics. And B regulates KEAP1-NRF2 signaling, drives metabolic rewiring, CpG methylomic and transcriptomic reprogramming contributing to the overall antitumor effect in CRC cell model. In addition to the regulation effects of FX and B via NRF2 signaling pathways, UA as a natural pentacyclic
triterpenoid carboxylic acid phytochemical also known to possess antioxidant, anti-inflammatory, and cancer-preventive/anti-cancer effects. We found UA protects against Pten (phosphatase and tensin homologue deleted on chromosome 10; one of the most frequently mutated/deleted tumor suppressor genes which is mutated in 30-63% of primary prostate cancer (PCa)), KO-induced tumorigenesis at different stages of PCa. Epigenomic CpG methyl-seq revealed UA attenuated Pten KO-induced differentially methylated regions (DMRs) profiles. Transcriptomic RNA-seq showed UA abrogated Pten KO-induced differentially expressed genes (DEGs) of PCa related oncogenes’ Has3, Cfh and Msx1 overexpression indicating UA plays a crucial role in Pten KO-mediated gene regulation and its potential consequences on cancer prevention. Pathway analysis revealed UA elicits stronger protective effect on Pten KO-induced inflammatory- and cancer-associated signaling pathways modulation at early stage (12-week age) compared to late stage (20-week age) of PCa. Associative analysis of DEGs and DMRs identified the mRNA expression of tumor suppressor genes BDH2, and oncogenes Ephas, Isg15 and Nos2 were correlated with the promoter CpG methylation status in the early-stage comparison groups indicating UA could regulate the tumor promoter/suppressor genes through modulating their promoter methylation at early stage of prostate tumorigenesis. Metabolomic study showed UA attenuated Pten KO-regulated cancer-associated metabolism like purine metabolism/metabolites correlating with RNAseq findings, glycolysis/gluconeogenesis metabolism, as well as epigenetic-related metabolites pyruvate and lactate indicating UA plays a critical
role in \textit{Pten} KO-mediated metabolic and epigenetic reprogramming and its consequences on cancer development. Given that oxidative stress and inflammation-mediated epigenetic, metabolomic and transcriptomic reactions are important (micro) environmental factors in malignancy transformation, understanding the role of redox and inflammation as well as the metabolic metabolisms in epigenetic regulation could bring novel insights in cancer prevention. In summary, the phytochemicals used in current studies including FX, B and UA exert the antioxidant and cancer prevention effects via rewiring the metabolomic and reprogramming epigenomic and transcriptomic profiles in different cancer cell and animal models, and Nrf2 play roles in these regulations via regulating the redox hemostasis. The detailed mechanisms of these corresponding regulation effects were investigated in the following specific chapters.
PREFACE

This dissertation is to support the Degree of Doctor of Philosophy in Pharmaceutical Science at Rutgers, The State University of New Jersey. It documents my research work conducted from September 2017 to September 2022 under the guidance of Dr. Ah-Ng Kong in the Department of Pharmaceutics. The work presented here is original, except for where suitable references are made to previous work.

The dissertation consists of five chapters. All five chapters contain manuscripts that are either published or intended to be submitted for publication in a journal indexed by PubMed Central. Chapter 1 contains parts published in *Current Opinion in Toxicology* as a review article. Chapter 2 is published as an original research article in the *American Association of Pharmaceutical Science Journal*. Chapter 3 is published as an original research article in the *Molecular Nutrition & Food Research*. Chapter 4 is a manuscript submitted to *FASEB Journal* as an original research article which is under review now. Chapter 5 is published as an original research article in the *Journal of Pharmacokinetics and Pharmacodynamics*. 
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This thesis is dedicated to my parents, the two most important people in my life. Your unfailing support helped me overcome the most desperate circumstances. The love and support from my whole family finally goes a great deal in achieving this endeavor.
DEDICATION

To My parents
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Chapter 1 Epigenetics/epigenomics and cancer prevention by phytochemicals

1.1 Introduction
Cancer is a complex chronic disease and cancer development is a multistep process (1, 2). In a simplified manner, it would involve initiation, promotion, and progression/metastasis (3, 4). Recent evidence suggests that many chronic illnesses including cancer are driven by epigenetics/epigenomics caused by environmental factors impinging upon the underlying genetic information (4-7). Since advanced metastasized cancers are resistant to radiation and chemotherapeutic drugs, prevention of early stages of cancer by relatively non-toxic dietary phytochemicals would be logical.

1.2 Role of epigenetics/epigenomics during the “long process” of cancer development
During the long process of cancer development particularly during the “promotion”

1Part of this chapter has been published in Cancer Prevention Research 14 (2), 151-164.
2Keywords: Isothiocyanates (ITCs), Cancer Chemoprevention, Epigenetics/epigenomics, skin, colon, lung, breast, prostate cancer.
3Abbreviations: ITCs, Isothiocyanates; SFN, Sulforaphane; GS, glucosinolates; EMT, Epithelial-Mesenchymal Transition; NSCLC, Non-Small Cell Lung Carcinoma; hTERT, human Telomerase Reverse Transcriptase; OSCC, Oral Squamous Cell Carcinoma; NQO1, NAD(P)H: quinone oxidoreductase; HO-1: hemeoxygenase 1; NRF2, Nuclear Factor Erythroid-2-related Factor-2; NF-κB, Nuclear Factor Kappa B; DNMT-1, DNA methyltransferase 1; EGFR, Epidermal Growth Factor Receptor
stage, epigenetics has been postulated to play an important role in driving cellular transformation such as stem cells in forming benign microscopic tumor (8). Feinberg et al. has shown since the 1980s that most if not all tumors could be associated with widespread losses and some gains of DNA methylation throughout the genome (9, 10). This is reviewed recently and elaborated on the role of epigenetics during cancer development (6, 11). However it is still unclear how epigenetics would be integrated with the different hallmarks of cancer as discussed by Weinberg et al. (12).

Epigenetics or in a more global context epigenomics is a very complex process involving modulation of epigenetic modifiers including ‘readers’, ‘writers’ and ‘erasers’ with involvement of multiple events including methylation, acetylation, ubiquitination (13). Accumulating evidence also suggests that multiple genetic and epigenetic alterations would occur concurrently during tumor development (6, 14). In this context, the epigenetic marks (DNA methylation, histone modifications, chromatin remodeling, and non-coding RNA) could be use as potential markers of different stages of cancer development including initiation, progression, and metastasis (15, 16).

From our recent UVB-induced skin carcinogenesis study, global CpG methylation changes more dramatically in early stages of 2 weeks as compared to 15 weeks and 25 weeks post UVB irradiation. The CpG methylome changes decreased as time progresses taken into account of the effect of aging (17). Imaging assisted evaluation of microscopic tumors may have suggested that during the early promotion stage of carcinogenesis, drastic epigenomic aberrations occurs
particularly with the progenitor stem cells driving phenotypic gene expression, may occur before mutational events (6). These epigenomic alterations would involve stem cell dysregulation blocking differentiation, dysregulation of multiple pathways including DNA repair and cell cycle, apoptosis/autophagy, cellular defense, and inflammatory pathways. However, the underlying mechanism of UVB-induced global epigenomic changes in the skin is unclear. Acutely, UVB triggers oxidative stress, inflammation and DNA damage (18, 19), which would be postulated to be some of the major drivers of epigenetic alterations.

Analyzing primary cutaneous melanoma samples for their global DNA methylation, found more than 98% loss of methylation and about 2% gain of methylation (20). In vitro study using melanoma cells showed that histone hypoacetylation is associated with downregulation of certain pro-apoptotic proteins, including Bak, Bim, and Bax, which belong to the BCL-2 family (21). Epigenetic reprogramming appears to play an increasing role in drug resistant melanoma (22). In the skin transformation model using epidermal JB6 cells, when JB6 cells were challenged with tumor promoter TPA (12-O-Tetradecanoylphorbol-13-acetate), significant alterations of DNA methylome coupled with transcriptome have been discovered (23). In a recent UVB-induced mouse carcinogenesis epigenomic study, about 60% of the DNA-methylated-regions (DMRs) showed inversed relationship between DNA methylation and RNA expression, i.e., hypermethylation coupled with suppression of transcription or hypomethylation coupled with promotion of transcription, while the other 40% of these DMRs did not show such relationship (23).
In breast cancer, many studies have shown strong association of aberrant DNA hypomethylation with cancer development (24). Histone modifications such as loss of H4K16 acetylation has been implicated as an early event in breast cancer development and is associated with altered level of NAD-dependent histone deacetylases (HDACs); NAD-dependent deacetylase sirtuin-1 (SIRT1) (25, 26).

In prostate cancer, up-regulation of EZH2 (Histone Methyltransferase, Enhancer of Zeste Homolog 2) appears common in both localized and metastatic prostate cancer and associated with poor prognosis (27, 28). In early prostate carcinogenesis, during the transition from benign prostate epithelium to inflammatory lesions, DNA hypermethylation is observed in the promoter regions of key tumor suppresser genes such as GSTP1, RASSF1A, and APC (29, 30). In TRAMP mice and TRAMP-C1 cell line, hypermethylation of Nuclear factor erythroid 2-related factor 2 (Nrf2)'s CpGs occurred (31), and global alteration of epigenomic DNA methylation was found with profiling using MeDIP-seq in TRAMP prostate tumor (32). In a PTEN deletion mouse prostate carcinogenesis model, PTEN deletion drives aberrations of DNA methylome and transcriptome in different stages of prostate carcinogenesis (33). Prostate cancer exhibited global alterations of histone modifications, including histone acetylation (H3K9, H3K18, and H4K12) and methylation (H3K4me2) (34). Gerhauser et al. (35) investigated the molecular evolution of 292 early-onset prostate cancer patients using Illuminia 450K methylation array identified some unique DNA methylation patterns of 500 most discriminatory CpG sites between the different cell types (basal, stromal, normal luminal and tumor luminal) and found that the
“purity-adjusted epigenetic prostate cancer index” (PEPCI) associated with increased Gleason score (36). By integrating the DNA methylation and RNA expression data from tumors diagnosed with early-onset, they were able to identify four robust subgroups which could stratify the patients into high- and low-risk groups (35).

Epigenetics plays an increasing role in the pathophysiology of colorectal cancer (CRC) (37). In an AOM-DSS induced colitis-accelerated colon cancer mouse model, differential CpG methylome and transcriptome occurs as compared to control mice (38). CpG island methylator phenotype (CIMP), has been identified as one of the molecular features of CRC (39, 40). Based on CIMP profiles, primary CRC may be clustered into three distinct but relatively homogeneous subclasses: CIMP1, characterized by intense methylation of multiple genes including MSI and BRAF mutations; CIMP2, increased methylation with age-related genes, and mutations in KRAS; and CIMP negative, characterized by rare methylation with p53 mutation (39). CIMP1 and CIMP2 phenotypes are more often expressed in the proximal colon; CIMP1 has a good prognosis, whereas CIMP2 has a poor prognosis (41). In general, CIMP appears to be associated with significantly worse prognosis in CRC patients (42).

1.3 Overview of epigenetic mechanisms and their role in cancer prevention

Epigenetic mechanism appears to provide a robust means for organisms to respond to environmental cues through changes in gene expression (43), working in concert with other more rapid responders including channels/receptors
mediated signaling. These post-translational modifications act in a coordinated fashion leading to chromatin conformational alterations that, in turn, regulate the genetic information accessed by transcription factors (44). The schematic representation of epigenetic pathways in stepwise carcinogenesis process and cancer prevention by the phytochemical isothiocyanates (ITCs) impinging upon and integrating with the different cellular processes are summarized in Figure 1.1. Epigenetic machinery would affect all the stages during carcinogenesis. Among the three epigenetic mechanisms, DNA CpG methylation is probably the most studied as reviewed by Feinberg (6). Specifically, DNA methylation entails the conjugation of methyl groups in cytosine (C) residues usually occur in CpG dinucleotide sequences, dispersed across the genome, leading to the formation of 5-methylcytosine (5mC) (45). DNA methylation is catalyzed by a class of enzymes known as DNA methyltransferases (DNMTs) the most predominate which include DNMT1, DNMT3A and DNMT3B. Unlike methylation, histone acetylation causes gene activation whereas histone methylation can result in either activation or repression of genes depending on the site of modifications (46). HDACs along with histone acetyltransferases (HAT), provide a significant mechanism of gene regulation involving removal and addition, of acetyl groups from an ε-N-acetyl lysine amino acid on histone proteins. The third epigenetic control would be regulated through non-coding-RNA-based mechanisms including micro-RNA. In particular, micro-RNA transcription can be regulated by both histone modification and DNA methylation, and micro-RNA themselves can, in turn, regulate key enzymes that enforce epigenetic remodeling (47).
Several chemotherapeutic drugs targeting epigenetic signaling have been approved by the FDA (14), and a growing list of dietary phytochemicals found naturally in food and plants have been studied targeting epigenetic mechanism during the carcinogenesis process for cancer prevention including modifications of histones and DNA methylations (48, 49). For instance, curcumin, a powerful anti-inflammatory/antioxidant agent, regulating multiple signaling pathways, is also found to modulate epigenetics/epigenomics pathways such as histones modifications, and DNA methylation (50, 51). Many natural products have been reported to inhibit the expression of HDACs, including curcumin (52, 53), ursolic acid (UA) (54), and sulforaphane (SFN) (55, 56). Triterpenoids such as cucurbitacin, ginsenoside Rh2, ginsenoside compound K and UA, have been shown to induce global hypomethylation, enhance hypermethylation/hypomethylation of the promoters of oncogenes and tumor suppressor genes, and modify miRNA by targeting DNMT1 (48). SFN could regulates DNA demethylation by down-regulating the expression of DNMT1 and DNMT3B, thus inducing the demethylation of the cyclin D2 gene promoter and its expression in cancer cells (57). Question remains as to how all these chemopreventive phytochemicals modulate the epigenetic machinery. Since many of these compounds are redox active, therefore, we would like to postulate that redox signaling triggered by these compounds could play a role in modulating the epigenetic machinery, but further study would be needed.

1.4 Role of redox signaling in the modulation of epigenetic machinery
Cellular oxidative stress occurs when the cellular antioxidant/reductive capacity is overwhelmed by the oxidative challenges (58, 59), and could play a vital role in epigenetic reprogramming (60). Many epigenetic marks, including histone methylation, histone acetylation, ADP-ribosylation and DNA methylation, may be directly associated with central metabolism through redox intermediates (60). One mechanism by which reactive oxygen species (ROS) can affect DNA methylation is through its action on DNMTs activity or expression (61). In an oxidizing redox chromatin microenvironment and in the absence of S-adenosyl methionine (SAM), DNMT3a and DNMT3b can catalyze the direct conversion of 5mC or 5-hydroxymethylcytosine (5hmC) to an unmodified C, although the exact mechanisms are still unclear (62). Ye et al. have proposed an iron–redox–methylation hypothesis (63) in which excess amount of irons perturbs DNA methylation by at least two possible mechanisms: first, oxidative stress depletes GSH and drives SAM/SAH cycle to produce more SAH, which inhibits DNMT activity; and second, iron directly inhibits DNMT activity. The methylation process can be reversed by DNA demethylases such as ten-eleven translocation enzymes (TETs) (64). ROS are also thought to regulate DNA methylation by targeting the expression and/or activity of the TETs. Knockdown of TET1 significantly increase hydrogen peroxide-induced apoptosis of cerebellar granule cells, and cerebellar granule cells from Tet1 KO mice are more sensitive to oxidative stress (65). In addition, mutagenic lesion of ROS-induced DNA damage of O6-methylguanine can inhibit binding of DNMT leading to hypomethylation with inhibition of methylation of adjacent cytosine nucleotides (66). Incorporation
of 8-OHdG and the oxidation by-product of 5-mC in the Methyl-CpG binding protein (MBP) recognition sequence resulted in significant inhibition of the binding affinity of MBP (67).

In addition to epigenetic regulation of DNA methylation, redox also participates in histone acetylation and histone methylation. The histone deacetylation is mediated HDACs. A study has shown that the redox-regulating protein Thioredoxin 1 (Trx1) regulates the nucleocytoplasmic shuttling of class II HDACs through a redox-dependent mechanism (68). By forming a multiprotein complex with DnaJb5, a heat shock protein 40, and TBP-2, a Trx1-binding protein, Trx1 reduces HDAC4, a class II HDAC, at Cys-667 and Cys-669, which are easily oxidized to form a disulfide bond in response to hypertrophic stimuli (69). Hu et al. showed that the catalytic activity of HDAC5 suppresses mitochondrial ROS generation and subsequent induction of NRF2-dependent antioxidant gene expression in cardiomyocytes (70). Collectively, there is strong evidence that ROS are capable to modulate chromatin accessibility by affecting histone acetylation state in particular via multiple modifications of HDAC expression and activity (71, 72). Histone acetylation is reversible and is regulated by a group of histone acetyltransferases (HATs) (73). Histone methylation is maintained by two classes of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMs) (74). HMTs and HDMs can also be subjected to redox regulation such that post-translational modifications of histone proteins by oxidants and environmental stresses can trigger gene transcription involved in chronic inflammatory events (75). Since many dietary chemopreventive
phytochemicals possess redox (76, 77) and epigenetic/epigenomic properties (78), question remains the direct linkage of phytochemical’s redox signaling and epigenetics/epigenomics.

1.5 Phytochemicals ITCs and cancer prevention

Isothiocyanates (ITCs) are a family of phytochemicals derived almost exclusively from plants, although marine sponges and fungi also have been reported to produce a few ITCs (79, 80). ITCs are synthesized and stored as glucosinolates (β-thioglucoside N-hydroxysulfates) as secondary metabolites in plants including the cruciferous. Glucosinolates are relatively stable in plant cell. However, when the plant tissue containing glucosinolates is damaged, as in the case of preparation (cutting, chopping, mixing) or chewing food, a β-thioglucosidase called myrosinase is released and hydrolyzed the glucosinolates to various metabolites, including the ITCs (81). When cruciferous are cooked before consumption, myrosinase is inactivated and glucosinolates transit to the colon where they are hydrolyzed by the intestinal microbiota to ITCs (81). By far the most studied ITC is sulforaphane (SFN), derived from broccoli (80), although other ITCs, including phenylethyl ITC (PEITC), benzyl ITC (BITC) and allyl ITC (AITC), also possess chemopreventive properties. Multiple molecular mechanisms are involved in the chemopreventive effect of ITCs. Below, we will focus on epigenetic mechanisms in in vitro and in vivo studies in skin, breast, lung, colon and prostate cancer, as well as some clinical chemoprevention studies with ITCs.
Skin cancer

The ITCs show chemopreventive effects against ultraviolet (UV)- and chemically-induced skin carcinogenesis (82) and the involvement of the epigenetics in epidermal carcinogenesis has been studied in melanoma (83). The activity of SFN on epigenetic regulation of skin cancer has been reported on PcG proteins’ (e.g. Bmi1 and EZH2) function by means of blocking tumor progression through their inhibition thus decreasing H3K27me3 level. (84). In squamous cell carcinoma (SCC), SFN treatment suppressed cancer progression and metastasis in vivo through reduction in arginine methylation at H3 (85). As discussed earlier, UVB would drive perturbation of CpG methylome at initiation, promotion and progression stages of skin carcinogenesis, but importantly, SFN would block carcinogenesis more effectively at early stages than late stages and would attenuate some of the perturbations of UVB-induced CpG methylome (86).

In cell culture model, SFN was also found to demethylate CpGs of NRF2 promoter, resulting in re-activation of NRF2-dependent expression of heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO-1), and UDP-glucuronosyltransferase 1A1 (UGT1A1) potentially leading to inhibition of TPA-induced transformation in epidermal JB6 cells (87). Such induced NRF2 expression was attributed to promoter hypo-methylation through down-regulation of DNMTs and inhibition of overall HDAC enzyme activity (87). Professor Paul Talalay who first isolated and identified SFN (88), and he also advanced the concept of redox/electrophile-mediated induction of phase 2 detoxifying genes (89). Oxidants and electrophiles including SFN could modify cysteine’s sulfhydryl
groups of Kelch-like ECH-associated protein-1 (KEAP1), prevent proteasomal degradation, allow newly synthesized NRF2 to enter the nucleus, and enhance NRF2-target gene transcription (reviewed in (90-92)). Interestingly, however, electrophile such as SFN and oxidant such as H₂O₂ may also regulate NRF2 via redox sensitive cysteine on the Nuclear Export Signal (NES) (93) as well as post-transcriptional/translation control of NRF2’s Internal Ribosomal Entry Sites (IRESs) (94). Question remains, how these different redox signaling of NRF2 would operate under different redox environments such as different redox agents, acute versus chronic redox agent’s stimulating conditions and pathological conditions.

Lung cancer
Several studies show an inverse relationship between consumption of dietary ITCs, and lung cancer risk (95). In vitro cell culture study shows the involvement of SFN and miR-616-5p in EMT and NSCLC metastasis (96). miR-616-5p directly targets GSK3β and decreased its expression, whereas SFN decreased miR-616-5p by histone modification and followed by inactivation of the GSK3β/β-catenin signaling pathway and inhibition of EMT in NSCLC cells (96). SFN inhibits HDAC activity and increases acetylated histones H3 and H4 in A549 and H1299 lung cancer cells (97). In lung cancer stem cells (CSCs) miR-19a and miR-19b expression are up-regulated and SFN suppresses miR-19 and Wnt/β-catenin pathway resulting in inhibition of lung CSCs (98). In human lung cancer A549 cells, SFN epigenetically demethylates the CpG sites of the miR-9-3 promoter
and reactivates miR-9-3 expression (99). Similarly, SFN was found to up-regulate miR-214 and mediate downregulation of c-MYC in non-small cell lung cancer, potentially results in inhibition of cancer stem-like cell properties and cisplatin resistance (100).

Colon cancer

In colon cancer model, SFN suppressed tumor development in Apc(min) mice, increased acetylated histones in the polyps, and inhibited HDAC in APC(min) mice (101). SFN treatment in human colon adenocarcinoma Caco-2 cells showed increased activation of NRF2 by demethylation of the NRF2 promoter region and reducing expression of DNMT1. In polyposis in rat colon (Pirc) model, single oral administration of SFN and structurally related long-chain ITCs decreased HDAC3 expression and increased pH2AX levels in adenomatous colon polyps (103). HCT116 and SW480 colon cancer cells study showed that SFN or its analogs altered HAT/HDAC activities and histone acetylation status, lowered the expression of HDAC3, P300/CBP associated factor (PCAF) and lysine acetyltransferase 2A (KAT2A/GCN5), and attenuated homologous recombination (HR)/non-homologous end joining (NHEJ) repair activities (103). A 0.12% PEITC-enriched mouse-diet reduced mucosal and submucosal inflammation possibly via modulating regulating NFκB proteins and NFκB mRNA expression was inversely correlated with tri-methylation of lysine 27 on histone 3 near its promoter region in a time-dependent manner (104). Interestingly, colon cancer cells treated with low-dose PEITC for >1 month exhibited stable
alterations in expression profile of epigenetic writers/erasers and chromatin-binding of HDACs and Polycomb-group (PcG) proteins (105). Sustained PEITC exposure not only blocked HDAC protein binding to euchromatin but was also associated with hypomethylation of PcG target genes that are typically hypermethylated in cancer (105). In human colon cancer cells ITCs inhibit HDAC enzyme activity and increase HDAC protein turnover and ITCs enhanced the acetylation and subsequent degradation of critical repair proteins, such as CtIP (106).

Breast cancer

Studies show that SFN modulates breast cancer risk at multiple stages of carcinogenesis through different biologic mechanisms including epigenetic modulations (107). SFN inhibited in dose- and time-dependent of hTERT in both MCF-7 and MDA-MB-231 human breast cancer cells. DNMT1 and DNMT3a, were decreased in SFN-treated breast cancer cells suggesting that SFN may repress hTERT by impacting epigenetic pathways (108). In combination studies, SFN and EGCG activated ERα MDA-MB-231 cells which was associated with significant reduction of DNMTs expression and activity, as well as of HDAC activity (109). Synergistic effect on MDA-MB-231 xenograft tumor growth, with combination of SFN and LSD1 inhibitor (110). In transplacental breast cancer chemoprevention study, SFN containing broccoli sprout diet upregulates tumor suppressor genes p53 and p16INK4a and downregulates tumor-promoting genes TERT and c-Myc potentially as a result of inhibition of HDAC1 expression (111).
SFN in combination with genistein downregulates HDAC2, HDAC3 and KLF4 protein expression and reduced tumor volumes/sizes (112).

Prostate cancer
In prostate cancer, SFN reactivated and induced its downstream antioxidant stress pathway in TRAMP-C1 cells through epigenetic modification of \textit{NRF2} promoter, and could play a role in chemoprevention (113). The expression of long noncoding RNAs (lncRNAs) in prostate cancer cells was altered and SFN restored the altered expression of lncRNAs (114). Increased SFN consumption in mice with prostate cancer cell xenografts showed reduced HDAC enzyme activity in prostates, and peripheral blood mononuclear cells (115). SFN inhibited the expression and activity of human telomerase reverse transcriptase (hTERT). SFN treatment selectively decreased HDAC activity, and Class I and II HDAC protein’s expression increased acetylated histone H3 at the promoter for P21, induced p21 expression and increased tubulin acetylation in prostate cancer cells (116).

1.6 Cancer chemoprevention by phytochemical ITCs via non-epigenetic signaling pathways
Numerous studies have shown that many non-epigenetic mechanisms by which ITCs exert their biological anti-carcinogenic activities have been being well investigated including induction of carcinogen detoxication (117), enhanced DNA damage repair (118, 119), anti-inflammatory pathway through \textit{NF-κB} regulation
elimination of cancer stem cells (121) and other cells (122), inhibition of cell cycle progression (123), induction of caspase/apoptosis (124), induction of autophagy (125), and mitogen-activated protein kinase (MAPK) signaling pathway (126), among others. As discussed above, the most studied and most relevant signaling pathway in the context of cancer prevention elicited by ITCs would be the NRF2 mediated signaling pathway. As reviewed by many scientists, and discussed above, ITCs react with sulfhydryl groups of C residues of KEAP1, releasing NRF2 from KEAP1 binding and then NRF2 translocating to the nucleus, partnering with sMafs and binding to the ARE found in many antioxidant/detoxifying enzymes including glutathione S-transferases (GSTs), thioredoxin, NQO-1, HO-1, among others (127-131). Many studies have also shown that the ITCs are potent anti-inflammatory agents. SFN significantly reduced the nuclear translocation of the pro-inflammatory transcription factor NF-κB in pancreatic acinar cells, downregulating the expression of NF-κB target genes that code for pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and inflammatory enzymes cyclooxygenase-2 (COX-2), prostaglandin E (PGE) synthase, and inducible nitric oxide synthase (iNOS) (120, 132). ITCs can also modulate the activity of phase I metabolizing enzymes (133-135), such as downregulating CYP3A2 mRNA expression, as well as the activity of benzyloxyquinoline debenzylase, a marker of CYP3As and upregulating CYP1A1 and CYP1A2 mRNA expression and the activity of ethoxyresorufin-O-deethylase (EROD), a marker of CYP1A1/2 activities (136). Some procarcinogens require activation by
phase I enzymes in order to become active carcinogens capable of binding DNA and forming cancer-causing DNA adducts (137). Inhibition of specific CYP enzymes involved in carcinogen activation has been found to prevent the development of cancer in animal models (138). In addition, ITCs have been shown to modulate the expression of cell cycle regulators, cyclins and cyclin-dependent kinases (CDK), and trigger apoptosis in cancer cell lines (139). For example, in a mouse model of CRC, PEITC administration reduced both the number and size of polyps and these alternations were associated with activation of the CDK inhibitor, p21, inhibition of various cyclins (A, D1, and E), and induction of apoptosis (140). ITCs also have been shown to suppress the formation of capillary-like structures from human umbilical endothelial cells and likely inhibit the expression of hypoxia inducible factors (HIFs) that control angiogenesis in endothelial cells and malignant cell lines inhibition of angiogenesis (141). As discussed above for BITC (142), SFN has been shown to induce autophagy in both *in vitro* cell culture models and *in vivo* models associated with decreased cell proliferation, alterations in protein levels of autophagy regulators Atg5 and phospho-mTOR (125, 143). Some other anticancer pathways such as inhibition of cell migration and invasion (144-146), have also been reported. Question remains how these non-epigenetic signaling pathways would be linked and integrated with epigenetic mechanisms elicited by ITCs in the context of cancer prevention necessitates further study.

**1.7 Pharmacokinetics and Pharmacodynamics of phytochemical ITCs**
Pharmacokinetics (PK) and pharmacodynamics (PD), a quantitative science, integrate and evaluate the dose/concentration-response relationships in the body following the administration of drugs (147, 148). Despite many reports studying the PK/PD of ITCs, there are not many showing the “direct quantitative relationship” between the dose/concentration of ITCs and the biological/pharmacological response(s) in in vivo animal models or in humans. In absorption, after oral administration of SFN in the rats the peak concentration (Cmax) were reported to be attained in 1 h, indicating rapid absorption (149). However, increasing the dose of SFN from 0.5-5 mg/kg, the oral absorption rate constant (ka) decreased and Cmax did not increase proportionally to the dose, suggesting non-linear absorption kinetics (149). In human, SFN was absorbed rapidly and achieved Cmax in 1 h after the consumption of broccoli sprout products (150, 151). Comparison of the area under the blood/plasma concentration time curve (AUC) AUC\textsubscript{0-24} values between the intravenous (IV) and oral treatment groups, at low dose (2.8 μmol/kg) shows an absolute bioavailability of 82%, which decreased to 20% at the higher dose (28 μmol/kg) (149).

In drug distribution, a rapid decrease was observed in the plasma concentration of SFN after both oral and IV dosing indicating rapid and extensive cellular uptake into various tissues (149). This is consistent with studies showing ITCs achieve very high intracellular concentrations as a result of their interaction with GSH (152-154). Such extensive intracellular localization explains the very large apparent volume of distribution (Vd) of 102 L/kg after IV injection in rat.
Additionally, the $V_d$ of SFN decreased dramatically from 102 to 42 L/kg with increasing doses from 0.5 to 5.0 mg/kg (149), showing non-linear $V_d$. In another study of SFN PK, the volume of distribution at steady state (Vss) was around 3.7 L or 14.8 L/Kg after an IV dose of 25 mg/kg of SFN (155) and this result is consistent with Hanlon et al.’s findings (149).

Freeze-dried aqueous extract of broccoli sprouts inhibited bladder cancer development induced by N-butyl-N-(4-hydroxybutyl) nitrosamine significantly and dose-dependently, with over 70% of the ITCs present in the extract were excreted in the urine as ITC equivalents (ITCs + dithiocarbamates) within 12 h after a single oral dose, indicating substantial urinary excretion (156). Urinary concentrations of ITC equivalents were 2 to 3 orders of magnitude higher than those in plasma (156). In a cross-over human study of glucoraphanin-rich (GRR) versus SFN-rich (SFR), urinary excretion of SFN and its metabolites (in approximately 12-hour collections after dosing), was substantially greater with the SFN-rich (mean = 70%) than with GRR rich (mean = 5%) beverages (157). These studies again suggest that substantial renal excretion of ITC or its metabolites occur.

In terms of PD, several studies have reported the increased levels of Nrf2-targeted enzymes GSTs and NQO1 in plasma (158) and saliva (159) in humans volunteers after consuming cruciferous vegetables. Similarly, administration of SFN-rich preparations to healthy subjects led to increased mRNA or protein levels of NQO1 and GSTs in skin punch biopsies, blood cells, and buccal scrapings (160-162). As a proof of conceptual study, after IV administration of 25
mg/kg of SFN, moderate increase in mRNA (2-5 folds) of HO-1, NRF2, and NQO1, while significant increase (> 5 folds) for GSTT1, GPx1, and Maf in rat lymphocytes (155). These PK/PD effects of SFN could be modeled with indirect response (IDR) model directly linking plasma SFN concentrations with NRF2-mediated gene expression response in lymphocytes (155). Future studies involving integrating acute PK/PD and long-term cancer preventive effects of ITCs in human would be needed.

1.8 Ongoing clinical studies/trials of phytochemicals and chemoprevention

Pre-clinical carcinogenesis studies of dietary ITCs have demonstrated extraordinary chemopreventive efficacy. Several clinical trials have illustrated that ITCs, administered orally as glucosinolate precursors or directly in its bioactive compound such as SFN, PEITC have preventive effects against cancers (163) (164). Specifically, SFN and PEITC have been studied in some clinical studies and briefly summarized in Table 1.1 (165-171). More ongoing ITCs clinical trials are listed in clinicaltrials.gov. In one particular clinical study involving male patient with recurrent prostate cancer, none of the patients experienced PSA doubling, a marker for disease severity, after serving SFN daily for 20 weeks (172). HDAC expression and activity inhibition has been used as a biomarker in some trials (173, 174) and one clinical study with healthy subjects showed that HDAC activity was significantly inhibited in PBMCs as early as 3 hours after consumption of 68 g of broccoli sprouts (115). Study using SFN in breast cancer patients showed an average decrease of 80.39 pmol/min/mg protein in blood
HDAC activity from pre- to post-intervention (169). A clinical study of PEITC as an inhibitor of metabolic activation of a tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) for cancer prevention, the NNK metabolic activation ratio was significant reduced by 7.7% (171). ITCs have shown promising efficacy in various clinical cancer chemoprevention trials, however, future clinical trials involving epigenetic endpoints are warranted.

1.9 Conclusion and future prospective
ITCs found abundantly in the human diet and are derived from cruciferous vegetables, are highly promising cancer preventive agents. Multiple and multi-targeted mechanisms, including epigenetic reprogramming, could be involved in the preventive and therapeutic effects of ITCs in cancer. Gene silencing through hypermethylation of CpG of tumor oncogenes/proto-oncogenes, gene activation through hypomethylation of CpG of tumor suppressor genes, and other modifications including phosphorylation, acetylation, ubiquitination are potential cellular targets of epigenetic regulation by ITCs. Genome-wide DNA methylation analysis might help to better understand the targeted tissue specificity and global events in the regulation by ITCs. Extensive research on the extrapolation of preclinical to clinical dosages of ITCs for prevention studies needs to be further refined. Understanding of the PK/PD of parent/metabolites of the ITCs, its tissue levels and modulation of signaling mechanisms including epigenetic mechanisms is warranted in human studies.
Cancer is a complex chronic disease and cancer development is a multistep process (1, 2, 12, 175, 176). The ITCs as discussed above belongs to a very powerful class of biologically active food components, they are relatively non-toxic at low physiological and even higher pharmacological doses, with good oral bioavailability (149, 157), powerful anti-oxidative stress/anti-inflammatory (80) and possess epigenetic modifying properties as well as with great anti-cancer efficacy in many *in vitro* cell culture models and *in vivo* animal models. The exact molecular mechanism of how the ITCs as well as many other dietary chemopreventive phytochemicals modifying the epigenetic machinery is currently unclear. However it is highly likely through redox biological process playing an important role (60) analogous to the kinases/caspases activation as we have discussed back in the 1990s (177-179). Additionally, it would be tempting to speculate that the ITCs particularly SFN and PEITC, although differ in some aspects of signaling pathways at same micromolar concentrations, SFN is a more potent *NRF2* activator than PEITC, and SFN activates *ERK* more strongly compared to JNK, whereas PEITC would be reversed (126, 180, 181), they would be considered as “general” cancer preventive agents not unlike the chemotherapeutic agents such as platinum-based or the taxane-based drugs. Analogous to chemotherapeutic agents, for maximal efficacy, the ITCs would need to be used in combination with other cancer preventive agents including other less toxic dietary phytochemicals, natural products or drugs such as NSAIDs, SERMs, aromatase inhibitors, and 5-α-reductase inhibitors (182) targeting different signaling pathways. Together with other considerations and
initiatives such as immunoprevention (183, 184) (185), the long-term control and prevention of progression of tumors to advanced and metastatic states may soon to be realized.
Figure 1.1 Schematic representation of epigenetic pathways in stepwise carcinogenesis process and chemopreventive effects of phytochemical isothiocyanates (ITCs).

Major epigenetic mechanism involved Histone modification, DNA modifications, and miRNA regulation. The role of ITCs in modifying/reversing of these epigenetic mechanisms and signaling pathways including reactive oxygen/nitrogen species (RO/NS), xenobiotic metabolizing enzymes (XMEs), DNA damage/repair, cellular kinases, onco/proto-oncogenes, inflammatory, matrix metalloproteinases (MMPs), epithelial-mesenchymal transition (EMT) biomarkers, and immune suppression, among others, in the process of carcinogenesis via initiation, promotion and progression. (↑ activation, ↓ inhibition, ⊥ blocking)
Table 1.1 Summary of completed and ongoing clinical trials using phytochemicals SFN and PEITC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Cancer type</th>
<th>Experiment detail</th>
<th>Outcome/Ongoing targets</th>
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<tbody>
<tr>
<td>SFN/Broccoli Sprout Extract</td>
<td>Lung</td>
<td>291 healthy subjects; broccoli sprout beverages; GR (600 μmol) and SFN (40 μmol); 84 days 50 healthy subjects; SFN-rich broccoli sprout beverage; 7 days 30 healthy subjects, young smokers; broccoli diet; 250 g/day; 10 days 72 former smokers with lung cancer; SFN; 120 μmol/time, 2 times/day; 12 months</td>
<td>Increases urinary excretion of benzene (61%) and acrolein (23%) 20-50% ↑ in excretion levels of glutathione conjugates of acrolein, crotonaldehyde and benzene after SFN treatment DNA repair activity ↑ in PBMC Targets: lung cancer chemoprevention with SFN in former smokers</td>
</tr>
<tr>
<td>PEITC/Watercress</td>
<td>Lung</td>
<td>11 healthy smokers; watercress;</td>
<td>↑ Urinary NNAL plus NNAL-Gluc (33.5%)</td>
</tr>
<tr>
<td>SFN, sulforaphane; PEITC, phenethyl isothiocyanate; GR, glucoraphanin; PBMC, Peripheral blood mononuclear cells; PSA, prostate-specific antigen; HDAC, Histone Deacetylase; NNAL-glu, 4-(methylnitrosamo)-l-(3-pyridyl)-l-butyl /3-D-glucopyranosiduronic acid; NNK, Nicotine-derived nitrosamine ketone; DCIS, Ductal carcinoma in situ; ↑, increase/activation; ↓, decrease/inhibition.</td>
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<tr>
<td>56.8 g / meal for 3 meals/day; 3 days 82 healthy smokers; PEITC 10 mg 4 times/day for 5 weeks</td>
<td>↓ NNK metabolic activation ratio (7.7%)</td>
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Chapter 2 NRF2 regulates metabolic rewiring and epigenetic reprogramming in mediating cancer protective effect by Fucoxanthin \(^{4,5,6}\)

2.1 Introduction

The skin, as the largest organ and outermost layer of the body, is continually exposed to environmental insults including UV irradiation, tumor promoters and environmental pollutants resulting in increasing skin cancer incidence worldwide (186). The mitochondria of skin cells have been ascribed to produce copious amounts of ROS when exposed to tumor promotors such as UV irradiations (187) and TPA (188) that could result in direct or indirect free radical damages to nucleotides, proteins, and lipids, promoting DNA damage, genetic instability and ultimately carcinogenesis (189, 190). Increasing evidence supporting redox imbalance and aberrant ROS are closely linked to the oncogenesis of skin cancers (191). FX, the most abundant xanthophyll carotenoids phytochemicals, is known for its protective functions against oxidative stress and inflammation, as well as modulating effect on redox and epigenetic signaling (192, 193). Previous studies shown that the phytochemicals including FX regulates ROS status via

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\(^{5}\)Keywords: Fucoxanthin; Nuclear Factor Erythroid-2 Like 2 (Nrf2/Nef2l2); Reactive oxygen species (ROS); Epigenetic; Metabolic rewiring.

\(^{6}\)Abbreviations: UV, Ultraviolet; ROS, Reactive oxygen species; TAP, 12-O-tetradecanolyphorbal-13-acetate; FX, Fucoxanthin; Nrf2/Nef2l2, Nuclear Factor Erythroid-2 like 2; ARE, Antioxidant Response Element.
Nrf2-ARE signaling pathways as well as protection against tumor promoter TPA-induced cellular transformation of mouse epidermal JB6 cells potentially via epigenetic CpG demethylation and reactivation of Nrf2 (194).

TPA is the most widely used tumor-promoting agent in experimental skin carcinogenesis model, which has been shown to generate a vast amount of ROS and oxidative stress through protein kinase C (PKC)-mediated activation of the stress-activated protein kinase (SAPK) pathway (195). ROS-induced oxidative stress regulates multiple redox signaling pathways that ultimately impacts on cellular metabolic rewiring (196). For instance, AMP-activated protein kinase (AMPK), the energy sensor in the cells, is activated in response to oxidative stress and promotes metabolic reprogramming (197). The altered mitochondrial metabolism and metabolic reprogramming have been considered a hallmark of cancer, since it can also regulate processes that are associated with cell proliferation, migration and invasion (198). Otto Warburg, a German physiologist and nobel laureate, first described in 1931 that cancer cells exhibit an altered metabolism, metabolizing glucose anaerobically, even in the presence of oxygen, with an increase in lactate production, thus supporting cell survival and growth (199). Metabolic rewiring is fundamental for the survival of transformed tumor cells in a hostile environment with limited nutrients, low oxygen levels and immune surveillance and for supporting accelerated cell proliferation and enhancing other biological survival processes of tumor cells (198, 200). ROS-induced epigenetic modifications are often manifested by global hypomethylation of the genome and an abnormal hypermethylation in the CpG island region of
genes. Thus, overproduction of ROS causes DNA/histone modifications resulting in the deactivation of the tumor suppressor genes and the activation of oncogenes by regulating DNA methyltransferases (DNMTs) and HDACs (201). Most recently, increasing in vitro (202) and in vivo (203) evidences suggest that mitochondrial metabolism could play an important role in skin cancer and that mitochondrial metabolism/metabolites are tightly coupled to the basic epigenetic machinery (204). Previous studies reported the epigenetic modifications such as DNA methylation and histone acetylation are sensitive to cellular metabolic status (205). Strong molecular link between metabolic reprogramming and epigenetic modifications through key metabolic intermediates, such as nicotinamide adenine dinucleotide (NAD), α-ketoglutarate (aKG), S-adenosyl methionine (SAM), and Acetyl-CoA (AcCoA), which are co-factors for the epigenetic enzymes and work as hubs between epigenetic processes and oxidative stress responses have been reported (60, 206, 207).

The NRF2-ARE signaling axis plays a critical role in many phytochemical-mediated cellular defense against oxidative and chemical stresses via induction of cellular defense and antioxidant enzymes (92). Studies have shown that the higher the presence of the phase II detoxifying/antioxidant enzymes including HO-1, NQO1, NADPH and SOD in the tissue, the less susceptible the tissue is to cancers. Nrf2 and its interaction with AREs increase the transcription of these enzymes (208). Previous in vivo studies have shown that Nrf2 KO mice exhibit significantly lower levels of cellular defense in various tissues (209), with an increased risk of developing carcinogen-induced skin (210) and colorectal (211)
cancers, among others. In the context of the skin, for instance, we have previously reported the critical role of *Nrf2* in the classical 2-stage skin carcinogenesis model induced by chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) and tumor promoter TPA in *Nrf2* KO and WT mice (212, 213). In addition, *NRF2* also has been reported to promote the synthesis of GSH (214). As the most abundant antioxidant molecule in cells, GSH are highly involved in the conversion of hydrogen peroxide to water as a hydrogen donor via the enzyme glutathione peroxidase. In this ROS scavenging process, the oxidized GSH (GSSG) can be reduced to GSH by consumption of NADPH, which would further rewire the interconnected cellular metabolism including pentose phosphate pathway, TCA cycle, etc. (215)

However, the role of FX and *Nrf2* in metabolic rewiring, epigenomic reprogramming, and transcriptomic network in blocking pro-tumorigenic signaling and elicit cancer-protective effects remain unknown. With that, we would like to deploy the multi-omics approaches to further investigate the upstream signaling events elicited by FX, the role of *Nrf2* in the context of metabolic rewiring, epigenetic reprogramming and transcriptomic contributing to the overall FX’s protective effects in TPA-induced ROS-mediated cellular transformation (Appendix 2.1). We aimed to reveal the underlying intricated biological connectivity between metabolomic, epigenomic and transcriptomic regulation by *Nrf2* signaling and FX in mouse epidermal JB6 cells.

### 2.2 Materials and methods
2.2.1 Chemicals and Reagents
FX was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Versene, trypsin-EDTA, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), puromycin, Minimum essential medium (MEM) was obtained from ThermoFisher Scientific (Lot No. 2110772). Dimethyl sulfoxide (DMSO) was ordered from Sigma-Aldrich (St. Louis, MO, USA). TPA was purchased from Alexis Biochemicals (San Diego, CA, USA). The MTS ((3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium) reagent CellTiter 96® AQueous One Solution was obtained from Promega (Madison, WI, USA). Glutathione assay kit (Item No. 703002) was obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.2.2 Cell Culture and Cell Viability Test
Mouse skin epidermal JB6 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). JB6 cells were cultured in MEM with 5% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5 % CO₂ atmosphere. JB6 cells transfected with shMock and shNrf2 KD were established using Lentivirus-mediated short hairpin RNAs (shRNAs) and maintained in MEM supplemented with 5% FBS and 2 μg/mL puromycin as described previously (87). Assessment of cytotoxicity of FX on JB6 cells, the MTS cell viability assay was proceeded. JB6 cells were plated in a 96-well plate at a density of 4 × 10³ cells per well overnight and then treated with either 0.1 % DMSO as vehicle control or various concentrations of FX for 1, 3, or 5 days in MEM medium supplemented
with 1% FBS. The cell culture medium was changed every other day. To test cell viability, the CellTiter® 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used followed by the manufacturer’s instructions.

2.2.3 Flow Cytometry Analysis of Intracellular ROS
The effect of FX on TPA-induced intracellular ROS was examined by flow cytometry (Beckman Coulter, Brea, CA) and the method was adapted from our previous publication (216). Briefly, 2 × 10^5 Nrf2 WT and KD JB6 cells were plated in 6-cm plates for 24 h and then treated with 0.1% DMSO, 10 ng/mL TPA, 10 μM FX alone and 10 ng/mL TPA + 10 μM FX, respectively for 24 h. ROS was measured by using 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen, Carlsbad, CA). Cells were incubated with 5 μM CM-H2DCFDA in serum-free medium for 40 min at 37 °C and then washed three times with 1 mL PBS. Treated cells were harvested, and the cell-associated fluorescence intensity were then analyzed using flow cytometry.

2.2.4 Measurement of Oxidized GSSG to Reduced GSH Ratio
The quantification of total glutathione (GSH + GSSG) and oxidized GSSG were performed with Glutathione Assay Kit of Cayman Chemical (Item No. 703002) which uses 2-vinylpyridine in deproteinized samples as GSH masking agent, and the reduced GSH will be calculated by using total GSH − GSSG. The assay involves optimized enzymatic recycling method using GSH reductase and
Ellman’s reagent (DTNB). Briefly, $2 \times 10^6$ Nrf2 WT and KD JB6 cells were harvested after 0.1% DMSO, 10 ng/mL TPA and 10 ng/mL TPA + 10 μM FX treatments. Cell pellets were sonicated in 0.5 mL 50 mM MES buffer containing 0.2 M 2-(N-morpholino) ethanesulphonic acid, 0.05 M phosphate and 1 mM EDTA, pH 6.0 and then the supernatant will be deproteinized by adding 0.5 mL metaphosphoric acid (0.1 g/mL) and 50 μL of 4 M triethanolamine solution. Then the samples were assayed for total GSH (both oxidized and reduced). Quantification of GSSG, exclusive of GSH, was accomplished by first derivatizing GSH with 10 μL of 1 M 2-vinylpyridine for 1 h. The total GSH and GSSG concentrations were measured after mixing 50 μL samples with 150 μL Assay Cocktail by measuring the absorbance at 415 nm and comparing it with standard curve for GSH and GSSG.

2.2.5 LC-MS Metabolomic Analysis

LC/MS metabolomic analysis was performed in Metabolomics Shared Resources, Rutgers Cancer Institute of New Jersey (CINJ) as reported previously (217, 218). $1.5 \times 10^6$ Nrf2 WT and KD JB6 cells were seeded in 6-cm cell culture dishes for overnight and then treated with 0.1% DMSO, 10 ng/mL TPA and 10 ng/mL TPA + 10 μM FX. The metabolites were extracted with 1 mL cold 40:40:20 methanol:acetonitrile:water solution with 0.5% formic acid, followed by 5 min incubation on ice and sequentially neutralized with 50 μL 15% NH₄HCO₃. Scrap cells from the plates and the mixtures were centrifuged at a speed of 15,000 g for 10 min at 4 °C. The cleared supernatant was then used for LC-MS analysis. LC
separation was performed on a XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters) coupled with a Waters XBridge BEH XP VanGuard cartridge (2.1 mm x 5 mm, 2.5 μm particle size, 130 Å pore size) guard column. The solvent A prepared by water/acetonitrile (95:5, v/v) with 20 mM NH₃AC and 20 mM NH₃OH at pH 9; and solvent B prepared by acetonitrile/water (80:20, v/v) with 20 mM NH₃AC and 20 mM NH₃OH at pH 9 in the following solvent B percentages over time: 0 min, 100%; 3 min, 100%; 3.2 min, 90%; 6.2 min, 90%; 6.5 min, 80%; 10.5 min, 80%; 10.7 min, 70%; 13.5 min, 70%; 13.7 min, 45%; 16 min, 45%; 16.5 min, 100%. The flow rate was set to 300 μL/min with an injection volume 5 μL. The column temperature was set at 25 °C. MS scans were obtained in both positive and negative ion modes with a resolution of 70,000 at m/z 200, in addition to an automatic gain control target of 3 × 10⁶ and m/z scan range of 72 to 1000.

2.2.6 Isolation of Nucleic Acids and Next-generation Sequencing (NGS)

JB6 cells were seeded in 10-cm cell culture dishes at a density of 2 × 10⁵ cells/dish for overnight and then treated with the 0.1% DMSO, 10 ng/mL TPA and 10 ng/mL TPA + 6.3 μM FX for 5 days. The cell culture medium dissolved with compounds was changed every two days and cells were harvest at day 5. Total RNA and DNA were extracted using an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and concentration of extracted RNA and DNA were determined with an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer. 3 μg RNA and 3 μg DNA of each sample was used to
perform RNA-seq and SureSelect Methyl-seq, respectively. The library preparation and sequencing were performed by RUCDR Infinite Biologics. Briefly, the RNA library was built with the Illumina TruSeq RNA preparation kit (Illumina, San Diego, CA, USA) and then sequenced on an Illumina NextSeq 500 instrument with 75 bp single-end reads, generating 30-40 million reads per sample. The DNA samples were further processed with the Agilent Mouse SureSelect Methyl-seq Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, USA). Bisulfite conversion was performed using an EZ DNA Methylation-Gold Kit (Zymo Research, USA). And then DNA sequencing was also performed on the Illumina NextSeq 500 instrument with 76-bp single-end reads, generating 30-40 million reads per sample. Further details of the general aspects of the RNA-seq and DNA Methyl-seq procedures have been described previously (219).

2.2.7 Bioinformatics Analyses

RNA-seq

Cutadapt, a command-line program produced by second-generation sequencers (220), was used to remove the Illumina Universal Adapter sequence. Hierarchical indexing for spliced alignment of transcripts (HISAT2) was adapted for aligning the reads to the mouse genome (mm10) (221), and remove PCR duplicates. Genomic features with overlapping reads were counted by featureCounts (version 1.5.1) (222) and then data were further analyzed for differential expression with DEGSeq (version 1.36.0) in R (version 3.4.0) (223).
**DNA SureSelect Methyl-seq**

Bismark (version 0.15.0) alignment algorithm was applied to align the DNA reads to the *in silico* C-T converted mouse genome (mm10). And the DMRfinder, an efficient tool to identify differentially methylated regions from Methyl-seq data, was used to extract methylation counts and cluster CpG sites into differentially methylated regions (DMRs) (224). Methylation differences > 10% with *P*-values < 0.05 were considered as significant. Genomic annotation was performed with ChIPseeker (version 1.10.3) in R (version 3.4.0) (225).

2.2.8 Ingenuity Pathway Analysis (IPA)

Isoforms with false discovery rates (FDR) adjusted *P* value (q value) < 0.05 and log2 fold changes > 1.0 or < -1.0 were applied for the IPA analysis (IPA 4.0, Ingenuity Systems, www.Ingenuity.com). The input genes were mapped to the IPA knowledge base, and the biological functions, networks, and pathways mediated by TPA +/- FX were identified.

2.2.9 Quantitative Real-time Polymerase Chain Reaction (RT-PCR) Validation

The first-strand cDNA was synthesized from 1 μg extracted RNA using SuperScript III First-Strand cDNA Synthesis System (Invitrogen, Grand Island, NY, USA). The mRNA expression levels were determined by the Applied Biosystems 7900HT Fast Real-Time PCR System. The primers were designed
and ordered from Integrated DNA Technologies (IDT, Coralville, Iowa, USA) (Appendix 2.2).

2.2.10 Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation (ChIP) assay was performed using MAGnify Chromatin Immunoprecipitation System (Invitrogen, Grand Island, NY) following the manufacturer's protocol. In brief, freshly prepared 37% formaldehyde at a final concentration of 1% was added to fix the 1.5×10^6 JB6 cells for 10 min at room temperature, then the excess formaldehyde was quenched by addition of 1.25 M glycine. After washing twice with ice cold PBS, cells were resuspended in cell lysis buffer containing protease inhibitor cocktail II. The samples were sonicated in ice cold water using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ) to shear the cross-linked DNA to an average length of 300-500 bp and then centrifuged at 12,000 rpm to collect the clear supernatant. The chromatin solutions were then diluted 10 folds with dilution buffer provided by the kit, and 10 μL of each sample was used as input control. Diluted chromatin solutions were then incubated with protein A magnetic Dynabeads® coupled to the specific antibody against H3K27Ac, H2K27Me3 (Cambridge, MA, USA), or nonspecific Mouse/Rabbit IgG for 2 hours at 4°C. The immunoprecipitated complex-magnetic beads were collected using magnetic separator (DynaMag™-PCR Magnet) and washed, following manufacturer's instructions. The pellets were then incubated with proteinase K in ChIP Reverse Crosslinking Buffer for 15 minutes at 55°C to elute immunocomplex and reverse cross-linking. After reversing cross-linking,
DNA was purified according to manufacturer's instructions. 1 μL of each purified DNA was used as template for PCR amplification using the designed primers for Ubiquitin Like With PHD And Ring Finger Domains 1 (Uhrf1) and Nrf2 genes (Appendix 2.1). The amount of ChIP-purified DNA was quantified by RT-PCR assay using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA).

2.2.11 Statistical analysis

The results are presented as the mean ± SEM or SD and P-value ≤ 0.05 was considered statistically significant. Statistical analysis was carried out using Student’s t test (two-tailed unpaired) for two groups and one-way ANOVA followed by Dunnett’s post hoc test for multiple groups within GraphPad Prism. Metabolomic pathways analysis and pathway enrichment analysis were performed within the Web-based inference MetaboAnalystR 5.0 (https://www.metaboanalyst.ca/).

2.3 Results

2.3.1 Cytotoxicity of FX in JB6 Cells

MTS assay was performed to determine the cytotoxic concentrations of FX in JB6 cells. Treatment with FX showed time- and dose-dependent effect on the JB6 cell viability (Figure 2.1a). The viability of JB6 cells that were treated with 10 μM of FX for 24 h was ~90%; therefore, the dose (10 μM) was used in the subsequent short-term ROS flow cytometry, GSH assay, metabolomic and ChIP
assays. The cell viabilities following the 6.3 μM FX treatment was ~90% after the 5-day treatments. The dose (6.3 μM) of FX was utilized in the subsequent next generation sequencing (NGS) studies.

2.3.2 FX Attenuates TPA-Induced ROS Through Nrf2 Signaling Pathway in JB6 Cells

Previous study from our lab found that FX induced the Nrf2-ARE-luciferase and upregulated the mRNA and protein levels of Nrf2 in HepG2-C8 cells transfected with ARE-luciferase reporter (194). In this context, we examined the antioxidant effect of FX on TPA-induced Nrf2 WT and KD JB6 cells. TPA significantly induced the ROS levels in both Nrf2 WT and KD JB6 cells. FX completely abrogated TPA-induced ROS in Nrf2 WT but not Nrf2 KD JB6 cells. 10 μM FX alone decreased the ROS level by 59% and 28% in Nrf2 WT and KD JB6 cells respectively compared to their corresponding control (Figure 2.1b), indicating the ROS regulation by FX in JB6 cells are highly associated but not completely dependent on the Nrf2 signaling pathway.

2.3.3 FX Regulates the Oxidized Glutathione disulfide (GSSG) to Reduced GSH Ratio in Nrf2 WT and KD JB6 cells

The ratio of oxidized GSSG to reduced GSH in biological samples is a frequently used parameter in monitoring oxidative stress (226). TPA significantly increased the GSSG/GSH ratio by ~1.5 fold while FX completely attenuated the TPA-induced modulation in Nrf2 WT JB6 cells. In addition, FX also alleviated TPA-
induced ROS level in Nrf2 KD JB6 cells but not significantly as that in Nrf2 WT JB6 cells. These results indicated that Nrf2 signaling may partially contribute to the FX-mediated ROS regulation in TPA-induced JB6 cells (Figure 2.1c).

2.3.4 FX and Nrf2 Drive Metabolic Rewiring

To unravel the potential underlying molecular links between ROS-induced metabolic reprogramming and epigenetic modifications as well as the role of Nrf2 signaling pathway and the impact of TPA and FX on mitochondrial metabolic pathways and metabolites, Nrf2 WT and Nrf2 KD JB6 cells were treated with 0.1% DMSO or TPA +/- FX. The Nrf2 expression levels in Nrf2 KD JB6 cells were further validated by Western Blot (Figure 2.2a). Principal Component Analysis (PCA) revealed a clear separation for each treatment, showing significant differential metabolic profile between them, with the most significant difference between Nrf2 WT and Nrf2 KD groups (Figure 2.2b). A total of 144 metabolites were identified under positive and negative ion modes, of which 72 showed significant differential levels between Nrf2 WT and KD groups covering 12 significantly modulated metabolic pathways (Figure 2.2c-d). Pathway analysis (Figure 2.2c) combined with pathway enrichment analysis (Figure 2.2d) and pathway topology analysis revealed that the TCA cycle, GSH metabolism, Warburg effect, glycolysis, nicotinate and nicotinamide metabolism, among others, were significantly altered in Nrf2 KD JB6 cells. In addition, the SAM/SAH ratio was reported to regulates DNA and histone methylation and dictates methyltransferase activity in vivo (227). We found that SAM and SAM/SAH ratios
were significantly elevated after TPA treatment and FX attenuated TPA-induced modulation in JB6 WT cells while FX did not alleviate TPA-induced SAM and SAM/SAH regulation in Nrf2 KD JB6 cells. Nrf2 KD in JB6 cell showed higher SAM and SAM/SAH level (Figure 2.2e) indicating that Nrf2 signaling pathway plays an important role in FX-mediated mitochondrial metabolites involved in epigenetic basic machinery. These results suggest that the alterations of mitochondrial metabolism/metabolites are impacted by Nrf2 WT and KD and treatments with TPA and FX, also pointing to the down-stream impact on epigenetic reprogramming, to be discussed below.

2.3.5 FX Drives Epigenetic CpG Methylation Reprogramming

Differentially methylated regions (DMRs) profiled from DNA Methyl-seq were used to represent the epigenomic modulations by TPA and FX in JB6 cells. More than 50% DMRs were located in the promoters and distal intergenic regions (Figure 2.3a). The methylation differences > 20% with P-value < 0.01 of specific genes were applied for the cutoff of DMRs. Briefly, 400 DMRs (176 hypermethylated and 224 hypomethylated) in TPA vs control group and 319 DMRs (165 hypomethylated and 154 hypermethylated) in TPA+FX vs TPA group were filtered (Figure 2.3b). 69 DMRs were found to be hypo- or hypermethylated by TPA and completely reversed by co-treatment with FX and top 32 regulated DMRs were plotted (Figure 2.3c).
2.3.6 Differentially Expressed Genes (DEGs) Regulated by FX

The DEGs in RNA-seq analysis were used to present the transcriptomic modulations of TPA +/- FX in JB6 cells. The PCA and Euclidean distance clustering (Figure 2.4a) clarified that the control group to be clearly separated from the TPA +/- FX groups indicating that exposure of JB6 cells to TPA +/- FX resulted in significantly regulated gene expression. The DEG profiles after treatment with TPA +/- FX were further analyzed and plotted with the cutoff p-value < 0.01 coupled to log2 fold change ≥ 2.0 or ≤ -2.0 (Figure 2.4b). Compared to control group, 872 and 357 genes were significantly up- and down-regulated by TPA respectively. Compared to the TPA group, 303 and 413 genes were significantly up- and down-regulated respectively, when FX was used with TPA (Figure 2.4c). Within these 2 comparisons, FX offsets 382 TPA-modulated (276 genes were up-regulated, while 106 were down-regulated) genes (Figure 2.4c) and top 64 of these opposing regulated genes (DEGs) between TPA vs. control and TPA+FX vs. TPA were plotted (Figure 2.4d, TPA-modulated gene expression shown on the outer edge, while reversal of gene expression by FX shown in the inner circle). Besides that, RNA-seq results revealed that TPA significantly increased the *Uhrf1* (a key epigenetic regulator by bridging DNA methylation and chromatin modification) and *Dnmt1* expression level by 7.3- and 3.6-fold; while FX almost completely abrogated TPA-induced gene modulations. Previous studies have reported that *Uhrf1* is required for recruitment of *Dnmt1* to DNA methylation sites (228, 229) indicating that the potential underlying mechanism of FX regulation on epigenomic profiles may highly associate with
**Uhrf1-Dnmt1** pathway. In addition, FX also alleviated TPA-induced inhibition of *Nrf2*-target genes such as *Ho-1*, *Nqo-1*, Ferritin light chain 1 (*Ftl1*) and Glutamate-Cysteine Ligase Catalytic Subunit (*Gclc*), and FX completely blocked TPA-induced activation of *Keap1* (a negative regulator of *Nrf2*) which would enhance *Nrf2* signaling and dampen oxidative stress.

2.3.7 Integrated Methyl-seq and RNA-seq Analysis Identifies DNA Methylation and Gene Expression Patterns

The current dogma of DNA methylation implicates regulation of transcription of many downstream target genes in mammalian cells (230). The correlation between DEGs and DMRs in the promoter regions were further analyzed to examine the underlying linkage between DNA methylation and transcriptomic gene expression. A cutoff threshold methylation difference ≥ 10% coupled to log2-fold change of gene expression difference ≥ 1 or ≤ -1 was applied to the selection (Figure 2.5a). A total 15 and 9 DMRs in the promoter regions were negatively correlated with their corresponding gene expression in TPA vs control and TPA+FX vs TPA groups, respectively (Figure 2.5a). Besides these filtered DMRs, TPA also decreased the CpG methylation ratio in the *Uhrf1* promoter region (chromosome 17, start:56303940 – end:56304324; NM_001111078) by 48% but FX completely reversed the TPA-induced methylation, and which was highly correlated with the RNA expression of *Uhrf1*, indicating that the DNA CpG methylation in *Uhrf1* promoter region is critical in regulating transcription of *Uhrf1* gene expression. CHIP-RT-PCR of *Uhrf1* promoter region will be validated below.
2.3.8 Signaling Pathway Analysis of JB6 Cells Treated with TPA +/- FX

A total of 12,772 and 12,904 genes regulated by TPA vs. control and TPA+FX vs. TPA groups, respectively (quantitated by RNA-seq) were conducted by Ingenuity Pathway Analysis (IPA). Among the top 37 shared pathways, 32 were significantly activated by TPA but attenuated by FX in TPA treated cells, while 5 pathways were inhibited by TPA and FX reversed the TPA regulated pathways (Figure 2.5b). Interestingly, IPA shows TPA-induced inhibition of tumor suppressor p53 signaling pathway was significantly offset by FX suggesting that FX could play an important role in protecting skin cells against TPA-induced DNA damage, oxidative stress and inflammation modulated by p53 pathway. Moreover, blockade of the TPA-induced AMPK, one of the central regulators of cellular and organismal metabolism, signaling pathway by FX indicating that FX may play critical roles in regulating growth and reprogramming metabolism as well as cellular processes such as autophagy and cellular polarity (231). FX attenuated AMPK signaling pathway also further block glycolysis metabolism which has been considered as an attractive anticancer strategy (212, 232). Numerous signaling pathways related to cancer-, cell cycle- and inflammatory-related signaling pathways including MAPK, G2/M DNA damage checkpoint regulation and G1/S checkpoint regulation pathways were also highly modulated by TPA and FX (Figure 2.5b). These modulated pathways may reveal new windows of therapeutic opportunity against skin cancers.
2.3.9 Validation of RT-PCR and ChIP Assay

RT-PCR and CHIP assay were performed to validate the transcriptomic and epigenomic profiles from the above NGS analysis and to better understand the molecular mechanism underlying these regulations. 50 μM of FX significantly increased the mRNA expression of Nrf2. FX increased the mRNA expression levels of Ho-1 by up to 12 folds in a dose-dependent manner (Figure 2.6a). In addition, FX also exerted a significant effect on Dnmt1, Dnmt3a, Hdac1 and Hdac3 (Figure 2.6b). Methylation markers on lysine residues in histone proteins have a key role in regulating chromatin structure and gene transcription. Multiple lysine residues like H3K27 may be mono-, di- or tri-methylated, giving rise to a very complex histone methylation code (233). CHIP assay showed that FX significantly alleviated TPA inhibition of enrichment of H3K27me3 (tri-methylation marker of lysine 27 on histone H3 protein) in the promoter regions of Uhrf1 (Figure 2.6c). ChIP assay coupled with NGS analysis from above, indicated that FX restored Uhrf1 expression by regulating the H3K27Me3 enrichment and CpG methylation status in the promoter region, consequently, may enhance the recruitment of Dnmt1 and thereby induce the epigenetic reprogramming and alteration. Both TPA and FX exerted no effect on the enrichment of H3K27Ac (acetylation marker of the lysine residue at N-terminal position 27 of the histone H3 protein) in the Uhrf1 promoter region. On the other hand, FX significantly decreased the H3K27Me3 enrichment in the Nrf2 promoter region indicating that FX promotes the epigenetic histone demethylation of Nrf2 promoter (Figure 2.6c), therefore, upregulate the Nrf2 gene expression since this tri-methylation is highly
associated with the downregulation of nearby genes via the formation of heterochromatic regions (234).

2.4 Discussion

Previous studies suggested that dietary cancer chemopreventive phytochemicals including curcumin (235), green tea (236) and FX (194) are effective in blocking both chemical- and UVB-induced skin carcinogenesis through Nrf2 anti-oxidative signaling. However, the molecular mechanisms by which these chemopreventive agents regulate the mitochondrial metabolism, epigenomic/CpG methylation and transcriptomic as well as the role of Nrf2 in regulating the cellular activities remain unclear. Therefore, in our current study, we performed studies with metabolomics, epigenomic and transcriptomic to uncover dysregulated pathways by tumor promoter TPA and reversed by FX, as well as the role of Nrf2 in FX-mediated cancer prevention effect.

Recent evidence suggests strong molecular link between metabolic rewiring and epigenetic reprogramming through key metabolic intermediates, such as SAM, NAD+, aKG and AcCoA, which work as hubs between epigenetic processes and oxidative stress responses (237). In cancer cells, aberrant ROS and mutations that activate oncogenes or inactivate tumor suppressor genes may also affect multiple signaling pathways that results in mitochondrial metabolic reprogramming and altered bioenergetics (238). Previous studies demonstrated that mammalian cells against oxidative stress through producing SAM to maintain mTORC1 signaling activity, thus helping cells survive (239). In current
study, FX significantly abrogated TPA-induced SAM production as well as SAM/SAH ratio (Figure 2.2e). In addition, Nrf2 KD in JB6 cells also significantly altered the mitochondria metabolisms and metabolites that are co-factors of epigenetic enzymes (Figure 2.2b-e). For instance, the epigenetic related metabolic pathways such as TCA cycle, GSH metabolism, Warburg effects, glycolysis and nicotinate and nicotinamide metabolism, among others, as well as the metabolites like SAM, SAM/SAH were significantly regulated by Nrf2 KD in JB6 cells. These metabolites are tightly linked to the basic epigenetic machinery DNA/histone modifications, chromatin remodeling and modulating phenotypic gene expression with high connectivity between ROS, mitochondrial metabolites, and epigenetics (60, 204, 240, 241). More specifically, SAM is the universal donor of methyl groups to both DNA and histone methyltransferase enzymes, and the changes in methionine metabolism altering levels of SAM can directly influence trimethylation of H3K4 (Histone H3 lysine K4) and consequently modulate gene expression (242). The intermediary metabolite SAH is the byproduct of SAM during methyltransferase reactions (243), which is a potent inhibitor of both DNA and histone methyltransferases. Thereby, the SAM/SAH ratio serves as a biosensor of the cellular metabolic state influencing the activity of methyltransferase enzymes that culminate in chromatin changes in response to cellular alternations (244). Therefore, TPA-induced SAM and SAM/SAH elevation would promote DNA and histone methylation, and FX could abrogate the TPA-mediated epigenetic regulation (Figure 2.2e). Importantly, the regulation effect of FX on TPA-induced SAM/SAH have been diminished in Nrf2 KD cells.
(Figure 2.2e) indicating \textit{Nrf2} signaling plays a critical role in FX-mediated oxidative stress-induced epigenetic modulations.

Methyl-seq and RNA-seq analysis revealed that TPA significantly decreased the CpG methylation ratio in the \textit{Uhrf1} promoter region by 48\% but FX completely blocked the TPA-induced CpG methylation which correlates with the increased RNA expression of \textit{Uhrf1}. This result suggests that epigenetic mechanism may constrains \textit{Uhrf1} expression by adapting CpG methylation of the promoter regions to maintain either gene silencing or gene expression. Since methylation markers such as mono-, di- or tri-methylation on H3k27 have a key role in regulating chromatin structure and gene transcription (233). So, the enrichment of H3K27Me3 in \textit{Uhrf1} promoter regions were quantified by ChIP assay and revealed that FX completely offset the TPA-induced epigenetic regulation (Figure 2.6c), which is in agreement with the RNA-seq and Methyl-seq results. Previous studies show that \textit{Uhrf1} is essential for the maintenance of DNA methylation by \textit{Dnmt1}, and \textit{Uhrf1} has been shown to recruit \textit{Dnmt1} to replicating DNA by the ability of its SET and RING-associated (SRA) domain to bind to hemi-methylated DNA (245, 246). In this context, FX may regulate the H3K27me3 enrichment and CpG methylation in \textit{Uhrf1} promoter region to enhance the recruitment of \textit{Dnmt1} and thereby leading to regulation of DNA methylation and gene expression. In the current study, the enrichment of H3K27me3 in \textit{Nrf2} promoter region was significantly decreased by FX indicating that FX could also contribute to the epigenetic CpG demethylation in \textit{Nrf2} promoter region in regulating \textit{Nrf2} expression and its downstream genes like \textit{Ho-1} expression (Figure 2.6a),
contributing to the redox regulation. This result supports our earlier finding that
FX promotes the epigenetic demethylation of CpG sites in Nrf2 promoter and blocks tumor promoter TPA-induced transformation of JB6 cells (194).

IPA pathways analysis revealed that TPA inhibits tumor suppressor p53 signaling pathway and it was significantly offset by FX. Vousden et al. (247) reported that p53 is regulated by various stresses, hypoxia, heat shock and oncogenic assault and it has a well-established role in protecting against cancer development (248). Genomic and mutational analyses documenting inactivation of p53 in more than 50% of human cancers suggesting the important role of redox/p53 signaling modulated by TPA and blockage by FX. Our current study shows that FX reverses TPA-induced MAPK signaling pathways. MAPK families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis (249) which are also highly altered in many cancers (250). FX also blocks TPA-induced AMPK signaling pathway (Figure 2.5b) indicating FX plays important roles in regulating cell growth and reprogramming metabolism as well as cellular processes such as autophagy and cell polarity which are mediated by AMPK pathway. AMPK may act in cancer cells as a metabolic gatekeeper that functions to establish metabolic checkpoints that limit cell division, and its loss of function to enhance both tumorigenesis and tumor progression (251). One of characteristic features of cancer cell metabolism is aerobic glycolysis (Warburg effect), a shift from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, even in the presence of oxygen (252). Since AMPK has been reported to negatively
regulates the Warburg effect in cancer cells and suppresses tumor growth in vivo (251), this may further contribute to the FX-mediated metabolic rewiring.

2.5 Conclusion

In conclusion, our study integrates the latest Methyl-seq, RNA-seq and LC/MS/MS technologies in dissecting the potential underlying mechanism of epigenomic CpG methylation, mRNA transcriptomic gene expression and mitochondrial metabolic rewiring in mouse epidermal JB6 cells after exposure to tumor promoter TPA and the impact of FX treatment and Nrf2 knockdown. The results provide the experimental evidence for the metabolomic, epigenomic, and transcriptomic effects of FX in TPA-induced skin cancer model and the role of Nrf2. We observed that FX rewires metabolic profile, reprograms epigenetic CpG methylation, driving alterations of phenotypic gene expression, de-/activated signaling pathways in the TPA-induced JB6 cellular transformation model. Nrf2, the master regulator of anti-oxidative stress response, would play an important role in the above processes by mediating redox signaling that significantly rewired the metabolic pathways and metabolites, which are highly involved in epigenetic reprogramming. Thus, the anticancer potential of FX is exhibited through reactivation of Nrf2-ARE defense pathways, metabolic rewiring and epigenetic reprogramming in TPA-induced epidermal JB6 cells, making it a promising drug candidate for the prevention of skin cancer.
Figure 2.1 FX-mediated cell viability in JB6 cells and regulation of ROS and GSSG/GSH ratio.

(a) Cell viability of the JB6 cells after FX treatment. JB6 cells were treated with control and various concentrations of FX ranging from 6.3 to 50.0 μM for 1-, 3- and 5-day. Cell viability was determined by the MTS assay; (b) Intracellular ROS levels in Nrf2 WT and Nrf2 KD JB6 cells after FX alone and TPA+/- FX treatment. The ROS levels were compared with the WT control group manifested as the fold changes; (c) GSH assay to measure oxidized GSSG/reduced GSH ratios. Nrf2 WT and KD JB6 cells were harvested after control, TPA and TPA+FX treatment.
Total GSH (both reduced GSH and oxidized GSSG) and oxidized GSG were measured by Glutathione Assay. The reduced GSH and oxidized GSSG/reduced GSH were manually calculated. All the data are presented as the means ± SEM of three independent experiments. *, P < 0.05 and **, P < 0.01 indicate significant differences between the treatment groups and the control group (0.1% DMSO). Student’s t test was used to calculate the significance of the differences compared with the control.
Figure 2.2 Metabolite profiles of Nrf2 WT and Nrf2 KD JB6 cells treated with control and TPA+/-FX.

(a) Reduced Nrf2 protein expression in shNrf2 JB6 cells compare to shMock control JB6 cells. The quantification plot data was from three independent replicates. β-actin was used as the housekeeping protein for the normalization; (b) PCA plot of metabolomic profiles in Nrf2 WT and Nrf2 KD JB6 cells after control and TPA+/-FX treatment; (c-d) Metabolism pathway analysis and pathway enrichment analysis of Nrf2 KD versus Nrf2 WT JB6 cells; (e) Epigenetic associated metabolites SAM levels and SAM/SAH ratios in Nrf2 WT versus Nrf2 KD JB6 cells after TPA+/-FX treatment. The data are presented as the means ±SEM of three independent experiments. *, P < 0.05 and **, P < 0.01 indicate significant differences between the compared groups. The student’s t test was used to calculate the significance of the differences compared with the control.
Figure 2.3 DNA methylation profile in JB6 cells regulated by TPA +/- FX.

(a) Distribution of annotated DMRs by genomic features including Distal Intergenic, Promoter (<=1kb, 1-2kb and 2-3kb), 1st Intron and other regions as shown in the Figure 2.3a legend. Each DMR has at least three CpG sites; (b) Venn diagrams comparing the upregulated and downregulated DMRs in TPA vs. control and TPA+FX vs. TPA comparison groups; (c) Heatmap showing promoter methylation changes in the top 32 regulated genes shared by both TPA vs. control and TPA+FX vs. TPA comparisons.
Figure 2.4 mRNA transcriptomic profiles in JB6 cells regulated by TPA +/- FX.

(a) PCA of transcriptomic profiles and Dendrogram of the gene expression profiles clustered by Euclidean distance; (b) MA plots showing DEGs in response to TPA vs. control group and TPA+FX vs. TPA group with cutoffs of \( p<0.01 \) and \( \log_2(\text{fold change}) \geq 2.0 \) or \( \leq -2.0 \); (c) Venn diagrams comparing the upregulated and downregulated genes in TPA vs. Control and TPA+FX vs. TPA comparison groups; (d) Circos plot of top 64 DEGs that appeared in completely opposite direction on the TPA vs. control group and the TPA + FX vs. TPA group. Gene expressions of TPA vs. control are shown on outer edge. Reversal of gene expression modulations from FX is presented by the inner circle.
Figure 2.5 Correlation of DEGs and DMRs and Ingenuity Pathway Analysis (IPA).

(a) Correlation analysis between gene expression and DNA methylation. 15 and 9 DMRs (genes located in the upper left and bottom right corners) in the promoter regions were negatively correlated with their corresponding gene expression in TPA vs. control and TPA+FX vs. TPA comparison groups. The DMR locations (gene features: Region, Body, Downstream and Promoter) are indicated by the colors; (b) Heatmap showing the top 37 regulated pathways shared in both comparisons (TPA vs. control and TPA+FX vs. TPA).
Figure 2.6 RT-PCR, ChIP assay validation.

(a) RT-PCR of mRNA expression of Nrf2, Ho1, (b) Dnmt1, Dnmt3a, Hdac1 and Hdac3; (c) ChIP assay to detect the enrichment of H3K27Me3 and H3K27Ac in Uhrf1 and Nrf2 promoter region; the data are presented as the means ± SEM of three independent experiments. *, P< 0.05, and **, P < 0.01 represent significant differences between comparison groups.
Chapter 3 Butyrate Drives Metabolic Rewiring and Epigenetic Reprogramming in Human Colon Cancer Cells

3.1 Introduction

Colorectal cancer (CRC) is one of the most diagnosed and important cause of cancer-related deaths in the US and worldwide (253). Accumulation of genetic and epigenetic/epigenomic alterations in colon epithelial cells transform them into adenocarcinomas and also serves as a crucial driving factor in CRC progression and metastasis (254). There are three epigenetic mechanisms including histone acetylation, DNA methylation, and non-coding microRNAs that are responsible for modifying the expression of critical genes associated with physiologic and pathologic processes (255). Specifically, DNA methylation, with the addition of a methyl group to 5-cytosine in the CpG dinucleotide, is one of the most common epigenetic mechanisms associated with aberrant gene expression in cancer.

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8Keywords: Colorectal cancer; Epigenetic; Metabolomics; Nuclear Factor Erythroid-2 Like 2 (NRF2); Sodium Butyrate

9Abbreviations: B, Butyrate; NaB, Sodium Butyrate; CRC, colorectal cancer; NGS, next generation sequencing; DMRs, differentially methylated regions; DEGs, differentially expressed genes; TCA, tricarboxylic acid; αKG, α-ketoglutarate; AcCoA, acetyl-CoA; SAM, S-adenosyl methionine; NAD, nicotinamide adenine dinucleotide; HDACs, histone deacetylases; HDACi, HDAC inhibitor; NRF2, Nuclear Factor Erythroid-2 like 2; KEAP1, Kelch-like-ECH-associated protein 1; HO1, Heme Oxygenase 1; NQO1, NAD(P)H dehydrogenase [quinone] 1; ARE, Antioxidant Response Element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DNMT1, DNA Methyltransferase 1; HAT1, Histone Acetyltransferase 1; KDM1A, Lysine Demethylase 1A; KDM1B, Lysine Demethylase 1B; TET1, Tet Methylcytosine Dioxygenase 1; ABCA1, ATP binding cassette A1; EGR3, Early growth response protein 3
instance, CpG hyper-/hypo-methylation in DNA promoter regions is believed to play a crucial role in regulating gene expression, perhaps by interfering transcription factor binding (256). In addition, histone tail acetylation enhances the accessibility of a gene to the transcription machinery, whereas deacetylated tails are highly un-charged and tightly associated with the DNA backbone, thus limiting accessibility of genes to transcription factors (257). Interestingly, increasing evidences suggest that cellular metabolism could play an important role in cancers and that the cellular metabolism/metabolites are tightly linked to the basic epigenetic machinery (204). Previous studies reported the epigenetic modifications such as DNA methylation and histone acetylation are sensitive to cellular metabolic status (205). Strong molecular link between metabolic rewiring and epigenetic modifications through key metabolic intermediates, such as aKG, AcCoA, NAD of the TCA cycle and SAM of methionine cycle which are co-factors for the epigenetic enzymes and work as hubs between epigenetic processes and therapeutic modalities (60, 206, 207). Therefore, modulation of epigenetic such as DNA de-/methylation, histone de-/acetylation as well as transcriptomics through regulating metabolomics may prevent diseases and protective against CRC development and metastasis (258).

The KEAP1-NRF2-ARE signaling axis mediates cellular defense against oxidative stresses via induction of cellular defense and multiple downstream cytoprotective genes (92). In this KEAP1-NRF2 axis, NRF2 is sequestered by the inhibitor KEAP1 and is transcriptionally inactive until KEAP1 is modified by sulfhydryl modification such as ROS and dissociated from NRF2. This
dissociation allows NRF2 to form transcriptionally active complexes with other proteins, such as v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAFs) in the nucleus, transcriptionally activates cytoprotective and antioxidative genes, to remove oxidative insults like ROS (259). However, recent accumulating evidence suggests that NRF2 has a contradictory role in cancers (260). Aberrant activation of NRF2 is highly associated with poor prognosis. The constitutive activation of NRF2 in various cancers including CRC (261, 262) induces pro-survival genes and promotes cancer cell proliferation by repression of cancer cell apoptosis, and enhancement of self-renewal capacity of cancer stem cells. In addition, NRF2 was proven to contribute to the chemoresistance of cancer cells as well as inflammation-induced carcinogenesis dramatically (260). For example, the in vitro and in vivo studies from Pawel et al. reported that the NRF2 KO cancer cells have significantly reduced proliferation phenotype and more sensitive to chemotherapeutic agents, such as cisplatin and carboplatin (263). More importantly, NRF2 has been reported to induces metabolic rewiring via modulating the cellular intermediary metabolisms and mitochondrial functions (264). It is either involved directly in the regulation of several key metabolic genes, or it affects their expression indirectly through crosstalk with other transcription factors (264). For example, NRF2 has been shown to inhibits lipogenesis, supports β-oxidation of fatty acids, facilitates flux through the pentose phosphate pathway (PPP), and increases nicotinamide adenine dinucleotide phosphate (NADPH) regeneration and purine biosynthesis, therefore, these findings suggest NRF2 directs metabolic rewiring which may further
influences the process of epigenetic associated metabolisms or the synthesis of the relevant metabolites such as TCA cycle, methionine metabolism and their respective metabolites (265).

As an important dietary constituent, dietary fiber ensures the potential carcinogens are removed from the colon and the microbiota within the colon converts the fiber into short chain fatty acids (SCFAs) by the process of fermentation (266). These SCFAs serve as a major source of energy for the colon cells. Butyrate (B), constitutes about 20–30% of the SCFAs, is the predominant energy providing source (267). B has received particular attention for its multiple beneficial effects including inhibit tumor-cell growth as well as its biological activity in many human CRC cells including HCT116 cell line (268).

The CRC microenvironment induced by colorectal tumor lesions shows significantly different from the normal intestinal environment (269). CRC cells are more sensitive to SCFAs than normal intestinal epithelial cells, suggesting that SCFAs affect tumor cells through some certain pathways (270). Previous studies have demonstrated that the elevated colonic concentration of B works as an important mediator in the observed protective effect of fermentable dietary fibers against CRC (271). B is also recognized for its potential to act on secondary chemoprevention by slowing cell growth and activating apoptosis in colon cancer cells, but it can also act as primary chemopreventive agent (272). The anticancer mechanisms of B are multiple, but many of these are related to its regulation effects on epigenetics as HDAC inhibitor (HDACi) with subsequent regulatory effects on gene expression (273). There is a growing interest in dietary
HDACi, in particular B because its impact on epigenetic mechanisms will lead to more specific and efficacious therapeutic strategies in the prevention and treatment of cancers (273). In terms of the metabolomic regulations, B has been reported to play a crucial role in maintaining the homeostasis of host metabolism and gut microbiome diversity (274). For instance, previous study investigated the effect of NaB on TCA cycle enzymes activity in the brain of rats subjected to an animal model of mania induced by D-amphetamine and found B exerts protective effects against the D-amphetamine-induced TCA cycle enzymes' dysfunction (275).

However, the role of the B in regulating NRF2 signaling as well as metabolic rewiring, CpG methylomic reprogramming, and transcriptomic network in blocking pro-tumorigenic signaling and elicit anti-cancer effects in CRC remain unknown. So, in the current study we aimed to reveal the underlying intricate biological connectivity between metabolomic, epigenomic and transcriptomic regulation as well as the critical role of NRF2 in B-mediated anti-cancer effect in human CRC HCT116 cells (Appendix 3.1).

3.2 Materials and methods

3.2.1 Cell culture and reagents

Sodium butyrate (NaB) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Versene, trypsin-EDTA, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), puromycin, Dulbecco’s Modified Eagle’s Medium (DMEM) were purchased from Gibco Laboratories (Grand Island, NY, USA). Dimethyl sulfoxide
(DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-azadeoxycytidine (5-aza) and trichostain A (TSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The MTS reagent ((3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium) CellTiter 96® Aqueous One Solution was obtained from Promega (Madison, WI, USA). Methanol (99%), and formic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and pure water were purchased from Honeywell Burdick & Jackson (Muskegon, MI). Isotope labeled L-methionine (Methyl-13C) was purchased from Cambridge Isotope Laboratories, Inc (Tewksbury, MA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotech (Rockford, IL, USA).

The human hepatocellular HepG2-C8 cell line was previously established by stable transfection with the pARE-TI-luciferase construct (276). Human colorectal carcinoma HCT116 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2-C8 cells and HCT116 cells were routinely cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5 % CO₂ atmosphere.

3.2.2 Cell Viability Test

HCT116 cells were seeded in a 96-well plate at a density of 4 × 10³ cells/well for overnight and then treated with either 0.1 % DMSO as vehicle control or various concentrations of NaB for 1-, 3-, 5-day in DMEM medium supplemented with 1% FBS. The cell culture medium dissolved with NaB was changed every other day.
To test cell viability, the MTS assay was performed followed by the manufacturer's instructions.

3.2.3 Dosage information
Previous studies reported that the physiological range of B concentrations in the intestinal lumen is between 1 and 10 mmol/l of the food content \((277)\), which, assuming a daily production of 9 l of the intestinal content, corresponds to 9–90 mmol/day, i.e. 1–10 g/day. So, all of the doses of B used in current study are achievable through regular diet (low concentration) or via consuming supplements (moderate and high concentrations).

3.2.4 Luciferase Reporter Activity Assay
Previous established HepG2-C8 ARE-luciferase reporter transfected cells were utilized to examine the effects of NaB on the \(NRF2\)-ARE pathway. The \(NRF2\) luciferase reporter stable HepG2 cells have been well developed for studying the \(NRF2\) associated pathway and were also well validated by various antioxidant molecules in previous studies \((278, 279)\). HepG2-C8 cells were plated in 12-well plates at a density of \(1 \times 10^5\) cells/well for 24 h, and then treated with 0.1% DMSO or various concentrations of NaB in DMEM supplemented with 1% FBS for 24 h. ARE-luciferase activity was determined using the luciferase activity assay kit (Promega, Madison, WI, USA). The reporter lysis buffer was used to lysate the cells and 10 µL of the cell lysate supernatant were measured for \(NRF2\)-ARE activity using a Sirius luminometer (Berthold Detection System
The results were normalized against the protein concentration as determined by the Bicinchoninic Acid (BCA) protein assay. The results are expressed as an inducible fold change compared to the control group.

3.2.5 Quantitative Real-time Polymerase Chain Reaction (RT-PCR)
HCT116 cells were plated in 6-well plate at a density of 3 x 10^5 cells/well overnight and then treated with either vehicle control, 1 mM, 5 mM and 10mM NaB for 24h. mRNA was extracted using mRNA extraction kit from Thermo Fisher Scientific (Cat No. K0732). The first-strand cDNA was synthesized from 1 μg extracted RNA using SuperScript III First-Strand cDNA Synthesis System (Invitrogen, Grand Island, NY, USA). To determine the RNA expression of specific genes, the cDNA was used as the template for real time PCR using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA). The mRNA expression was calculated as the fold change with normalization to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the 2^-ΔΔCT method while GAPDH was used as an internal loading control. The primers were designed and ordered from Integrated DNA Technologies (IDT, Coralville, Iowa, USA) (Appendix 3.2).

3.2.6 Protein Lyses Preparation and Western Blotting
HCT116 cells (1x10^6) treated with vehicle control and various concentrations of NaB for 24h and cells were harvested using RIPA buffer supplemented with protein inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations of each
cleared lysates were measured using the BCA method (Pierce, Rockford, IL). Total 45μg proteins of each sample were separated by 4-15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) followed by blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. Then the membrane was sequentially incubated with specific primary antibodies and then HRP-conjugated secondary antibodies. The blots were visualized by SuperSignal enhanced 66 hemiluminescence (ECL) detection system and recorded using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). Primary antibodies were purchased from different resources: anti-DNA Methyltransferase 1 (DNMT1) and anti-GAPDH were purchased from Cell Signaling (Boston, MA); anti-NRF2, anti-KEAP1, anti-Heme Oxygenase 1 (HO1), anti-MYCL proto-oncogene (c-MYC), and anti-NAD(P)H dehydrogenase [quinone] 1 (NQO1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3.2.7 Isolation of Nucleic Acids and Next-generation Sequencing (NGS)
HCT116 cells were plated in 10cm cell culture plates at a density of $2 \times 10^5$ cells/dish for overnight and then treated with the vehicle control, 1mM NaB for 5 days. The cell culture medium dissolved with 1mM NaB was changed every other day and cells were harvest at day 5. RNA and DNA were extracted using an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). Total four RNA samples (n=2) and two DNA samples (n=1) were subject to RNA-seq and SureSelect
Methy-seq respectively. Each RNA or DNA sample was pooled by 3 independent biological replicates. Previous NGS studies from our lab showed that the pooled DNA and RNA collected from 3 plates of cells and combined into one DNA-seq and RNA-seq sample were not much variation between different plates of cells (280, 281). 3 μg RNA and 3 μg DNA of each sample was used for NGS. The library preparation and sequencing were performed by RUCDR Infinite Biologics. Briefly, the RNA library was built with the Illumina TruSeq RNA preparation kit (Illumina, San Diego, CA, USA) and then sequenced on an Illumina NextSeq 500 instrument with 75 bp single-end reads, generating 30-40 million reads per sample. The DNA samples were further processed with the Agilent Mouse SureSelect Methyl-seq Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, USA). Bisulfite conversion was performed using an EZ DNA Methylation-Gold Kit (Zymo Research, USA). And then DNA sequencing was also performed on the Illumina NextSeq 500 instrument with 76-bp single-end reads, generating 30-40 million reads per sample (219).

3.2.8 Bioinformatics Analyses

**RNA-seq**

Cutadapt, a command-line program produced by second-generation sequencers (220), was used to remove the Illumina Universal Adapter sequence. Hierarchical indexing for spliced alignment of transcripts (HISAT2) was adapted for aligning the reads to the mouse genome (mm10) (221), and remove PCR duplicates. Genomic features with overlapping reads were counted by featureCounts
and then data were further analyzed for differential expression with DEGSeq (version 1.36.0) in R (version 3.4.0) (223).

**DNA SureSelect Methyl-seq**

Bismark (version 0.15.0) alignment algorithm was applied to align the DNA reads to the in-silico C-T converted mouse genome (mm10). And the DMRfinder, an efficient tool to identify differentially methylated regions from Methyl-seq data, was used to extract methylation counts and cluster CpG sites into differentially methylated regions (DMRs) (224). Methylation differences > 10% with P-values < 0.05 were considered as significant. Genomic annotation was performed with ChIPseeker (version 1.10.3) in R (version 3.4.0) (225).

3.2.9 Ingenuity Pathway Analysis (IPA)

Isoforms with false discovery rates (FDR) adjusted P value (q value) < 0.05 and log2 fold changes > 2.0 or < -2.0 were applied for the IPA analysis (IPA 4.0, Ingenuity Systems, [www.Ingenuity.com](http://www.Ingenuity.com)). The input genes were mapped to the IPA knowledge base, and the signaling pathways mediated by NaB were identified.

3.2.10 LC-MS Metabolomic Analysis

LC-MS metabolomic analysis was performed in Metabolomics Shared Resources, Rutgers Cancer Institute of New Jersey (CINJ) as our previously reported (282, 283). 3 ×10⁶ HCT116 cells were seeded in 10-cm cell culture dishes (n=3) and
cultured in dialyzed FBS DMEM medium overnight. For the stable isotope labelling of methyl group, methionine free DMEM media will be used, and supplemented with 2 mM $^{13}$C-methionine as we have performed with other isotopes previously (218). Cells were then be treated with vehicle control (DMSO) vs various concentrations of NaB for 24 hrs. The cells were scraped after PBS wash for 3 times and centrifuged at 4 °C, 15,000 g for 10 min. Then metabolites were extracted with 1 ml cold 40:40:20 methanol:acetonitrile:water solution with 0.5% formic acid, and then followed by 5 min incubation on ice and sequentially neutralized with 50 μL 15% NH$_4$HCO$_3$. The cleared supernatant was then used for LC-MS analysis. LC separation was performed on a Xbridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters) coupled with a Waters Xbridge BEH XP VanGuard cartridge (2.1 mm x 5 mm, 2.5 μm particle size, 130 Å pore size) guard column. The solvent A prepared by water/acetonitrile (95:5, v/v) with 20 mM NH$_3$AC and 20 mM NH$_3$OH at pH 9; and solvent B prepared by acetonitrile/water (80:20, v/v) with 20 mM NH$_3$AC and 20 mM NH$_3$OH at pH 9 in the following solvent B percentages over time: 0 min, 100%; 3 min, 100%; 3.2 min, 90%; 6.2 min, 90%; 6.5 min, 80%; 10.5 min, 80%; 10.7 min, 70%; 13.5 min, 70%; 13.7 min, 45%; 16 min, 45%; 16.5 min, 100%. The flow rate was set to 300 μL/min with an injection volume 5 μL. The column temperature was set at 25°C. MS scans were obtained in both positive and negative ion modes with a resolution of 70,000 at m/z 200, in addition to an automatic gain control target of 3 × 10$^6$ and m/z scan range of 72 to 1000.
3.2.11 DNA Extraction and Bisulfite Genomic Sequencing TA Cloning

HCT116 cells were treated with vehicle control, 1mM NaB and 5mM NaB treatment for 5 days and the medium dissolved with the compounds were changed every 2 days. For the positive control group, HCT116 cells were treated with 500 nM 5-aza in every other day. On day 4, for the 5-aza and TSA combination treatment, 100 nM TSA was added to the 5-aza containing medium. Cells were then harvested on day 5 for genomic DNA isolated with the QIAamp® DNA mini Kit (Qiagen, Valencia, CA). Then 500 ng DNA was denatured and utilized to bisulfite conversion using EZ DNA Methylation Gold Kits (Zymo Research Corp., Orange, CA) following the manufacturer’s instructions. The converted DNA was amplified by PCR using Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) with primers (Forward: 5’- GAAAGAAAGAAAGAAAAGAAAAG-3’, Reverse: 5’- CACCCAAAAATAAAAATAACACCC-3’) that amplify the 62 CpGs located between -291 and 337 of human KEAP1 gene (284) with the translation start site defined as +1. The first 12 CpGs located between -291 to -89 in KEAP1 promoter region were counted as the methylation ratio of these 12 CpGs have been proven to regulate the KEAP1 gene expression in CRC (285). PCR products were cloned into pCR4 TOPO vector using a TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids from at least ten colonies of each treatment group were selected using QIAbprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced (Genwiz, Piscataway, NJ).
3.2.12 Statistical analysis

The results are presented as the mean ± SEM or SD and P-value ≤ 0.05 was considered statistically significant. Statistical analysis was carried out using Student’s t test (two-tailed unpaired) for two groups and one-way ANOVA followed by Dunnett’s post hoc test for multiple groups within GraphPad Prism. Metabolomic pathways analysis and pathway enrichment analysis were performed within the Web-based inference MetaboAnalystR 5.0 (https://www.metaboanalyst.ca/).

3.3 Results

3.3.1 Cytotoxicity of NaB in HCT116 Cells

Treatment with NaB showed a time- and dose-dependent effect on the HCT116 cell viability (Figure 3.1a). Determination of an ideal treatment duration and concentration involves a trade-off between toxicity and efficacy. The viability of HCT116 cells that were treated with 1mM NaB for 1-, 3- and 5-day were 97.4%, 93.2% and 89.5%, respectively, therefore 1mM of NaB was used in the subsequent 5-day next generation sequencing (NGS) so that maximal cell viability was achieved; 1mM and 5mM of NaB were used for the 5-day bisulfite sequencing TA cloning studies. In addition, the cell viability of HCT116 after low (1mM), moderate (5mM) and high (10mM) doses treatment for 24 h were 97.4%, 81.5% and 73.2%, respectively. These doses were further utilized for the metabolomic, RT-PCR, western blot analysis, as stated accordingly. In addition,
as aforementioned the physiological range of B concentrations in the intestinal lumen is between 1 and 10 mM (286, 287) which can further convince our doses.

3.3.2 NaB Regulates NRF2 signaling and NRF2-target Gene Expression

To investigate the mechanism underlying therapeutic efficacy of NaB, we used HepG2-C8 cells that were stably transfected with the ARE-luciferase reporter. NaB inhibits NRF2-ARE luciferase activity at the NaB concentration range from 4 to 10 mM (Figure 3.1b) while lower NaB concentration (<2mM) slightly increased the luciferase activities but not significant (these low concentrations are easily achievable in in vivo situation, including human) (288). In addition, RT-PCR revealed NaB significantly decreased the mRNA expression levels of NRF2 and NRF2-target genes including NQO-1, HO-1, PRDX1, TXN1 and TXN2, while increased the NRF2 negative regulator KEAP1 mRNA levels compared to control (Figure 3.1c and 3.1d). The protein levels measured by Western Blot were further validated showing that NaB decreased NRF2, NOQ-1 and HO-1, while increased KEAP1 protein expression (Figure 3.1e). These results indicate that NaB may be act as a potent NRF2 inhibitor in CRC cells and could be a promising therapeutic agent against advanced cancers.

3.3.3 NaB Drives Epigenetic CpG Methylation Reprogramming

DNA Methyl-seq profiled differentially methylated regions (DMRs) were used to show the epigenomic modulations by NaB in HCT116 cells as we have reported previously in other systems (280, 283). More than 50% DMRs were detected in
the promoters and distal intergenic regions (Figure 3.2a). The methylation differences > 10% of specific genes were applied for the cutoff of DMRs. Briefly, 1,100 DMRs located in the promoter and intron regions were filtered which included 493 hypermethylated and 607 hypomethylated after NaB treatment (Figure 3.2b). Interestingly, DNA methyl-seq analysis also revealed that NaB decreased the CpG methylation ratio in the KEAP1 promoter region from 94% to 85% indicating NaB may regulates the KEAP1 gene expression through modulating its promoter methylation. The methylation of KEAP1 promoter will be validated through bisulfite genomic sequencing TA cloning as below.

3.3.4 NaB Regulates Differentially Expressed Genes (DEGs)

DEGs obtained from RNA-seq analysis were utilized to show the transcriptomic regulations of NaB in HCT116 cells. The PCA (Figure 3.2c) showed that the NaB treatment group to be clearly separated from the vehicle control indicating that exposure of HCT116 cells to NaB significantly induced gene expression alterations. The DEG profile was further analyzed and plotted with the cutoff p-value < 0.01 coupled to log2 fold change ≥ 2.0 or ≤ -2.0 (Figure 3.2d). The MA plot showing that 3,917 and 783 genes were significantly up- and down-regulated after NaB treatment, respectively, compared with control group. Besides the overall mapping, RNA-seq analysis also further validated that NaB inhibited the NRF2 and significantly induced the KEAP1 transcription levels which are highly in agreement with the RT-PCR and Western Blot results above. RNAseq also shows that NaB significantly inhibited NRF2 target genes HO-1 and GCLM gene
expression by 92% and 69% respectively (Figure 3.2e). Moreover, NaB also significantly decreased the cancer marker MYCL proto-oncogene (c-MYC) gene expression by 64%. The c-MYC protein expression level was also validated by western blot (Figure 3.2f).

3.3.5 NaB Regulates Signaling Pathways of HCT116 Cells
A total of 4,700 genes (3,917 up- and 783 down-regulated) filtered with the thresholds p-value < 0.01 coupled to log2 fold change ≥ 2.0 or ≤ -2.0 from the RNAseq dataset were further used to conduct the Ingenuity Pathway Analysis (IPA). These highly regulated genes covered 83 significantly modulated signaling pathways (P ≤ 0.05). The top regulated signaling pathways with -log(p-value) > 2.0 and an absolute values z-score >2.0 were further plotted as shown in the Figure 3.3a. Among these 83 highly regulated pathways, 77 were significantly activated by NaB while 6 pathways were inhibited by NaB. Interestingly, IPA shows NaB significantly activated AMPK, one of the central regulators of cellular and organismal metabolism, signaling pathway indicating that NaB may play critical roles in reprogramming energetic metabolism as well as cellular processes such as autophagy and cellular polarity (231). Other cancer- and cell cycle-related signaling pathways including Tumor Microenvironment Pathway and Cell Cycle Control of Chromosomal Replication pathways were also highly regulated NaB. The totality of these modulated signaling pathways may contribute to the biological effects of B and provide new windows of therapeutic opportunity against CRC.
3.3.6 Integrated Methyl-seq and RNA-seq Analysis Identifies DNA Methylation and Gene Expression Patterns

The current dogma of DNA methylation implicates regulation of transcription of many downstream target genes in mammalian cells (230). The correlation between DEGs and DMRs in the promoter regions were conducted to discover the underlying linkage between DNA methylation and transcriptomic gene expression. A cutoff threshold methylation difference ≥ 10% coupled to log2-fold change of gene expression difference ≥ 2 or ≤ -2 was applied to the selection. A total 444 DMRs in the promoter regions were negatively correlated (85 genes promoter methylation increased with downregulated mRNA expression; 359 gene promoter methylation decreased with upregulated mRNA expression) with their corresponding gene expression after NaB treatment. To have a better visualization, the negatively correlated genes filtered with threshold methylation difference ≥ 15% coupled to log2-fold change of gene expression ≥ 5 or ≤ -5 were used for the starburst plot (Figure 3.3b). Interestingly, this associative analysis revealed that NaB decreased the tumor suppressor ABCA1’s promoter methylation by 11% and increased the gene expression by 6.5-fold compared to control. In addition, the promoter methylation and gene expression of EGR3 were increased by 11% and decreased by 94% respectively, compared to control. These results indicated that NaB may regulate the tumor promoter/suppressor genes through modulating the it’s promoter methylation.
3.3.7 NaB Drives Cellular Metabolomic Rewiring

To unravel the potential underlying molecular links between metabolic reprogramming and epigenetic modifications as well as the impact of NaB on epigenetics through regulating mitochondrial metabolic pathways and metabolites, the samples collected from HCT116 cells treated with vehicle control or 1mM, 5mM and 10mM of NaB for 24h were used to perform the metabolomic analysis. PCA revealed a clear separation for each treatment, showing NaB induced significant differential metabolic profile with a dose-dependent manner, with the most significant difference between control and 10mM NaB groups (Figure 3.4a). A total of 111 metabolites were identified under positive and negative ion modes of which top 30 regulated metabolites (one-way ANOVA computed P value) across 4 groups were further plotted as in figure 3.4b. In addition, the metabolites regulated by 10 mM NaB were filtered with the thresholds p-value < 0.05 and log2 fold changes > 2.0 or < -2.0. Pathway analysis (Figure 3.4c) combined with pathway enrichment analysis (Figure 3.4d) with these filtered metabolites from control vs. 10mM NaB comparison group revealed that the epigenetic associated metabolism such as TCA cycle and methionine metabolism were significantly altered in 10 mM NaB treated HCT116 cells. These results suggested that the alterations of mitochondrial metabolism/metabolites are impacted by NaB and also pointing to the downstream impact on epigenetic reprogramming, to be discussed below.
3.3.8 NaB Regulates Epigenetics via Cellular Metabolism

Mitochondrial TCA cycle and metabolites are tightly linked to the basic epigenetic machinery DNA/histone modifications, chromatin remodeling and modulating phenotypic gene expression (60, 240, 289). Some of the TCA metabolites are cofactors utilized by the epigenetic enzymes that catalyze the post-translational epigenetic modification including AcCoA, the acetyl (-COH) donor for histone acetylation with the HATs catalyzation; aKG, the cofactor along with the histone lysine demethylase (KDMs) and ten eleven translocation (TETs) enzymes for histones and DNA demethylation respectively (289), participate in the biological function of the basic epigenetic machinery (Figure 3.5a). NaB significantly activated the TCA cycle and elevated the metabolites levels in a dose-dependent manner (Figure 3.5b). The AcCoA, aKG and citrate were increased by ~ 1.9-, 4.7- and 12.5-fold after 10 mM NaB treatment respectively (Figure 3.5c). Besides the NaB-induced metabolic regulation, RNAseq also revealed that the transcription levels of epigenetic genes HAT1 (Histone Acetyltransferase 1), KDM1A (Lysine Demethylase 1A), KDM1B (Lysine Demethylase 1B) and TET1 (Tet Methylcytosine Dioxygenase 1), which couple to the above cofactors for the epigenetic regulation, were significantly activated (Figure 3.5d) after NaB treatment. The metabolomic along with the transcriptomic results from RNAseq indicated that NaB would potentially promote histone acetylation, histone demethylation and DNA demethylation via the contribution of NaB-induced metabolic rewiring.
In addition to the TCA cycle, the epigenetic associated methionine cycle metabolites like SAM which was also highly regulated by NaB (Figure 3.6a). SAM, as the universal donor of methyl groups to both DNA and histone methyltransferase enzymes, coupled to DNMTs are tightly linked with basic epigenetic methylation reactions like CpG and histone methylation driving epigenetic reprogramming (290). To monitor the methyl group transfer and synthesis of SAM after NaB treatment, the stable isotope tracing with $^{13}$C-methionine (methyl group donor) experiment was performed. The labeled SAM (m+1) and methionine (m+1)/SAM (m+1) ratio were significantly decreased and increased respectively, after NaB treatment (Figure 3.6b) indicating the decreased transfer potential of the methyl groups to DNA and histones. Furthermore, the intermediary metabolite SAH which is the byproduct of SAM during methyltransferase reactions (243) is a potent inhibitor of DNMTs. The SAM/SAH ratio serves as a biosensor of the cellular metabolic state influencing the activity of methyltransferase enzymes that culminate in chromatin changes in response to cellular challenges (244). NaB inhibited SAM/SAH ratios indicated the reduced cellular methylation potential (291). Moreover, both mRNA and protein expression of DNMT1 (Figure 3.6c) were significantly decreased indicating that the intermediary metabolites coupled to epigenetic transcription enzymes mediated by NaB may contribute to the regulation of epigenetics.
3.3.9 NaB Regulates CpG Methylation in Keap1 Promoter Region

Previous study showed that KEAP1 gene silencing is highly associated with its promoter hypermethylation of the first 12 CpG (−291 to −89) and the KEAP1 promoter regions also have significant different methylation status between CRC and normal cells (285). Therefore, bisulfite sequencing was conducted to further investigate if NaB treatment would demethylate the first 12 CpGs in KEAP1 promoter region and contribute to the gene and protein expression. Figure 3.7 shows that the 12 CpGs were hypermethylated in HCT116 cells in the control group (methylation ratio 91.7%). Compared to control, treatment with NaB (1.0 mM, 5.0 mM) and the combination of 5-aza (500 nM)/TSA (100 nM) (Positive control) significantly decreased the methylation ratio to 80.0%, 77.7% and 62.5%, respectively. This suggests that NaB has demethylation potential on the promoter of KEAP1 gene and results in the increase of KEAP1 gene expression, consequently, attenuates NRF2 biological functions. Furthermore, the bisulfite sequencing result also aligned with the DNA Methyl-seq data which further validated the regulatory effect of NaB on KEAP1-NRF2 signaling axis.

3.4 Discussion

Previous studies reported the anti-tumor effect of NaB in many cancers including CRC through various mechanisms including it can acts as HDACi for the epigenetic modulation and regulates gene expression (273). NaB also has been used as dietary cancer chemopreventive phytochemicals blocking chemical-induced carcinogenesis through regulating NRF2 anti-oxidative signaling in
normal cells (292, 293). However, the molecular mechanisms by which NaB regulate the mitochondrial metabolism, epigenomic/CpG methylation and transcriptomic as well as the role of \( \textit{NRF2} \) in regulating the CRC cell activities remain unclear. So, in our current study, we performed studies with metabolomics, epigenomic and transcriptomic to uncover underlying intricated biological connectivity between NaB-induced metabolomic, epigenomic and transcriptomic regulations, as well as the role of \( \textit{NRF2} \) signaling in NaB-mediated anti-cancer effect in human CRC cells.

The \textit{KEAP1-NRF2} signaling is one of primary pathway in maintaining cellular homeostasis in order to respond adaptively to oxidative stress. Under basal condition, \( \textit{NRF2} \) interacts with two molecules of \textit{KEAP1} to activate Cullin 3 (Cul3)-based E3 ligase complex-mediated \( \textit{NRF2} \) ubiquitination reaction (294). Once \( \textit{NRF2} \) is ubiquitinated, it will be degraded and maintained at a low level in the cytoplasm (295). When cells are exposed to oxidative stress or chemopreventive agents, the cysteine residues of \textit{KEAP1} are modified which can promote the detachment of \( \textit{NRF2} \) from \textit{KEAP1} and allow the \( \textit{NRF2} \) to translocate into nucleus and binds to ARE in the upstream promoter region of multiple genes (260). In cancer cells, aberrant activation of \( \textit{NRF2} \) have been widely reported especially in CRCs and it’s highly associate with \textit{KEAP1} expression levels. \( \textit{NRF2} \) overexpression in cancers further promote cell proliferation and enhance the chemoresistance of cancer cells (260). Previous study reported 8 of 10 CRC cell lines had hypermethylated CpG islands in the \textit{KEAP1} promoter region. HT29 CRC cells with a hypermethylated \textit{KEAP1} promoter resulted in decreased mRNA
level. These results suggested that methylation of the *KEAP1* promoter regulates its mRNA level. Furthermore, aberrant *KEAP1* promoter methylation was detected in 53% of tumor tissues from 40 surgical CRC specimens, indicating that cancerous tissue showed increased methylation of the *KEAP1* promoter region, conferring a protective effect against cytotoxic anticancer drugs (285). In this context, NaB significantly decreased the *KEAP1* promoter methylation (Figure 3.7) which can promote the *KEAP1* mRNA and protein expression levels and then further decrease the *NRF2* mRNA and protein levels as well as the *NRF2*-ARE signaling pathway (Figure 3.1), and contribute to the NaB-mediated cancer therapy. Based on these, NaB (as a *NRF2* inhibitor in advance cancers) also can be used in combination with other chemotherapy agents to alleviate the chemoresistance and strengthen the anti-cancer effects.

In addition to the regulation effect on *NRF2* signaling, NaB also upregulates the tumor suppressor gene *ABCA1* and downregulates the tumor promote gene *EGR3* potentially through modulating their corresponding promote methylation. Previous studies showed *ABCA1* is a known tumor suppressor (296) and downregulation of *ABCA1* expression causes high intracellular cholesterol levels, which creates an environment conducive to tumor progression (297). *ABCA1* transporter is directly suppressed by miR-200b-3p and upregulation of miR-200b-3p was also observed in cancers. Therefore, it is proposed that one of the mechanisms of cancer cell proliferation and metastasis in cancers is via miR-200-3p-directed inhibition of the *ABCA1* transporter (298). Moreover, Arnon et al, reported *EGR3* has been implicated in cancer cell migration which makes it
highly associated with tumor progression and it's expression level also have been used as a prognostic marker in various cancers including breast carcinomas (299) and CRC(300). In colon cancer cell lines, EGR3 has binding sites in several genes related to the cancer therapy agent 5-fluorouracil resistance which support that EGR3 may have a protective function against chemotherapy (301). Moreover, previous studies indicated that that c-MYC amplification and overexpression was showed in approximately 10 and 70 % in CRC, respectively (302). And c-MYC gene can promote tumorigenesis in CRC, mediate the critical role in the CRC progression and highly involve in chemotherapy resistance (303). Deregulation of c-MYC by NaB (Figure 3.2f) would be promising to against CRC and alleviate chemoresistance. So, the findings in current study may indicate that NaB can be used as a potential anti-cancer therapeutics or in combination with other anti-cancer drugs for cancer therapy, via regulation of the cancer markers.

Recent evidence suggests strong molecular link between metabolic rewiring and epigenetic reprogramming through key metabolic intermediates, such as SAM, aKG and AcCoA, which can be used as therapeutic targets for cancer therapy (237). In cancer cells, aberrant mutations activate oncogenes or inactivate tumor suppressor genes may affect multiple signaling pathways that results in mitochondrial metabolic reprogramming and altered bioenergetics (238). Moreover, ROS are known as an important progenitors in carcinogenesis, including CRC (304). ROS are commonly higher in CRC cells than their normal counterpart cells (305) which has been reported to induced cellular oxidative
stress and regulate multiple redox signaling pathways that ultimately impacts on cellular metabolic homeostasis (196). In current study, NaB regulates metabolic pathways such as TCA cycle and methionine metabolism as well as the metabolites like SAM, SAM/SAH, AcCoA in HCT116 cells (Figure 3.4-3.6). These metabolites are tightly linked to the basic epigenetic machinery DNA/histone modifications, chromatin remodeling and modulating phenotypic gene expression with high connectivity between mitochondrial metabolites, and epigenetics (60, 204, 240, 241). Specifically, SAM is the universal donor of methyl groups to both DNA and histone methyltransferase enzymes, and the changes in methionine metabolism altering levels of SAM can directly influence trimethylation of H3K4 (Histone H3 lysine K4) and consequently regulates gene expression (242). The intermediary metabolite SAH is the byproduct of SAM during methyltransferase reactions (243), which is a potent inhibitor of both DNA and histone methyltransferases. Thereby, the SAM/SAH ratio serves as a biosensor of the cellular metabolic state influencing the activity of methyltransferase enzymes that culminate in chromatin changes in response to cellular alternations (244). The methyl group isotope labeling study showed that NaB decreased transfer potential of methyl groups to DNA and histones (Figure 3.6b). Besides the metabolic regulation, NaB also modulates the epigenetic regulatory enzymes/genes such as DNMT1 (Figure 3.6c), \textit{HAT1}, \textit{KDM1A}, \textit{KDM1B} and \textit{TET1} (Figure 3.5d). These results indicated that NaB coordinately regulates epigenetic associated genes and metabolites which can further reveal the underlying mechanism of NaB-mediated anti-cancer effect.
In conclusion, our study integrates the latest Methyl-seq, RNA-seq and isotope labeling LC/MS/MS technologies in dissecting the potential underlying mechanism of epigenomic CpG methylation, mRNA transcriptomic gene expression and mitochondrial metabolic rewiring in HCT116 CRC cells after exposure to cancer therapeutic compound NaB. The results provide the experimental evidence for the metabolomic, epigenomic, and transcriptomic effects of NaB in CRC cells. We observed that NaB rewire metabolic profile, reprograms epigenetic CpG methylation, driving alterations of phenotypic gene expression, de-/activated signaling pathways in HCT116 cells. NaB decreased NRF2 gene expression through regulating the KEAP1 promoter methylation, which further regulates NRF2-target genes and contribute the NaB-mediated cancer therapy. Thus, the anticancer potential of NaB is exhibited through regulation of KEAP1/NRF2-ARE pathways, metabolic rewiring and epigenetic reprogramming in HCT116 cells, making it a promising drug candidate for the treatment of CRC.
Figure 3.1 NaB-mediated cell viability and regulation of NRF2/NRF2-target genes in HCT116 cells.

(a) Cell viability of the HCT116 cells after various concentrations of NaB treatment for 1-, 3- and 5-day. Cell viability was determined by the MTS assay; (b) The luciferase activity after NaB treatment in HepG2 cells transfected with the ARE-luciferase reporter vector. The activity was normalized based on the protein concentrations in the BCA protein assay and shown as fold change compared to control group; (c) NRF2 and KEAP1 mRNA expression measured by RT-PCR. The expressions were shown as fold change compared to control group; (d) NRF2-target genes mRNA expression measured by RT-PCR. The expression levels were shown as fold change compared to control group; © NRF2, KEAP1,
HO1 and NQO1 protein expression quantified by Western Blot after NaB treatment. The protein expressions were shown as fold change compared to control group and GAPDH was used as the housekeeping protein for the normalization. All the data are presented as the means ± SEM of three independent experiments. *, P < 0.05 and **, P < 0.01 indicate significant differences between the treatment groups and the control group (0.1% DMSO). Student’s t test was used to calculate the significance of the differences compared with the control.
Figure 3.2 DNA Methyl-seq and RNA-seq profiles in HCT116 cells regulated by NaB.

(a) Distribution of annotated DMRs by genomic features including Distal Intergenic, Promoter (<=1kb, 1-2kb and 2-3kb), 1st Intron and other regions as shown in the Figure 3.2a. Each DMR has at least three CpG sites; (b) Overall DMRs in response to 1mM NaB with cutoffs of methylation difference $\geq 10\%$ or $\leq -10\%$. “+” represents Intron, “*” represents Promoter, “0” represents the absolute methylation difference <10%, “1” represents the methylation increased by >10% and “2” represents the methylation decreased by >10%; (c) PCA of transcriptomic profiles in control and NaB treatment groups; (d) MA plots showing overall DEGs in response to 1mM NaB with cutoffs of $p<0.01$ and log2(fold change).
change) ≥ 2.0 or ≤ -2.0; © NRF2 and NRF2-target genes mRNA expression reported from RNA-seq analysis. The mRNA expressions were shown as fold changes compare to control group; (f) c-MYC mRNA expression reported from RNA-seq and protein expression quantified by western blot after NaB treatment. GAPDH was used as the housekeeping protein for the protein normalization. The RNA-seq data are presented as the means ± SEM of two independent biological replicates. The western blot protein levels are presented as the means ± SEM of three independent experiments. *, P < 0.05 and **, P < 0.01 indicate significant differences between the treatment groups and the control group (0.1% DMSO). Student’s t test was used to calculate the significance of the differences compared with the control group.
Figure 3.3 Ingenuity Pathway Analysis (IPA) and Correlation of DEGs and DMRs.

(a) Top 30 regulated pathways in HCT116 cells after NaB treatment; (b) Correlation analysis between gene expression and DNA methylation. Total 43 DMRs (located in the upper left and bottom right corners) were negatively correlated with their corresponding gene expression with the cutoff of methylation difference $\geq 15\%$ or $\leq -15\%$ coupled to gene expression log2(fold change) $\geq 5.0$ or $\leq -5.0$. 9 of 43 DMRs were located in the promoter region. The DMR locations (gene features: Region, Body, Downstream and Promoter) are indicated by the colors.
Figure 3.4 Metabolomic profiles of HCT116 cells treated with NaB.

(a) PCA plot of metabolomic profiles in HCT116 cells after control and 1mM, 5mM and 10mM NaB treatment; (b) Top 30 regulated metabolites after NaB treatment; (c) Metabolism pathway analysis and (d) pathway enrichment analysis of HCT116 cells after 10mM NaB treatment.
Figure 3.5 NaB upregulates epigenetic associated TCA metabolites and epigenetic genes.

(a) Schematic of connectivity between TCA cycle and epigenetic enzymes. The enzymes involved are highlighted in red; (b) Heatmap showing metabolites of TCA cycle in HCT116 cells after treated with 10mM NaB for 24h (n=3); (c) Quantified TCA metabolites shown as fold changes compare to control group (n=3); (d) Epigenetic gene expression reported from RNA-seq analysis (n=2). *, P < 0.05 and **, P < 0.01 indicate significant differences between the treatment groups and the control group (0.1% DMSO). Student’s t test was used to calculate the significance of the differences compared with the control group.
Figure 3.6 NaB regulates the methionine metabolism and decreases transfer potential of the methyl groups to DNA and histones.

(a) Schematic of connectivity between methionine metabolism and epigenetic enzymes and the regulation effect of NaB on methionine cycle metabolites (n=3). The enzyme involved is highlighted in red; (b) Quantified methionine metabolism metabolites and isotope labeling of methionine to trace the methyl group transfer in HCT116 cells after NaB treatment; (c) Quantified DNMT1 gene and protein expression by RT-PCR and western blot respectively. *, P < 0.05 and **, P < 0.01 indicate significant differences between the treatment groups and the control group (0.1% DMSO). Student’s t test was used to calculate the significance of the differences compared with the control group.
Figure 3.7 Effect of NaB on KEAP1 promoter methylation in HCT116 cells.

Cells were treated with 1.0 or 5 mM NaB for 5 days, and then genomic DNA was extracted from the treated cells. A combination treatment of 5-aza (500 nM)/TSA (100 nM) was used as positive control, while TSA was added 24 h before cell collection. The methylation pattern of the first 12 CpGs located at -291 and 337 in KEAP1 promoter region, where the translational starting site is considered as +1 was determined. Black dots indicated methylated CpGs while open circles indicate unmethylated CpGs. At least ten clones were picked randomly and sequenced from each of the three independent experiments.
Chapter 4 Pten regulates metabolic, epigenomic, transcriptomic and protection by triterpenoid ursolic acid in different stages of prostate cancer in prostate specific Pten KO mice10, 11, 12

4.1 Introduction

Prostate cancer (Pca) is one of the most commonly diagnosed cancer and the third leading cause of cancer-related death in men in the United States (306). The phosphatase and tensin homologue (PTEN) tumor suppressor on chromosome 10 and the phosphoinositide 3-kinase (PI3K) signaling axis it restrains are among the most commonly altered pathways in primary human Pca (307, 308). Inactivation of PTEN is identified in ~20% of primary prostate tumor tissue samples at radical prostatectomy and in 50% of castration-resistant tumors. Loss of PTEN function results in activation of the PI3K-AKT (RAC-alpha serine/threonine-protein kinase) pathway, which is highly linked with adverse oncological outcomes, making PTEN a potentially useful genomic marker to distinguish indolent from aggressive disease in patients with clinically localized tumors. In addition, deletion of Pten has been highly linked to inflammation, in prostate-specific Pten KO mice, the expression of CXCL8/IL-8, a pro-inflammatory chemokine promoting tumorigenesis was significantly increased.

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11Keywords: Prostate cancer (Pca); PTEN; Ursolic acid (UA); Epigenetic; Metabolomic; Cancer prevention.
12Abbreviations: Pca, Prostate Cancer; PTEN, phosphatase and tensin homologue; UA, Ursolic acid; PI3K, phosphoinositide 3-kinase; NRF2, Nuclear factor E2-related factor 2
(309). So, the cellular regulatory role of PTEN points to the fact that PTEN signaling pathway as well as PTEN mutation-mediated biological alternations could be used as a primary target in cancer chemoprevention and therapy. Cellular metabolism is the set of chemical reactions that occur in living organisms in order to allow organisms to grow and reproduce, maintain their structures, and respond to environmental changes. Cancer metabolism is an essential aspect of tumorigenesis, as cancer cells have increased energy requirements in comparison to normal cells. Thus, an enhanced metabolism is needed in order to accommodate tumor cells’ accelerated biological functions, including increased proliferation, vigorous migration during metastasis, and adaptation to different tissues from the primary invasion site (310). The characteristic metabolic hallmark of tumor metabolism is aerobic glycolysis or the Warburg effect. Unlike normal cells that produce energy mostly through the oxidation of pyruvate in the mitochondria, cancer cells predominantly produce energy via enhanced glycolysis in the cytosol, even under aerobic conditions. Aerobic glycolysis in cancers is the combined result of oncogenes, tumor suppressors, a hypoxic microenvironment and others (311). Understanding the complex cancer energy metabolism will help to develop new approaches in early diagnosis and cancer therapy. This study will cover the tumor suppressor gene PTEN-mediated metabolism, including the analysis of altered enzymatic gene expressions that are involved in cancer-related purine metabolism, and discuss current strategies of targeting metabolic pathways such as pyruvate metabolism for cancer treatment.
Epigenetics is the study of genomic alterations, such as DNA methylation and histone modifications, which alter DNA accessibility and chromatin structure, thus influencing patterns of gene expression, but without altering the genetic code itself (312). Epigenetic dysregulation occurs at every phase of Pca and plays critical role in Pca initiation, progression, and treatment resistance. These aberrations are responsible for silencing tumor-suppressor genes, activating oncogenic drivers, and driving therapy resistance (313). Specifically, CpG hyper-/hypo-methylation in DNA promoter regions is believed to play a key role in regulating gene expression, perhaps by interfering binding of transcription factors (256). Previous studies reported the epigenetic silencing of PTEN gene in melanoma with high percentages of PTEN methylation were associated with low PTEN transcription levels in melanoma, therefore, resulting in increased tumorigenesis (314). Interestingly, increasing evidence suggests that cellular metabolism plays an important role in cancers and that the cellular metabolism/metabolites are tightly linked to the basic epigenetic machinery (204). Epigenetic modifications including DNA methylation and histone acetylation are sensitive to cellular metabolic status (205). Strong molecular link between metabolic rewiring and epigenetic modifications through key metabolic intermediates, such as aKG, AcCoA of the TCA cycle and SAM of methionine cycle which are co-factors for the epigenetic enzymes and work as hubs between epigenetic processes and therapeutic modalities (60, 206, 207). So, modulation of epigenetic such as DNA de-/methylation, histone de-/acetylation as well as
transcriptomics through regulating metabolomics may prevent diseases and protective against cancer development and metastasis (258).

Ursolic acid (UA) is a natural pentacyclic triterpenoid carboxylic acid derived from medical herbs, fruits and vegetables (e.g. cranberry, blueberry, apple peels and mushrooms) and exerts a wide range of biological activities, including antioxidant, anti-inflammatory, cancer prevention as well as anti-tumor (315-317). UA has been reported to suppress the proliferation and induce apoptosis in both in vitro tumor cells and in vivo animal models, therefore, to inhibit tumor promotion, metastasis and angiogenesis (318). Our recent studies reported that UA decreased the CpG methylation of Nrf2 promoter region, which was associated with the Nrf2 activation in mouse epidermal JB6 cells (319). In addition, UA has been reported to regulate several cellular metabolites and metabolism-related signaling pathways including SAM, methionine, glycolysis, and nucleotide sugars metabolism which are tightly coupled to epigenetic machinery and cancers (283).

Lodi et al. (320) also reported that combinatorial treatment with natural compounds including UA in prostate cancer inhibits prostate tumor growth and leads to key modulations of cancer cell metabolism.

Our previous study (321) involved crossing the probacin (Pb)-Cre promoter transgenic mice and Pten-flox allelic mice to obtain prostate-specific Pten-/ (KO) mouse prostatic adenocarcinoma model, and found that Pten deletion drives prostate prostatic intraepithelial neoplasia (PIN), inflammation reactions, increases prostate size and regulates the downstream signaling changes. (Appendix 4.1) and also reprograms global changes in DNA CpG methylation
and transcriptomic gene expression which are highly associated with several inflammatory and immune molecular pathways, such as NF-kB signaling, IL-6 signaling and PI3K in B lymphocytes signaling, during Pca development. However, the role of the UA in regulating metabolic rewiring, CpG methylomic reprogramming, and transcriptomic network in blocking PTEN deletion-mediated biological alterations and elicit cancer prevention/anti-cancer effects in Pca remains unknown. So, as a continuation of our Pten deletion study, previous Pten WT vs KO (321) incorporated with current UA treated Pten KO datasets were reanalyzed to examine 1) the role of Pten deletion in the cancer prevention/anti-cancer function of UA in prostate-specific Pten KO prostatic adenocarcinoma mouse model; 2) the underlying intricate biological connectivity between metabolomic, epigenomic and transcriptomic regulation by UA.

4.2 Materials and methods

4.2.1 Chemicals and animal diets

UA was purchased from Toronto Research Chemicals (North York, ON, Canada) and blended into AIN-93M rodent diet (Research Diet, Inc. New Brunswick, NJ, USA) at a final concentration of 0.1 % (w/w). The diet was stored at 4°C throughout the animal experimentation period. All mice were housed in our animal facility in accordance with the protocol approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). All mice were maintained under standard 12-h light/12-h dark cycles with water and diet provided ad libitum unless otherwise specified.
4.2.2 Animal studies

A mouse model that uses the Pb-Cre promoter and Pten-flox has been commonly used to examine the role of Pten deletion in prostate tumorigenesis (322, 323). Previous studies reported that in prostate-specific Pten KO mice, at week 10, the low-grade prostatic intraepithelial neoplasia (PIN) was observed; at week 12, the high-grade PIN would appear, and adenocarcinoma developed starting from week 12 to week 20 (at week 20, 100 % of the mice developed adenocarcinoma) (324, 325). Pb-Cre4 mice (strain: B6.Cg-Tg(Pbsn-cre)4Prb/Nci) and Pten(flox/flox) mice (C;129S4-Ptentm1Hwu/J) obtained from National Cancer Institute and Jackson Laboratories, respectively, were used to crossbreed and generate the prostate-specific Pten KO mouse (326, 327). F2 generation prostate-specific Pten KO male offspring (Pb-cre/Pten(flox/flox)) were generated by crossing male Pb-Cre4 mice with female Pten(flox/flox) mice. The detailed prostate Pten KO breeding scheme was presented in our previous publication (321). For simplicity, Pb-cre/Pten(flox/flox) mice are referred to as Pten KO, and Pten(flox/flox) mice are considered as Pten WT. The mice were genotyped using PCR, and only the male mice that were Cre carriers and homozygous Pten(flox/flox) were used for the treatments. The detailed primers used for the Pb-Cre4 and Pten genotyping were also reported in our previous study (321).

4.2.3 Experimental procedure

Pten KO mice were randomly assigned to four experimental groups (n=5). Briefly, 6-week (wk)-old Pten KO mice were fed with either AIN-93M control diet or 0.1 %
UA diet for 6 (age: 12 wk old) and 14 wk (age: 20 wk old) and then sacrificed by CO₂ asphyxiation. Mouse prostates were collected immediately and either dissected into ventral & lateral prostate (VLP) and dorsal & lateral prostate (DLP) and snap-frozen and then stored at -80°C for epigenomics, biomarkers and metabolomics analyses.

4.2.4 Isolation of nucleic acid and next-generation sequencing (NGS)

Total RNA and DNA were extracted from prostate tissues using an AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and concentration of the extracted RNA and DNA were determined using an Agilent 2100 Bioanalyzer and a NanoDrop spectrophotometer, respectively. 8 RNA samples and 8 DNA samples [2 groups (Pten KO, Pten KO+UA) * 2 time points (6&14 wk) * 2 (n=2)] were subjected to RNA-Seq and SureSelect Methyl-Seq, respectively. Library preparation and sequencing were performed by RUCDR Infinite Biologics. The DNA samples were further processed using an Agilent Mouse SureSelect Methyl-Seq Target Enrichment System (Agilent Technologies Inc., Santa Clara, CA, USA) and sequenced on an Illumina NextSeq 500 instrument with 76-bp single-end reads, resulting in the generation of 30-45 million reads per sample. For RNA-Seq, 75-bp paired-end reads produced approximately 30 million reads per sample. The further details of the DNA-seq and RNA-seq procedures were described previously (219).
4.2.5 Bioinformatics analyses

**RNA-seq**

Cutadapt (220) was used to remove the Illumina Universal Adapter sequence. Hierarchical indexing for spliced alignment of transcripts (HISAT2) was adapted for aligning the reads to the mouse genome (mm10) and remove PCR duplicates (221). Genomic features with overlapping reads were counted by featureCounts (version 1.5.1) (222) and then data were further analyzed for differential expression genes (DEGs) with DEGSeq (version 1.36.0) in R (223).

**DNA SureSelect Methyl-seq**

The DNA reads were aligned to the *in silico* bisulfite-converted mouse genome (mm10) using the Bismark (version 0.15.0) alignment algorithm (328). And the DMRfinder (version 0.1) was used to extract methylation counts and to cluster CpG sites into differentially methylated regions (DMRs) (224). Each DMR contained at least three CpG sites. Methylation differences greater than 0.1 (10%) with *P*-values < 0.05 were considered statistical significance. Genomic annotation was performed with ChIPseeker (version 1.10.3) in R (225).

4.2.6 Ingenuity Pathway Analysis (IPA)

DEGs with false discovery rates (FDR) adjusted *P* value (q value) < 0.05 coupled to log2-fold change >1.0 or <-1.0 were subjected to IPA (IPA 4.0, Ingenuity Systems). The input DEGs were mapped to the IPA knowledge base, and the biological functions, networks, and pathways related to *Pten* WT vs. KO
(reanalyze from our published datasets) (321) and *Pten* KO vs KO+UA treatment in different time points (6 & 14 wk) were identified.

### 4.2.7 LC-MS Metabolomic Analysis

LC-MS metabolomic analysis was performed in Metabolomics Shared Resources, Rutgers CINJ as our previously reported (329-331). Prostate tissues from three mouse groups (*Pten* WT, KO and KO+UA) were subjected to organic extraction of cellular metabolites for metabolomic analysis. Briefly, 15-30 mg tissue were weighed and pulverized with Yttria Grinding ball using CryoMill at 20 Hz for 2 min to ensure completely homogenized tissue. Then metabolites were extracted with (tissue weight (mg) * 40)/2 ul volume of cold 40:40:20 methanol:acetonitrile:water solution with 0.5% formic acid, and then followed by 5 cycles of ice cold sonication with 30s ON and 30s OFF using Bioruptor UCD-200 sonication machine and another 10 minutes incubation on ice, and then sequentially neutralized with 50 μL/1mL extraction buffer of 15% NH₄HCO₃. The cleared supernatant was then used for LC-MS metabolomic analysis. And the tissue pellets were further lysate with RIPA buffer for the protein extraction which will be used for the LC-MS metabolomic data normalization.

LC separation was performed on a Xbridge BEH Amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130 Å pore size; Waters) coupled with a Waters Xbridge BEH XP VanGuard cartridge (2.1 mm x 5 mm, 2.5 μm particle size, 130 Å pore size) guard column. The solvent A prepared by water/acetonitrile (95:5, v/v) with 20 mM NH₃AC and 20 mM NH₃OH at pH 9; and solvent B prepared by
acetonitrile/water (80:20, v/v) with 20 mM NH₃AC and 20 mM NH₃OH at pH 9 in
the following solvent B percentages over time: 0 min, 100%; 3 min, 100%; 3.2
min, 90%; 6.2 min, 90%; 6.5 min, 80%; 10.5 min, 80%; 10.7 min, 70%; 13.5 min,
70%; 13.7 min, 45%; 16 min, 45%; 16.5 min, 100%. The flow rate was set to 300
μL/min with an injection volume 5 μL. The column temperature was set at 25°C.
MS scans were obtained in both positive and negative ion modes with a
resolution of 70,000 at m/z 200, in addition to an automatic gain control target of
3 × 10⁶ and m/z scan range of 72 to 1000.

4.2.8 Dosage information/Dosage regimen
Animal diets containing UA (≥95% purity, Sigma-Aldrich U6753) 0.1% w/w kg
diet were prepared in AIN-93M control diets and the specially prepared diet will
exclude the use of antioxidants to avoid interference. The oxidation will be
prevented by frequent change of diet and storing it in a nitrogen atmosphere in
the cold. The average daily consumption of feed for an adult 25 g mouse is 3-5 g,
corresponds to 3-5 μg/day. Previous studies used 0.1%-2% UA-supplemented
diets for the long-term treatment (≥ 6 weeks) in both health and disease animal
models (332) and the toxicity studies indicated that the animals received daily
doses of 1000 mg/kg/day via oral gavage for 90 days does not result in toxic
effects and concluded that the no-observed-adverse-effect-level (NOAEL) for UA
is likely to be higher than 1000 mg/kg/day. A clinical pharmacokinetic (PK) and
safety study in healthy adult volunteers of UA at single oral doses up to 1000 mg
also found no serious adverse event (333). In terms of the pharmacological
effects, previous animal studies reported that 0.1%-2% UA-supplemented diets could initiate the therapeutic potential of UA (332). In conclusion, based on these reports that the dose used in the current animal study is non-toxic and would potentially show pharmacological effects of UA.

4.2.9 Statistical analysis
Statistical significance was tested with one-way ANOVA followed by Dunnett’s post hoc test for differences among multiple experimental groups and with Student’s t-test for differences between two experimental groups. The values are presented as the mean ± standard deviation (SD). $P$-values ≤ 0.05 were considered statistically significant.

4.3 Results
4.3.1 Overview of DMRs Regulated by UA in Pten KO Mice Prostate
Our previous histology analysis showed that almost all glands of the 12 & 20 wk-old Pten prostate-specific KO mice developed to low/high-grade PIN. The severity of PIN and inflammation development gradually increased as Pten KO mice aged (321). The size of prostates in Pten KO and UA treated Pten KO mice at different time points (12wk & 20wk age or 6wk & 14wk treatment) were shown in figure 4.1E. Briefly, deletion of Pten resulted in progressively enlarged prostate lobes in an age-dependent manner and the sizes of prostates significantly different between Pten KO and Pten KO+UA mice.
To identify the overall regulatory effect of UA on Pten KO-induced DNA methylation alterations, we performed single base-pair resolution CpG Methylation-seq with DNA prostatic samples from Pten KO and KO+UA mice. Sequencing reads were aligned to an in-silico C-T converted mouse genome (mm10) and deduplicated. Individual CpG sites were clustered into DMRs, and the average methylation ratio for each DMR was calculated. We then collected DNA methylation data for a total of 137,897 DMRs. These DMRs were further annotated with gene features using ChIPseeker. As shown in Appendix 4.2a, majority of the DMRs were detected in the promoters and the distal intergenic regions (> 3 kb upstream of the transcription start site (TSS) or downstream of the 3’ untranslated region (UTR)). Similarly, analysis of the distribution of DMRs based on number of CpGs and region showed that the number of CpG sites in the promoter, gene body, and downstream regions were much greater than that in other regions (Appendix 4.2b). We next compared the DNA methylation levels in Pten KO and KO+UA groups. As shown in Appendix 4.2c, no significant methylation difference was observed among each individual treatment group. However, CpG methylation in the promoters was much lower than that in other regions for these groups. PCA revealed a clear separation for each treatment groups, showing that there were clear differences between Pten KO mice and Pten KO+UA groups (Appendix 4.2d). The overall DMRs in Pten WT mice were reported in our previous study (321) which will be reanalyzed with Pten KO and KO+UA datasets to uncover the role of Pten deletion in the epigenetic regulation
function of UA in prostate-specific Pten KO prostatic adenocarcinoma mouse model, to be discussed below.

4.3.2 UA Drives Epigenetic CpG Methylation in Pten KO Mouse Prostate

To examine the epigenetic regulatory effects of UA on Pten KO mice after 6 and 14 wk treatments, we compared the CpG Methylseq profiles between the Pten WT, KO and KO+UA groups. Firstly, the DMR regulations of Pten KO and UA protective effect on Pten KO-mediated CpG methylation were identified by comparing Pten WT vs. KO and KO vs. KO+UA counterparts with the threshold methylation difference ≥ 10%, P-value ≤ 0.05 (Figure 4.1a). Briefly, in 6 wk counterpart, Pten KO significantly increased 3968 and decreased 5330 DMRs while UA reversed 1514 (1514/3968) and 2121 (2121/5330) DMRs, respectively, indicating the epigenetic regulatory effect of UA on Pten KO-mediated CpG methylation. In 14 wk counterpart, Pten KO exacerbate the epigenetic modulation with extra hypomethylated DMRs (14109) compared to 6 wk counterparts, while UA reversed 4262 (4262/14109) Pten KO-regulated DMRs. These results indicate the later stage of Pca drives severe epigenetic CpG methylation which may be responsible for silencing tumor-suppressor genes and activating oncogenic drivers, etc. (313), while UA partially reversed the Pten KO-induced epigenetic alterations in both early and late stage (6wk & 14 wk treatment or 12 wk & 20wk age). Based on these findings, to further explore the effect of UA on Pten KO-mediated methylation modulations at different Pca stages (6wk vs. 14wk treatment or 12wk vs. 20wk age in current study), a cutoff with higher
thresholds of methylation difference ≥ 20% coupled to \(P\)-value ≤ 0.05 were used to identify and plot the UA-regulated DMRs by comparing \(Pten\) KO mice with \(Pten\) KO+UA mice (KO vs. KO+UA) at different stages (Figure 4.2b-c). Specifically, 307 DMRs were hypermethylated and 924 DMRs were hypomethylated after UA treatment for 6 wk. 274 and 957 DMRs were hyper- and hypomethylated, respectively, in response to the 14 wk UA treatment. Of the DMRs regulated by UA treatment, a total of 1038 DMRs were found to affect methylation in same directions after both 6 and 14 wk treatment (Figure 4.1d). Specifically, 194 DMRs that were hypermethylated in both 6 wk (194/307) and 14 wk (194/274) UA treatment. And almost all of the hypomethylated DMRs in 6 wk (844/924) and 14 wk (844/957) UA treatment groups were completely overlapped indicates that UA possesses the stable CpG methylation on \(Pten\) KO mice prostate.

4.3.3 UA Regulates Differentially Expressed Genes (DEGs) in \(Pten\) KO Mouse Prostate

To identify gene expression changes in \(Pten\) KO mice as well as \(Pten\) KO mice treated with UA for 6 and 14 wk, RNA-seq was performed with RNA samples extracted from \(Pten\) WT, KO and KO+UA mice prostate tissues. Appendix 4.3a shows that the counts and the raw gene expression overviews across the \(Pten\) KO and KO+UA groups were measured within the similar magnitude. PCA (Appendix 4.3b) and Euclidean distance clustering (Appendix 4.3c) showed the \(Pten\) KO groups to be clearly separated from 12 and 20 wk UA treatment groups.
The DEGs overview of \textit{Pten} WT mice were reported in our previous publication (321). To examine the effect of UA treatment on \textit{Pten} KO-mediated gene regulation, the DEG profiles between \textit{Pten} WT vs. KO and KO vs. KO+UA were further analyzed and plotted with the cutoff p-value ≤ 0.01 coupled to log2 fold change ≥ 1.0 or ≤ -1.0 (Figure 4.2a). When comparing the 6 wk groups, the MA plot showing that 2,398 and 803 genes were significantly up- and down-regulated after \textit{Pten} KO respectively (\textit{Pten} KO vs. WT); while 1,157 and 1,658 genes were significantly up- and down-regulated respectively, when \textit{Pten} KO mice treated with UA diet (\textit{Pten} KO vs. KO+UA). Within these 2 comparisons in the 6 wk counterparts, UA offsets 1,741 \textit{Pten} KO-modulated (1,576 genes were up-regulated, while 165 were down-regulated after \textit{Pten} KO) genes (Figure 4.2b). Similarly, the gene expression profiles in the 14 wk counterparts were shown in figure 4.2c-d. In addition, 14 wk \textit{Pten} KO exacerbate the gene regulation with an increased DEGs compared to that in 6 wk counterpart. However, 6 wk UA treatment elicited stronger gene regulatory effect compared to the 14 wk counterparts manifested as more filtered DEGs (Figure 4.2a&c). These results indicate that UA possesses more potent gene regulatory effects on the early stages of Pca which may elicit stronger cancer protective effects. Notably, UA significantly reversed or suppressed the \textit{Pten} deletion-induced Pca related oncogenes such as \textit{Has3}, \textit{Cfh} and \textit{Msx1} overexpression (Figure 4.2e) indicating UA plays a crucial role in \textit{Pten} KO-mediated gene regulation and its potential consequences on cancer prevention.
4.3.4 UA Regulates Pten KO-induced Signaling Pathway

The regulated genes shown in Figure 4.2a&c which were filtered with the thresholds p-value < 0.01 coupled to log2 fold change ≥ 1.0 or ≤ -1.0 from the RNAseq datasets were further used to conduct canonical pathway analysis using IPA to uncover potential biological functions of Pten deletion the treatment effect of UA. Briefly, Pten KO significantly (-logP<1.3 or P<0.05) regulated 163 and 193 signaling pathways in 6 and 14 wk groups, respectively, compared to their corresponding Pten WT mice, while UA modulated 167 and 39 signaling pathways after 6 and 14 wk treatment respectively. These results indicate that the signaling pathways in later stage (14 wk treatment or 20 wk age) of Pca are highly modulated compared to early stage (6 wk treatment or 12 wk age), while UA exerts stronger signaling protective effects in early stage rather than late stage. To further explore the effects of UA on Pten KO-mediated signaling pathways, the shared pathways between Pten WT vs. KO and KO vs. KO+UA comparison groups in 6 & 14 wk were filtered. Top 25 shared signaling pathways were further plotted with their ‘activation z scores’ (Figure 4.3a). Among the 6 wk counterpart, 126 and 11 signaling pathways were significantly activated and inhibited by Pten KO while UA completely offsets these Pten KO-mediated signaling pathways. For the 12 wk counterparts, UA only reversed 34 Pten KO-mediated signaling pathways (30 upregulated and 4 downregulated). These results indicate that the effects exerted by UA treatment on the pathways were stronger at 6 wk than that at 14 wk, consistent with earlier findings. Interestingly, blockade of the Pten KO-induced AMPK, one of the central regulators of cellular
and organismal metabolism, signaling pathway by UA treatment indicating that UA could play critical roles in regulating growth and reprogramming metabolism as well as cellular processes such as autophagy and cellular polarity in Pten KO mice prostate (231). UA-mediated AMPK signaling inhibition may further block glycolysis metabolism which has been considered as an attractive anticancer strategy (212, 232). Other inflammatory response and immune response pathways, such as NF-kB signaling, iNOS signaling, PI3K in B lymphocytes signaling, LXR/RXR activation, IL-6 signaling, IL-8 signaling, CD28 signaling in T helper cells and iCOS-iCOSL signaling in T helper cells, among others were also highly regulated in a reverse manner by UA in Pten KO mice. It was not surprising that these findings suggested the inhibitory effects of UA on the level of inflammation induced by Pten deletion at 6 wk are stronger than those at 14 wk by affecting inflammatory response and immune response pathways. In this context, the totality of these modulated signaling pathways would contribute to the biological effects of UA and provide new windows of therapeutic opportunity against Pca in early stages of tumor development.

4.3.5 Integrated Methyl-seq and RNA-seq Analysis Identifies DNA Methylation and Gene Expression Patterns

The current dogma of DNA methylation implicates regulation of transcription of many downstream target genes in mammalian cells (230) and DMRs have been shown to play an important role in the transcriptional control of many crucial genes. It is generally accepted that the promoter CpG methylation ratio of DMRs
is negatively associated with their transcription of downstream genes. The correlation between DEGs and DMRs induced by UA treatments were further analyzed to examine the underlying linkage between DNA methylation and transcriptomic gene expression. A cutoff threshold of $\geq 0.1$ (10%) or $\leq -0.1$ (10%) for DNA methylation changes combined with log2-fold change $\geq 2.0$ or $\leq -2.0$ for gene expression were applied for the correlation analysis (Figure 4.3b). Briefly, with the 6 wk groups, a total of 55 DMRs in the promoter regions were negatively correlated (32 genes' promoter methylation increased with downregulated mRNA expression; 23 gene promoter methylation decreased with upregulated mRNA expression) with their corresponding gene expression after UA treatment comparing Pten KO control diet versus UA treated. These negatively correlated genes were further used for the starburst plot and the negatively correlated genes were highlighted in the blue boxes (Figure 4.3b). Interestingly, this associative analysis revealed that UA significantly decreased the tumor suppressor gene 3-hydroxybutyrate dehydrogenase 2 (BDH2)'s (334) promoter methylation by 11.2% (from 17.4% in Pten KO to 6.2% in Pten KO+UA) and increased the gene expression by 2.7-fold comparing the Pten KO at 6 wk. In addition, the promoter methylation of Pca relevant oncogenes ephrin type-A receptor 2 (Ephas) (335), interferon-stimulated gene 15 (Isg15) (336) and nitric oxide synthase (Nos2) (337) were increased by 15.7%, 26.3% and 25.0% respectively, while the gene expression were decreased by 86.4%, 83.0% and 98.0% respectively, compared to Pten KO at 6 wk (Figure 4.3c). These results indicated that UA could regulate the tumor promoter/suppressor genes through
modulating their promoter methylation at the early stage of Pca. Collectively, these results suggest an important subset of genes associated with Pca development and the chemopreventive activity of UA was identified through investigation of the correlation between mRNA expression and DNA CpG methylation.

4.3.6 UA Attenuates Pten KO-Driven Cellular Metabolomic Reprogramming

To unravel the potential underlying molecular links between metabolic reprogramming and epigenetic modifications as well as the impact of UA on Pten KO-modulated epigenetic effects on carcinogenesis through regulating mitochondrial metabolic pathways and metabolites, the prostate tissues collected from Pten WT, KO and KO+UA mice were used to perform the LC-MS metabolomic analysis. A total of 205 metabolites were identified of which top 30 regulated metabolites (one-way ANOVA computed P value) across 3 groups (n=5 in each group) were further plotted (Figure 4.4a). Pathway analysis (Figure 4.4b) combined with pathway enrichment analysis between Pten WT vs. KO and KO vs. KO+UA comparison groups revealed that both Pten deletion and UA treatment significantly reprogram the cancer associated metabolisms including purine metabolism, glycolysis/gluconeogenesis as well as the epigenetic related metabolism such as pyruvate metabolism (Figure 4.4b and Appendix 4.4 and 4.5). These results suggested that Pten KO and UA alter the mitochondrial metabolism/metabolites, and also linking the down-stream impact on epigenetic reprogramming and Pca, to be discussed below.
4.3.7 *Pten* KO and UA Regulates Epigenetics and Pca via Cellular Metabolism

Purine or purine metabolism is the most abundant and most critical metabolic substrate for all living organisms by providing essential components for DNA and RNA which is tightly linked to cancers (338). UA treatment completely reversed the *Pten* KO-upregulated cancer-relevant metabolisms including purine metabolism/metabolites (Figure 4.4c). In the context of purine metabolism, RNASeq revealed that the transcription levels of purine biosynthesis pathway catalyzing enzymes including Adenylosuccinate Synthase (*Adss*), *Adss1*, Phosphoribosylaminimidazole Carboxylase And Phospho-ribosyl-aminimidazole-succino-carboxamide Synthase (*Paics*) and Guanine Monophosphate Synthase (*Gmps*) were also upregulated by *Pten* KO and attenuated by UA, which are highly in agreement with the metabolomic profiles. The metabolomic along with the transcriptomic results from RNASeq indicated that UA would potentially exert its cancer preventive or therapeutic effects via the attenuation/reversal of *Pten* KO-induced metabolic rewiring. Therefore, these results provide the evolving landscape of purine synthesis inhibitors/purine metabolism enzyme inhibitors can be potentially exploited for cancer prevention/treatment for Pca.

The Warburg effect is an alteration in the metabolism of most cancer cells that enables them to convert glucose into lactate, even in the presence of abundant oxygen; a process known as aerobic glycolysis (199). *Pten* KO significantly regulated the Glycolysis/Gluconeogenesis (a reverse glycolysis pathway) (Figure 4.4b) indicating the effect the *Pten* KO in reprogramming cancer-associated
cellular metabolism. Recent research reports that the aberrant metabolism in cancer is not only involved in maintaining a high proliferative rate or survival but also have consequences that impact epigenetic mechanisms such as DNA methylation, histone post-translational modifications, triggering oncogenes activation or loss of tumor suppressor genes expression resulting in tumor development (339). In current study, UA significantly blocked the Pten KO-regulated pyruvate metabolic metabolites including pyruvate and lactate (Figure 4.5b) which are highly linked with to the basic epigenetic machinery (340, 341). Advances in understanding of prostate cancer metabolism might help to explain many of the biological responses such as epigenetic modulations that are induced by treatment, which might, in turn, lead to the attainment of more effective therapeutic effects.

4.4 Discussion
Carcinogenesis is caused by a cumulative and multistage process that primarily consists of initiation, promotion, and progression stages. PTEN alteration is strongly implicated in Pca development (342). To achieve Pten prostate-specific deletion, we crossed PtenloxP/loxP mice (327) to the ARR2 Probasin (Pb)-Cre transgenic line, PB-Cre4, in which the Cre recombinase is under the control of a modified rat prostate-specific probasin (PB) promoter (326). Since Cre-mediated recombination event is a unidirectional process, cells with Cre-mediated gene deletion are likely to increase and accumulate over time (325). Previous studies also demonstrated that Pten KO lead to a significant shortened latency of PIN
formation and results in prostate cancer progression to a metastatic stage which are also in agreement with our previous (321) and current findings (Figure 4.1e). *PTEN*-controlled signaling pathways are frequently altered in human Pca and the major function of *PTEN* relies on its phosphatase activity and subsequent antagonism of the pro-growth phosphatidylinositol 3′-kinase (PI3K)/AKT pathway (343, 344). Alterations in Pten/PI3K/AKT and p53 signaling pathways can impact cellular metabolism (345). The PI3K pathway has been shown to play a major role in tumor proliferation and survival for a wide variety of human cancers (346). Activation of PI3K results in the downstream activation of AKT and stabilization of hypoxia-inducible factor (HIF)-1. The PI3K enzyme itself antagonizes the tumor-suppressor PTEN, and the loss of PTEN increases glycolysis by activation of AKT and HIF-1 (238). Interestingly, we found UA significantly decreased the PI3K/AKT Signaling in Pten KO mice (P<0.05) indicating the potential Pca cancer prevention effects of UA.

Recent studies also suggest that *PTEN* may function through AKT-independent mechanisms (62, 63). Chronic inflammation is now known to contribute to several forms of human cancers, with an estimated 20% of adult cancers including Pca attributable to chronic inflammatory conditions. Men with chronic inflammation in non-cancerous prostate tissue may have nearly twice the risk of actually having prostate cancer (347). In current study, IPA pathway analysis revealed that Pten deletion significantly modulates the inflammation response pathways such as NF-kB signaling, iNOS signaling, PI3K in B lymphocytes signaling, IL-6 signaling, IL-8 signaling and CD28 signaling in T helper cells, among others. Most
importantly, UA treatment offset these *Pten* KO-induced inflammatory signaling responses (Figure 4.3a) indicating the potential anti-inflammatory effects of UA which would further contribute to its cancer prevention effect. Specifically, NF-κB activation associated with inflammation are known to contribute to prostate cancer malignancy. Inflammatory signals have also been associated with the development of castration resistance and resistance against other androgen depletion strategies, which is a major therapeutic challenge (348). Chronic inflammation also has been reported highly associated with epigenetic alterations mediated by DNA and histone modifications, thus driving changes in the expression of many inflammation-related genes, such as IL1R1, IL-1β, cyclooxygenase-2 (COX2), CXCL14, CCL25, CXCL6, IL13, IL17C, and IL4R (349-351). Epidemiological data also indicate that patients diagnosed with chronic inflammatory prostatitis have an increased risk of developing Pca at a later age (321). Hence, our results underscore the importance of inflammation/anti-inflammation in Pca progression/prevention and provide a plausible explanation based on whole-genome methylation and transcription profiling.

Mutations that activate oncogenes or inactivate tumor suppressors can significantly affect activities of metabolic enzymes and have a key role in aerobic glycolysis and other metabolism of cancer (238, 352). AKT stimulates glycolysis by increasing the expression and membrane translocation of glucose transporters, and also by phosphorylation of glycolytic enzymes, such as hexokinase (HK) and phosphofructokinase (PFK) (238, 353). Moreover, AKT
activates mammalian target of rapamycin (mTOR), which indirectly affects other metabolic pathways by activating HIF-1, even under normoxic conditions (353). P53 has been shown an inhibitory effect on glycolysis by upregulating the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), which decreases fructose-2,6-bisphosphatase (Fru-2,6-P2) by dephosphorylation (354). Increased expression of TIGAR results in a decreased level of Fru-2,6-P2 and a decreased glycolytic rate (355). Hence, loss of p53 induced by Pten deletion at 14 wk time point (Figure 4.3a) may shifts metabolism from mitochondrial respiration towards glycolysis. In current study, metabolomic analysis revealed UA blocks Pten KO-induced AMPK signaling pathway indicating UA plays important roles in regulating cell growth and reprogramming metabolism as well as cellular processes such as autophagy and cell polarity which are mediated by AMPK pathway (356). AMPK may act in cancer cells as a metabolic gatekeeper that functions to establish metabolic checkpoints that limit cell division, and its loss of function would enhance tumorigenesis and tumor progression (251). In addition, AMPK signaling also has been reported to regulates the Warburg effect, one of characteristic features of cancer cell metabolism as aforementioned, in cancer cells and suppresses tumor growth in vivo (65). UA-mediated AMPK signaling inhibition may also further regulates glycolysis metabolism which has been considered an attractive anticancer strategy (212, 232). The defining distinction between neoplastic cells and their normal counterparts is the unregulated and increased rate of growth of the former. Purines are basic components of nucleotides in cell proliferation which provide essential
components for DNA and RNA which is also tightly linked with cancers (338). Under the conditions with higher requirement for purine nucleotides, such as dividing cells and tumor cells, the de novo biosynthetic pathway is fundamental to replenish the purine pool. Nucleotide synthesis such as purine is a frequently limiting factor of proliferation, therefore, purine biosynthesis and its associated mitochondrial pathways have been targeted by chemotherapeutic agents for decades (357). The typical approach is by direct inhibition of this pathway using purine antimetabolites, analogs of nucleotides, or their precursors acting as competitive inhibitors. These have been proven to be effective treatments acting to stall DNA replication or cause apoptosis via DNA damage, and they reflect a significant percentage of currently available cancer treatment. Another class of purine antimetabolites, the purine deoxynucleoside analogs fludarabine, cladribine, clofarabine, nelarabine, and pentostatin are US Food and Drug Administration (FDA)-approved drugs for the treatment of cancers (358). However, none of these currently approved drugs target purine biosynthesis directly; instead, they target upstream’s input availability and downstream’s utilization of synthesized purines. While several biosynthetic inhibitors are in development, a better understanding of the precise molecular mechanisms of these agents and identification of new enzyme and metabolite targets is crucial for improving options for cancer treatment (359). So, the regulation effects of UA on Pten KO-mediated purine metabolism (Figure 4.4b-c) along with its regulations on the biosynthesis catalyze enzymes including Adss, Adss1, Paics
and Gmps may provide new therapeutic targets on the purine metabolism for cancer prevention and treatment.

Recent studies show that the aberrant metabolism in cancer is not only involved in maintaining a high proliferative rate or survival but also have consequences in epigenetic reprogramming through key metabolic intermediates, such as pyruvate, lactate, AcCoA and aKG which work as hubs between epigenetic processes and oncogenes activation or loss of tumor suppressor genes expression (237). In many cancer cells, pyruvate is usually processed into lactate, which is actively transported to the extracellular matrix due to the upregulation of monocarboxylate 1 (MCT1) and giving the cells a high glycolytic rate. In current study, Pten KO significantly decreased the pyruvate and increased lactate production while UA treatment completely offsets Pten KO-modulated pyruvate and lactate (Figure 4.5b) indicating the blockade effect of UA on Pten KO-promoted pyruvate to lactate conversion. Lactate plays a key role in regulating gene transcription by inhibiting the histone HDAC enzymes, promoting hyperacetylation in nucleosomes and active transcriptional state (360). Some studies also mentioned that the histone H4 acetylation levels increase when cells are treated with lactate, promoting changes in gene expression that favors the cancer establishment (361). In breast cancer, the overproduction of lactate induces tumor growth by demethylation of HIF-1α in patients’ tissue (362).

In conclusion, our current study integrates the latest Methyl-seq, RNA-seq and LC/MS/MS technologies in dissecting the potential underlying mechanism of epigenomic CpG methylation, mRNA transcriptomic gene expression and
mitochondrial metabolic rewiring in *Pten* prostate-specific deletion mice after treatment with dietary chemopreventive phytochemical UA. The results provide the experimental in vivo evidence for the interconnectivity of metabolomic, epigenomic, and transcriptomic effects of UA in *Pten* prostate-specific KO mouse model. UA rescues the metabolic profile, reprograms epigenetic CpG methylation, driving alterations of phenotypic gene expression, de-/activated signaling pathways in prostate-specific *Pten* KO mice. The correlative analyses of multi-omics data revealed the underlying intricated biological connectivity between metabolomic, epigenomic and transcriptomic regulation by UA. Thus, the overall cancer prevention/anticancer effect of UA may exhibit through regulation of metabolic rewiring and epigenetic reprogramming, making it a promising drug candidate for the prevention and treatment of Pca.
Figure 4.1 DNA Methyl-seq profiles in Pten WT, Pten KO and UA treated Pten KO mice for 6- and 14-weeks.

(a) Venn diagram showing the DMRs in response to Pten WT vs. KO and Pten KO vs. KO+UA after 6-week and 14-week. The cutoff threshold of methylation difference ≥ 10% or ≤ -10%, P-value ≤ 0.05; (b) MA plots showing DMRs in response to Pten KO mice treated with UA at 6 and 14 week with cutoffs of methylation difference ≥ 20% or ≤ -20% coupled to p<0.05; (c) Heatmap comparing the DMRs in response to Pten KO mice treated with UA for 6- and 14-week; (d) Venn diagrams showing the overlapped hyper- and hypo-methylated genes after 6- and 14-week of UA treatment in Pten KO mice; © Representative photo of prostates obtained from Pten KO and Pten KO treated with UA at 6- and 14-week time points.
Figure 4.2 mRNA transcriptomic profiles regulated by Pten KO and the protective effects of UA on Pten KO-mediated gene regulation.

(a,c) MA plots showing DEGs in response to Pten WT vs KO and Pten KO vs KO+UA at 6-week (a) and 14-week (c) with cutoffs of \( p < 0.01 \) and \( \log_2(\text{fold change}) \geq 1.0 \) or \( \leq -1.0 \); (b,d) Venn diagrams comparing the upregulated and downregulated genes in Pten WT vs KO and Pten KO vs KO+UA comparison groups at 6-week (b) and 14-week (d); © Prostate cancer-relevant oncogenes reported from RNA-seq analysis. The mRNA levels were shown as RNAseq reads per million in different groups (Pten KO, WT and KO+UA) at 6- and 14-week time points. All the data are presented as the means ±SEM of three independent experiments. *, \( P < 0.05 \) and **, \( P < 0.01 \) indicate significant differences between two groups. Student’s t test was used to calculate the significance of the differences between groups.
Figure 4.3 Ingenuity pathway analysis (IPA) and correlation analysis of DEGs with DMRs.

(a) Top 25 shared signaling pathways which modulated by Pten KO and reversed by UA treatment at 6- and 14-week time points; (b) Correlation analysis between gene expression (DEGs) and DNA methylation (DMRs) in Pten KO vs. KO+UA groups at different time points. The DMRs which were negatively correlated with their corresponding gene expression with the cutoff of methylation difference ≥ 10% or ≤ -10% coupled to gene expression log2(fold change) ≥ 2.0 or ≤ -2.0 were selected as show in the blue boxes (locate in the upper left and bottom right corners). The DMR locations (gene features: Region, Body, Downstream and Promoter, etc.) are indicated by the colors. (c) mRNA expression levels of tumor suppressor genes/oncogenes and their corresponding promoter CpG methylation ratio (red font numbers) in Pten KO and KO+UA mice. All the data are presented as the means ±SEM of three independent experiments. *, P < 0.05 and **, P <
0.01 indicate significant differences between two groups. Student’s t test was used to calculate the significance of the differences between groups.
Figure 4.4 Metabolomic profiles of prostate samples from Pten WT, KO and UA treated Pten KO mice.

(a) Top 30 regulated metabolites after Pten KO and UA treatment (n=5); (b) Metabolism pathway analysis of Pten WT vs KO and Pten KO vs KO+UA comparison groups; (c) The major metabolites quantification of purine metabolism in Pten WT, KO and UA treated Pten KO mice. All the data are presented as the means ±SEM of three independent experiments. *, P < 0.05 and **, P < 0.01 indicate significant differences between two groups. Student’s t test was used to calculate the significance of the differences between groups.
Figure 4.5 Quantified mRNA expression and metabolites levels.

(a) Quantified purine metabolism catalyze enzyme mRNA expression levels (the mRNA levels were shown as RNAseq reads per million) in Pten WT, KO and UA treated Pten KO mice at different time points; (b) Quantified epigenetic associated metabolites in Pten WT, KO and UA treated Pten KO mice groups at 20 wk. *, P < 0.05 and **, P < 0.01 indicate significant differences between two groups. Student’s t test was used to calculate the significance of the differences between groups.
Chapter 5 Pharmacokinetics and pharmacodynamics of three oral formulations of curcumin in rats\textsuperscript{13, 14, 15}

5.1 Introduction

Curcumin (diferuloylmethane, CUR) is a yellow pigment presents in the dried rhizomes of \textit{Curcuma longa} (turmeric), and turmeric is also commonly used as a dietary spice in some Asian countries. Furthermore, turmeric (\textit{Curcuma longa}) is often used in traditional Indian and Chinese medicines for the treatment of various diseases \textsuperscript{(363)} and commonly used in the US as botanical dietary supplement. CUR has been shown as the significant phytochemical responsible for the beneficial effects of turmeric. Many studies have investigated the important role of CUR in the prevention and treatment of many diseases, including cancer \textsuperscript{(364, 365)}, anxiety, cardiovascular disease \textsuperscript{(366)}, osteoarthritis, and metabolic disturbances such as diabetes mellitus and obesity \textsuperscript{(367, 368)}. Also, many in vivo studies have demonstrated that CUR can be involved in various cellular signal transduction pathways and inhibits carcinogenesis such as the GI tract, including the colon, esophagus, stomach and liver \textsuperscript{(369)}. The wide range of biological activities of turmeric has been attributed to CUR \textsuperscript{(370)}. However, its experimental biological benefits in clinical trials have faced many

\textsuperscript{13}Part of this chapter has been submitted to an international journal.

\textsuperscript{14}**Keywords:** Curcumin; pharmacokinetics/pharmacodynamics; Nrf2; oxidative stress, formulation

\textsuperscript{15}**Abbreviations:** Cur, Curcumin; PK/PD, Pharmacokinetic/pharmacodynamic; COG, curcumin-O-glucuronide; PBPK, Physiological-based pharmacokinetic;
barriers since the low bioavailability of CUR after oral administration might have contributed and confounded its pharmacodynamic (PD) responses in human subjects (371) and in rodents (372). Previously, scientists have performed preclinical and clinical studies with CUR. For instance, LC-MS/MS study showed that maximum levels of CUR in the plasma of rats receiving a single oral dose of 500 mg CUR per kg body weight were around 0.06 μg/mL (373, 374). By comparing with an intraperitoneal (IP) dosing, the oral bioavailability of CUR was estimated as about 1%. Clinical trials in humans indicate that the systemic bioavailability of orally administered CUR is relatively low (375-377) and that mostly the metabolites of CUR, instead of CUR itself, are detected in the plasma or serum following oral administration (378, 379). These results are similar with our previous study showing, the plasma levels of parent CUR were below the detection limit of 0.1 ng/ml by HPLC-ITMS/MS/MS and only the metabolite, curcumin-O-glucuronide (COG) was detected as early as 30 minutes after oral administration (380). This poor bioavailability can be explained by its poor absorption due to low water solubility (<0.1 mg/ml), limited tissue distribution and extensive metabolism in the intestines and liver (381). Therefore, the poor bioavailability of CUR appears to be the principal barrier in achieving adequate blood circulating levels to give rise to desirable PD effects.

Glucuronidation is the major phase II conjugation reaction of many xenobiotics, including polyphenolic CUR (382, 383). With the addition of the glucuronic acid group of uridine-5'-diphosphoglucuronic acid (UDPGA), CUR molecules become more hydrophilic and are therefore more readily eliminated. This glucuronidation
process is catalyzed by a superfamily of UDP-glucuronosyltransferases (UGTs), which are membrane-bound enzymes localized at the luminal side of the endoplasmic reticulum (ER). The glucuronidation pathway of CUR contributed greatly to the overall poor oral bioavailability observed in both preclinical models and human subjects and many formulations of CUR have been developed and studied. While many studies on CUR have been focused on improving its bioavailability and investigating its potential effects on disease, to date, few studies have compared the Pharmacokinetics (PKs) and PDs of commercially marketed CUR. Additionally, no studies have been performed to study the PK/PD of CUR of different marketed formulations and its pharmacological effect on antioxidant gene activity.

CUR exerts both direct and indirect antioxidant effects by sweeping away ROS and stimulating the expression of cytoprotective proteins through the nuclear factor Nrf2-Keap1 signaling pathway. Nrf2 is an important regulator of the cellular response to oxidative stress. In this context, CUR can activate Nrf2-mediated downstream antioxidant genes such as HO-1 (383, 384), NQO-1 (372) and glutathione S-transferase P1 (GSTP1) (Figure 5.1). The ability of CUR to suppress inflammatory genes has also been widely reported in both human and rat studies. KO of Nrf2 attenuates CUR’s antioxidant and anti-inflammatory activities in mouse macrophages (385), suggesting the important role Nrf2 is playing in CUR’s biological responses. Hence, in our current study, we aimed to examine the PK/PD of commercially marketed CUR products (GNC and Vitamin Shoppe) and CUR from Sigma on the antioxidant response in preclinical rat
model integrated with PK/PD modeling approach. In addition, Physiological based pharmacokinetic (PBPK) modeling, which is a compartmental and flow-based type of PK model which could facilitate the simultaneous description of drug concentration changes over time in each organ (386). The compartment may not be limited to entire organs, and often PBPK models may contain nested compartments that represent different cell types within an organ, and even different organelles within a cell. These levels of hierarchical complexity permit modeling of molecularly-driven events, such as specific metabolic pathways. In our current study, the Advanced Compartmental Absorption and Transit (ACAT™) physiologically based PK (PBPK) model in GastroPlus will be utilized to help examine and enhance the understanding of the oral absorption process and PK of CUR. This kind of simulation with preclinical PK parameters could be potentially extrapolatable from rats to humans, and may inform dosing recommendation and the design of clinical trials.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

CUR (98% purity as standard for quantification; Lot C7727), ethanol (99%), and formic acid (98%) were purchased from Sigma-Aldrich (St. Louis, MO). CUR-d6 (>98%, Santa Cruz Biotechnology, TX), acetonitrile (ACN) and pure water for the LC mobile phase were purchased from Honeywell Burdick & Jackson (Muskegon, MI). Deionized water was obtained from a Milli-Q system (EMD Millipore™ Direct-QTM3, Millipore, Bedford, MA). Heparin sodium injections (1000 U/ml)
were purchased from Baxter Healthcare Corporation (Deerfield, IL), and sodium chloride injections (0.9%) were purchased from Hospira Inc. (Lake Forest, IL). GNC Herbal Plus® Turmeric Curcumin capsules (GC, Item #181212) were purchased from GNC, Vitamin Shoppe Curcumin C3 Complex capsules (VC, Item # DB-7025) was purchased from Vitamin Shoppe and Sigma curcumin powder (SC) was purchased from Sigma (Lot C1386). HPLC analysis of commercial formulations showed the content of individual curcuminoids mainly contains CUR, demethoxycurcumin, and bisdemethoxycurcumin. This was in close agreement with the manufacturer’s product data sheet that listed CUR content of 65 to 70% with the major impurities being demethoxycurcumin and bisdemethoxycurcumin. The CUR purity of three formulations was shown in Appendix 5.1.

5.2.2 Animal treatment and sample collection
The scheme of experimental design was shown in the Appendix 5.2. Briefly, four adult female Sprague-Dawley (SD) rats (250–300 g) per group with cannulated jugular veins were purchased from Hilltop Laboratories (Scottsdale, PA). The rats were housed and acclimatized for three days in the Laboratory Animal Service facility at Rutgers University and had full access to food and water before the PK experiment. This PK study was carried out according to the animal protocol approved by the Institutional Animal Care and Use Committee of Rutgers University (01–016). Heparinized saline (50 U/ml) was used to flush the inserted cannulas and tubes. CUR was suspended in vehicle consisting of Cremophor,
Tween 80, ethanol and water at a volume ratio of 1:1:1:7. CUR at 250 mg/kg body weight was administered through oral gavage (PO). For the intravenous (IV) administration group, the same vehicle-suspended compound as the PO group was given as CUR 40 mg/kg. Blood samples (0.3 ml) were withdrawn through an exteriorized cannula at 0, 10, 20, 30, 40, 50, 60, 120, 240, 360, 480 and 720 min after the oral and IV administration of CUR. The cannula was flushed with one volume (0.3 ml) of heparinized saline solution immediately after each collection. All blood samples were centrifuged at 2,500 rpm and 4°C for 10 min to isolate plasma samples and then stored at -80°C for subsequent triple-stage ion trap mass spectrometry coupled with high-performance liquid chromatography (HPLC-ITMS/MS/MS) analysis.

5.2.3 Sample preparation

CUR samples in rat plasma were prepared by a novel protein precipitation extraction (PPE) coupled with size exclusion chromatography (SEC) method according to our previously published method (387). Briefly, PPE was carried out with cold ACN (−20°C). Aliquots of the original rat plasma samples (100 µl) and CUR standard samples in blank SD rat plasma (100 µl, Innovative Research, MI) spiked with CUR-d6 as an internal standard (IS) were mixed with 300 µl of cold ACN and whirled for 30 s on a bench vortex. The resulting mixture was subject to an SEC procedure. It was centrifuged at 4°C and 16,000 g for 25 min. Then, 300 µl of sample supernatant was carefully transferred to an OMEGA NANOSEP 3 K size exclusion cut-off tubes (cut-off 10 kD, Pall Corporation, NY) and centrifuged
at 4°C and 16,000 g for 30 min. Filtered CUR with internal standard (IS; curcumin-d6) samples were then added to 300 µl auto sample vials (Life Technologies, NY). Calibration standards were prepared by spiking a series of CUR working standard solutions and the IS into 100 µL of blank rat plasma, and then the same extraction procedure as that used for the plasma samples above was followed. We have previously reported this novel HPLC–ITMS/MS/MS analysis method performed by using a Thermo Finnigan LTQ mass spectrometer coupled with a Thermo Finnigan Surveyor HPLC System (Thermo Fisher Scientific Inc., San Jose, CA) (387). Chromatographic separation was performed by a Zorbax Eclipse XDB C18 column (3.5 μm, 4.6 × 50 mm, Agilent Technologies, Santa Clara, CA). Briefly, aqueous mobile phase A was composed of 0.1% formic acid in water, and organic mobile phase B was ACN. The column oven and autosampler temperatures were 40°C and 4°C, respectively. The mobile phase was 25:75 (A: B, v/v) for the first 0.8 min at a flow rate of 250 μL/min followed by 15:85 (A: B, v/v) for 1.1 min at a flow rate of 250 µL/min and then was transitioned to 5:95 (A: B, v/v) at a flow rate of 400 µL/min in 2 min. The mobile phase was maintained at 5:95 (A:B, v/v) at 400 µL/min for an additional 4 min and then returned to 25:75 (A: B, v/v) at 250 µL/min for 3 min. The total run time for the present analysis was 11 min. The injection volume of the sample was 25 µl. The ITMS system was operated in a positive ESI ion source. The analytes were detected using consecutive reaction monitoring (CRM) mode. Data acquisition, peak integration and quantitation were achieved by the Xcalibur Data System (Thermo Electron, San Jose, CA, USA). The lower limit of detection was
1 ng/mL and upper limit of detection was 3000 ng/mL. The plasma calibration curve was linear, with $r^2 > 0.99$.

5.2.4 Measurement of mRNA expression in leukocytes

Total RNA was extracted from leukocytes according to the protocol of the PicoPure® RNA Isolation Kit. RNA concentrations were quantified using an Infinite M200 NanoQuant spectrophotometer (Tecan, Mannedorf, Switzerland). First-strand cDNA was synthesized from 300 ng of RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). RT-PCR was conducted using SYBR Green reagent on a QuantStudio5 Real-Time PCR system (Thermo Fisher Scientific, Rockford, IL). The relative mRNA levels were calculated using the $\Delta\Delta$Ct approach (388), and GAPDH was used as a reference for gene expression normalization. PD data were presented as the gene fold change at each time point against their respective expression in the vehicle control arm.

5.2.5 PK/PD model development

This study investigated the PK of three different CUR formulation preparations in 4 groups of Female Sprague-Dawley rats 0-12 h after a single oral gavage or IV dose. CUR was administered at a dose of 250 mg/kg (oral) or 40 mg/kg (IV) to each rat. Plasma samples were quantified for free CUR and its glucuronidation conjugate, COG, since CUR predominantly undergoes glucuronidation in the gut wall (382). Noncompartmental analysis (NCA) was first conducted on the time
course of CUR plasma concentration with Phoenix WinNonlin (Certara USA, Inc., Princeton, NJ, USA). And then the NCA PK parameters such as the area under the curve (AUC), clearance (CL), the mean residence time (MRT) and the volume of distribution (Vss) were estimated using Phoenix WinNonlin. The oral and IV CUR plasma concentrations versus time t were analyzed by a two-compartment (2CM) PK models (Figure 5.2a) and plasma levels of the COG were fitted with one-compartment (1CM) PK model. The parent CUR and metabolite COG PK model differential equations are as follows:

2 CM oral and IV

\[
\frac{dA_a}{dt} = -ka \cdot A_a; \quad A_{a0} = \text{oral dose} \tag{1}
\]

\[
\frac{dA_c}{dt} = ka \cdot A_a \cdot F + \frac{CL}{V_c} \cdot Ap - \left( \frac{CL}{V_c} + \frac{CL_d}{V_c} \right) \cdot A_c - k_{met} \cdot A_c; \quad A_{c0} = IV \text{ dose or 0} \tag{2}
\]

\[
\frac{dA_p}{dt} = \frac{CL_d}{V_p} \cdot Ap + \frac{CL_d}{V_p} \cdot A_c; \quad A_{p0} = 0 \tag{3}
\]

1 CM metabolite (COG)

\[
\frac{dA_{met}}{dt} = k_{met} \cdot A_c - \frac{CL_{met}}{V_{met}} \cdot A_{met} \quad A_{met0} = 0 \tag{4}
\]

ka is the first-order absorption rate constant, and F is the oral CUR bioavailability.

The oral and IV datasets were fitted simultaneously with a two-compartment
(2CM) model. Eqs. 1-3 show the differential equations for the 2CM IV and oral dosing, where $V_c$ represents the volume of distribution in the central compartment and $V_p$ is the volume of distribution in the peripheral compartment. $CL$ represents the total clearance from the central compartment and $CL_d$ represents the clearance from inter-compartmental distribution clearance. $A_c$ represents the amount of CUR in the central compartment and $A_p$ represents the amount of CUR in the peripheral compartment; thus, the plasma concentration $C_p$ equals $A_c/V_c$. Eq 4 described the differential equation for the 1 CM metabolite (COG), where $k_{met}$ is the rate constant of COG input, $V_{met}$ is the volume of distribution of COG, $CL_{met}$ represents the clearance of COG, $A_{met}$ represents the amount of COG in the compartment.

Gene expression of antioxidant genes ($Nrf2$, $Ho-1$, $Nqo-1$) was measured as PD response ($E$) and described by an indirect response (IDR) model with baseline (Figure 5.2a) (389, 390). The plasma CUR concentration is linked to the rate of change of the signal induction by the Hill function. The initial biologic signal effector parameter $mRNA_0$ relates to the rise or fall of plasma CUR concentrations to the time course of changes in antioxidant gene expression, where $mRNA_0$ is the initial condition and is defined as $mRNA0 = 1$, which represents the gene expression before CUR administration. Finally, an IDR model with the stimulation of input ($kin$) by CUR was applied to describe mRNA gene formation (PD) (391). The differential equations are shown in Eqs. (5-6).

$$\frac{dmRNA}{dt} = kin \cdot \left(1 + \frac{Emax}{EC50+C} \cdot C\right) - kout \cdot mRNA; \quad mRNA_0 = mRNA0 \quad (5)$$


\[ \text{mRNA}_0(t=0) = \frac{\text{kin}}{\text{kout}} = 1 \]  

(6)

\( \text{kin} \) is the zero-order rate constant for the production of antioxidant genes’ mRNA, \( \text{kout} \) is the first-order rate constant for the degradation of mRNA, and \( \text{mRNA}_0 \) is set as 1 as the initial condition before CUR administration. \( \text{Emax} \) is the maximum ability of CUR to stimulate signal initiation. \( \text{EC50} \) represents the concentration of CUR resulting in 50% of the maximum induction. \( \text{Cp} \) is the CUR plasma concentration driven by the PK parameters obtained from the final 2CM PK model used as input variables to predict drug concentrations of CUR (C) in plasma for PD modeling. PK/PD models were conducted using R software (Version 3.5.2) with Ubiquity modeling platform. The code of the modeling differential equations are provided in the Appendix 5.3. Each of the oral formulation datasets was fitted using the same structural model and separate sets of data were estimated.

The above PK/PD models were performed using R software (Version 3.5.2) installed with the PK/PD Model Development and Deployment package (Ubiquity) which tested with Strawberry Perl. All the PK parameters were estimated using the maximum likelihood method. The variance model was defined as \( VAR_i = \left( \sigma_1 + \sigma_2 \cdot Y(\theta, t_i) \right)^2 \), where \( VAR_i \) is the variance of the \( i^{th} \) data point, \( \sigma_1 \) and \( \sigma_2 \) are the variance model parameters, and \( Y(\theta, t_i) \) is the \( i^{th} \) predicted value from the PK model. The goodness of fit was assessed by system convergence, Akaike
Information Criterion, estimator criterion value for the maximum likelihood estimation method, and visual inspection of residuals and fitted curves.

5.2.6 PBPK model simulation by GastroPlus

To simulate intestinal absorption and metabolism, GastroPlus™ software (Simulations Plus, Lancaster, CA) embedded with the Advanced Compartmental Absorption Transit (ACAT) model was used to obtain the simulated plasma concentration-time profile as shown in Appendix 5.4. In our study, the permeability values were predicted by using different permeability models (patented by Affymax, Inc.US patent 6,043,027) for human jejunum effective permeability ($Peff$) based on CUR molecular structure with ADMET® Predictor. Then $Peff$ was used to convert the permeability in rat in GastroPlus. All the physicochemical parameters (pKa, LogP, diffusion coefficient, solubility and permeability), as well as the systemic PK properties (central volume of distribution, blood to plasma ratio (B:P ratio) and unbound fraction (%Fu)) used in rat PBPK model building were obtained using ADMET® Predictor (Simulations Plus), which is a built-in module within GastroPlus®. The total clearance ($CL$) value of formulated CUR was from the estimated PK parameters from the observe data. The predicted parameters from the 2CM PK model were used as input parameters to perform the simulation. The PBPK modeling was performed using Advanced Compartmental Absorption and Transit (ACAT) model to predict the $Cp$ vs time PK profile of CUR. This process was validated by comparing the simulated PK profile and properties of CUR to our animal experimental data after
oral absorption. Lastly, to better explain the variance of PK profile and evaluate the absorption kinetics among the three oral formulations, the deconvolution of the in vivo PK data was performed with Phoenix WinNonlin. The objective of Phoenix WinNonlin’s deconvolution is to estimate the cumulative amount and fraction absorbed over time for the individual formulation utilizing the PK profile dataset and dose for each profile.

5.3 Results

5.3.1 CUR Pharmacokinetics (PK)

The PK of CUR was first analyzed by noncompartmental analysis (NCA) and the results are shown in Table 5.1. The Cmax of SC formulation is 17.79 ng/ml, which was higher than the GC (12.6 ng/ml) and VC (9.92 ng/ml) formulations. The plasma concentration (Cp) versus time (t) profiles of CUR and its metabolite COG following oral dosing of 250 mg/kg and IV administration of 40 mg/kg parent CUR are presented in Figure 5.3. The Cp vs. t profiles after the oral and IV CUR administrations showed a biexponential decay that could be well captured by the 2CM models, as shown in Figure 5.3. By co-fitting the oral and IV datasets, the IV dose informed the parameters of 2CM model, whereas the oral route provided the necessary data for estimating the parameters associated with the oral absorption (ka) part of the model. The 2CM oral and IV PK parameter estimations are summarized in Table 5.2. The PK profile of the metabolite COG fitted reasonably well with 1CM model and the PK parameters are summarized in Appendix 5.5.
The deconvolution of the three different oral CUR formulations with their *in vivo* data indicated different cumulative input amount (Figure 5.2b). The SC formulation which has the highest cumulative input and more sustained absorption correlated well with the higher *AUC* and *Cmax* PK profile. Comparison between the absorption rate (*ka*) and cumulative input profiles shows that the absorption rate correlates with the deconvolution cumulative input profile. Compared to GC and VC, SC shows higher absorption rate and also has faster cumulative input profile than the other two formulations as shown in Figure 5.2b. The bioavailability (*F*) of the three formulations is shown in Table 5.2, and the %CV of the predicted parameters is within a reasonable range. The *F* of CUR from Sigma, SC is 3.1%, which is much higher than that of GC (0.9%) and VC (0.6%). The relative importance of the blood levels of free CUR with respect to the pharmacological effects will be discussed below. The plasma CUR concentration (PK) is linked to the rate of change of the biomarker induction (PD) and the PK/PD modeling fitted simultaneously within R as described in the PD section below.

5.3.2 PD of antioxidant gene expression

The mRNA levels of the PD response genes were assessed by RT-qPCR. The time course of the Phase II/antioxidant genes *Nrf2*, *Ho-1*, and *Nqo-1* in leukocytes for each formulation is shown in Figure 5.4. The mRNA expression levels increased over time, and the peak time for gene induction was approximately 1.5-3 h following the oral administration of 250 mg/kg CUR.
Furthermore, the fold changes of gene expression level of *Nrf2* was 1.5, *Ho-1* was 1.6, and *Nqo-1* was 2.1, approximately. Subsequently, the mRNA levels declined gradually to baseline (Figure 5.4). An indirect response (IDR) model (389) was used to estimate the quantitative aspects of the mRNA expression profiles. Antioxidant gene expression reached its highest point before 3 h after oral treatment with CUR, and the estimated PD parameters for phase II genes are presented in Table 5.3. mRNA0 is fixed as 1 as the initial condition before CUR administration. The maximum effect (*Emax*) of CUR to stimulate *Nrf2* is 1.74, *Ho-1* is 2.02 and *Nqo-1* is 2.24. The production antioxidant genes’ rate (*kin*) for the three oral CUR formulations are 4.24, 4.86 and 5.53.

5.3.3 PBPK model simulation by GastroPlus

The aim of GastroPlus study was to simulate the preclinical PK behavior of different oral CUR formulations using an Advanced Compartmental Absorption and Transit (ACAT) PBPK model incorporating physicochemical properties from GastroPlus. All the parameters used for the simulation are listed in Table 5.4. For the total CL of CUR used in this PBPK simulation was estimated from the observed PK data (Table 5.2). The observed GC and VC plasma concentration-time profiles appear to be well-captured by the proposed ACAT PBPK model as shown by the simulated curves (Figure 5.5). SC formulation shows higher observed *Cp*-time PK profile than GC and VC formulations. The observed and simulated PK parameters of the different oral CUR formulations are listed in
Table 5.5. The simulated PK parameters reasonably predict the observed PK parameters.

5.4 Discussion

CUR is a common dietary phytochemical and botanical health supplement widely consumed because of its purported antioxidant and anti-inflammatory properties for a variety of diseases, such as arthritis, metabolic syndrome, and cancer (392). CUR is pharmacologically more active than its conjugated metabolites and therefore it is assumed that the blood levels of free CUR would better reflect its pharmacological activity (382, 389-392). Previously, studies from our laboratory and from other scientists have shown that CUR could activate Nrf2, a master transcription factor regulating cellular protective gene expression as shown in Figure 5.1 (393). Although there are a plethora of studies supporting CUR’s health benefits, its fairly low oral bioavailability presents a potential barrier for CUR to reach sufficient in vivo concentrations to achieve an adequate biological response. CUR undergoes substantial gut and hepatic first pass metabolisms after oral administration, which would contribute to the low systemic bioavailability (375, 377). To solve this problem, many scientists have developed and tested different formulations to hopefully achieve more favorable in vivo responses. This include administering CUR intravenously through liposomes (394) or micronized CUR (395). Another common approach is to formulate CUR with piperine, which can enhance the bioavailability of CUR due to its potential in inhibiting intestinal and hepatic UGT metabolism (396). In our current study, we
compared 3 oral formulations, GC, VC and SC versus IV and all three oral formulations have measurable levels of free CUR with fairly high levels of CUR COG metabolite. Furthermore, the plasma concentration of CUR and the expression of phase II/antioxidant genes in leukocytes were measured simultaneously. Similar to the results of previous studies (371), the plasma levels of CUR in our current study were fairly low (0-18 ng/ml), and the CUR metabolite COG concentrations were fairly high. For many of later time points, the parent CUR concentrations were lower than the limit of quantification (LLOQ) (<= 0.1 ng/ml) are not shown in Figure 5.3. COG was detected at an earliest time point of 10 min after CUR administration and achieved maximal concentrations at approximately 1 h (Figure 5.3). Thus, the metabolism of CUR appears to occur very rapidly, forming the glucuronide conjugate. As shown in Table 5.1, the NCA PK parameters \( C_{\text{max}} \) and \( AUC \) of SC formulation are better than GC and VC. And the SC formulation of CUR tested in this current study demonstrated enhanced absorption compared to GC and VC formulations. To investigate the variation of CUR absorption phase, deconvolution of in vivo PK data was performed with Phoenix WinNonlin. The differences between each oral formulation as shown in Figure 5.2b could be due in part to disintegration/dissolution variation, potential impact of the different formulation on UGT metabolism and transport at the absorption site, or other potential factors that could contribute to this phenomenon. Unlike the SC powder, the commercial GC and VC capsules would contain additives (inactive ingredients such as magnesium stearate, microcrystalline cellulose and etc.) formulated with excipients which may affect
the bioavailability of GC and VC as compared to SC. Some reports discussed that magnesium stearate interferes with the body’s ability to absorb the contents of the medication capsules (397). Some other studies also reported that the dosage form related factors can affect drugs absorption. The major factors in this category are dissolution rate, particle size, polymorphism, amorphism, lipophilicity, ionization state, solvates, hydrates, and available surface area, and formulation factors including manufacturing, pharmaceutical ingredients, product age and storage conditions (398). Setthacheewakul et al. showed that the formulations of CUR are more stable with a particle size of about 30 nm with 10 – 14 folds higher absorptions compared to the same oral dose of native CUR administrated in Wistar-strain rats (399). In Figure 5.2b, the total cumulative absorption of the three oral CUR formulations is < 0.2 mg/kg, which is much less than the oral dose of 20 mg/kg. This result could be due in part to: a large portion of CUR may be unabsorbed; CUR molecules (parent and metabolites) are excreted unchanged in the feces either as unabsorbed or through biliary excretion; and or other factors (400). Thus, we chose to present here the simplest approach where only the cumulative CUR input are simulated with the in vivo PK data used as input. Despite not representing a unique solution, we believe our analysis indicates that absorption/PBPK modeling could be considered as an approach to guide formulation development for some new drugs at early stages.

CUR metabolite COG can be explained by the important role that UGTs play in the metabolism of CUR. Glucuronidation is a major pathway in CUR metabolism
in the rats, particularly in the intestine. Several isoforms, such as UGT1A1, UGT1A7, UGT1A9, and UGT1A10, in human liver and human intestinal microsomes have been reported to be responsible, although the majority of UGT isoforms are able to catalyze CUR glucuronidation (383). The PK profiles of oral, IV CUR and COG were well described by a 2CM and a 1CM (Figure 5.3) in agreement with previous works (395, 401, 402). The bioavailability ($F$) of GC, VC, and SC was 0.9 %, 0.6% and 3.1%, suggesting the 2 commercially available products GC and VC are quite similar in their F. It would be interesting to find out the bioavailability and PK/PD of other commercially available CUR products currently on the market.

The beneficial effects of CUR could be explained in part via its actions on Nrf2 (402, 403). Numerous studies have observed the role of the activation of Nrf2 signaling by CUR in preventing diseases in animal models. For instance, in UVB-induced skin cytotoxicity models, CUR up-regulated Nrf2 and Ho-1 to reduce UVB cytotoxicity and oxidative stress (404). CUR activates Nrf2 and increases antioxidant and anti-inflammatory activity to protect against cardiac injury (405). CUR has also been observed to inhibit the transformation of cells from normal to tumor, and inhibit the synthesis of a protein thought to be instrumental in tumor formation (406). By blocking the inflammatory molecule NF-κB, CUR blunts cancer-causing inflammation, slashing levels of inflammatory cytokines throughout the body (407, 408). CUR also interferes with production of dangerous advanced glycation end products that trigger inflammation, which can lead to cancerous mutation (409). CUR alters cellular signaling to enhance
healthy control over cellular replication, which tightly regulates the cellular reproductive cycle, helping to stop uncontrolled proliferation of new tissue in tumors (410). To better understand the impact of different oral formulations of CUR on the biological responses and CUR’s antioxidant effects, Nrf2-mediated Phase II/antioxidant gene expression was measured. Nrf2, Ho-1, and Nqo-1 expression level increased over time, and these changes were well-captured with an IDR model (Figure 5.4). Irrespective of the oral formulations, once CUR is absorbed, the systemic PK would drive the PD profile of each biomarker and can be modeled simultaneously with the shared PD model and the results are shown in Table 5.3. The levels of these three biomarkers Nrf2, Ho-1 and Nqo-1 reached maximum peak values at approximately 1.5 to 3 h and then returned to baseline over time. It appears that all three CUR oral formulations exert similar PD response on the Nrf2-mediated antioxidant effects, and these PD responses would potentially contribute to the overall health beneficial effects of CUR. This current PK/PD model represent out first attempt to characterize the antioxidant effects of CUR at the gene transcription level. Our current model can describe the observed dataset and provides certain flexibility to capture the dynamics of gene expression in different treatment groups. While this study used the relative gene fold change (normalized to each time point with the control time point \((t=0)\) as a PD marker, the circadian effects of mRNA expression may need to be considered in some situations. Although mechanistic consideration was incorporated in the current model, overall, it is still an empirical model. In the future, a more mechanistic based model could be further developed by including
the intermediate molecules involved in the signal transduction at a series of time points, as reported in previous publication (411).

The PBPK model in GastroPlus was utilized to enhance the understanding of oral absorption and PK of CUR. By inputting CUR’s physicochemical properties and the *in vivo* PK parameters (Table 5.4), the observed GC and VC PK data were fairly well-captured by the simulated PK profiles but not as well with SC (Figure 5.5). The reason for the differences are not clear, could be due in part to the vehicle (Cremophor, Tween 80, ethanol and water) used to suspend the CUR powder for SC versus capsules for GC and VC. Additional study with more marketed CUR products would be needed. Utilizing the simplified PBPK ACAT model, reasonably good predictions and better understanding of the role of physicochemical properties of CUR may play in the intestinal absorption kinetic processes resulting in the CUR PK profile as observed in the when compared to preclinical experimental study observations. Furthermore, many scientists have assessed the ability of PBPK models, more specifically the GastroPlus oral ACAT model, to predict oral absorption and exposure in preclinical species. For example, one study applied the GastroPlus oral ACAT model to a large dataset of 623 compounds from discovery projects. And the in vivo IV parameters were included into the model while the PO data served as observation control. Finally, the GastroPlus ACAT model provided reasonable oral absorption prediction (412). The PBPK (ACAT) model for oral absorption, where the disposition and elimination were described by fitting a compartmental PK model to an in vivo IV profile, have also been studied in many other compounds: Parrott and Lavé (413)
and Jones et al. (414) evaluated such a model in rats respectively for 3 and 8 compounds. Generally, the predictions were reasonable, and when they were not, the model allowed hypothesis testing and an improved understanding of oral absorption.

This type of simulation exercise would inform dosing recommendations and or the design of clinical trials when evaluating CUR for a new indication. The model is readily implemented in GastroPlus and could be applied for drugs with similar metabolism pathways at various stages of drug development to evaluate if altered PK may occur in a gastrointestinal disorders population based on healthy subject data. In addition, a better and more ideal approach would be to conduct a comprehensive PBPK/PD study with the different organs and quantifying the PK and PD profiles.

To date, there are limited preclinical and clinical studies that have investigated the PK/PD of different commercially marketed formulations of CUR, a herbal medicinal product often taken as an over-the-counter botanical supplement. The presence of COG, the glucuronide metabolite of CUR, is due to intestinal metabolism by UGTs. Despite the very low concentrations of parent CUR in the rat blood/plasma, the antioxidant modulatory responses were observed in plasma for all 3 oral formulations, as shown by the increased gene expression levels of *Nrf2*, *Ho-1*, and *Nqo-1*. In summary, in our current study we conducted the PK and PD assessment after the IV and oral administrations of 3 different CUR products. The anti-oxidative properties of CUR as measured by the gene expression of Nrf2-mediated response in leukocytes would serve as potential
surrogate PD biomarkers, implicating the potential health beneficial effects of the marketed CUR products, including the cancer chemopreventive effects. Moreover, the PK-PD modeling effort reasonably describes and links the PK and PK/PD of the three CUR products. Thus, this study presents a feasible approach to apply the PK/PD and PK/PD modeling and simulation in preclinical and clinical studies of botanical dietary supplements in general, including the understanding of a drug's initial acute pharmacological response.
Figure 5.1 Nrf2 transcription factor regulates the expression of antioxidant and Phase II detoxifying genes.

CUR will disrupt Keap1-Nrf2 binding and activates Nrf2, which then migrates into the cell nucleus and binds to the Antioxidant Response Element (ARE) present in the 5’ flanking region of many Nrf2-mediated genes. ARE then upregulates a variety of antioxidant enzymes and Phase II detoxifying genes.
Figure 5.2 Scheme of PKPD model and deconvolution of observed PK profile.

(a) Schematic of two-compartment model that describes CUR PK following both IV and oral doses and PK-PD model of CUR-mediated induction of antioxidant/phase II detoxifying genes. Vc is the central compartment; Vp is the peripheral compartment; CLd is inter-compartmental clearance between the central and peripheral compartment; CL is the total clearance from the central compartment. Cp represents the plasma concentration; ka is First-order absorption rate constant; F is bioavailability; kin is the zero-order rate constant for the production of antioxidant genes’ mRNA, kout is the first-order rate constant for the degradation; Emax is the maximum ability of CUR to stimulate
signal initiation; \textit{EC50} represents the concentration of CUR resulting in 50\% of the maximum induction. (b) Average fraction input vs. time profiles for GC, VC, SC formulations estimated by deconvolution. Cumulative CUR input v.s. time, single oral dose.
Figure 5.3 Plasma concentration-time profile of oral, IV curcumin and CUR-O-glucuronide (COG).

Concentration-time profiles of PO CUR (solid line) and IV CUR (dash line) as fitted by a two-compartment model. CUR-O-glucuronide (COG) of oral administration PK profile described by the one compartment model (dot line). GC, VC and SC represent CUR from GNC, Vitamin Shoppe and Sigma, respectively. Experimental observation data are shown as the mean +/- SD of plasma concentrations in 4 rats. The solid line represents the R software predicted curves after 250 mg/kg (oral) while the dash line represents the 40 mg/kg (IV) doses of CUR.
Figure 5.4 PD response of CUR with induction of antioxidant gene expression and PKPD modeling with IDR model for GC (a), VC (b) and SC (c).

Phase II detoxifying/antioxidant gene expression including Nrf2, Ho-1, and Nqo-1 induced by CUR and captured by IDR model. The red triangles represent the mean of the observed data in rat leukocytes, and the blue lines represent the model prediction.
Figure 5.5 PBPK model using Gastroplus® for GC, VC and SC CUR in rat.

Simulation of plasma profile for oral dosing with ADMET Predictor using physicochemical parameters such as pKa, logP, effective permeability ($P_{eff}$), solubility and estimated clearance from experimentally observed data from Table 1. The blue solid line represents the mean concentration for the simulated population by GastroPlus. The different symbols denote the mean values (n=4) of the observed data.
### Table 5.1 Parameters estimates of CUR pharmacokinetics with Noncompartment model in rat plasma by Phoenix WinNonlin

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Description</th>
<th>Estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>Maximum concentration observed</td>
<td>12.6</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>Time to reach maximum concentration</td>
<td>0.33</td>
</tr>
<tr>
<td>$AUC_{0-12h}$ (ng/ml*h)</td>
<td>Area under the curve 0 to 12h</td>
<td>268.9</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (ng/ml*h)</td>
<td>Area under the curve 0 to infinity</td>
<td>268.9</td>
</tr>
<tr>
<td>$AUMC_{0-12h}$ (ng/ml*h2)</td>
<td>Area under the first moment curve 0 to 12h</td>
<td>456.9</td>
</tr>
<tr>
<td>$AUMC_{0-\infty}$ (ng/ml*h2)</td>
<td>Area under the first moment curve 0 to infinity</td>
<td>457.1</td>
</tr>
<tr>
<td>$MRT$ (h)</td>
<td>Mean residence time</td>
<td>1.7</td>
</tr>
<tr>
<td>$CL$ (L/h/kg)</td>
<td>Total clearance</td>
<td>112.4</td>
</tr>
<tr>
<td>$V_{ss}$ (L/kg)</td>
<td>Volume of distribution in steady state</td>
<td>62.8</td>
</tr>
</tbody>
</table>
Table 5.2 Pharmacokinetics parameters predicted from Phoenix WinNonlin compartment models.

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Description</th>
<th>Estimation</th>
<th>IV (CV%)</th>
<th>oral (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td>VC</td>
</tr>
<tr>
<td>( V_c ) (L/kg)</td>
<td>Volume of distribution in central compartment</td>
<td>43.6 (0.6)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_p ) (L/kg)</td>
<td>Volume of distribution in peripheral compartment</td>
<td>278.4 (9.9)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CL ) (L/h/kg)</td>
<td>Elimination clearance</td>
<td>172.4 (8.9)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CL_d ) (L/kg)</td>
<td>Inter-compartment clearance</td>
<td>53.2 (6.6)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( k_a ) (h(^{-1}))</td>
<td>First-order absorption rate</td>
<td>-</td>
<td>3.1 (11.9)</td>
<td>1.8 (16.7)</td>
</tr>
<tr>
<td>( F ) (%)</td>
<td>Bioavailability</td>
<td>-</td>
<td>0.9 (3.5)</td>
<td>0.6 (8.0)</td>
</tr>
</tbody>
</table>

*oral and IV shared parameter
Table 5.3 Pharmacodynamic parameters of mRNA expression estimated from indirect response (IDR) model

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Definition</th>
<th>Nrf2 (CV%)</th>
<th>Ho-1 (CV%)</th>
<th>Nqo-1 (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{in} )</td>
<td>zero-order rate constant for the production of mRNA</td>
<td>4.24 (2.4)</td>
<td>4.86 (4.1)</td>
<td>5.53 (1.5)</td>
</tr>
<tr>
<td>( E_{max} )</td>
<td>Maximum ability of CUR to stimulate signal initiation.</td>
<td>1.74 (1.2)</td>
<td>2.02 (14.5)</td>
<td>2.24 (0.3)</td>
</tr>
<tr>
<td>( EC_{50} )</td>
<td>Concentration of CUR gives 50% of the maximum response</td>
<td>3.87 (0.1)</td>
<td>4.32 (3.2)</td>
<td>4.03 (0.2)</td>
</tr>
<tr>
<td>mRNA0</td>
<td>Initial condition (Fixed)</td>
<td>1.00</td>
<td>1.00 (Fixed)</td>
<td>1.00 (Fixed)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculated Parameter</th>
<th>Definition</th>
<th>Nrf2</th>
<th>Ho-1</th>
<th>Nqo-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{out} )</td>
<td>First-order rate constant for the degradation of mRNA</td>
<td>4.24</td>
<td>4.96</td>
<td>5.53</td>
</tr>
</tbody>
</table>

*Percentage coefficient of inter-individual variability of the estimation*
Table 5.4 Physicochemical and metabolism related in vitro and in vivo characteristics of CUR used in GastroPlus simulation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>368.4</td>
<td>ADMET®</td>
</tr>
<tr>
<td>MW (g/mol)</td>
<td></td>
<td>Predictor</td>
</tr>
<tr>
<td>Ionization coefficient</td>
<td></td>
<td>ADMET®</td>
</tr>
<tr>
<td>pKa</td>
<td>9.5, 8.96, 8.32</td>
<td>Predictor</td>
</tr>
<tr>
<td>Partition coefficient</td>
<td></td>
<td>ADMET®</td>
</tr>
<tr>
<td>logP</td>
<td>3.29</td>
<td>Predictor</td>
</tr>
<tr>
<td>Blood to plasma ratio</td>
<td>1.09</td>
<td>Predictor</td>
</tr>
<tr>
<td>B:P ratio</td>
<td></td>
<td>ADMET®</td>
</tr>
<tr>
<td>Unbound fraction</td>
<td>6.29</td>
<td>Predictor</td>
</tr>
<tr>
<td>Elimination clearance</td>
<td>172.4</td>
<td>Experiment Data</td>
</tr>
<tr>
<td>Dose</td>
<td>250</td>
<td>Design</td>
</tr>
<tr>
<td>Diff. Coeff (cm^2/s x 10^5)</td>
<td>0.67</td>
<td>Predictor</td>
</tr>
<tr>
<td>S+Peff (cm/s*10^4)</td>
<td>5.14</td>
<td>Predictor</td>
</tr>
<tr>
<td>Solubility (mg/ml) at PH=6.11</td>
<td>0.045</td>
<td>Predictor</td>
</tr>
<tr>
<td>CL (L/h/kg)</td>
<td></td>
<td>Experiment Data</td>
</tr>
<tr>
<td>Dose (mg/kg, Oral)</td>
<td></td>
<td>Design</td>
</tr>
</tbody>
</table>
Table 5.5 Observed and simulated pharmacokinetics parameters of different CUR formulations.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observed Values</th>
<th>Simulated Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC</td>
<td>VC</td>
</tr>
<tr>
<td>$AUC$ (ng/ml*h)</td>
<td>20.0 10.7 45.6</td>
<td>26.7</td>
</tr>
<tr>
<td>$Cmax$ (ng/ml)</td>
<td>12.6 9.92 17.8</td>
<td>13.7</td>
</tr>
<tr>
<td>$Tmax$ (h)</td>
<td>0.3 0.3 0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>$F$ (%)</td>
<td>0.9 0.6 3.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Summary and Future Perspective

Biological redox signaling plays an important role in many diseases. Redox signaling involves reductive and oxidative mechanisms. Oxidative stress occurs when reductive mechanism overwhelms oxidative challenges. Cellular oxidative stress occurs when ROS exceed the cellular reductive/antioxidant capacity. Endogenously produced ROS from mitochondrial metabolic TCA cycle coupled with electron-transport-chain or exogenous stimuli trigger cellular signaling events leading to homeostatic response or pathological damage. Recent evidence suggests that ROS also modulate epigenetic machinery driving gene expression by affecting DNA methylation/demethylation, histone acetylation/deacetylation or histone methylation/demethylation. Interestingly, ROS-induced oxidative stress regulates multiple redox signaling pathways that ultimately impacts on cellular metabolic rewiring. And epigenetic modifications such as DNA methylation and histone acetylation are sensitive to cellular metabolic status. Strong molecular link between metabolic reprogramming and epigenetic modifications through key metabolic intermediates including NAD, SAM, and AcCoA which are co-factors for the epigenetic enzymes and work as hubs between epigenetic processes and oxidative stress responses have been reported (60, 206, 207). More, importantly, many health beneficial phytochemicals possess redox capability that counteract ROS either by directly scavenging the radicals or via inductive mechanism of cellular defense antioxidant/reductive signaling pathway including KEAP1-NRF2 and among others (92). In addition, the phytochemicals also have been reported possess
epigenetic modifying ability (415). So, this dissertation summarized the latest advances on the interactions between redox signaling, mitochondrial metabolism, epigenetics and redox active phytochemicals (e.g. FX, B, UA and Curcumin) and the future challenges of integrating these events in human health.

Chapter 1 of this dissertation summarized the role of epigenetics/epigenomics in the prevention of early stages of various cancers by phytochemicals with an emphasis on isothiocyanates (ITCs). Chapter 2 deploys the multi-omics approaches to investigate the role of Nrf2 in the context of metabolic rewiring, epigenetic reprogramming and transcriptomic contributing to the overall FX’s protective effects in TPA-induced ROS-mediated cellular transformation. Chapter 3 reveals the underlying intricate biological connectivity between metabolomic, epigenomic and transcriptomic regulation as well as the critical role of NRF2 in B-mediated anti-cancer effect in human CRC HCT116 cells. Chapter 4 examines the role of the ursolic acid (UA) in regulating metabolic rewiring, CpG methylomic reprogramming, and transcriptomic network in blocking the phosphatase and tensin homologue (Pten), a tumor suppressor gene, deletion-mediated biological alterations and how UA elicits cancer prevention/anti-cancer effects in prostate-specific Pten KO prostatic adenocarcinoma mouse model. Chapter 5 is an animal study that was conducted to quantitively assess the antioxidant and anti-inflammatory effects of Curcumin via pharmacokinetics (PK)/pharmacodynamics (PD) modeling approach. Altogether, these chapters cover a range of dietary phytochemicals and their antioxidant and epigenetic effects in different cell and animal disease models. The findings presented in these chapters could provide
future directions on the use of dietary phytochemicals in diseases such as cancers and its interplay with metabolomics as well as epigenetics.
Appendix 2.1: The study design scheme of chapter 2
Appendix 2.2: RT-PCR and ChIP-RT-PCR primers.

<table>
<thead>
<tr>
<th>RT-PCR Primers</th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>AGCAGGACTGGGAAGTT</td>
<td>TTCTTTTTCAGCGAGGAGA</td>
</tr>
<tr>
<td>Ho1</td>
<td>CCTCAGCAGGAAATCATC</td>
<td>CCTCGTGGAGACGCTTTACATA</td>
</tr>
<tr>
<td>Dnmt1</td>
<td>CTATCGCATCGTCGATAAA</td>
<td>GTTGTAGGACCTGTGGGTATT</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>AGCGTCACAGAAGCATAC</td>
<td>GGCGGTAGAACATCAAAGAG</td>
</tr>
<tr>
<td>Hdac1</td>
<td>AGTCTGTACACTACGAGG</td>
<td>TGACGAGGAAATGAGTAC</td>
</tr>
<tr>
<td>Hdac3</td>
<td>CATCGCCTGCTTACTCAT</td>
<td>AAGGCATTAAGCCTCTGGTC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CGTTCAATACCCCAGCCATG</td>
<td>GACCCGTCACCAGAGTCC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ChIP-qPCR primers</th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIP-Uhrf1</td>
<td>TGAGAGGAGGAGTGAAGAGA</td>
<td>GTAAGAGGCTAAGCGTGAG</td>
</tr>
<tr>
<td>ChIP-Nrf2</td>
<td>ACAGCGAGGAGGAGATCAA</td>
<td>CCAGTGGAGAGAGAGAG</td>
</tr>
</tbody>
</table>


Appendix 3.1: The study design scheme of chapter 3.
### Appendix 3.2 RT-PCR primers

<table>
<thead>
<tr>
<th>RT-PCR Primers</th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>NRF2</em></td>
<td>GTTGCCCACATTCCAAATC</td>
<td>CGTAGCCGAAGAAACCTCAT</td>
</tr>
<tr>
<td><em>KEAP1</em></td>
<td>AACCGAACCTTCAGCTAC</td>
<td>CAGCTGGGATGTCCTCATATTT</td>
</tr>
<tr>
<td><em>NQO1</em></td>
<td>GGAAGAAACGCGCTGGGAATA</td>
<td>AGAATCGCTGCTGAAAGTTTAG</td>
</tr>
<tr>
<td><em>HO1</em></td>
<td>TCTTGGCTGGCTTTTC</td>
<td>CATAGGCTCCTCCTCCTTTC</td>
</tr>
<tr>
<td><em>PRDX1</em></td>
<td>CCACGGAGATCATTGCTTTCA</td>
<td>AGGTTGATTTGACCCATGCTAGAT</td>
</tr>
<tr>
<td><em>TXN1</em></td>
<td>GTGAAGCAGATCGAGAGGAAG</td>
<td>CGTTGGCTGAGAAGTCAACTACTA</td>
</tr>
<tr>
<td><em>TXN2</em></td>
<td>CTGGTGGCCTGACTGTAACAC</td>
<td>TGACCACTCGGTCTTGAAGAT</td>
</tr>
<tr>
<td><em>DNMT1</em></td>
<td>CCCCTGAGCCCTACCGAAT</td>
<td>CTCGCTGGAGTGGAAGTTGT</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>CAAGAGCACAAGAGGAAGAG</td>
<td>CTACATGGCAACTGTGAGGAG</td>
</tr>
</tbody>
</table>
Appendix 4.1: Pten KO drives prostate prostatic intraepithelial neoplasia (PIN), inflammation reactions, increases prostate size and regulates the downstream signaling changes.
Appendix 4.2 DNA overall methylation profile in Pten KO mice and UA treated Pten KO mice at different time points. (a) Distribution of annotated DMRs by genomic features including Distal Intergenic, Promoter (<=1kb, 1-2kb and 2-3kb), 1st Intron and other regions. Each DMR has at least three CpG sites; (b) Distribution of DMRs by number of CpG sites and region; (c) Average methylation levels of DMRs based on gene regions for samples in the Pten KO and Pten KO+UA groups after 6 & 14 wk treatment; (d) Principal component analysis (PCA) of CpG epigenomic profiles for Pten KO and Pten KO+UA groups after 6 & 14 wk treatment.
Appendix 4.3 Overall transcriptomic profile in *Pten* KO mice and UA treated Pten KO mice at different time points. (a) RNA-seq analysis revealed the distribution of DEGs by number of genes and normalized annotated data for the *Pten* KO and *Pten* KO+UA groups at 6 week and 14 week; (b) Principal component analysis (PCA) of transcriptomic profiles and (c) Dendrogram of the gene expression profiles clustered by Euclidean distance of *Pten* KO and *Pten* KO+UA groups at 6- and 14-week.
Appendix 4.4: *Pten* KO-mediated metabolic pathways (*Pten* WT vs. KO).

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>Match Status</th>
<th>P-Value</th>
<th>-log (P)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimidine metabolism</td>
<td>14/39</td>
<td>0.01004</td>
<td>1.9983</td>
<td>0.46819</td>
</tr>
<tr>
<td>Purine metabolism</td>
<td>16/66</td>
<td>0.03355</td>
<td>1.4743</td>
<td>0.36611</td>
</tr>
<tr>
<td>Thiamine metabolism</td>
<td>2/7</td>
<td>0.038306</td>
<td>1.4167</td>
<td>0.66667</td>
</tr>
<tr>
<td>Glycolysis / Gluconeogenesis</td>
<td>8/26</td>
<td>0.039255</td>
<td>1.4061</td>
<td>0.29964</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td>6/22</td>
<td>0.047501</td>
<td>1.3233</td>
<td>0.2493</td>
</tr>
</tbody>
</table>
Appendix 4.5: The metabolic pathway regulation of UA on \textit{Pten} KO mice (\textit{Pten} KO vs. KO+UA).

<table>
<thead>
<tr>
<th>Pathway Name</th>
<th>Match Status</th>
<th>P-Value</th>
<th>-log (P)</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis and degradation of ketone bodies</td>
<td>1/5</td>
<td>0.0023529</td>
<td>2.6284</td>
<td>0.0</td>
</tr>
<tr>
<td>Purine metabolism</td>
<td>16/66</td>
<td>0.027991</td>
<td>1.553</td>
<td>0.36611</td>
</tr>
<tr>
<td>Galactose metabolism</td>
<td>5/27</td>
<td>0.034057</td>
<td>1.4678</td>
<td>0.10943</td>
</tr>
<tr>
<td>Pentose and glucuronate interconversions</td>
<td>4/18</td>
<td>0.03708</td>
<td>1.4309</td>
<td>0.20312</td>
</tr>
<tr>
<td>Valine, leucine and isoleucine degradation</td>
<td>6/40</td>
<td>0.038708</td>
<td>1.4122</td>
<td>0.07502</td>
</tr>
<tr>
<td>Valine, leucine and isoleucine biosynthesis</td>
<td>5/8</td>
<td>0.038737</td>
<td>1.4119</td>
<td>0.0</td>
</tr>
<tr>
<td>Cysteine and methionine metabolism</td>
<td>7/33</td>
<td>0.045512</td>
<td>1.3419</td>
<td>0.33048</td>
</tr>
</tbody>
</table>
Appendix 5.1: Content of curcuminoids analysis

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Curcumin (std)</th>
<th>demethoxycurcumin (std)</th>
<th>bisdemethoxycurcumin (std)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>67.05% (0.92%)</td>
<td>11.38% (0.03%)</td>
<td>1.71% (0.01%)</td>
</tr>
<tr>
<td>VC</td>
<td>66.59% (1.17%)</td>
<td>15.68% (0.40%)</td>
<td>1.81% (0.04%)</td>
</tr>
<tr>
<td>SC</td>
<td>68.32% (0.89%)</td>
<td>18.27% (0.40%)</td>
<td>5.54% (0.14%)</td>
</tr>
</tbody>
</table>
Appendix 5.2: Experimental design of project 5.
Appendix 5.3

#Abbreviation note

#ODE: Ordinary differential equation. Differential equations convert to ODEs
#Abs1, Abs2 and Abs3 represent GC, VC and SC initial oral dose, respectively.
#K_a1, K_a2 and K_a3 represent absorption rate constant of GC, VC and SC, respectively.
#K_met represents rate constant of metabolite (COG)
# F1, F2 and F3 represent bioavailability of GC, VC and SC, respectively.
# A_pl_c_oral1, A_pl_c_oral2, A_pl_c_oral3 and A_pl_c_iv represent the plasma concentration of GC, VC, SC and IV, respectively.
#R1, R2 and R3 represent the gene biomarker response of GC, VC and SC, respectively.

# Representing the system simply as ODEs #

-------------------------------------------------------------------

<ODE:Abs1>   -Abs1*k_a1
<ODE:A_pl_c_oral1>   Abs1*k_a1*F1  - A_pl_c_oral1*k_el - A_pl_c_oral1*k_12 +A_pl_p_oral1*k_21 - A_pl_c_oral1*k_met
<ODE:A_pl_p_oral1>  A_pl_c_oral1*k_12 -A_pl_p_oral1*k_21
<ODE:A_pl_met_oral1> k_met*A_pl_c_oral1 -kel_met*A_pl_met_oral1

<ODE:Abs2>   -Abs2*k_a2
<ODE:A_pl_c_oral2>   Abs2*k_a2*F2  - A_pl_c_oral2*k_el - A_pl_c_oral2*k_12 +A_pl_p_oral2*k_21 - A_pl_c_oral2*k_met
\[
\begin{align*}
\text{<ODE:A\_pl\_p\_oral2> } & \quad A_{pl\_c\_oral2}k_{12} - A_{pl\_p\_oral2}k_{21} \\
\text{<ODE:A\_pl\_met\_oral2> } & \quad k_{met}A_{pl\_c\_oral2} - kel_{met}A_{pl\_met\_oral2} \\
\text{<ODE:Abs3> } & \quad -Abs3k_{a3} \\
\text{<ODE:A\_pl\_c\_oral3> } & \quad Abs3k_{a3}F3 - A_{pl\_c\_oral3}k_{el} - A_{pl\_c\_oral3}k_{12} + A_{pl\_p\_oral3}k_{21} - A_{pl\_c\_oral3}k_{met} \\
\text{<ODE:A\_pl\_p\_oral3> } & \quad A_{pl\_c\_oral2}k_{12} - A_{pl\_p\_oral3}k_{21} \\
\text{<ODE:A\_pl\_met\_oral3> } & \quad k_{met}A_{pl\_c\_oral3} - kel_{met}A_{pl\_met\_oral3} \\
\text{<ODE:A\_pl\_c\_iv> } & \quad -A_{pl\_c\_iv}k_{el} - A_{pl\_c\_iv}k_{12} + A_{pl\_p\_iv}k_{21} \\
\text{<ODE:A\_pl\_p\_iv> } & \quad A_{pl\_c\_iv}k_{12} - A_{pl\_p\_iv}k_{21} \\
\text{<ODE:R1> } & \quad kin (1 + (EmaxA_{pl\_c\_oral1}/Vc)/(EC50 + A_{pl\_c\_oral1}/Vc)) - koutR1 \\
\text{<ODE:R2> } & \quad kin (1 + (EmaxA_{pl\_c\_oral2}/Vc)/(EC50 + A_{pl\_c\_oral2}/Vc)) - koutR2 \\
\text{<ODE:R3> } & \quad kin (1 + (EmaxA_{pl\_c\_oral3}/Vc)/(EC50 + A_{pl\_c\_oral3}/Vc)) - koutR3
\end{align*}
\]
Appendix 5.4: Scheme of the PBPK modeling development.
## Appendix 5.5: COG PK parameters

<table>
<thead>
<tr>
<th>COG Parameters</th>
<th>PK Description</th>
<th>Estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{met}$</td>
<td>Rate constant of COG input</td>
<td>3.121 (11.7)</td>
</tr>
<tr>
<td>$CL_{met}$</td>
<td>Total clearance of metabolite COG</td>
<td>197.4 (37.6)</td>
</tr>
<tr>
<td>$V_{met}$</td>
<td>Volume of distribution</td>
<td>87.42 (21.3)</td>
</tr>
</tbody>
</table>
Reference


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