EPIGENETIC REGULATION OF NRF2 AND URSOLIC ACID IN SKIN CARCINOGENESIS

By

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Cellular and Molecular Pharmacology

Written under the direction of

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New Brunswick, New Jersey

October 2018

ABSTRACT OF DISSERTATION

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Non-melanoma skin cancer (NMSC) is the most common cancer in the United States with over 4.0 million new cases diagnosed in 2012. An increase of exposure to environmental pollutants and sunlight are among the causative agents of the disease. Ursolic acid (UA), a well-known natural triterpenoid found in abundance in fruit peels, cranberries, and blueberries has been shown to possess beneficial health effects against a multitude of disorders including, cardiovascular, neurological, and oncological disorders. However, epigenetic modulation of UA in skin carcinogenesis is still poorly understood. Increasing evidence suggests epigenetics play an important role in the development and progression of cancer including NMSC. Among its many anti-cancer activities, UA has also been shown to have the ability to modulate epigenetic mechanisms *in vitro* and thus presents an attractive candidate to target the underlying epigenetic mechanisms of skin carcinogenesis. Preliminary data in our laboratory and that of others points to epigenetic regulation of key genes at the center of UA activities: 1) Skin carcinogenesis is enhanced in Nrf2 (-/-) mice; 2) The expression Nrf2 and its target gene HO-1 is reduced in skin tumors of Nrf2 (+/+) mice; 3) UA inhibits TPA-induced ear edema and tumor promotion; 4) Hypermethylation of the promoter region of Nrf2 resulting in reduced expression and its target genes is closely associated with prostate tumor progression; 5) Dietary phytochemicals epigenetically modify the hypermethylation of the Nrf2 promoter region and inhibit TPA-induced

transformation. Based on the aforementioned observations, it was hypothesized UA can suppress skin carcinogenesis, in part, through the epigenetic regulation of Nrf2 signaling. We demonstrated for the first time that UA restores the expression of Nrf2 by demethylating CpG islands in the Nrf2 promoter in mouse epidermal cells resulting in an increase in the expression of cytoprotective detoxifying/antioxidant enzymes and suppression of tumor promoter-induced cell transformation. Furthermore, we demonstrate UA is able to suppress skin carcinogenesis *in vivo* using a relevant and novel B[a]P/TPA skin carcinogenesis model. The long-term goal of this research is to understand some of the underlying epigenetic mechanisms driving skin carcinogenesis and to develop safe and effective strategies to prevent/treat NMSC using phytochemicals such as UA who possess anti-cancer properties.

ACKNOWLEDGEMENT

I wish to extend my sincerest gratitude to my advisor Dr. Ah-Ng Tony Kong for his unfaltering support, guidance, and encouragement throughout my studies. Your support of my graduate endeavors as well as my professional development endeavors have helped shaped the scientist I have become.

I would also like to express my gratitude to my committee members Dr. Joseph Bertino, Dr. Li Cai, and Dr. Fang Liu for their invaluable guidance throughout the course of my studies and their critical review of this dissertation.

My graduate studies would not have been complete without the previous and current members of Dr. Kong's laboratory. I wish to express my gratitude to Dr. Hyuck Kim, Dr. Francisco Fuentes, Dr. Jonghun Lee, and Dr. Zhengyuan Su whom I collaborated on several studies with during my graduate career. I'd also like to thank Dr. Wenji Li, Dr. Renyi Wu, Dr. Chao Wang, Dr. Ran Yin, Dr. Shan Su, and Dr. Linbo Gao for their support of projects at the end of my graduate studies. The laboratory atmosphere wouldn't have been the same without my fellow graduate students Dr. Yuqing Yang, Dr. Dr. Sarandeep Boyanapalli, Dr. Ximena Paredes-Gonzalez, Dr. Yue Guo, Dr. Ying Huang, Dr. Chengyue Zhang, Dr. David Cheng, and Mr. Doug Pung.

I'd further like to thank my first student in the laboratory Fayrouz Merhom who showed me the meaning of mentorship. The experience fueled my passion in mentoring. With that, I'd like to thank my mentor Dr. Jerome Langer for his support and tremendous guidance from the start of my graduate studies.

The studies described in this dissertation wouldn't have been possible without the additional support of Dr. Yaoping Lu, whom I consulted with for the animal studies described, and without the financial support of the Department of Pharmaceutics, UMDNJ Society of Research Scholars, iJOBS, NIH IMSD Pipeline Grant, the School of Graduate Studies and the Division of Life Sciences.

Finally, I'd like to express my sincerest gratitude to my family and friends. Their tremendous understanding and support throughout the years has made this possible. My sincerest gratitude to my mother, Arlene Velez; your years of support and guidance throughout my life have shaped who I am today. I am appreciative of all that you've done and all you continue to do.

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Introduction[1,2]

[1] A portion of this chapter has been published in *AAPSJ* as a review paper: **Ramirez CN** *et al*., (2017). *In vitro-in vivo* dose response of ursolic acid, sulforaphane, PEITC, and curcumin in cancer prevention. *AAPSJ.* Dec 20;20(1):19.

[2]Keywords: triterpenoids, isothiocyanates, curcumin, chemoprevention, phytochemicals

1 Dietary Phytochemicals in cancer prevention

1.1 Introduction

Cancer is now the leading cause of death in 21 states in the US (1). Cancer is a chronic disease that could be prevented (2, 3). Cancer development can take about 10-30 years to develop, from initiation, promotion to progression (Figure 1) (4). Thus, the slow development of the disease potentially allows the intervention in the progression of cancer into advanced stages and metastases (Figure 2). Recent evidence suggests epigenetic alterations precede genetic mutations during cancer development. As such, naturally occurring phytochemicals have been shown to have the ability to activate the anti-oxidative stress, Nrf-2 mediated pathway, anti-inflammatory networks as well as others (5, 6), resulting in blocking cancer initiation, promotion and/or progression, in many *in vitro* and *in vivo* models (5). These pathways may be directly or indirectly regulated through epigenetic modulation by natural dietary phytochemicals. Among these, some of the most promising chemopreventive agents include ursolic acid (UA), sulforaphane (SFN), phenethylisothiocyanate (PEITC), and curcumin. The dose by which these phytochemicals produce their chemopreventive effects will be explored in this review

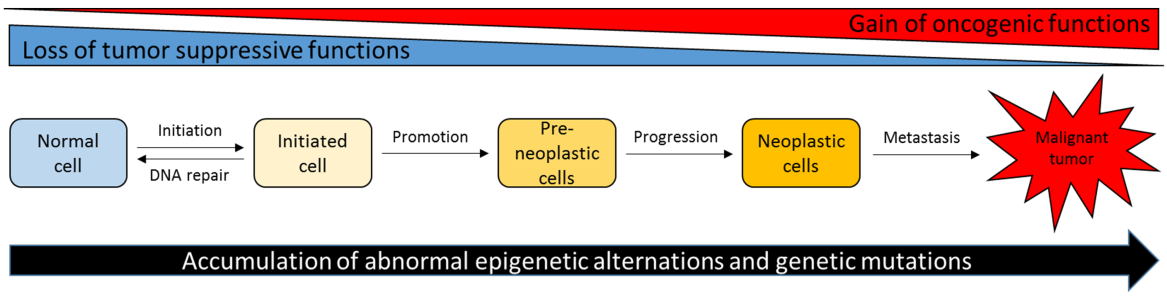


Figure 1. Development of carcinogenesis with aberrant epigenetics and genetics changes. The accumulation of genetic mutations and epigenetic alterations along with the loss of suppressive functions and the gain of oncogeneic functions permits the progression of normal cell initiation to metastasis.

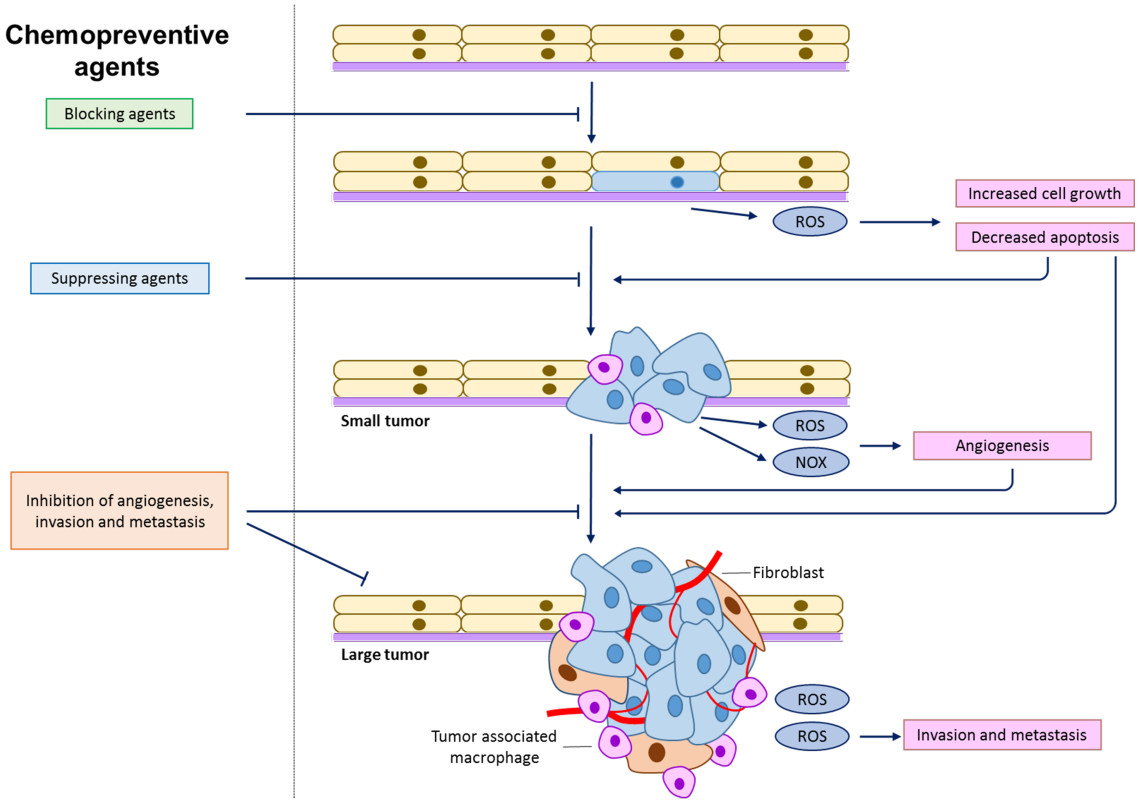


Figure 2. Illustration of chemopreventive agents on different carcinogenesis stages. Chemopreventive agents can intervene at the initiation stage, the promotion phase, and the progression phase of carcinogenesis.

1.2 The importance of dose in the *in vitro* and *in vivo* chemopreventive effects of phytochemicals

1.2.1 Triterpenoids

Triterpenoids are a natural class of compounds produced through the cyclization of squalene widely used in Asian medicine. Approximately 20,000 sources of triterpenoids exist in nature (7). This class of compounds has been shown to have anti-inflammatory and anti-cancer properties. Specifically, the triterpenoids, oleanolic acid (OA, 3B-hydroxyolean-12-en-28oic acid) (8) and ursolic acid (UA, 3B-hydroxy-urs-12-en-28-oic-acid), an isomer of OA, have shown great promise in these areas. The anti-cancer, anti-inflammatory, and chemopreventive effects of these compounds vary with dose and are explored here

UA is found in blackberries, blueberries, holy basil, thyme, lavender, catnip, peppermint leaves, olive oil, rosemary and apple peels (7). It has been shown to modulate a number of pathways implicated in the progression and the survival of cancer. The same can be said of OA, a triterpenoid found in ginseng root and the olive plant, bearberries, heather, three leaved caper, reishi, Chinese elder, and Sodom's apple (9). UA and OA are often found in combination and share many of their pharmacological properties (10). In addition to its anti-inflammatory and anti-cancer effects, OA possesses a wide range of pharmacological activities such as anti-viral, anti-microbial, anti-parasitic, anti-diabetic, and anti-analgesic (9) effects.

A multitude of experiments have shown UA is able inhibit proliferation and induce apoptosis of a variety of cell lines and in various animal models. Triterpenoids have been shown to exert their anti-inflammatory properties through the modulation of ROS and the attenuation of iNOS, COX-2 and NF-κB, a key factor in controlling transcription of DNA, cytokine production and cell survival. In the T lymphoma Hut-78

cells, UA inhibited proliferation and induced early apoptosis at 10-80 μM, with the highest effect achieved at 80 μM. This was noted in the downregulation of NF-κB p65, and p50 proteins and the upregulation of caspase-8, caspase-3, and caspase-9. In addition, COX-2 mRNA also decreased in the presence of UA (11). UA and OA have been shown to inhibit the proliferation of non-small cell lung cancer A549 cells in a nude mouse model at low and high doses of 50 and 100 mg/kg bw, respectively. At 100 mg/kg bw UA significantly inhibited the growth of the cells noted in tumor weight. Further investigations, noted UA and OA increased expression of Bid and decreased the protein levels of MMP-2, Ki-67, and CD34 (12). Additionally, UA has been shown to inhibit proliferation and induce apoptosis in MTC-SK cells, a medullary thyroid carcinoma cell line) at 10 μM and 20 μM *in vitro* (13). Additionally, UA and OA have been shown to induce apoptosis through a multitude of pathways in prostate cancer. These include the activation of JNK and inhibition of Akt pathways in PC-3 cells at 80 μM (14), and the down regulation of Bcl-2 in PC-3 and LNCaP prostate cancer cells at 55 μM and 45 μM respectively (15).

The anti-inflammatory capabilities of UA have been shown to be expansive in a number of experimental models. UA has been shown to inhibit tumor promotion by 12-O-tetradecanolphorbol-13-acetate (TPA) in a two-stage skin carcinogenesis ICR mouse model at 2 μmol applied topically prior to application of TPA. UA reduced TPA-induced inflammation and decreased the gene expression of IL-1, IL-22, and Cox-2 inflammatory genes. In addition, UA reduced binding of NF-Kβ, Egr-1, and AP-1 (12). Furthermore UA has recently been shown to inhibit cell growth and proliferation of pancreatic cell lines AsPC-1, MIA, PaCa-2, and Panc-28 cells at 5-20 μM *in vitro* (16). UA suppressed

NF-Kβ activation and was able to suppress its target genes in Panc-28 cells *in vitro*. UA's anti-cancer effects were further confirmed in an orthotopically implanted pancreatic cancer model in which UA inhibited pancreatic cancer at a dose of 250 mg/kg bw given orally daily. Moreover, UA and OA have been shown to prevent ROS-induced hepatocellular carcinoma *in vivo* in a male Wistar rat model at an oral dosage of 20 mg/kg bw (17) and skin cancer through the attenuation of chemically induced ROS and protect against DNA damage induced hydrogen peroxide at concentrations 5 and 10 μM in murine keratinocyte Ca3/7 cells (18). Furthermore, UA has been shown to induce anti-inflammatory activity at 5 μM through the suppression of NF-Kβ in activated T cells, B cells and macrophages (19). UA's anti-inflammatory role was further solidified in a study demonstrating UA was able to reduce NF-KB activation and the release of cytokines at 10 μM and 50 μM in human colon cancer COLO 205 cells (20). The study extended their findings in a (DSS)-induced acute murine colitis treated model. When induced and treated with either UA 10 mg/kg or 20 mg/kg disease activity decreased (20).

UA has demonstrated anti-oxidative activity through the modulation of several pathways. When colorectal cancer Caco-2 cells were treated with UA it resulted in the normalization of antioxidant levels and protection against oxidative damage at 5 μM and 10 μM and (21). Furthermore, UA and OA have attenuated H_2O_2 and in neuroblastic PC12 cells at 20 μM and 40 μM (22).

A recent study demonstrates UA's role in epigenetic modulation in which UA increased phosphorylation of SAPK/JNK pathway in human non-small cell lung cancer H1299 and A549 cells *in vitro* at a concentration of 30 μM. Further investigations demonstrated UA was able to decrease the expression of SP1 and in turn regulate

DNMT1 and EZH2 expression in H1299 and A549 cells (23). UA has also been reported to increase the acetylation of histone H3 and inhibit HDAC activity *in vitro* (24).

UA and OA have been shown to promote the differentiation of glioma, melanoma, and thyroid cancer cell lines, A375, U87, and ARO cell lines respectively, through the inhibition of endogenous reverse transcriptase (RT) at 10, 15, and 20 μM (25). Furthermore, UA has been shown to induce the differentiation of HL60, U-937, and THP-1 leukemic cells at 10, 20 and 30 μM via the activation of the ERK1/2 MAPK pathway (26). UA inhibits proliferation and induces apoptosis of ovarian epithelial cancer SKOV sphere cells at 12.5-50 μg/mL. In addition, UA downregulates the expression of EMT markers including Snail, Slug, Twist, vimentin, N-cadherin and fibronectin. These effects translated *in vivo* in a SKOV3 sphere cell xenograft athymic nude BALB/c-nu mouse model at 60 mg/kg bw (27).

Triterpenoids demonstrate their anti-cancer activities at a concentration range of 5 μM to 80 μM *in vitro* and 10-250 mg/kg *in vivo*. The variations in concentrations can be attributed to pharmacological effects related to cell line, assay system, animal model and source of compounds. While there are less than a handful of clinical studies evaluating human. A clinical study evaluating the effect of 150 mg of UA given orally once a day for 12 weeks on metabolic syndrome, insulin sensitivity, and inflammation lead to a transient remission in 50% of patients (28). Another study evaluating UA at 50.94 mg for use in sarcopenia demonstrated a significant increase in the right-handgrip of female subjects in comparison to the control group (29). Overall, triterpenoids hold great promise in the area of chemoprevention and as such are being synthetically modified in order to increase potency *in vivo*.

1.2.2 Isothiocyanates (ITCs)

Numerous epidemiological and pharmacological studies suggest a correlation between the consumption of cruciferous vegetables and a reduced cancer risk in humans (30, 31). Over 200 naturally-occurring glucosinolates are found in cruciferous vegetables (32), which consist of a β-D-thioglucose group, a sulfonated oxime group, and a side chain derived from methionine, phenylalanie, tryptophan, or branch-chained amino acids (33). Interestingly, the chemopreventive effects are mostly attributed to the isothiocyanate (ITC)-containing compounds rather than their glucosinolate precursors. ITCs, converted by myrosinase mediated hydrolysis from glucosinolate, are characterized by the sulfur containing N=C=S functional group with a wide structural diversity. Ally isothiocyanate (AITC) from cabbage, mustard, and horseradish; benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) from watercress and garden cress; and sulforaphane (SFN) from broccoli, cauliflower, and brassicas have been mostly studied against a variety of human malignancies (34). In cell culture models, micromolar concentrations of ITCs have shown potent anti-cancer effects through different mechanisms *in vitro* (35, 36). Several pharmacokinetic studies have provided evidence that the concentration range is achievable *in vivo*. For example, in a pharmacokinetics study of PEITC in rats, it demonstrated that plasma concentration of PEITC could reach 9.2 and 42.1 μM after an oral dose of 10 and 100 μmol/kg body weight in rats (37). Interestingly, it was also found that PEITC was highly bound to serum protein in the rats with the protein-binding ratio around 98.1% and was not concentration-dependent. The high plasma concentration was due to the high oral bioavailability, which was 115 and 93% at doses of 10 and 100 μmol/kg (37). Compared to PEITC, SFN is relative less

associated with protein binding and the binding ratio did not increase with time (38). In an in vitro study, the initial protein binding by PEITC was almost 3-fold higher than that of SFN. Four hours after incubation, cellular protein binding of PEITC became 6-fold higher than that of SFN (38). In an *in vitro* setting, PEITC also modified bovine serum albumin (BSA) covalently to a greater extent than SFN occurring exclusively at cysteine residue (38). Oral administration of 50 μmol SFN in rats resulted in a peak plasma concentration of 20 μM at 4 h (39). In a chemopreventive study using the ApcMin/+ mouse model, SFN inhibited adenoma formation with a steady-state concentration of 3-13 nmol/g (roughly equivalent to 3-10 μM) in the gastrointestinal tract (40).

In clinical studies, there are several reports showing that the ITCs could potentially impact in the prevention of cancer. After receiving 1 week of PEITC treatment (10 mg in 1 mL of olive oil, 4 times per day), tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) metabolic activation ratio was reduced by 7.7% in a clinical trial containing 82 smokers, which suggests that PEITC could be a potent inhibitor of lung carcinogenesis in smokers (41). In a double-blinded, randomized, placebo-controlled clinical trial, 78 senior patients with increasing PSA levels after radical prostatectomy were given 60 mg sulforaphane 3 times daily for 6 months, a much lower plasma PSA level were found in the sulforaphane treated group, which potentially suggests a promising treatment in recurrence of prostate cancer after prostatectomy (42). A randomized controlled clinical study consisting of 54 women subjects revealed a mean 81.7 g/d intake of cruciferous vegetable, enriching of SFN, for over 4 years (August 2009 to December 2013) was associated with a lower level of Ki-67, a cellular marker for proliferation, in breast ductal carcinoma in situ tissue, which

strengthen the correlation of cruciferous vegetable consumption and lowering breast cancer risk (43).

The chemopreventive effect of ITCs is considered to be associated with their ability to induce the expression of phase II drug metabolism/detoxifying enzymes. It has been extensively documented that SFN exerts potent activation of phase II/antioxidative gene expression in both *in vitro* and *in vivo* studies (44). In rats, 40 μmol/kg/day SFN treatments were found to increase glutathione-S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) activities in the duodenum, forestomach, and bladder tissues (45). In hepatocytes, SFN induced UGT1A1 and GSTA1 mRNA expression and protected cells against the 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP, commonly found in cooked meat and considered as risk factors for cancer)-DNA adduct formation (46). Similarly, PEITC was found to induce hepatic phase II enzymes, resulting in decreased PhIP-DNA adduct levels in rat tissues (47). In mice, 12 h after an oral dose of PEITC, upregulation of several GST isozymes in the liver were identified using a microarray approach (48). Markedly, a number of studies on ITCs suggest that the induction of phase II/antioxidant enzymes is NF-E2-related factor-2 (Nrf2) dependent (48-50).

Mechanistic studies demonstrated that ITCs activate the Nrf2 pathway by modifying Nrf2-Keap1 interactions. Using a liquid-tandem mass spectrometry approach, Hong et al. provided evidence that SFN can directly react with the thiol groups of Keap1. The formation of SFN-Keap1 thionoacyl adducts releases Nrf2 from the Nrf2-Keap1-Cul3 degradation complex; this stabilization of cellular Nrf2 consequently results in Nrf2 nuclear translocation and activation (51). On the other hand, PEITC may induce the

Nrf2/ARE signal through a different mechanism potentially mediated by mitogen-activated protein kinases (MAPKs). It has been reported that PEITC induces ARE activity through the attenuation of c-Jun N-terminal kinase-1 (JNK1) and extracellular signal-regulated kinase (ERK) inhibitors (50). In the same study, *in vitro* kinase assays showed that JNK1 and ERK2 directly phosphorylate Nrf2 protein. Collectively, PEITC increased the phosphorylation of ERK1/2 and JNK1/2 in cells, which, in turn, caused phosphorylation of Nrf2 and subsequent release from Keap1 binding, and resulted in translocation activation of the Nrf2/ARE pathway. To note, Nrf2-deficient mice have shown increased susceptibility in carcinogenesis models and less effective towards preventive treatment (52-54). Therefore, transcriptional induction of Nrf2/ARE mediated phase II enzymes would be considered as an important mechanism for the chemopreventive effects of ITCs.

Inactivation of the NF-κB pathway by ITCs is another important mechanism that can contribute to their anti-cancer activities. Experimental evidence suggests that ITCs stabilize IκB by inhibiting its phosphorylation and degradation, resulting in a reduction in nuclear translocation of p65 (a subunit of NF-κB) and NF-κB activation. In PC-3 cells, both SFN (20 and 30 μM) and PEITC (5 and 7.5 μM) strongly inhibited nuclear translocation of p65, with the concomitant decreased expression of NF-κB regulated genes such as Bcl-XL, cyclin D1, and vascular endothelial growth factor (VEGF) (55). Correspondingly, PEITC and SFN were found to inhibit lipopolysaccharide (LPS)-induced NFκB luciferase activity in human colorectal cancer HT-29 cells, which was also mediated through the inhibition of IκKβ phosphorylation (56). In addition, SFN was proposed to interact with glutathione and other redox regulators like Ref-1 and

thioredoxin, which in turn indirectly impairs the NFκB-DNA binding ability (57). Another study by Heiss et al suggested SFN directly interacts with Cys residues of NFκB subunits by forming dithiocarbamate, which results in decreased DNA binding abilities (58). Collectively, these findings indicate that redox modulation and thiol reactivity play certain roles in regulating NFκB-dependent transcription by SFN. Interestingly, studies on the crosstalk between Nrf2 and NFκB signaling have shown that Nrf2 downstream targets may inhibit of NFκB nuclear translocation (59, 60). Accordingly, pre-treatment of SFN (25 mg/kg per day) mitigated dextran sodium sulphate (DSS)-induced acute colitis *in vivo*, while increased expression of Nrf2-dependent genes and reduced expression of inflammatory were observed in colon tissues (61). Similarly, SFN restored the number of sunburn cells to basal levels in Nrf2 WT but not Nrf2 knockout (KO) mice after UV irradiation. The inflammatory markers were lower in SFN treated Nrf2 WT tissues compared to Nrf2 KO tissues (62). These results suggest activation of Nrf2 by SFN can, in part, contribute to the suppression of proinflammatory signaling pathways.

Given that epigenetics lies on the molecular interface between genetics and environmental factors, there is a growing interest in evaluating the potential of dietary phytochemicals to block or reverse epigenetic abnormalities in cancer development. In a recent study, Wong et al. reported the effects of SFN on promoter DNA methylation profiles in prostate epithelial cells (PrEC), androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cells (63). SFN treatment was found to decrease the DNMT levels in all the tested cell lines. Although SFN showed complex effects on genome-wide DNA methylation patterns among normal prostate epithelial and prostate cancer cells, the genes of altered methylation status were functionally similar within a

single cell line (e.g. cell migration, cell adhesion etc.). In various *in vitro* and *in vivo* studies, SFN or PEITC treatments appeared to down-regulate DNMT activity, thereby resulting in promoter demethylation of epigenetically silenced genes, with the concomitant change of gene expressions (reviewed by (64, 65). Interestingly, DNA demethylation in a promoter region is often found to be associated with local relaxing of histone structure, although the precise mechanism remains to be elucidated. For example, in mouse prostate cancer TRAMP-C1 cells, SFN (1.0 and 2.5 μM) restored the epigenetically suppressed Nrf2 levels by reversing the hypermethylation status of the Nrf2 promoter region via inhibition of DNMT activities, as well as HDACs (66). In mouse epidermal JB6 P+ cells, this change in methylation pattern by SFN is associated with increased Nrf2 level and a phenotype more resistant to TPA-induced neoplastic transformation (67).

On the other hand, HDACs are often upregulated in cancers therefore HDAC inhibition is considered as an important strategy in cancer prevention and therapy. Molecular docking experiments have shown the metabolite of SFN and several structural related ITCs directly interact with the HDAC catalytic core to inhibit the enzyme activity (68). In a clinical study, a single dose of 68 g of broccoli sprouts (containing ~105 mg of SFN) inhibited HDAC activity significantly in peripheral blood mononuclear cells (PBMC) 3 and 6 hours post consumption (69). Incubation of BPH-1, LNCaP, and PC3 prostate cancer cells with 15 μM SFN significantly reduces HDAC expression by 30–40%, which is accompanied by a 50–100% increase in the acetylation of histones, as well as G2/M arrest of cell development and induction of apoptosis in a caspase-dependent manner (70).

Epigenetic upregulation of p21 gene expression by PEITC was found to be associated with chromatin remodeling, which compromises dynamic changes in both histone acetylation and methylation (71). To note, PEITC also exhibits the dual functions of CpG demethylation, HDAC inhibition and epigenetic regulation of various genes (72, 73). Last but not least, the anti-cancer effects of ITCs may be partially attributed to their ability to regulated miRNA. Using an oligonucleotide approach, we identified top altered miRNAs upon 2.5 μM PEITC treatment in prostate cancer cells. Among them, miR-194 was a primary target of PEITC which was able to suppress cell invasion (74).

ITCs have been shown to exert cytoprotection via the activation of Phase II enzymes within the Nrf2 pathway. Rat aortic smooth muscle cells treated with SFN at 0.25–5 μM, resulted in the increase of phase 2 antioxidant enzymes in a concentration-dependent manner. Furthermore, when pre-treated with SFN (0.5, 1, and 5 μM), the cells were protected from oxidative and electrophilic cytotoxicity induced by xanthine oxidase (75). Incubation with SFN, BITC, and PEITC (0-10 μM) protected against oxLDL-induced endothelial damage in a dose-dependent manner through the induction of Nrf2's target gene heme oxygenase-1 (HO-1). In addition, the expression of NF-κB, ICAM-1, VCAM-1, and E-selectin were decreased (76). Human peripheral blood mononuclear cells (PBMC) treated with PEITC (1-10 μM) for 24 h, increased the detoxification enzymes GPX1 (3.7-fold increase by 1 μM PEITC treatment) and SOD2 (7.3-fold increase by 10 μM PEITC treatment) (77).

Furthermore, SFN inhibited breast CSCs at concentrations 1-5 μM *in vitro* which is much lower than the concentration needed to induce apoptosis (78). In an *in vivo* xenograft model, where 5-week-old female NOD/SCID mice with a xenograft of

SUM159 cells received daily injections of 50 mg/kg SFN for 2 weeks, breast CSCs were found to be inhibited mainly due to the down-regulation of Wnt/β-catenin self-renewal pathway (79). SFN is also effective in the treatment of leukemia by enhancing the differentiation of leukemic cells. When human promyelocytic leukemia cells were treated with 0.2-100 μM SFN, SFN induced differentiation in the leukemic cells to granulocytic and macrophagic lineages. This process was mediated mainly through PKC (80).

GATA-3 is a marker for luminal progenitor cell differentiation and can actively promote the differentiation of cancer cells (81, 82). When PyMT transgenic mice were treated with PEITC (8 mmol/kg bw), the progression of tumor size was delayed and there were smaller tumors compared to the control. These findings were accompanied by a low expression of ERα, FOXA1 and GATA-3 (83). PEITC also inhibited CSC growth *in vitro* (84). PEITC transformed LNCaP floating spheres into prostate cancer stem cells (PCSC) due to the enhancement of H3K4 acetylation, the inhibition of DNMT1 and activation of GSTP1. After androgen deprivation, the PCSCs differentiate into neuroendocrine cells with decreased proliferation, expression of the androgen receptor, and PSA (85)

Extensive studies have shown ITCs are able to inhibit the growth of cancer cells via arresting the cell cycle through the regulation of cell cycle proteins, cyclin-dependent kinase activity, tubulin polymerization and histone acetylation, and the induction of apoptosis (86). PEITC induces extrinsic apoptosis pathway through stimulating death receptors and Fas (87, 88) and the intrinsic apoptosis pathway by regulating BCL2, BID and BAX (89-91). In addition, PEITC can induce G0/G1 arrest via p53 and G2/M cell arrest in a p53 independent manner (90, 92).

PEITC (0 to 10 μM) significantly inhibited human laryngeal carcinoma Hep-2 cell growth and enhanced apoptosis with G2/M cell cycle arrest in a dose- and time-dependent manner while no effect was observed in the growth of normal human bronchial epithelial cells (93). Treating human non-small cell lung cancer L9981 cells with BITC (7.5 and 10 μM) and PEITC (12.5 and 20 μM) resulted in apoptosis through the stimulation of caspase-3 and cell cycle arrest at the G2/M phase via cyclin B1 regulation (94). BITC and PEITC also inhibited the growth of lung cancer L9981 cells with IC50 5.0 and 9.7 μM respectively by suppressing Akt and NF-KB, enhancing ROS production, and reducing GSH (95).

SFN can inhibit the growth of cancer cells by causing cell cycle arrest and apoptosis induction. SFN can stimulate the intrinsic apoptosis pathway by activating BCL-2 and suppressing inhibitors of apoptotic proteins (IAPs) (96). Treating DU145 and PC-3 prostate cancer cells with SFN (10, 20, and 40 μM) enhanced cytochrome c levels by producing more ROS leading to apoptosis (97). Additionally, in human bladder cancer T24 cells, SFN arrested the cell cycle in G0/G1 phase via the p27 pathway (98). It also induced G2/M cell cycle arrest by stimulating p21 pathway and suppressing Cdc2/Cyclin B1 (99, 100).

SFN can inhibit cell growth and induce apoptosis in a dose dependent manner. Incubation of A549 cells treated with 30 μM SFN induced G2/M arrest via p21 pathway (101). Upon treating Caco-2 cells with various concentrations of SFN, 25 μM SFN had the greatest effect on enhancing UGT1A expression via Nrf2 pathway, while 75 μM SFN induced G1/G2 arrest and apoptosis via decreasing bcl-2 level and enhancing bax (102). When treating colorectal cancer (CRC), higher concentrations of SFN (12.5 and 25 μM)

produced apoptosis through decreasing caspase-3 and increasing caspase-2, -3, -8, and -9. The low dose SFN generated a mitotic delay (103). SFN inhibited the growth and induced apoptosis in a dose- and time-dependent manner in MDA-MB-231 human breast cancer cells, whereby 30 μM SFN induced apoptosis by increasing caspase-3 and reducing BCL-2. Furthermore, it induced S and G2/M cell-cycle arrest by upregulating $p21^{WAF1}$ and $p27^{KIP1}$ expression and down-regulating cyclin A, cyclin B1 and CDC2 levels (104).

In summary, for ITCs, in the context of *in vitro* cell line dose response, it appears it is dependent on the cell line, biomarker measured, and the chemical structure of the ITC, among others. Nevertheless, there is a dose-dependency of dose response. For instance, in human hepatoma cell line HepG2-C8 expressing the ARE-luciferase reporter, SFN increases ARE activity at concentrations up to 35 μM (105). Beyond 35 μM, ARE activity decreases due to cellular toxicity. This higher dose-dependent cellular toxicity could be blocked by adding exogenous glutathione (GSH). Interestingly, at lower doses of SFN, GSH attenuated ARE activity, however, at higher dose level, GSH enhances ARE activity, due to blockade of caspase 3 activation and apoptosis. These dose-dependency effects of SFN are quite similar to phenolic antioxidants butylated hydroxyanisole (BHA) and its metabolite *tert*-butylhydroquinone (tBHQ) (106, 107), although SFN in general is more potent by about one order of magnitude. From the above discussion, in our experience, it appears that SFN would activate epigenetic events in low micromolar concentrations, then it would activate Nrf2 signaling in low tenths micromolar and activation of caspases/apoptosis around fifty-one hundred micromolar concentrations. We have also reviewed this dose-dependency effects previously (108).

1.2.3 Curcumin

Polyphenols are a group of compounds that have at least one aromatic ring with one or more hydroxyl functional groups attached (109). Natural polyphenols, which are widely present in foods and beverages from plant origin (110), are another category of phytochemicals that have been extensively studied for their health beneficial effects in many diseases, including cancer. It is well accepted that their potent antioxidant and anti-inflammatory activities largely contribute to their anticancer efficacy. In addition, experimental evidence suggests dietary polyphenols are able to modulate molecular targets and signaling pathways regulating detoxification enzymes, cell survival, proliferation, differentiation, migration, and angiogenesis. (111).

While flavonoids and phenolic acids account for over 90% of all the natural polyphenols, curcumin, the bright yellow colored polyphenol rich in rhizomes of *Curcuma longa* (turmeric) has a distinct chemical structure. Curcumin is considered a highly promising chemopreventive agent since it fulfills several ideal characteristics such as low toxicity, affordability, and easy accessibility. Numerous studies using cell lines and animal models have demonstrated curcumin is effective in inhibiting tumor growth, which warranted clinical trials to test its safety and efficacy. However, phase I/II clinical trials showed poor bioavailability of curcumin in humans. Oral administration at doses up to 8 g resulted in undetectable levels of curcumin in blood (112). It does not seem practically possible to reach the *in vitro* effective dose of curcumin in humans. Efforts have been made to circumvent the bioavailability challenge by chemical structure modifications (curcumin analogs) and diverse delivery systems (liposome, nanoparticles, and conjugates). However, 17 out of 49 curcumin double-blinded placebo-controlled

clinical trial showed efficacy. Another 27 clinical trials of curcumin pointed to the therapeutic benefits (113). Curcumin doses ranged from 180 mg/day to 3,000 mg/day have been used in human. Under the treatment with standard chemotherapy protocols, the bioavailable curcuminoid preparation (180 mg/day) for a period of 8 weeks as adjuvant therapy in cancer patients with solid tumors can significantly improve quality of life and suppress systemic inflammation (114). In addition, in curcumin (total 3 g/day) with external-beam radiation therapy of up to 74 Gy patients with prostate cancer group, plasma total antioxidant capacity significantly increased and the activity of superoxide dismutase decreased compared with those at baseline (115). The clinical results still support the use of curcumin as an effective cancer preventive agent, particularly, in several colorectal cancer trials (116, 117). The interactions between curcumin and the host body system are expected to be more complicated. The following sections will focus on the relationship between the exposure and the response of curcumin.

An important molecular switch through which curcumin may mediate its health benefits is the transcription factor nuclear factor 2-related factor (Nrf-2). Curcumin has been shown to induce reactive oxygen species (ROS) scavenging enzymes. ROS is a bi-functional cellular molecule in cancer cells. It can drive DNA mutations in carcinogenesis, and it can trigger mitochondrial apoptosis. In a study where astrocytes were treated with 5-15 μM curcumin expression of NQO1 and GST, members of phase II detoxification enzymes, increased significantly. Moreover, HO-1 mRNA and protein expression were elevated after a 6 h incubation with 5–25 μM curcumin. However, higher concentrations of curcumin (50–100 μM) caused a substantial cytotoxic effect with no change in HO-1 protein expression (118). And in renal epithelial cells, curcumin

stimulated the expression of Nrf-2 in a dose- and time-dependent manner (119). Conversely, curcumin is able to generate cellular ROS to drive mitochondrial apoptosis to treat malignancies (120). Despite paradoxical roles in regulating cellular ROS, the overall anticancer effect of curcumin has been clearly shown in a number of studies.

Curcumin is a traditional remedy for inflammatory diseases (121).The anti-inflammatory effects of curcumin have been postulated on the basis of a number of *in vitro* and *in vivo* studies(122, 123). Curcumin dose-dependently increased the number of pre-apoptotic and apoptotic cells in phorbol myristate acetate (124) and stimulated human neutrophilic granulocytes (125). The application of curcumin significantly inhibited the activity of neutrophilic granulocytes in a rat model of arthritis (an inflammatory arthropathy), which confirmed the anti-inflammatory properties of curcumin *in vivo*. Moreover, a curcumin injection given to mice prior to an intraperitoneal LPS administration led to an inhibition of LPS-induced increased MCP-1 (monocyte chemoattractant protein 1) mRNA levels (126). LPS-induced mRNA and protein levels of MCP-1 and interleukin-8 (IL-8) were reduced by curcumin treatment in human renal epithelial cells HK-2. Furthermore, curcumin prevented LPS-induced NF-kB DNA binding (126).

The cytoprotective effect of curcumin has been well studied. 20 μM curcumin has been reported to protect human proximal tubule HK-2 cells from apoptosis and necrosis induced by Shiga toxin (127). Interestingly, the protective effect of curcumin against stx1 and stx2-induced injury on HK-2 cells is not related to its anti-oxidative properties. Curcumin can attenuate palmitate-induced apoptosis in MIN6 pancreatic β-cells through PI3K/Akt/FoxO1 and mitochondrial survival pathways (128). In this study, 10 μM

curcumin improved cell viability and enhanced glucose-induced insulin secretory function. Curcumin treatment neutralizes ROS generated by palmitate induction. The epithelial-to-mesenchymal transition (EMT) of mature tubular epithelial cells in kidney is considered to contribute to the renal accumulation of matrix proteins associated with diabetic nephropathy. Studies suggest 20 μM of curcumin protects renal tubular epithelial cells from high glucose-induced EMT through Nrf2-mediated upregulation of HO-1 (129). Alinejad et al. demonstrated a combination of safranal, thymoquinone and 50 μg/mL of curcumin can block glucose/serum deprivation (GSD)-induced cell death and has the potential to be used for management of cerebral ischemia and neurodegenerative diseases (130). Theracurcumin is a highly bioavailable curcumin analog. It has been found that 10 μM of both theracurcumin and curcumin may have potential protective effects against sodium nitroprusside-induced cytotoxicity by free radical-scavenging and iron-chelating activities (131). Curcumin modulates peroxisome proliferator-activated receptor- γ signaling, which is a key molecule in the etiology of bronchopulmonary dysplasia (BSD). *In vivo* studies showed curcumin, when given daily at 5 mg/kg bw intraperitoneally, effectively protected against short-term and long-term hyperoxia-induced lung injury. Curcumin prevented hyperoxia-induced increases in cleaved caspase-3 and the phosphorylation of Erk1/2. Molecular effects of curcumin, both structural and cytoprotective, suggest that its actions against hyperoxia-induced lung injury are mediated via Erk1/2 activation and that it is a potential intervention against bronchopulmonary dysplasia (BPD) (132).

Curcumin has been found to alter the differentiation of many different cells. *In vitro* studies have shown that 0.5 μM curcumin increases the differentiation rate of

neurons in neural stem cells via Wnt signaling pathway (133). It's also reported curcumin can enhance EB directed differentiation of H-9 human embryonic stem cells (hESCs). 10 μM of curcumin significantly increased gene expression of cardiac specific transcription factor NKx2.5, cardiac troponin I, myosin heavy chain, and endothelial nitric oxide synthase during ES cell differentiation through modulation of the nitric oxide-cyclic GMP pathway (134). Myeloid-derived suppressor cells (MDSC) accumulate in the spleen and contribute to tumor growth, angiogenesis, and progression. Curcumin treatment inhibited cell proliferation and colony formation of cancer cells and decreased the secretion of murine IL-6 by MDSCs in a co-culture system. In addition, polarized MDSCs toward a M1-like phenotype with an increased expression of CCR7 and decreased expression of dectin 1 (135). Also, 20 μM curcumin inhibited differentiation of adipocytes and cardiac fibroblasts. Adipocyte differentiation is a key process in determining the number of mature adipocytes in the development of obesity. Curcumin has been reported to have an anti-adipogenic function both in 3T3-L1 murine cells and in human primary preadipocytes (136). The differentiation of cardiac fibroblasts (CFs) into myofibroblasts and the subsequent deposition of the extracellular matrix is associated with myocardial fibrosis following various types of myocardial injury. Treatment with 20 μM curcumin effectively suppressed TGF-β1-induced CF differentiation via Smad-2 and p38 signaling pathways. These findings suggest curcumin may be a potential therapeutic agent for the treatment of cardiac fibrosis (137, 138).

Studies in our laboratory suggest that curcumin increases activity of activator protein (AP-1)-luciferase in a concentration-dependent manner at 1-25 μM in HT-29 cells transfected with an AP-1- luciferase reporter gene. The protein expression of endogenous

cyclin D1, a gene that is in downstream of AP-1, increased with 10 μM curcumin treatment (139). Additionally, we found 10 and 50 μM curcumin inhibited LPS-induced NF- κ B-luciferase activity in HT-29 cells stably transfected with a NF- κ B-luciferase construct (127). We found 2.5 and 5 μM curcumin inhibited colony formation of HT-29 cells, whereas, inhibition of colony formation failed in stable knockdown of deleted in lung and esophageal cancer 1 (DLEC1) cells. Furthermore, we observed 5 μM curcumin up-regulated the mRNA expression of DLEC1 and decreased CpG methylation of the DLEC1 promoter in HT-29 cells. We further discovered 5 μM curcumin down-regulated protein expression of DNA methyltransferases and subtypes of histone deacetylases, such as HDAC4, 5, 6 and 8 (140).

Furthermore, treatment with 50 μM curcumin induced apoptosis in colon, leukemia, breast, hepatocellular and ovarian carcinoma cell lines. However, curcumin failed to display cytotoxicity in cell lines established from lung, kidney, cervix, prostate and CNS malignancies. The mechanism of curcumin-mediated apoptosis was determined to be related to the generation of ROS. The addition of N-acetyl cysteine (109), a ROS scavenger, during curcumin treatment resulted in the disappearance of apoptosis. Additionally, curcumin's failure to exhibit cell death in some cell lines is due to the overexpression of Hsp70 in the cells which protect cells from apoptosis (141). Several studies investigated the relationship of ROS level-effect and apoptosis-induction of curcumin. Different dosage effects of curcumin on cell death types in a human osteoblast cell line were explored. Curcumin at concentrations lower than 25 μM caused apoptosis in human osteoblasts HFOb 1.19 cells, through the activation of JNK and cleavage of caspase-3, PARP and PAK2. However, 50-200 μM curcumin induced necrotic cell death

instead of apoptosis in human osteoblasts. In addition, 12.5-25 μM curcumin directly increased oxidative stress demonstrated by the use of the cell permeable dye 2', 7'-dichlorofluorescin diacetate (DCF-DA), an indicator for intracellular ROS, nevertheless, 50-200 μM curcumin had much less activity. Moreover, NAC or α-tocopherol (ROS scavengers) pre-treatment significantly decreased intracellular ROS levels and 12.5-25 μM curcumin-induced apoptosis to necrosis. Pre-treatment with antimycin or 2-deoxyglucose reverted apoptosis induced by 12.5-25 μM curcumin to necrosis which could induce ATP (a mediator of apoptosis versus necrotic cell death) depletion (142). Although curcumin caused cell death of HL-60 cells in a concentration- and time-dependent manner, its effects on ROS production differed with fluctuations in concentration. Curcumin at less than 25 μM decreased ROS production, while 50-100 μM enhanced ROS generation. Furthermore, the addition of antioxidant agents, ascorbic acid (ASA), NAC and glutathione (GSH), promoted the antioxidant and anti-cancer activities of curcumin at low concentrations (143). These studies were consistent with reports curcumin at low concentrations (<10M) prevents GSH depletion and higher concentrations decrease GSH levels (144). Proteasome inhibitors have been reported to cause apoptosis in cancer cells (18). Curcumin has been shown to demonstrate biphasic dose-response proteasome activity in human keratinocytes, specifically, 0.3 μM and 1.0 μM curcumin increased proteasome activity by 34% and 46%, respectively. However, curcumin at higher concentrations of 3 and 10 μM decreased proteasome activity by 32% and 46%, respectively (145). It was suggested the biphasic dose-response is through a homeostasis mechanism, in which a low dose of agents stimulates signaling pathways to protect the organism, whereas a high dose displays an inhibitory effect (146) (147). A

similar phenomenon has been observed for many natural compounds, such as resveratrol (148) (22), berberine (149), clove and cinnamon essential oils (150).

In a study where dose-dependent differences on DNA-damage and the p53 response of quercetin and curcumin, whose chemical structures are similar, in HT1080 cells (a human cell line with wild-type p53), 8 μM curcumin significantly increased the expression of phosphorylated H2AX, a biomarker of DNA damage, while as much as 20 μM, quercetin could displayed similar activities. Curcumin (4 and 7 μM, respectively) increased the protein expression of p53 and p-p53 (ser15) at lower concentrations than quercetin (30 and 20 μM, respectively). It was suggested even with similar chemical structures, the two natural compounds displayed different effects on DNA-damage response patterns in terms of dose and cell fate (151)

Curcumin is recognized as an epigenetic modulator and plays a major role in the prevention of disease. Studies in our laboratory demonstrated that 10 μM curcumin prevents prostate cancer progression via CpG demethylation in the promoter region of *Nrf2* in TRAMP-C1 cells (152). In HT-29 cells, curcumin inhibited anchorage-independent growth by decreasing CpG methylation of the promoter region of the tumor suppressor gene (153). After treatment with curcumin (2.5 and 5.0 μM) for 5 days, protein levels of DNMT1, DNMT3b, HDAC4, HDAC5, HDAC6, and HDAC8 decreased (140). In human prostate LNCaP cells, curcumin treatment decreased the methylation of CpG islands of *Neurog1* as well as the binding ability of methyl-CpG binding protein 2 (MeCP2). The expression of HDAC1, 4, 5, and 8 increased whereas the expression of HDAC3 and the total HDAC activity decreased upon 2.5 μM-curcumin treatment. ChIP analysis showed curcumin decreased the enrichment of H3K27Me3 in the *Neurog1*

promoter region (154). In breast cancer cell lines MCF7 and MDA MB 231, *DNMT* (i.e., *DNMT1*, *DNMT3a*, and *DNMT3b*) transcript levels and the protein levels of DNMT1, HDAC1, and MeCP2 decreased after treatment with 10 μM curcumin (155). In addition to curcumin, the curcumin analogue FN1 and liposomal-formulated curcumin (lipocurc) have been reported to have a protective effect on the development of disease (156, 157). Our previous work demonstrates FN1 is more potent than curcumin in activating the Nrf2-ARE pathway and inducing expression of Nrf2 and its downstream detoxifying enzymes. Not surprisingly, FN1 inhibited colony formation of prostate TRAMP C1 cells by decreasing the expression of Keap1 and CpG hypomethylation of the Nrf2 promoter (156). In a Park 7 (DJ-1)-knockout rat model of Parkinson's disease, lipocurc was found to improve the motor impairment and prevent neuronal apoptosis by targeting HDACs (157). Approximately 20-40% miRNAs are located close to CpGs and are suppressed by epigenetic mechanisms (158). Epigenetic compounds can induce upregulation of some miRNAs through reducing the percentage of CpG methylation of the promoters of miRNAs. An example is miR-203, a miRNA downregulated in bladder cancer. Restoration of miR-203 expression reduced cell viability, invasiveness and migration, and increased the number of cells in the G0–G1 phase of the cell cycle through Akt2 and Src signaling. Curcumin treatment (10 μmol/L) induced demethylation of miR-203 promoter and subsequent augmentation of miR-203 expression (159).

1.3 Pathways targeted in cancer chemoprevention

As discussed, triterpenoids, ITCs and curcumin exude their chemopreventive properties through a variety of signaling pathways (Figure 3)c. These pathways are explored in greater details in this section.

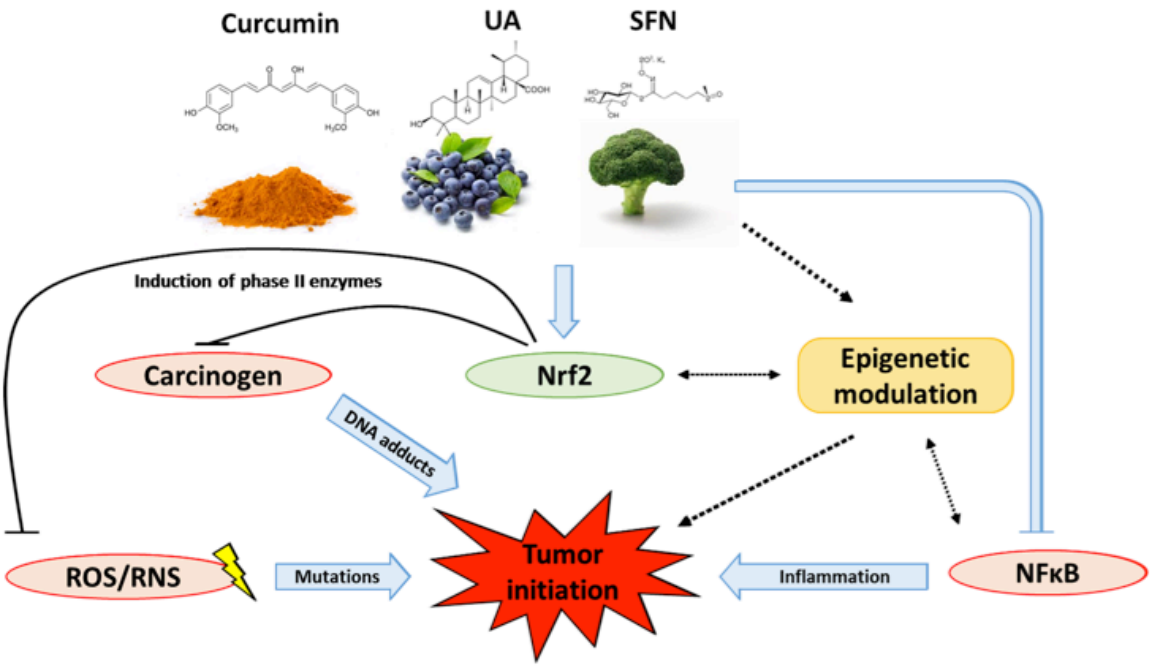


Figure 3. Schematic diagram on mechanisms of phytochemicals (curcumin, UA, SFN) inhibiting tumor initiation. Curcumin, UA, and SFN have the ability to epigenetically modulate Nrf2, which results in the inhibition of initiation and pro-inflammatory processes leading to the prevention of tumor initiation.

1.3.1 Nrf2-mediated ARE signaling

The anti-oxidant stress defense system is responsible for the direct inactivation or conjugation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS/RNS into less deleterious molecules. In response to reactive species the cell has implemented an antioxidant defense system encompassing both enzymatic and nonenzymatic mechanisms (160). The enzymatic system includes superoxide dismutases (SODs), catalase, and glutathione peroxidases (GPxs). These enzymes directly inactivate ROS/RNS. In addition to the direct inactivation of ROS/RNS, there are other antioxidant enzymes that facilitate the detoxification of ROS/RNS using reduction/conjugation

reactions and the recycling of thiols. The enzymatic soluble products of these reactions are easily excreted. These enzymes include phase II enzymes (e.g. NAD(P)H: quinone oxidoreductase, NQO-1; glutathione S-transferases, GST; UDP-glucuronosyl transferases, UGT; among others). These enzymes play an important role in this protective machinery as detoxifying enzymes that conjugate endogenous polar molecules to phase I metabolites, thereby facilitating xenobiotics (including carcinogens) elimination and excretion (127). Activation of these cytoprotective enzymes is important for maintaining cellular homeostasis towards environmental challenges. Activation of the genes encoding these enzymes and proteins are regulated in large part by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the NF-E2 family of nuclear basic leucine zipper (bZIP) transcription factors. Nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the NF-E2 family of nuclear basic leucine zipper (bZIP) transcription factors, is a master regulator controls the expression of phase II/antioxidant enzymes. Under normal conditions, Nrf2 is bound to Kelch-like erythroid cell-derived protein with CNC homology (82)-associated protein 1 (Keap1). Keap1, an adaptor protein for a Cullin 3 (Cul3)-based ubiquitin E3 ligase, sequesters Nrf2 in the cytosol and ensures its degradation by the proteasome. Upon oxidative stress, Nrf2 is released by Keap1 and translocates to the nucleus where it heterodimerizes with Maf and binds the ARE/EpRE of the antioxidant defense system genes. Unsurprisingly, Nrf2 has been shown to play an essential role in the protection of carcinogenic events and Nrf2 KO mice are susceptible to the initiation, promotion, and progression of cancer (161). As the master regulator of the antioxidant response, unsurprisingly, the induction of Nrf2 has become an attractive target in chemoprevention.

1.3.2 Anti-inflammation

Oxidative stress occurs when there is an imbalance between the anti-oxidant defense system and the production of ROS/RNS (162, 163). The general terms ROS and RNS are given to the reactive species generated from the interaction of free radicals such as superoxide anion, hydroxyl radical, and nitric oxide with metals, oxidants, and reductants found in cells. ROS/RNS are important cellular messengers involved in a number of physiological processes: cellular respiration, immune response, ion transport, apoptosis, neuromodulation, and transcription (164). While important secondary messengers, the activities of ROS/RNS can exhibit a double-edged sword. In addition to endogenous production, exogenous production of ROS/RNS can be initiated through UV radiation, environmental pollutants, lipid peroxidation, and inflammatory cytokines (165). The anti-oxidant stress defense system is responsible for the direct inactivation or conjugation of ROS/RNS into less deleterious molecules. An excess of ROS/RNS can cause an imbalance in the system and induce oxidative stress; a hallmark of a number of neurodegenerative diseases and, of most importance here, cancer (166, 167). The excess oxidative stress results in the induction of the NFK-B signaling cascade and thus, activation of cytokines and the production of acute inflammation. Nuclear factor kappa B (NFκB) is a transcription factor lays on the molecular node linking inflammation, cell survival, and cancer progression signals (168). NFκB is normally sequestrated by its cellular suppressor IκB in the cytosol. Upon activation, IκK phosphorylates IκB consequently leads to the degradation of IκB, accompanied with release and nuclear translocation of NFκB. A considerable number of NF-κB target pro-inflammatory genes have been shown to be involved in cancer development, including various cytokines,

chemokines, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) among others. The inability of the cells to eliminate the exogenous culprits results in chronic inflammation. The immune cells involved in the inflammation cascade continuously use ROS/RNS as a means to recruit more immune cells. The excess production can lead to the mutagenesis of oncogenes and tumor suppressing genes and, ultimately, the initiation of cancer.

1.3.3 Epigenetic modulation

The term epigenetics is defined as heritable changes in gene expression without changes in the integrity of the DNA sequence (169). Recently, numerous evidences have shown that initiation and progression of carcinogenesis involves aberrant epigenetic alterations. Unlike genetic mutations, changes on the epigenetic level are considered reversible. Given that epigenetics lies on the molecular interface between genetics and environmental factors, there is a growing interest in evaluating the potential of dietary phytochemicals that blocks or reverses the epigenetic abnormity in cancer development. Epigenetic alterations encompass DNA methylation, histone modifications, and microRNA (mRNA) expression changes. Epigenetic alterations such as DNA methylation and histone modifications have been shown to contribute to the development and progression of cancer (170). DNA methylation is the mostly characterized epigenetic event in many cancers (139), which occurs at the 5' position of the cytosine residues within CG dinucleotides through addition of a methyl group by DNA methyltransferases (DNMTs). CpG dinucleotides tend to be grouped in regions known as CpG islands in the promoters of genes. In normal cells, the majority of CpG islands remain unmethylated leaving an open structure for the transcriptional machinery to bind and induce expression.

In cancer cells, certain areas of the promoter region of tumor suppressor genes are hypermethylated leading to the silencing of the tumor suppressor genes (171, 172). In addition, DNA methylation can also serve as a binding site for proteins such as methyl CpG binding domain proteins (MBDs) and methyl CpG binding protein 2 (MeCP2). These proteins can interact with a co-repressor complex to repress transcription. The co-repressor complex includes proteins such as histone deacetylases (HDACs) involved in the modification of histones. Histone modification is tightly associated with DNA methylation. Histone modifications play an important role in chromatin structure. Chromatin is a densely packed macromolecular complex composed of histones, DNA, and non-histone proteins. Chromatin serves to package a large amount of material into the nucleus of a cell and to influence DNA replication. Histones play an essential role in chromatin structure and post-translational modifications of histones regulate gene expression. Some of these modifications include acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (160). These modifications typically occur at serine, lysine, and arginine resides of N-terminal histone tails. The enzymes responsible for these modifications include histone acetyltransferases (41), histone methyltransferases (HMTs), histone demethylases (HMTs), and HDACs. The influence of these modifications on chromatin structure can either activate or suppress transcription.

The interplay of DNA methylation with histone modifications, transcription factors, transcriptional coactivators, and DNA binding proteins determines the status of gene transcription (127). Epigenetic modifications such as DNA methylation and histone modifications have been shown to be a hallmark of cancers (173-177). The promoter

region of human GSTP1 is hypermethylated in approximately 7-100% of prostate cancer specimens (178-180). Aberrant epigenetic modifications have also been associated with the development and progression of skin cancer (49, 181-183). Thus, targeting the reversal of DNA methylation and histone modifications presents a novel strategy for the prevention and treatment of cancer. The FDA has already approved chemotherapeutics targeting DNMTs and HDACs (184). However, their usage has been limited by adverse events. Targeting epigenetic modifications for the prevention or treatment of cancers using dietary phytochemicals has become increasingly more attractive. Dietary phytochemicals may prevent cancer through epigenetic modifications (185-187).

1.3.4 Cancer Stem Cells and Apoptosis

Stem cells are characterized by their ability to differentiate into a heterogenous population of specialized cells, their ability to self-renew, and their ability to balance self-renewal and differentiation based on environmental needs (188). Two of the pathways demonstrated to be involved in stem cell regulation and differentiation include Sonic hedge hog and Notch signaling pathways (189). Similarly, cancer stem cells (CSCs) are able to self-renew and differentiate using common pathways (186). However, CSCs are able to form tumors when implanted into animals (190). For this reason, CSCs are often referred to as tumorigenic cells or tumor initiating cells and are fundamental to the initiation and relapse of many tumor types (190-192) CSCs were first identified in 1997 (193) where CD34+CD38- cells derived from leukemic patients were able to initiate cancer in immunodeficient mice. Currently, cancer stem cells have been identified in a number of cancers including breast and colon (194). Pathways implicated in cancer stem cell renewal include Wnt (195), janus kinase (Jak), bone morphogeneic protein (BMP),

and octamer-binding transcription (Oct-4) signaling pathways (194). Natural dietary compounds have been shown to regulate CSCs by increasing their sensitivity to chemotherapeutic agents, enhancing their differentiation, and inhibiting their self-renewal signaling (196, 197).

One of the most important processes involved in regulating the proliferation of cells is apoptosis. The process of apoptosis can be divided into intrinsic and extrinsic pathways. The intrinsic pathway is in large part controlled by Bcl-2 family members, while the extrinsic pathway, is mediated by tumor necrosis factor (TNF) family members. The extrinsic pathway is initiated with the respective ligand binding to death receptors such as TNF-related apoptosis-inducing ligand receptor (TRAILR) and FAS. Oligomerization of the receptors leads to the activation of caspase-8 and caspase-10, which cleave caspase-3 and caspase-7 and ultimately leads to apoptosis (198). The intrinsic pathway is activated when stress stimuli induces BCL-2 homology domain 3 (BH3)-only protein activation which leads to BAX and BAK activity and consequently mitochondrial outer membrane permeabilization (MOMP). This results in the release of cytochrome c which interacts with apoptotic protease activating factor 1 (APAF1), which activates caspase-9. Caspase-9 then activates caspase-3 and caspase-7, which leads to apoptosis (198). Cancer cells have developed mechanisms by which apoptosis is evaded through the mutation of essential genes involved in regulation of the process. A number of phytochemicals have been shown to induce apoptosis in cancer cells/in vivo models (Table 1).

Table 1. The diverse anti-cancer properties of phytochemicals are driven by dose and model system.

Cancer/ Model Type	Cell Line/Animal Model	Concentration/ Dose	Phytochemical	Reference	Process/es Affected
Bladder	T24 cells	5-20μM	SFN	Shan, Sun (98)	A[1]
Breast	SUM159 xeonograph mouse model	50mg/kg	SFN	Li, Fu (96), (127)	CSCs[2]
Breast	PyMT transgenic mice	8mmol/kg	PEITC	Singh and Singh (83)	CSCs[2]
Breast	MDA-MB-231	30μM	SFN	Kanematsu, Uehara (104)	A[1]
Colon	COLO 25	10μM, 50μM	UA	Chun, Kundu (199)	AI[3]
Colon	DSS-induced acute murine colitis model	10mg/kg, 20mg/kg	UA	Chun, Lee (20)	AI[3]
Colon	ApcMin/+ mouse model	3-13nmol/g	SFN	Hu, Khor (40)	A[1], AI[3]
Colon	(DSS)-induced acute colitis mouse model	25mg/kg/day	SFN	Wagner, Will (61)	NMAS[4]
Colorectal	Caco-2 cells	5μM, 10μM	UA	Ramos, Pereira-Wilson (21)	NMAS[4]
Colorectal	HT29 cells	25μM, 50μM	PEITC, SFN	Jeong, Kim (56)	A[1], AI[3]
Colorectal	Caco-2 cells	25μM, 75μM	SFN	Wang, Chen (102)	NMAS[4], A[1] (respectively)
Colorectal	CRC cells	12.5μM, 25μM	SFN	Chen, Tang (103)	A[1]
Gastro-intestinal related	Rat	40 μmol/kg/day	SFN	Munday and Munday (45)	NMAS[4]
Glioma	A375 cells	10μM	UA, OA	Bonaccorsi, Altieri (25)	CSCs[2]
Human study	PBMC	105mg	SFN	Myzak, Tong (69)	EM[5]
Laryngeal carcinoma	Hep-2	10μM	PEITC	Dai, Wang (93)	A[1]
Leukemia	HL60, U-937, THP-1 cells	10μM, 20μM, 30μM	UA	Zhang, He (26)	CSCs[2]
Leukemia	Promyelocytic leukemic cells	0.2-100μM	SFN	Fimognari, Lenzi (80)	CSCs[2]

Liver	Male Wistar rat model	20mg/kg	UA, OA	Gayathri, Priya (17)	AI[3], NMAS[4]
Liver	HepG2 cells and Hepatocytes	1-10µM	SFN	Bacon, Williamson (46)	NMAS[4]
Liver	HepG2 cells	2µM-20µM	SFN	Hong, Freeman (51)	NMAS[4]
LPS-stimulated Inflammation	RAW 264.7 cells	25µM, 50µM	SFN	Heiss and Gerhauser (57)	AI[3]
Melanoma	U87 cells	15µM	UA, OA	Bonaccorsi, Altieri (25)	CSCs[2]
Neuronal related	PC12 cells	20µM, 40µM	UA, OA	Tsai and Yin (22)	CSCs[2], NMAS[4]
Non-small cell lung	A549 Nude Mouse Model	50mg/kg, 100mg/kg	UA, OA	Cho, Rho (12)	A[1]
Non-small cell lung	H1299 and A549 cells	30µM	UA	Wu, Zhao (23)	EM[5]
Non-small cell lung	L9981 cells	12.5µM, 20µM	PEITC	Yan, Zhu (94)	A[1]
Non-small cell lung	L9981 cells	5µM, 9.7µM	PEITC	Wu, Zhu (95)	AI[3]
Non-small cell lung	A549 cells	30µM	SFN	Zuryn, Litwiniec (101)	A[1]
Ovarian	SKOV3 xenograft athymic BALB/c-nu mouse model	12.5-50µg/mL	UA	Zhang, Wang (27)	CSCs[2]
Pancreatic	AsPC-1, MIA, PaCa-2, Panc-28 cells	5-20µM	UA	Prasad, Yadav (16)	A[1], AI[3]
Pancreatic	Orthotopic Pancreatic Mouse Model	250mg/kg	UA	Prasad, Yadav (16)	A[1], AI[3]
Prostate	PC3 cells	80µM	UA	Zhang, Kong (200)	A[1], AI[3]
Prostate	PC3 cells, LNCaP cells	55µM, 45µM	OA	Kassi, Papoutsi (15)	A[1]
Prostate	PC3 cells	20µM & 30µM, 5µM & 7.5µM	SFN, PEITC	Xu, Shen (55)	AI[3]
Prostate	LNCaP and PC3 cells	15µM	SFN	Wong, Hsu (63)	EM[5]
Prostate	TRAMPC1	1µM, 2.5µM	SFN	Zhang, Su	EM[5]

	cells			(66)	
Prostate	LNCaP	0.5-1µM	PEITC	Wang, Beklemishe va (201)	A[1], EM[5]
Prostate	LNCaP, PC3	2.5µM	PEITC	Zhang, Shu (74)	EM[5]
Prostate	DU145 cells, PC3 cells	10µM, 20µM, 40µM	SFN	Singh, Srivastava (97)	A[1]
Prostate	BPH-1, LNCaP, PC3	15µM	SFN	Myzak, Hardin (70)	A[1], EM[5]
Skin	ICR Mouse Model	2µmol topical application	UA	Cho, Rho (12)	AI[3]
Skin	Ca3/7 cells	5µM, 10µM	UA, OA	Kowalczyk, Walaszek (18)	NMAS[4], AI[3]
Skin	JB6 P+ mouse epidermal cells	5µM	UA	Kim, Ramirez (202)	EM[5]
Skin	Nrf2 (+/+) and Nrf2 (-/-) mice	100nmol topical application	SFN	Saw, Huang (62)	NMAS[4], AI[3]
Skin	JB6 P+ mouse epidermal cells	2.5µM, 5µM	SFN	Su, Zhang (203)	EM[5]
T-cell lymphoma	Hut-78 cells	10-80µM	UA	Yang, Shi (11)	A[1]
Thyroid	ARO cells	20µM	UA, OA	Bonaccorsi, Altieri (25)	CSCs[2]
Thyroid	MTC-SK cells	10µM, 20µM	UA	Aguiriano-Moser, Svejda (13)	A[1]
Immune	Activated T cells, B cells, and macrophages	5µM	UA	Checker, Sandur (19)	AI[3]
Anti-oxidative Stress	Nrf2 (+/+) and Nrf2 (-/-) mice	40mg/kg	PEITC	Hu, Xu (48)	NMAS[4]
Neuronal	Astrocytes	5-15µM, 50-100µM	Curcumin	Scapagnini, Colombrita (118)	NMAS[4], A[1] (respectivel y)
Differentia tion	Neuronal stem cells	0.5µM	Curcumin	Chen, Wang (133)	CSCs[2]
Differentia tion	Embryonic stem cells	10µM	Curcumin	Mujoo, Nikonoff (134)	CSCs[2]
Bone	HFOb 1.9	25µM	Curcumin	Chan, Wu	A[1]

	cells			(142)	
Leukemia	HL60 cells	25µM	Curcumin	Chen, Wanming (143)	AI[3], NMAS[4]
Fibrosarcoma	HT1080 cells	8µM	Curcumin	Sun, Ross (151))	A[1]
Colon	HT29 cells	2.5µM, 5µM	Curcumin	Guo, Shu (140)	EM[5]
Prostate	LNCaP cells	2.5µM	Curcumin	Shu, Khor (154))	EM[5]
Prostate	TRAMPC1 cells	10µM	Curcumin	Khor, Huang (152)	EM[5]
Breast	MCF7 and MDA MB 231 cells	10µM	Curcumin	Mirza, Sharma (155)	EM[5]

[1]Apoptosis

[2] Cancer Stem Cells

[3] Anti-inflammation

[4] Nrf2-mediated ARE Signaling

[5] Epigenetic Modulation

1.4 Perspective

Cancer is one of the leading causes of death in the United States and around the world. Modern diagnostics and treatment regimens have improved patient care, but advanced metastasized cancers remain a challenge to treat. Hence alternative strategies have to be integrated into regimens to reduce the burden of cancer using relatively non-toxic phytochemicals and or pharmaceutical agents such as non-steroidal anti-inflammatory drugs (NSAIDs), selective estrogen receptor modulators (SERMs), aromatase inhibitors, HMG-CoA reductase inhibitors (statins), among others.

The idea of cancer prevention by dietary and nutritional phytochemicals can be further refined to “NutriPrevention” versus “Chemoprevention”. In 2013, the USDA

suggested “Myplate” replacing the previous “Food Pyramid”, whereby half of the plate/meal includes fruits and vegetables for healthy living. This could be defined as “NutriPrevention”, where low level phytochemicals would presumably effect and impact the epigenome of “healthy” cellular defense genetic pathways including the Nrf2-regulated anti-oxidative stress/antioxidant pathways and anti-inflammatory pathways discussed above. However, if one were to be exposed to high environmental risk factors such as smoking, “bad/unhealthy diets”, alcohol, environmental pollutants, occupational carcinogens, and/or other environmental factors/insults coupled with inherent genetics/epigenetics “stem cells” that could drive “initiated cells”, then it would logically require higher pharmacological doses of certain dietary phytochemicals and/or non-toxic pharmaceutical agents and this may be classified as “PharmacoPrevention”. During cancer remission, in order to prevent cancer from recurring, or high risk individuals with chronic inflammation diseases such as Inflammatory bowel disease (IBD), then one would use “ChemoPrevention” with relatively higher but non-toxic doses phytochemicals/botanicals alone and/or in combination with relatively nontoxic drugs such as NSAIDs, SERMs, HMG-CoA reductase inhibitors, among others. Analogously, these concepts could be applicable to other chronic diseases which are utilizing similar signaling pathways such as oxidative stress and inflammation.

1.5 Conclusions

In general, for many phytochemicals, it would appear much higher concentrations are required to elicit biological effects in *in vitro* cell culture models as compared to *in vivo* animal models. This phenomenon could be due to a variety of reasons. Most cell

lines are tumor cell lines, thus, behave quite differently to their *in vivo* counterparts. They may possess efflux transporters that can exclude compounds from entering the cells, have very different cellular signaling response pathways as compared to normal cells, lack of active metabolism processes forming potential active metabolites and lack of endocrine-paracrine signaling as compared to *in vivo*. However, for many epigenetic effects, it appears that lower concentrations of phytochemicals are able to elicit an epigenetic response such as it relates to CpG methylation, DNMTs or HDACs in cell culture models. Further *in vitro-in vivo* animal and human studies would be warranted to ascertain these observations.

Chapter II

Triterpenoid ursolic acid enhances Nrf2 expression in mouse epidermal cells through epigenetic modifications[3]

[3]A portion of this chapter has been published in *The Journal of Nutritional Biochemistry*: Kim H and **Ramirez C** *et al* (2016). Triterpenoid ursolic acid enhances Nrf2 expression in mouse epidermal cells through epigenetic modifications. J Nutr Biochem. 2016 Jul;33:54-62.

2.1 Introduction

UA is a lipophilic pentacyclic triterpenoid derived from apple peels, basil (*Ocimum basilicum*), blueberry (*Vaccinium* spp.), cranberry (*Vaccinium macrocarpon*), heather flower (*Calluna vulgaris*), labrador tea (*Ledum groenlandicum* Retzius), olive (*Olea europaea*), pear (*Pyrus pyrifolia*), and rosemary (*Rosmarinus officinalis*) (199, 200). UA exerts various biological effects, including anti-inflammatory, anti-atherosclerosis, anti-diabetic, anti-viral, and anti-cancer activities. Additionally, UA has the ability to decrease reactive oxygen species (ROS) toxicity and increase the activity of antioxidant enzymes (199). *In vivo* and *in vitro* studies have shown that UA inhibits benzo[a]pyrene (B[a]P)- and 7,12-dimethylbenz[a]-anthracene (DMBA)-induced tumor initiating activity, suppresses TPA-induced skin inflammation and tumor promotion in CD-1 and ICR mice, and induces apoptosis in M4Beu human melanoma cells (201-203). Additionally, UA hinders UVA-induced ROS production, lipid peroxidation, MMP-2 expression, and DNA damage in human keratinocyte HaCaT cells (204). Recently, studies have revealed that UA protects the brain against cerebral ischemia and protects the liver against CCl_4-induced damage in mice via the nuclear factor E2-related factor 2 (Nrf2) pathway (205, 206). Notably, we have previously shown that dietary phytochemicals, such as apigenin, curcumin, 3,3′-diindolylmethane, γ-tocopherol-rich mixture of tocopherols, sulforaphane, tanshinone IIA, Z-ligustilide and radix angelica, regulate Nrf2 activation via epigenetic modifications (66, 67, 152, 207-211); however, the effect of UA on the epigenetic regulation of Nrf2 has not been previously examined.

Skin cancer is one of the most prevalent malignant tumors, contributing to the increasing mortality rate of cancer in the US (212). An imbalance between the production

and removal of ROS in the epidermis and dermis may lead to skin tumorigenesis. Exposure to ultraviolet (UV) radiation, ozone layer depletion, excessive time spent outdoors, indoor tanning, and noxious environmental insults induce ROS overproduction (213). Cells contain a self-defense mechanism that removes ROS through the synthesis of detoxifying/antioxidant enzymes, which include HO-1, NQO1, uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT), and GST (214). Unfortunately, these antioxidant defenses have limited capacity and can be impaired during certain conditions, thereby leading to a redox imbalance that promotes the development of skin cancer.

The genes encoding cytoprotective detoxifying/antioxidant enzymes are controlled by the transcription factor Nrf2. Under homeostatic conditions, Nrf2 is bound to Keap1 in the cytoplasm. Nrf2 is targeted for polyubiquitination and proteasomal degradation through the formation of a Keap1- and Cullin 3-based-E3/Rbx1 ligase complex. Under stress conditions, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it binds to the AREs of target protective genes and activates transcription (215). Nrf2 has long been recognized as a pivotal player in the prevention of many diseases, including skin cancer. Nrf2 knockout mice are more susceptible to airway inflammation and asthma, striatal toxicity and behavioral dysfunction, colorectal carcinogenesis, gastric neoplasia, and skin carcinoma upon DMBA/TPA exposure compared with wild-type mice (52-54, 216, 217). Moreover, a recent study demonstrated that low Nrf2 expression is associated with the oncogenic transformation of mesenchymal stem cells and poor survival in patients with skin cutaneous melanoma, kidney clear cell carcinoma, and prostate cancers (218). Therefore, understanding the

molecular mechanisms by which Nrf2 expression can be altered to slow or prevent the progression of skin cancer is of great importance.

Frequent epigenetic changes during the early stages of tumorigenesis lead to genetic aberrations and promote cancer development (219). Epigenetics refers to changes in gene expression by DNA methylation and/or post-translational histone modification without alterations of the DNA sequence. The modifications to DNA and histones are driven by DNMTs and HDACs, respectively (220). DNA methylation occurs at the 5' position of cytosines within CpG dinucleotides found in CpG islands. The silencing of tumor suppressor genes by the hypermethylation of CpG islands within promoter regions is a hallmark of cancer. Such methylation in CpG islands impedes the binding of transcription factors and represses transcription. Moreover, protein complexes, such as the MBD family and HDACs, are recruited to specific loci where they alter the structure of the chromatin and facilitate gene silencing (220-222). As such, epigenetic modifications as preventive targets have been the focus of numerous studies in cancer, largely due to the notion that epigenetic changes are reversible and affect numerous cellular events in tumorigenesis. The FDA has approved four epigenetic agents for clinical use: the DNMT inhibitors 5-azacytidine (5-aza, azacytidine) and 5-aza-2′-deoxycytidine (decitabine) and the HDAC inhibitors suberoylanilide hydroxamic acid (vorinostat) and depsipeptide (romidepsin) (184). However, off-target action, drug resistance and their selective applicability to selective cancers have mitigated their use in treating cancer (184, 223). As a way of circumventing this challenge, natural compounds found in fruits, vegetables, teas, and medicinal plants have attracted considerable interest due to their ability to overcome oxidative stress and regulate epigenetic events at non-

toxic concentrations (5, 224-226). The aim of this study is to demonstrate the chemopreventive effect of UA and identify UA-induced epigenetic modifications in mouse epidermal cells. We demonstrated that UA activated the Nrf2 pathway by demethylating the Nrf2 promoter and reducing the expression of DNMTs and HDACs, resulting in the inhibition of TPA-induced cell transformation.

2.2 Materials and Methods

2.2.1 Reagents and Antibodies

Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/ml), versene, and Trypsin-EDTA were supplied by Gibco (Grand Island, NY). A Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay Kit was obtained from Promega (Madison, WI). Platinum Taq DNA polymerase was purchased from Invitrogen (Grand Island, NY). Tris-HCl precast gels, turbo transfer buffer, and PVDF membranes were obtained from Bio-Rad (Hercules, CA). Tris-Glycine-SDS running buffer was from Boston BioProducts (Ashland, MA). Super Signal enhanced chemiluminescent substrate, NE-PER Nuclear and Cytoplasmic Extraction Reagents, and BCA Protein Assay Kit were purchased from Thermo (Rockford, IL). Antibodies against Nrf2 (C-20), HO-1 (C-20), NQO1 (H-90), UGT1A1 (V-19), and actin (I-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA). Anti-acetyl histone H3 was from Millipore (Billerica, MA). The protease inhibitor cocktail, radioimmunoprecipitation (RIPA) buffer, and antibodies against HDACs (HDAC1, HDAC2, HDAC3, HDAC4 and HDAC6) were supplied by Cell Signaling Technology (Beverly, MA). The anti-HDAC8 antibody was obtained from Proteintech Group (Chicago, IL), and the anti-HDAC5, -HDAC7, -DNMT3a and -DNMT3b

antibodies were from Abcam (Cambridge, MA). Anti-DNMT1 was supplied by Novus Biologicals (Littleton, CO). All other chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

2.2.2. Cell culture

JB6 P+ mouse epidermal cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in MEM supplemented with 5% FBS and penicillin-streptomycin (100 units/ml) at 37°C under a 5% CO_2 atmosphere. JB6 P+ cells stably transfected with shMock and shNrf2-knockdown (KD) were maintained in the same medium as JB6 P+ cells and 2 μg/mL puromycin was added.

2.2.3. Cell viability assay

JB6 P+ cells were seeded at a density of 5×10^4 cells/well into 96-well plates in 5% FBS/MEM. After 24 h, the medium was removed, and the cells were treated with UA (1 and 2.5 μM) in 1% FBS/MEM, where 0.1% DMSO was used as the vehicle control group. The medium containing UA was changed every 2 days for 3 and 5 days. On the day of the assay, 20 μl of Cell Titer 96 Aqueous One Solution in 100 μl of 1% FBS/MEM was added to each well, and the cells were then incubated for 1 h at 37°C in a 5% CO_2 incubator. The absorbance was measured at 490 nm.

2.2.4. Anchorage-independent cell transformation assay

JB6 P+ cells (8×10^3/ml) were suspended in 1 ml of basal medium Eagle (BME) containing 0.33% agar and plated over 3 ml of a solidified BME consisting of 0.5% agar and 10% FBS in 6-well plates in the presence of TPA (20 ng/ml) alone or together with 1 or 2.5 μM UA. The cells were maintained at 37°C in a 5% CO_2 incubator for 2 weeks.

The cell colonies were photographed using a Nikon ACT-1 microscope (Version 2.20; LEAD Technologies) and counted using Image J (NIH, Bethesda, MD).

2.2.5 RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from JB6 P+ cells on days 3 and 5 after treatment using the RNeasy mini kit (Qiagen, Valencia, CA). For cDNA synthesis, 0.5 μg of total RNA was incubated with oligo $(dT)_{16}$ primers and MultiScribe reverse transcriptase (TaqMan reverse transcription reagents, Applied Biosystems, Grand Island, NY) with the following reaction conditions: 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. The qPCR was performed with an ABI ViiA7 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using synthesized cDNA, Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), and a pair of gene-specific primers. β-actin was used as an internal control gene. Each sample was prepared in triplicate and normalized to β-actin. The primers for each qPCR reaction are as follows: Nrf2, 5′-AGCAGGACTGGAGAAGTT-3′ (sense) and 5′-TTCTTTTTCCAGCGAGGAGA-3′ (antisense); HO-1, 5′-CCTCACTGGCAGGAAATCATC-3′ (sense) and 5′-CCTCGTGGAGACGCTTTACATA-3′ (antisense); NQO1, 5′-AGCCCAGATATTGTGGCCG-3′ (sense) and 5′-CCTTTCAGAATGGCTGGCAC-3′ (antisense); UGT1A1, 5′-GAAATTGCTGAGGCTTTGGGCAGA-3′ (sense) and 5′-ATGGGAGCCAGAGTGTGTGATGAA-3′ (antisense); β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3′ (sense) and 5′-CAATAGTGATGACCTGGCCGT-3′ (antisense)

2.2.6 Western blot analysis

JB6 P+ cells were seeded at a density of 1×10^5 cells in 100-mm dishes with 5% FBS/MEM. After 24 h, the cells were treated with either 0.1% DMSO, 5-azacytidine (5-aza, 250 nM), or each concentration of UA in 1% FBS/MEM. The medium containing each agent was changed every 2 days. The cells incubated with 5-azacytidine (5-aza) serving as a positive control were treated with trichostatin A (TSA, 50 nM) 24 h before harvest. On the day of the harvest, the cells were rinsed with cold PBS and resuspended in 100 μl of RIPA buffer containing a protease inhibitor cocktail and agitated on ice for 30 min. The cells were then centrifuged at 13,000 ×g for 15 min at 4°C, and only a clear supernatant was obtained. The total protein fraction (25 μg of protein) was separated by 4-15% Tris-HCl precast gels. The separated proteins were transferred onto PVDF membranes, which were blocked with PBS containing 0.05% Tween 20 (PBST) and 5% skim milk. After a sequential incubation of the membranes with the primary antibodies and the appropriate secondary antibodies, the immunoreactive bands were detected with the Super Signal enhanced chemiluminescent system and visualized using the Bio-Rad ChemiDoc imaging system (Bio-Rad, Hercules, CA). The band intensity was analyzed using Image J. The protein concentrations were determined using the bicinchoninic acid (BCA) assay.

2.2.7 DNA isolation and bisulfite genomic sequencing

Genomic DNA was isolated from each group of treated cells using the QIAamp DNA Mini Kit (Qiagen). Then, 500 ng of DNA was subjected to bisulfite treatment using the EZ DNA Methylation Gold Kit (Zymo Research, Irvine, CA). The converted DNA was amplified by touchdown PCR using bisulfite sequencing-specific primers for the first

15 CpG sites of the murine Nrf2 gene. Then, the PCR products were gel extracted using the DNA Extraction Kit (Qiagen) and cloned into pCR4 TOPO vectors (TA cloning kit, Invitrogen). Ten clones per group were sequenced using T7 primers (GeneWiz, South Plainfield, NJ). The sequences for the PCR are as follows: sense, 5′-AGTTATGAAGTAGTAGTAAAAA-3′; anti-sense, 5′-ACCCCAAAAAAATAAATAAATC-3′.

2.2.8 HDAC activity assay

Nuclear extracts from the treated cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents. HDAC activity was measured in nuclear extracts using Epigenase HDAC Activity/Inhibition Direct Assay Kit (Epigentek Inc, Farmingdale, NY) following the manufacturer's protocol.

2.2.9. Statistical analysis

All of the quantitative results are expressed as the mean values ± SD of three independent experiments. Statistical significance was determined by one-way ANOVA and a p value of

<0.05 was considered statistically significant in all analysis.

2.3 Results

2.3.1 UA inhibits the growth of JB6 P+ cells

We first examined the dose and time-dependent cytotoxicity of UA using mouse epidermal JB6 P+ cells. The cells were treated with six different concentrations of UA (0, 2.5, 0.5, 1, 2.5, 5 or 10 μM, final concentration) dissolved in DMSO (vehicle) for 3 and 5

days. Our previous studies and others have shown that it needs at least 3 days to have cells epigenetically altered (66, 67, 152, 207-211, 227, 228). UA was not cytotoxic up to 1 μM (Figure 1); however, at 2.5 μM the cell viability decreased approximately 23% in comparison with vehicle (0.1% DMSO). No difference was observed between 3 and 5 days of treatment, and concentrations greater than 2.5 μM were found to be toxic. Because cell viability was greater than 70% at ≤2.5 μM and cytotoxicity was not time-dependent, the cells were treated with 1 and 2.5 μM UA for 3 days to study the chemopreventive efficacy of UA in the subsequent experiments.

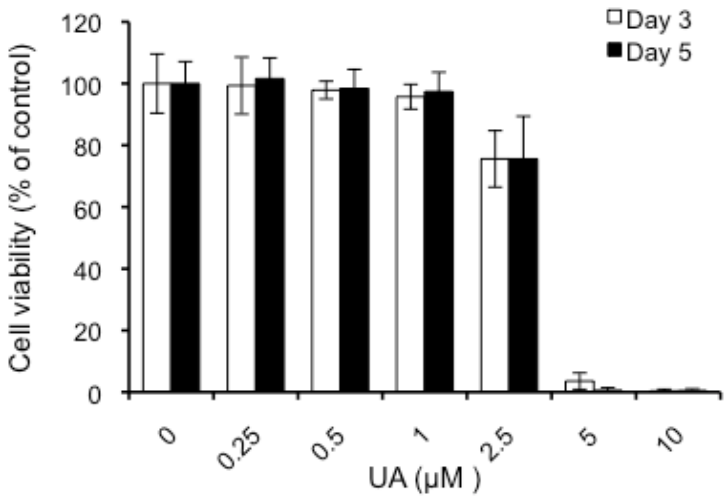


Figure 1. Effects of UA on the growth of JB6 P+ cells. Cells grown in a 96-well plate were treated with the indicated concentrations of UA, and cell viability was analyzed with an MTS assay after 3 and 5 days of treatment. The results are shown as the mean ± SD of triplicate experiments. *$p < 0.05$ compared with vehicle control (0.1% DMSO).

2.3.2 UA inhibits TPA-induced transformation of JB6 P+ cells

To determine whether UA exhibits anti-cancer and chemopreventive effects in skin, we studied the effects of UA on the tumor promotion of JB6 P+ cells induced by

TPA. The anchorage-independent cell transformation assay is an *in vitro* system that allows only cells transformed by tumor promoters such as TPA to grow and form colonies. As expected, the cells treated with TPA alone for 2 weeks developed a significant amount of colonies in the soft agar (Figure 2). The cells treated with TPA and 2.5 μM UA inhibited TPA-induced transformation by 30% compared with the cells treated with TPA alone. Incubation with 1 μM UA did not significantly inhibit transformation (16%). These results demonstrate the chemopreventive effects of UA against TPA-induced transformation in JB6 P+ cells.

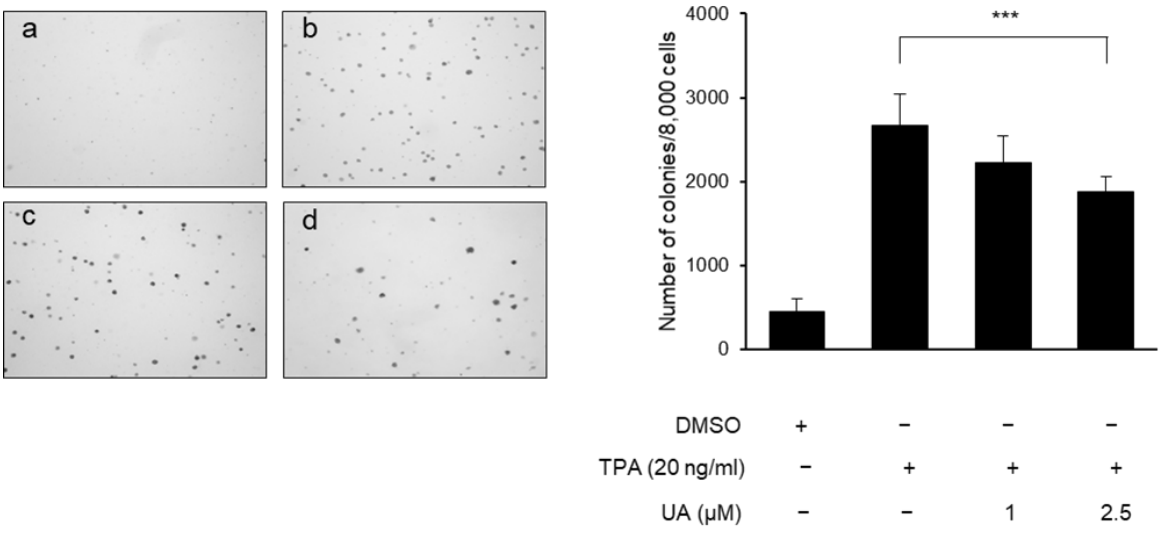


Figure 2. UA inhibits TPA-induced transformation in JB6P+ cells. Cells (8×10^3/ml) in 1 ml of BME containing 0.33% agar were maintained in the presence of DMSO (control, a), TPA alone (b), UA 1 μM plus TPA (c) and UA 2.5 μM plus TPA. After 2 weeks, the cell colonies were counted. The data are presented as the mean ± S.D. ***$p < 0.0001$ compared with TPA alone

2.3.3 UA upregulates Nrf2 and its downstream detoxifying/antioxidant target gene

TPA-induced ROS production stimulates the neoplastic transformation of JB6 P+ cells (229). To test whether UA inhibits TPA-induced transformation through the induction of detoxifying/antioxidant enzymes, we investigated the expression levels of HO-1, NQO1 and UGT1A1 at the mRNA and protein levels using qPCR and Western blotting. The cells treated with 2.5 μM UA showed an increase in HO-1, NQO1 and UGT1A1 mRNA expression, whereas 1 μM UA did not (Figure 3A). Similarly, protein expression was elevated by 2.5 μM UA treatment, but not by 1 μM UA (Figure 3B). Nrf2 is regarded as an essential regulator of cytoprotective detoxifying/antioxidant enzymes. As such, we then determined whether UA increases Nrf2 expression in JB6 P+ cells. As expected, 2.5 μM UA treatment increased Nrf2 expression; however, 1 μM UA did not result in a significant increase in Nrf2 expression. These results demonstrate that UA inhibits TPA-induced transformation of JB6 P+ cells by, at least in part, augmenting detoxifying/antioxidant enzymes, which is mediated by enhanced Nrf2 expression.

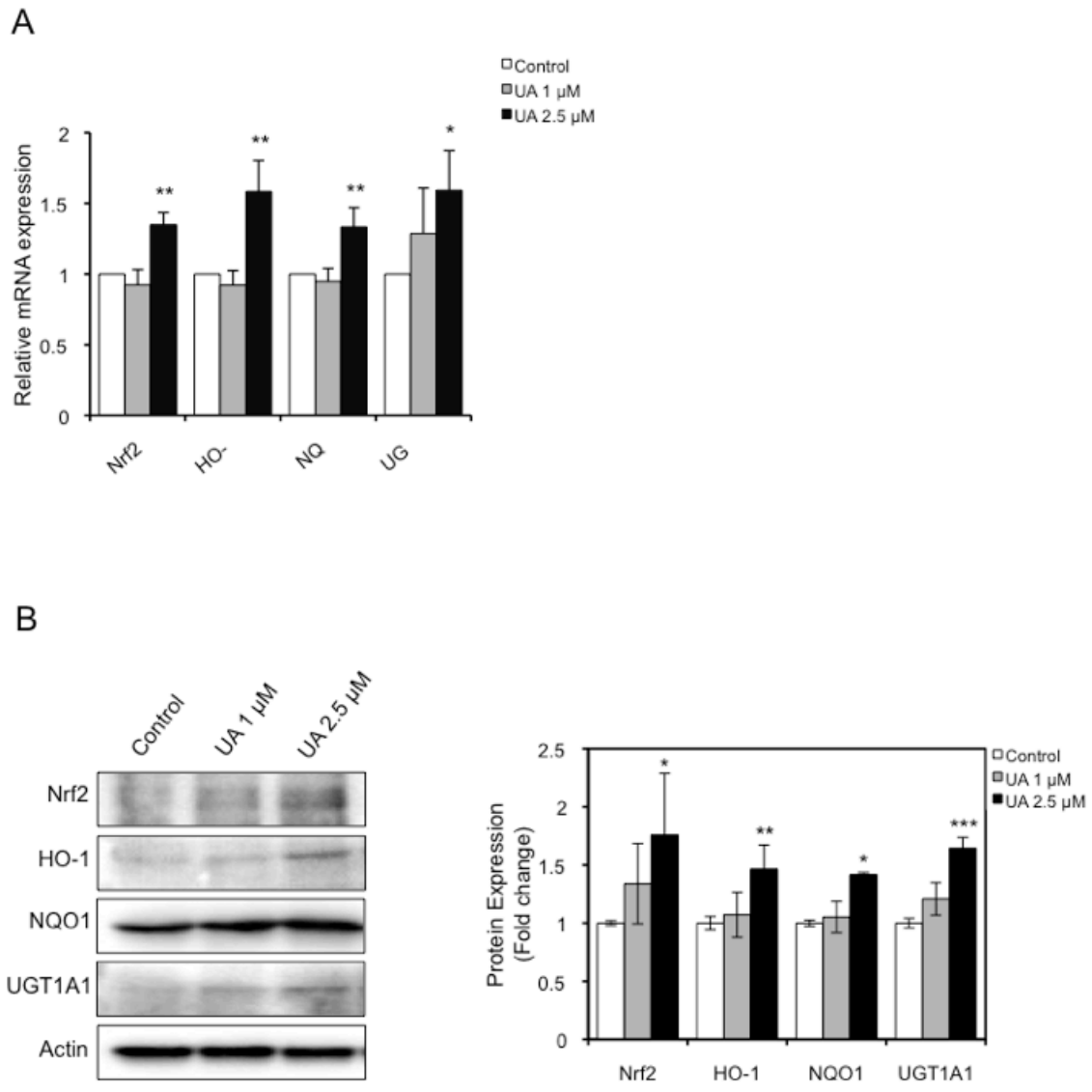


Figure 3. UA upregulates the expression of Nrf2 and its downstream target genes. Cells were treated with each concentration of UA for 3 days, and the total cells were divided for RNA and protein extraction. (A) Total 0.5 μg of RNA was reverse transcribed for cDNA synthesis. The cDNAs were then used to perform qPCR by adding SYBR Green and a pair of gene-specific primers. (B) Western blots and quantification of protein levels. The data shown were normalized to β-actin and expressed as the relative fold change compared with the control. The values are the mean ± SD of three independent

experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ compared with vehicle control (0.1% DMSO).

2.3.4 Expression of Nrf2 downstream target genes by UA is Nrf2 dependent

Next, we clarified whether Nrf2 is required for induction of cytoprotective detoxifying/antioxidant genes by UA treatment. We used Nrf2-Mock and Nrf2-KD stable JB6 P+ cells established in our laboratory (67). The basal expression of Nrf2 was decreased by about 70% in Nrf2-KD JB6 P+ cells compared with control Nrf2-Mock (Figure 4). 2.5 μM UA treatment significantly increased protein expression of Nrf2, HO-1, NQO1 and UGT1A1 in Nrf2-Mock JB6 P+ cells. Conversely, the inducing effects of 2.5 μM UA on the expression of Nrf2 downstream target genes was much smaller in Nrf2-KD compared to those in Nrf2-Mock treated; 30%, 52%, and 51% decrease of HO-1, NQO1, and UGT1A1, respectively. The results indicate that Nrf2 is a direct regulator driving expression of cytoprotective detoxifying/antioxidant genes by UA in JB6 P+ cells.

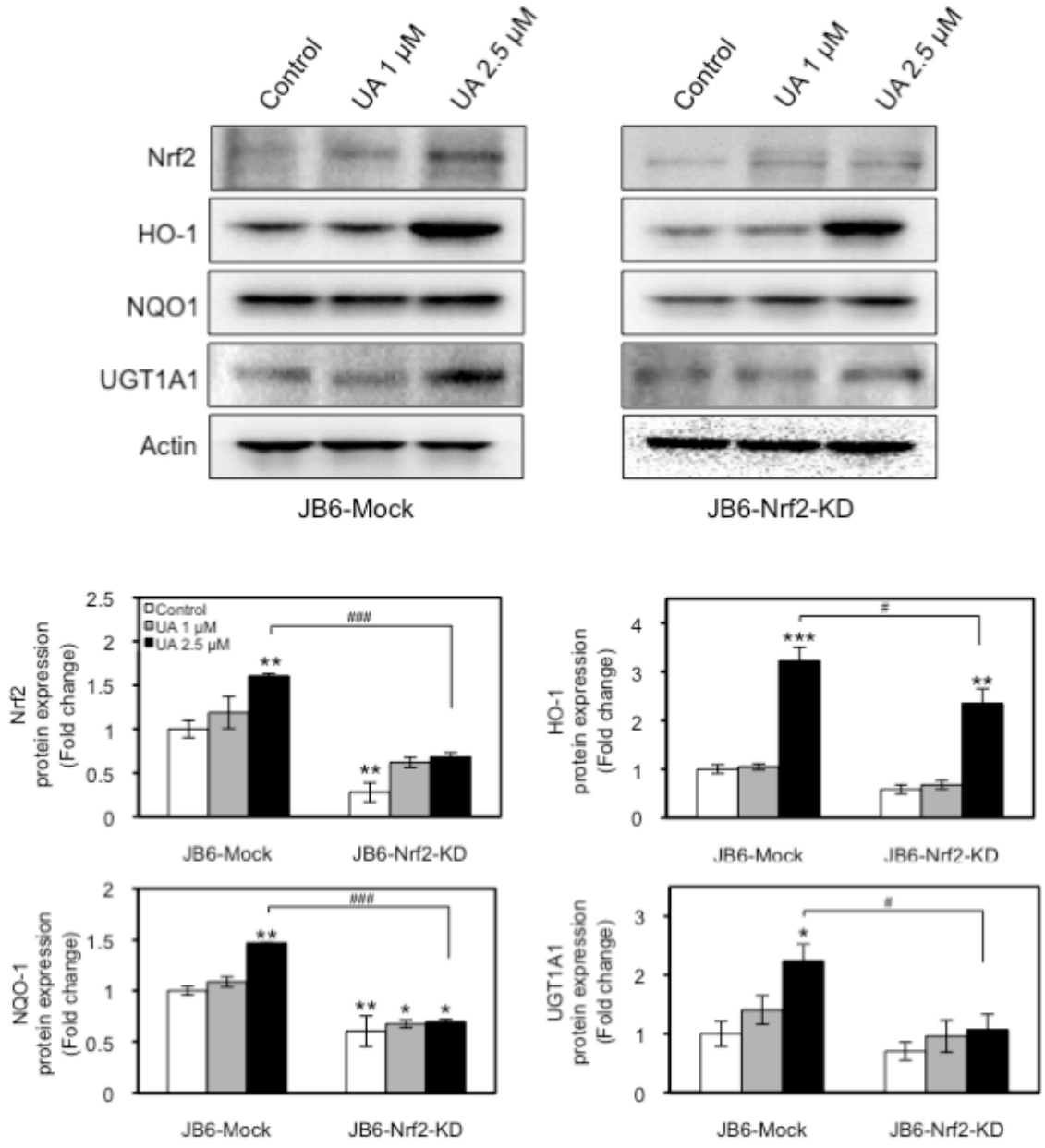


Figure 4. Nrf2 knockdown decreases expression of Nrf2 downstream target genes. Cells were treated with each concentration of UA for 3 days, and whole-cell extracts were prepared as described in Materials and Methods. Then, the proteins were subjected to Western blot to analyze the expression of Nrf2 and its downstream target genes indicated. The protein amounts were normalized to the levels of β-actin and expressed as the

relative fold change compared with JB6-Mock control. The values are the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$ *vs* JB6-Mock control. #$p < 0.05$, ###$p < 0.0001$ *vs* JB6-Mock UA 2.5 μM.

2.3.5 UA decreases Nrf2 promoter methylation

We previously showed that promoter demethylation of Nrf2 is an important epigenetic mechanism underlying Nrf2 activation in prostate cancer TRAMPC1 cells and JB6 P+ cells treated with phytochemicals (67, 152, 207, 209, 210). To determine whether Nrf2 was epigenetically regulated by UA, we determined the methylation status of the Nrf2 promoter using bisulfite genomic DNA sequencing. The first 15 CpG sites located between -1226 and -863 of the mouse Nrf2 gene promoter relative to the translation start site (+1) were analyzed (230). As previously reported, the Nrf2 promoter was hypermethylated in JB6 P+ cells (89.3%) (Figure 5). The cells treated with 5-aza and TSA, well-known inhibitors of DNA methylation and histone deacetylation, respectively, reduced methylation by 46.6%, which is similar to the results from previous studies (67). Treatment of JB6 P+ cells with 2.5 μM UA decreased methylation by 17% compared with JB6 P+ cells treated with vehicle. Treatment with 1 μM UA resulted in only a 7% decrease in methylation. These results are in accordance with (Figure 3), which shows that the levels of Nrf2 mRNA and protein were increased by 2.5 μM UA treatment, but they were unchanged by 1 μM UA treatment. These findings suggest that UA induces Nrf2 expression by altering the methylation status of the Nrf2 promoter.

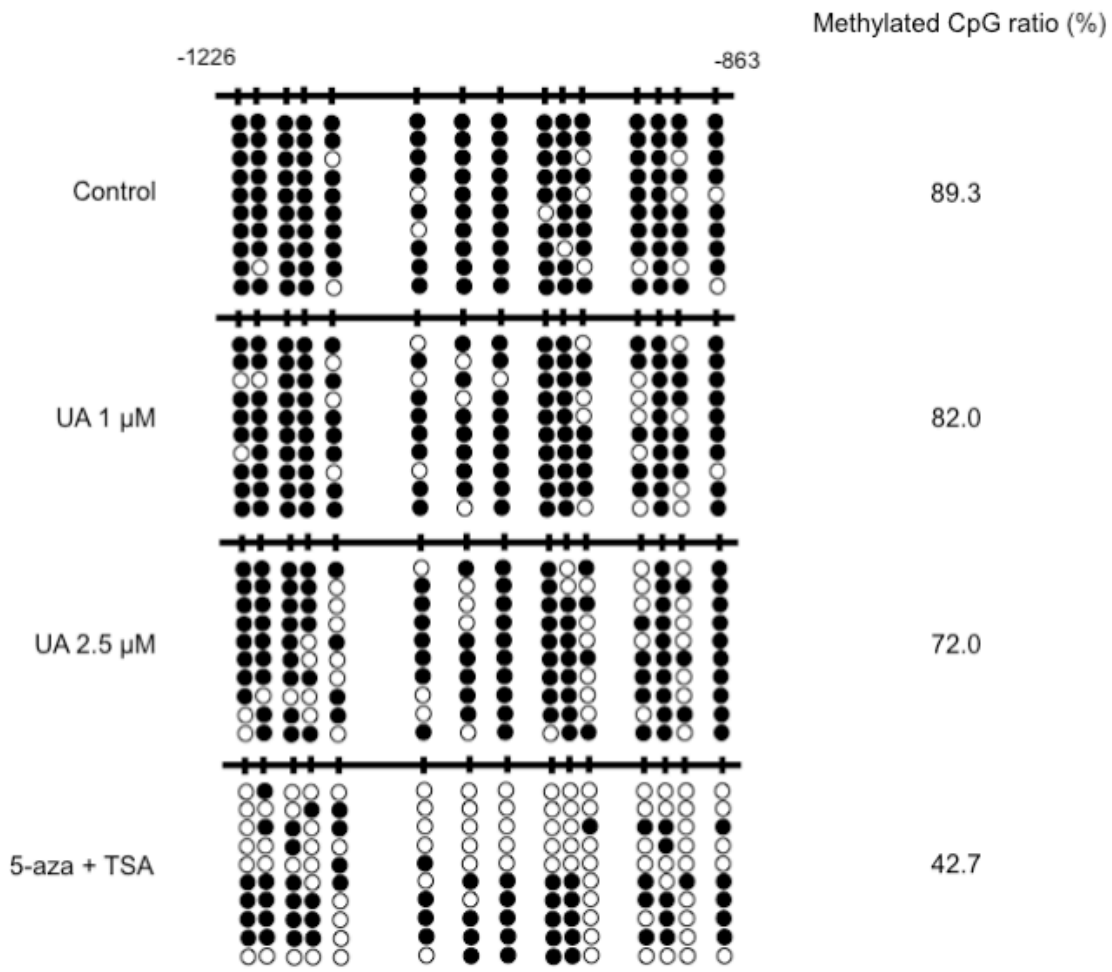


Figure 5. UA decreases Nrf2 promoter DNA methylation in JB6 P+ cells. The cells were treated with each concentration of UA for 3 days, and then the genomic DNA was isolated for bisulfite conversion. The methylation status of the first 15 CpG sites, the region between -1226 and -863 relative to the translational start site, within the promoter of Nrf2, was analyzed. Positive control cells were treated with 5-aza (250 nM) for 48 h and TSA (50 nM) for 24 h. Ten individual clones were analyzed. The filled and open dots indicate methylated and unmethylated CpG. The data are expressed as a percentage of the total number of methylated cytosines *vs.* total 15 CpGs of three independent experiments.

2.3.6 UA alters the levels of epigenetic modifying enzymes

To understand the mechanisms by which UA decreases Nrf2 promoter methylation in JB6 P+ cells, we determined an impact of UA on DNMTs and HDACs, which are involved in methylation-induced gene silencing (220). The family of DNMTs consists of three members, DNMT1, DNMT3a, and DNMT3b. Treatment with 2.5 μM UA resulted in a significant reduction in DNMT1 and DNMT3a protein levels (Fig. 6A). In addition, 1 μM UA treatment also slightly decreased both DNMT1 and DNMT3a. No significant difference was found between the effects of treatment with 2.5 and 1 μM UA. The DNMT3b protein levels were unaffected by UA treatment. HDACs are classified into four groups: Class I (HDAC1, 2, 3, and 8), Class II (HDAC4, 5, 6, 7, 9, and 10), Class III (SIRT1-7), and Class IV (HDAC 11). UA has previously been reported to increase histone acetylation by strongly inhibiting HDAC1, 3, 4, 5, and 6 (24). We examined the expression levels of HDAC 1, 2, 3 and 8 (Class I) and HDAC 4, 5, 6 and 7 (Class II). The protein expression levels of all HDACs were diminished in the JB6 P+ cells treated with 2.5 μM UA (Figure 6B and 6C). Among them, HDAC2 and 8 showed a dose-dependent reduction. The expression of HDAC4 was not affected by UA treatment and HDAC5 was not detected. The decrease of HDAC expression confirmed the inhibition of HDAC activity, whereas the levels of acetylated histone H3 (H3ac), an epigenetic marker for active genes, was increased by 2.5 μM UA treatment (Figure 6D). Taken together, these results indicate that UA-induced demethylation of the Nrf2 promoter is mediated by the negative regulation of epigenetic modification enzymes.

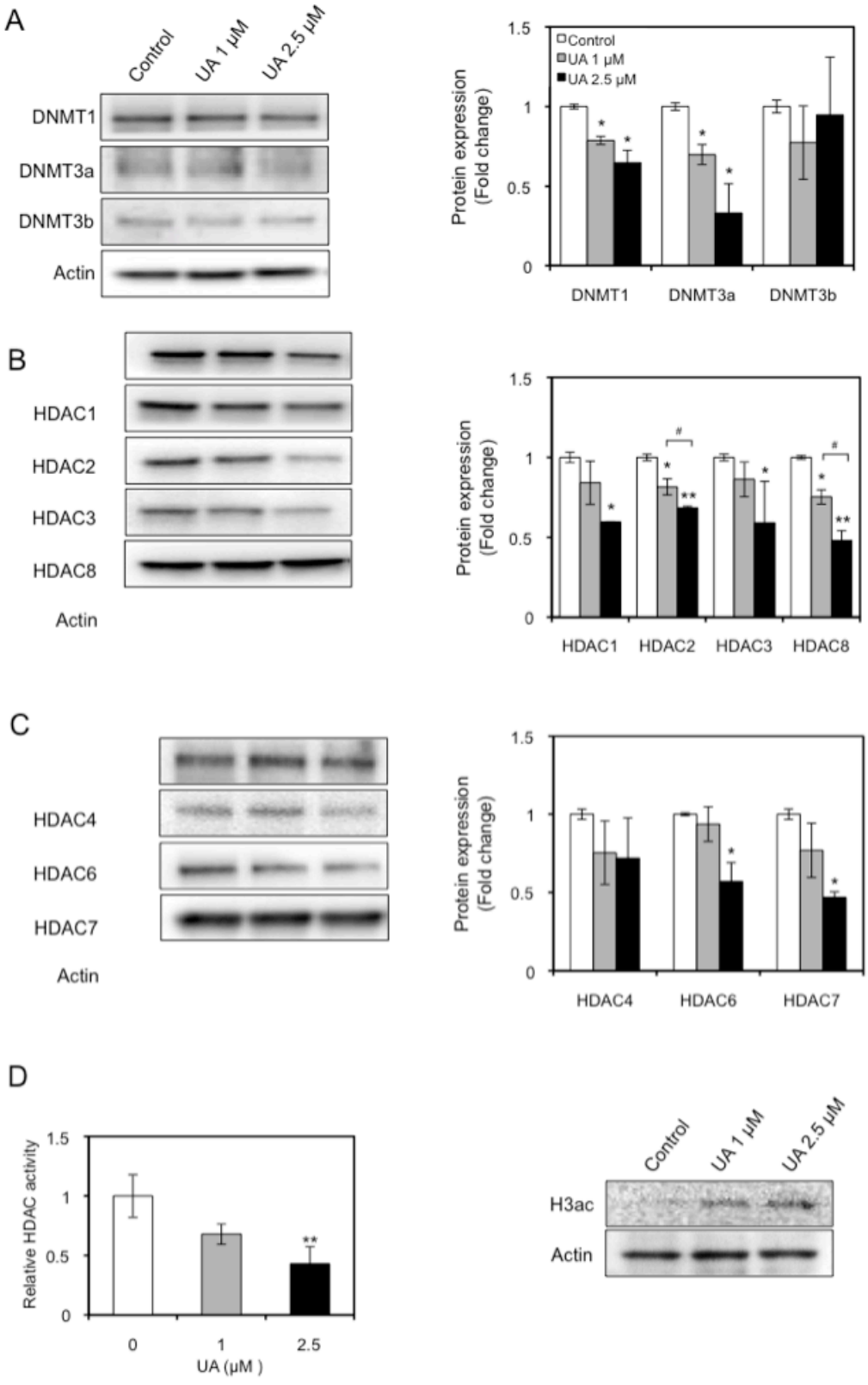

A
Control
UA 1 μM
UA 2.5 μM
DNMT1
DNMT3a
DNMT3b
Actin
Control
UA 1 μM
UA 2.5 μM
Protein expression (Fold change)
1.5
1
0.5
0
DNMT1
DNMT3a
DNMT3b
B
HDAC1
HDAC2
HDAC3
HDAC8
Actin
Protein expression (Fold change)
HDAC1
HDAC2
HDAC3
HDAC8
C
HDAC4
HDAC6
HDAC7
Actin
Protein expression (Fold change)
HDAC4
HDAC6
HDAC7
D
Relative HDAC activity
0
1
2.5
UA (μM)
Control
UA 1 μM
UA 2.5 μM
H3ac
Actin

Figure 6. UA decreases the expression of DNMTs (A), Class I and II HDACs (B & C), inhibits HDAC activity, and increases H3ac levels (D) in JB6 P+ cells. The cells were treated with the indicated concentration of UA for 3 days, and the total cell lysates and nuclear proteins were harvested at the end of the treatment. Total protein (25 μg per lane) was separated by SDS-PAGE, and the levels of each protein of interest were determined by Western blot analysis. The isolated nuclear extracts from each group were used to determine total HDAC activity. The protein amounts in Western blot analysis were normalized to the levels of β-actin and data are expressed as the relative fold change compared with the control. The values are the mean ± SD of three independent experiments. The figure H3ac is a representative of three individual experiments. *$p < 0.05$ and **$p < 0.001$ *vs* control. #$p < 0.05$ *vs* UA 1 μM.

2.4 Discussion

A variety of external stimuli continuously make contact with the skin and accelerate the formation of ROS, impairing cellular metabolism, signal transduction, and genomic stability, and ultimately contributing to the development of skin cancer (231). Therefore, inhibiting and/or reducing oxidative stress by ROS is crucial in preventing skin cancer. Many dietary phytochemicals eliminate ROS toxicity by inducing detoxifying/antioxidant enzymes via Nrf2 activation, which has led to a decrease in cancer development (5). We have previously shown that curcumin, 3,3′-diindolylmethane, and a γ-tocopherol-rich mixture of tocopherols, sulforaphane, Z-ligustilide and radix angelica regulate Nrf2 activation through an epigenetic pathway in a prostate cancer model (66, 152, 208, 209, 211). Moreover, studies have shown that a variety of natural compounds interact with epigenetic regulators (222). These studies

suggest that natural dietary compounds that are able to epigenetically regulate gene expression are promising chemopreventive agents. Our findings demonstrate that UA, a naturally occurring triterpenoid in fruits and plants, restores the expression of the epigenetically silenced Nrf2 gene by demethylating CpG islands of the Nrf2 promoter, leading to upregulated Nrf2 expression. As a result, the expression of its target genes increases. Subsequently, this results in the inhibition of the TPA-induced neoplastic transformation in JB6 P+ cells.

JB6 P+ mouse epidermal cells, unlike P- cells, are susceptible to tumor promoter-induced transformation and are a suitable *in vitro* model to study progression in carcinogenesis and the molecular mechanisms of cancer chemoprevention (232). Previous studies have used JB6 P+ cells to investigate whether dietary agents have the capacity to suppress transformation induced by tumor promoters (67, 210, 233-235). Thus, we treated JB6 P+ cells with UA to test the chemopreventive potential of UA in TPA-induced transformation. The inhibitory effects of UA on tumor promotion by TPA and B[a]P or DMBA/TPA have been described in mouse skin (201, 202, 236). Consistent with these reports, we found that UA was effective in inhibiting the transformation-inducing effects of TPA in JBP+ cells at a concentration in which the cytotoxicity was no more than 25% (Figure 1 and 2). The cumulative ROS production is detected in TPA-induced transformation (232). In addition, ROS inhibition by detoxifying/antioxidant enzymes attenuates TPA-induced transformation of JB6P+ cells (67, 237). Conversely, several reports indicate that UA remarkably reduces oxidative stress and increases the activity of antioxidant enzymes (238-240). We observed that the expression of HO-1 (antioxidant), NQO1, and UGT1A1 (detoxification) noticeably increased at both the

mRNA and protein levels in the JB6 P+ cells treated with UA; however, TPA activated AP-1, NF-κB, and ERK 1/2 as well (232, 241). Moreover, UA targets AP-1, NF-κB, and ERK 1/2 (200). Hence, our observations suggest that the inhibition of TPA-induced transformation of JB6 P+ cells by UA is partially reliant on ROS reduction through the accumulation of antioxidative/detoxifying enzymes. How UA alters the expression and activity of AP-1, NF-κB, and ERK in TPA-induced transformation remains to be elucidated.

The production of phase II detoxifying/antioxidant enzymes is an innate cellular event that provides protection against deleterious endogenous and exogenous substances. In general, the genes encoding such cytoprotective enzymes are postulated to be regulated in an Nrf2-dependent manner. Thus, Nrf2 is central to the prevention of deleterious diseases, such as skin cancer. We have provided evidence that TPA-induced cell transformation is increased in Nrf2-KD JB6 P+ cells. Furthermore, the inhibitory effect of sulforaphane on TPA-induced cell transformation is blocked upon Nrf2-KD (67). Many cancer chemopreventive agents acting via Nrf2 activation are phytochemicals. Some examples include carnosol, curcumin, epigallocatechin-3-gallate (EGCG), phenethyl isothiocyanate (PEITC), sulforaphane, and resveratrol (242, 243). In our study, UA elevated the levels of Nrf2 mRNA and protein (Figure 3). Additionally, Nrf2 deficiency in Nrf2-KD JB6 P+ cells lowered the effects of UA on the protein expression of detoxifying/antioxidant genes (Figure 4). These results imply that UA is a chemopreventive dietary phytochemical that targets Nrf2. Our data are strongly supported by recent findings demonstrating that UA-driven activation of Nrf2 protects mice from neuronal defects induced by cerebral ischemia, and hepatotoxicity and

fibrosis caused by CCl_4. (205, 206). Furthermore, an isomer of UA, oleanolic acid, and the synthetic oleanane triterpenoid CDDO (2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid), and its methyl (CDDO-Me) and imidazolide (CDDO-Im) derivatives have been shown to be potent Nrf2 inducers (244). Upregulated Nrf2 expression can in part be achieved by the increased half-life of Nrf2, which is mediated by the reduction of Keap1-dependent ubiquitin/proteasome degradation of Nrf2 (245). Keap1 is a suppressor protein of Nrf2. Under normal circumstances, Keap1 binds to Nrf2 and causes rapid Nrf2 degradation via polyubiquitination. By contrast, upon high oxidative stress, a subset of cysteine residues in Keap1 are modified, which perturbs the Keap1/Nrf2 interaction and hinders Nrf2 ubiquitination. This enables the accumulation and translocation of Nrf2 into the nucleus where it triggers the transcription of various phase II cytoprotective genes (242). Sulforaphane modifies cysteine 151 within the BTB (Broad complex, Tramtrack, and Bric-a-brac) domain of Keap1, which results in lowered Nrf2 ubiquitination/degradation and increased stabilization (246). Moreover, a previous study revealed that Keap1 allows common inducers of phase II genes to alter its cysteine sulfhydryl groups regardless of the inducers' structures (247). As such, UA might modify cysteine residues in Keap1, resulting in an increase of Nrf2, which facilitates detoxifying/antioxidant expression by binding to the AREs in the promoters of its target genes.

DNA hypermethylation is the most common epigenetic modification in degenerative diseases such as cancer. This modification influences the depression of tumor suppressor genes. To date, many genes have been shown to be silenced by CpG hypermethylation within the promoter region during tumor progression. For example, in

skin cancer, 14-3-3sigma (cell cycle), MGMT (DNA repair), RASSF1 (signal transduction), PTEN (apoptosis), and others have been shown to be hypermethylated (221). Thus, discovering compounds that are able to reduce hypermethylation is an attractive strategy for the prevention of skin cancer. Studies by our group and others have revealed that Nrf2 expression is altered by methylation of CpG sites in the Nrf2 promoter region (66, 67, 152, 207-211, 248). These studies suggest that the epigenetic modulation of Nrf2 is likely to be a critical mechanism for Nrf2 activation. The present study demonstrates that UA decreased the methylation of the Nrf2 promoter in JB6 P+ cells. Although the effects were not comparable with those of the well-known epigenetic inhibitors 5-aza and TSA in combination, 2.5 μM UA treatment showed similar efficacy to that of 2.5 μmol/L sulforaphane and 6.25 μM apigenin in JB6 P+ cells (20% decrease compared with control in both). Notably, UA induces the expression of SHP-1, a tyrosine-specific protein phosphatase silenced by methylation in leukemias and lymphomas, in human multiple myeloma U266 cells (249). These results suggest that UA has the potential to modulate DNA methylation, which is implicated in carcinogenesis. Concomitantly, we found that UA decreased the protein levels of DNMT1 and DNMT3a. DNMT1 preserves DNA methylation patterns across generations, whereas DNMT3a and 3b act as *de novo* methyltransferases (220). The levels of DNMT1, DNMT3a, and DNMT3b are upregulated in UVB-induced murine skin tumors, and DNMT3a and DNMT3b are increased in stage III and IV cutaneous melanoma patients (250, 251). Hence, our observations indicate that UA functions as a natural DNMT inhibitor to reduce DNA methylation in the skin. In cancer cells, DNMT1 and DNMT3b collaborate to maintain hypermethylation in the CpG islands of promoters (252); however, UA did

not have a significant effect on DNMT3b expression in JB6 P+ cells. This result may account for the weaker than expected inhibitory effect of UA on TPA-induced transformation and methylation of the Nrf2 promoter.

Hypermethylation in promoter regions provides binding sites for MeCP2, one of the MBD proteins, which subsequently recruits HDACs. HDACs remove acetyl groups from histones, mainly histone H3 and H4. This removal accelerates the formation of a compact chromatic structure, which drives the repression of transcription and causes gene silencing (220, 221). Because HDACs such as HADC1, HDAC2, HDAC3, HDAC6, and HDC8 are overexpressed in many cancers (253), the discovery of selective HDAC inhibitors has had significant implications for cancer therapy. As natural HDAC inhibitors for skin cancer, EGCG and grape seed proanthocyanidins have been reported to decrease the level of HDAC1 and HDAC activity, accompanied by reduced expression and activity of DNMTs in squamous cell carcinoma (221, 227). Recently, sulforaphane has been shown to reduce the protein levels of HDAC1-4 and 6 in human keratinocytes (254). Interestingly, UA from *Microtropis japonica* significantly decreases the protein levels of HDAC1, 3, 4, 5, and 6 in HL-60 myeloid leukemia cells (24). Similarly, in our experiments, UA downregulated all Class I HDACs, including HDAC1, 2, 3 and 8, and two from Class II HDACs, HDAC6 and 7 in JB6 P+ cells. Although HDAC4 expression did not decrease, similar results were found in JB6 P+ cells when treated with apigenin, sulforaphane, and tanshinone IIA (67, 207, 210). A decrease of HDAC expression was linked to a reduced HDAC activity and a dramatic increase of H3ac (Figure 6). Thus, UA-induced HDACs reduction results in a reduction of HDAC activity and, in turn, an enhanced acetylation of histone, which leads to epigenetic gene activation. Further, these

data, together with the DNMTs results, imply that UA prevents DNA hypermethylation through the regulation of DNMTs and HDACs, unlike 5-aza and TSA, which are only specific for the inhibition of DNA methylation and histone deacetylation.

2.5 Conclusion

In conclusion, this study demonstrated for the first time that UA restores the expression of Nrf2 by demethylating CpG islands in the Nrf2 promoter in mouse epidermal cells. The reduced expression of enzymes involved in DNA methylation and histone deacetylation and the increased level of histone acetylation mediated this alteration. The response to epigenetic alterations of Nrf2 by UA induced an increase in the expression of cytoprotective detoxifying/antioxidant enzymes, which resulted in the suppression of tumor promoter-induced cell transformation. Collectively, this data provided new insight into the function of UA as an epigenetic regulator for the prevention of skin cancer.

Chapter III

Ursolic acid suppresses skin carcinogenesis in a novel two-stage carcinogenesis model[4]

[4]A portion of this chapter is intended to be submitted as a research article.

3.1 Introduction

NMSC is the most common cancer in the United States and its incidence continues to rise (1, 255). Healthcare costs for the treatment and management of NMSC are approximately $650 million per year (255). As such, effective preventative measures are needed. The most common skin cancers are subdivided into NMSC and melanoma skin cancer. NMSC is made up of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC and SCC make up 98% of all skin cancer cases. The remainder is made up of the most severe form of skin cancer, melanoma. Causes for skin cancer include ultraviolet radiation and environmental pollutants. Occupational exposure to petroleum byproducts, organophosphate compounds, and arsenic are associated with the development of NMSC (256-259). Additional risk factors include family history or previous personal history, pigmentary characteristics (fair skin), immunosuppression (solid-organ transplant recipients), genetic disorders, therapeutic radiation exposure, and cigarette smoking (255, 259, 260). The most common treatment for NMSC is surgical management (261). Surgical treatment can be disfiguring and costly (262). The ideal preventative measure for skin cancer is to avoid exposure to the sun and environmental pollutants. In practice, it is nearly impossible to completely avoid these exposures. As such, secondary measures to prevent or suppress the promotion or progression of skin carcinogenesis using dietary phytochemicals is of great importance.

The process by which skin carcinogenesis occurs is a multi-faceted process that can be broken down into three distinct phases (263, 264). The first phase is the initiation phase. In this phase genetic mutations are acquired resulting in gene activation (v-Ha-ras oncogene) or gene inactivation (p53 tumor suppressor gene). The next stage is promotion.

This stage is characterized by increased DNA synthesis and inflammation, the genetic alteration of gene expression and/or protein activities, and the clonal expansion of initiated cells (265). The clonal expansion of initiated cells containing genetic mutations is reversible and is an area of great interest in chemoprevention. The accumulation of clonally expanded initiated cells containing mutations with a dysregulation of apoptosis, epigenetic changes in DNA methylation, and infiltration of activated leukocytes is irreversible and leads to the development of pre-neoplastic papillomas (264). Simultaneously, vascularization occurs to nourish the pre-neoplastic lesion with oxygen and nutrients. The occurrence of additional mutations and accumulation of chromosomal abnormalities leads into the final phase, progression. In this phase pre-neoplastic lesions are converted into carcinoma. It can take up to 10 or more years for initiated cells to become a pre-neoplastic lesion while progression to carcinoma can occur in less than a year (4, 266). For this reason, many chemopreventive strategies are focused on the intervention of dietary phytochemicals in initiation and promotion phases. The most relevant model to study both phases operationally and mechanistically is the two-stage chemical carcinogenesis mouse model (265).

Chemical carcinogenesis was first reported in the 1770s Dr. Percival Pott noted there was a high incidence of scrotal cancer associated with chimney sweeps (267). This led to the hypothesis environmental pollutants can cause cancer and was further evaluated by scientists using a variety of models. The mouse model was first introduced in the 1920s when a mouse previously treated with tar was wounded and developed tumors (265). This finding led to the development of the two-stage chemical carcinogenesis mouse model to study multi-stage carcinogenesis.

The well-established two-stage chemical carcinogenesis model describes the initiation, promotion, and progression of mouse skin cancer due to its many similarities to the evolution of human non-melanoma SCC (268-270). The model is typically generated with a single dose chemical carcinogen followed by repeated topical applications of a tumor promoter. Commonly used initiating agents include the polycyclic aromatic hydrocarbons 7,12-dimethylbenz(a)anthracene (DMBA) and benzo{a]pyrene (B[a]P). B[a]P is a commonly found environmental pollutant implicated in the induction of skin cancer (124, 271-273) and induces mutations in Hras1 resembling human skin cancer etiology (266-268). The initiating event is irreversible and tumor formation will only appear after promotion with a promoting agent such as TPA. The combination of initiating agent and promoting agent causes sustained hyperplasia and inflammation, which in turn, leads to the selective clonal expansion of benign papillomas (263, 274-278). These papillomas can progress to SCC as early as 20 weeks after the start of promotion and is largely dictated by mouse strain and the dose of initiating and promoting agents (279-283).

As described previously, skin carcinogenesis is driven by inflammation and oxidative stress. As a key regulator of oxidative stress and inflammation responses in the cell, Nrf2 has been shown to protect against the development and progression of several skin cancers (214). Previous studies in our laboratory have shown Nrf2 (-/-) mice are more susceptible to DMBA-induced skin carcinogenesis (54). In addition, aberrant epigenetic changes have been observed in the development and progression of skin cancer (181-183). Our laboratory has recently identified extensive gene methylation profiles of skin carcinogenesis in a genome-wide epigenome analysis of DMBA/TPA-

treated CD1 mice (284) noting gene methylation as an attractive therapeutic target for skin carcinogenesis. The FDA has approved chemotherapeutic agents inhibiting epigenetic modifying enzymes but their use has been plagued by toxicity (285, 286). Therefore, dietary phytochemicals with the ability to modulate epigenetics involved in the promotion and progression of skin cancer are needed.

UA has been reported to possess many beneficial health effects. These effects include anti-cancer activity in various cancers, such as skin cancer. Skin cancer is the most common cancer in the world. Nrf2 is a master regulator of anti-oxidative stress response with anti-carcinogenic activity against UV- and chemical-induced tumor formation in the skin. Recent studies show that epigenetic modifications of Nrf2 play an important role in cancer prevention. In the previous chapter we demonstrated that the epigenetic effects of the triterpenoid UA could potentially contribute to its beneficial effects, including the prevention of skin cancer. Therefore, the ability of UA to modulate Nrf2 epigenetically *in vitro* warrants further investigation *in vivo*.

The efficacy of dietary phytochemicals including UA has been heavily explored in the two-stage chemical carcinogenesis model in our laboratory as well as others due to tumor response reproducibility (54, 287). The model generates a spectrum of different stages of carcinogenesis ranging from premalignant papilloma to metastatic tumors with H-Ras mutations resembling human cancer development (278, 288, 289) and thus is a suitable animal model to study the role of UA in skin carcinogenesis.

In this study, we evaluated the ability of UA to suppress skin carcinogenesis in a B[a]P/TPA SKH-1 mouse model. To our knowledge this is the first time the combination of B[a]P/TPA has been evaluated in an SKH-1 mouse model. This study will contribute

to the exploration of dietary phytochemicals in the prevention of skin cancer. Future work will evaluate epigenomic changes in the various phases of skin cancer and identify potential epigenomic biomarkers during skin carcinogenesis for the development of novel therapeutic strategies.

3.2 Materials and Methods

3.2.1 Animals

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Rutgers, The State University of New Jersey (Protocol Number: 04-003).

3.2.1.1 Pilot Studies:

The SKH-1 mouse strain is homozygous WT for Nrf2 and was purchased from Charles River Laboratory (Bar Harbor, ME, USA). The SKH-1 Nrf2 (-/-) mice were generated by backcrossing SKH-1 Nrf2 (-/-) with SKH-1 WT mice as described previously ((290)). All animals were female. The genotype of each Nrf2 (-/-) animal was confirmed. DNA was extracted from the tail of each animal and was followed by polymerase chain reaction (PCR). Bands for Nrf2 (-/-) were visualized at 200bp by agarose gel electrophoresis, while WT mice display a band at 300bp as described previously (54). 7-8 week old female mice were used for the pilot studies. Mice were housed at the Rutgers Animal Facility, maintained under 12-h light/dark cycles, and provided *ad libitum* access to food and water.

3.2.1.2 Main Study:

5-6-week old female SKH-1 mice were purchased from Charles River Laboratory (Bar Harbor, ME, USA). Mice were housed as described in pilot studies. Upon their arrival, the 165 mice were weighed and randomized into experimental group cages to reduce any experimental bias. The mice were acclimatized for a period of 1 week. Enrichment was not provided in the animal cages to reduce any potential skin aggravation.

3.2.2 Chemicals

Acetone (HPLC grade), Ursolic Acid (UA) (U6753), Benzo[a]pyrene (B[a]P), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma, Aldrich. (St. Louis, MO, USA). All solutions for the study were made in acetone.

3.2.3 Experimental design of pilot studies

The right ear of each mouse received 20µL of the control group and the left ear of each mouse received 20µL of the treatment group. For each application 10µL was applied to the inner surface of the ear and 10µL was applied to the outer surface of the ear. Six hours after TPA application, the mice were sacrificed and a 6 mm diameter disc from each ear was removed with an ear punch and weighed.

Pilot Study 1

Ear edema was induced on the left ear of 7-week old female SKH-1 mice with 2.6 nmol/20µL TPA, 1.3 nmol/20µL TPA, or 0.65 nmol/20µL TPA. The right ear of each mouse received 20µL of acetone.

Pilot Study 2

Ear edema was induced on left and right ears of 7-week old female SKH-1 mice with 0.65 nmol/20µL TPA. 30 minutes prior to TPA application, acetone was applied to the right ear and 50nmol/20µL, 100 nmol/20µL, or 200 nmol/20µL UA was applied to the left ear.

Pilot Study 3

Ear edema was induced on the left and right ears of 7-week old female SKH-1 mice with 0.65 nmol/20µL TPA. 30 minutes or 15 minutes prior to TPA application, acetone was applied to the right ear and 200 nmol/20µL UA was applied to the left ear.

Pilot Study 4

Ear edema was induced on left and right ears of 7-week old female Nrf2 (+/+) or Nrf2 (-/-) SKH-1 mice with 0.65 nmol/20µL TPA. 30 minutes prior to TPA application, acetone was applied to the right ear and 200 nmol/20µL UA was applied to the left ear.

3.2.4 Experimental design of main study

165 mice were separated into four groups. The experimental study design is summarized in Table 1.

Group	No. of animals	Time of 2µM UA Treatment	Initiation (nmol B[a]P)	Promotion
1	12	None	200µL acetone	200µL acetone
2	51	None	200nmol + 100nmol	6.8nmol TPA
3	51	Early[1]	200nmol + 100nmol	6.8nmol TPA
4	51	Late [2]	200nmol + 100nmol	6.8nmol TPA

Table 1 Experimental Design. [1] UA applied one week prior and the day of B[a]P application. [2] UA applied 30 minutes prior to each TPA application.

Chemicals were applied topically to the dorsal skin of approximately 8-week old female SKH-1 mice. Group 3 was treated with 2 µmol/200µL UA twice a week prior to B[a]P application and prior to each application of B[a]P. Groups 2, 3, and 4 were given two applications of initiating doses of fresh B[a]P in 200µL of acetone. Once the first dose was completed, the second dose was given one week later. The initiating doses were followed by a week of rest to allow the B[a]P to have its effect and reduce exposure of personnel to the initiating agent. Following the week of rest, animals were treated with 2 µmol/ 200µL UA (groups 3 and 4) or 200µL of acetone (group 2) 30 minutes prior to receiving 6.8 nmol/200µL TPA (groups 2, 3, 4) at the same site twice weekly for 31 weeks. Negative control animals were treated with 200µL acetone only at start of topical applications (early UA application) for 34 weeks. Animals were sacrificed at the time-points described in Figure 1 to capture all phases of the skin carcinogenesis model.

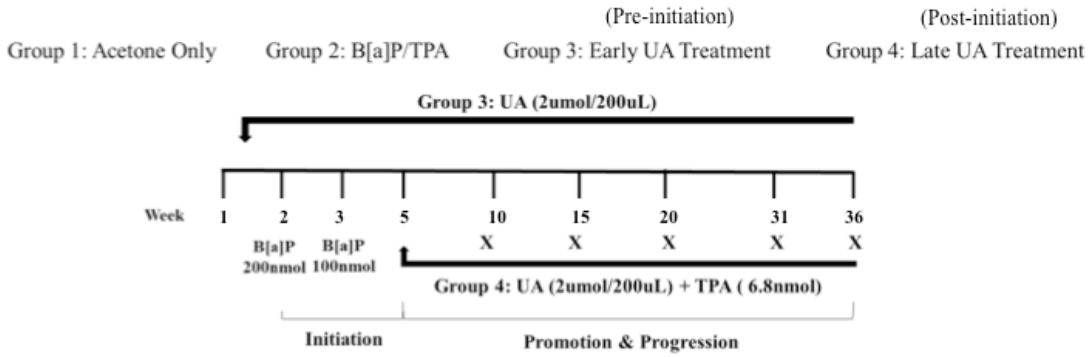


Figure 1. Study Design of B[a]P/TPA model in SKH-1 mice. B[a]P was applied twice at the age of 6 and 7 weeks. UA treatment started either 1 week before, prior to B[a]P initiation or after B[a]P initiation. TPA was applied twice a week starting 1 week after last B[a]P treatment for 31 weeks.

3.2.5 Preparation of skin specimens and histological examination

Skin samples (25 mm length and 5mm width) were obtained from the dorsal area of the mouse and were placed in 10% phosphate-buffered formalin at room temperature for 24-48 hours. The samples were then dehydrated in increasing concentrations (80, 95, and 100%) of ethanol, cleared in xylene, and embedded in Paraplast Plus (Fisher Scientific, Pittsburgh, PA, USA) using an automated platform (Leica). Paraffin blocks were submitted to Pathology Core Facility for further processing. The H&E sections were examined and imaged under a light microscope (Nikon Eclipse E600, Japan).

3.2.6 Data presentation and statistical analysis

The word tumor denotes papilloma, cyst, keratoacanthoma, carcinoma or sarcoma. The data are presented as the mean ± standard error of the mean (63), except as otherwise stated. Student's t-test was used to determine statistically significant differences between control and treatment groups. A p value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 SKH-1 mice are suitable for two-stage carcinogenesis investigation

To determine the optimal dose of TPA and UA to use in the main study three doses of TPA and three doses of UA were evaluated in an ear edema assay. Several two-stage carcinogenesis studies using TPA as the promoting agent have utilized concentrations between 5-15 nmol/200μL (265). As such, the following doses were evaluated in an ear edema assay: 0.65 nmol/20μL (1X), 1.3 nmol/20μL (2X), and 2.6 nmol/20μL (3X). All concentrations were found to result in a significant increase in ear edema in comparison to the acetone treated group (Figure 2A). Due to the possibility of

toxic effects resulting from the use of high doses, 0.65 nmol/20µL was selected for downstream ear edema assays. Preliminary studies in our laboratory demonstrated 2 µmol/200µL UA was the maximum tolerated dose for SKH-1 mice. For this reason the following doses of UA were evaluated in an ear edema assay: 50 nmol/20µL (1X), 100 nmol/20µL (2X) and 200 nmol/20µL (3X). While 100 nmol/20µL UA demonstrated only a 15% decrease in ear edema, a significant decrease of 37% was observed at 200 nmol/20µL UA (Figure 2B).

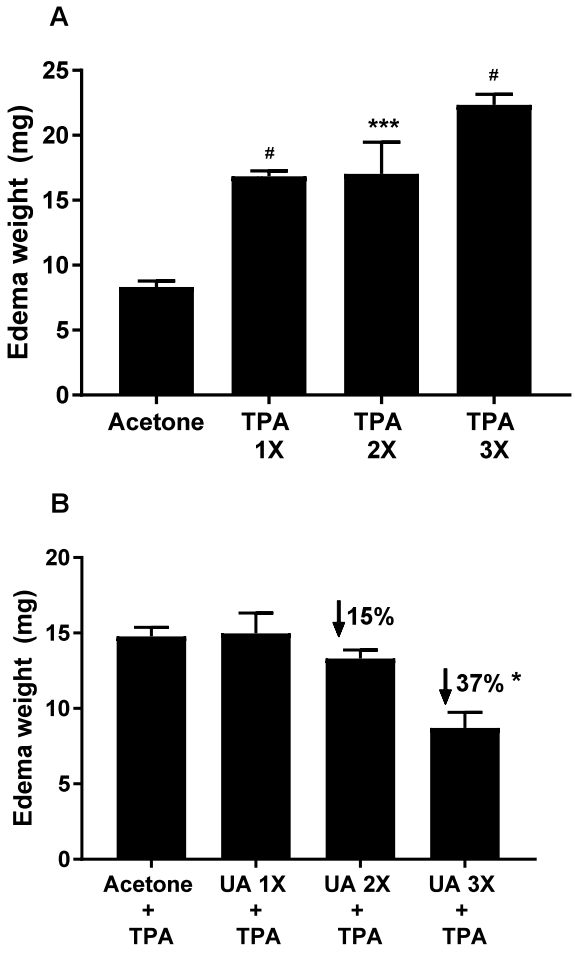


Figure 2. SKH-1 mice are suitable for use in two-stage carcinogenesis model. A) Nrf2 WT SKH-1 mice (n=3) were treated with or without three doses of TPA 0.65 nmol/20µL (1X), 1.3 nmol/20µL (2X), or 2.6 nmol/20µL (3X). Mice were sacrificed 6 hours after application. Ear punches were taken and average edema weight was determined. #$p < 0.00001$ and ***$p<0.0001$ indicates significant differences compared to vehicle-treated group. B) Nrf2 WT SKH-1 mice (n=3) were treated with acetone or three doses of UA 50 nmol/20µL (1X), 100 nmol/20µL (2X), or 3X=200 nmol/20µL (3X) 30 minutes prior to 0.65 nmol/20µL TPA application. Mice were sacrificed 6 hours after application and ear punches were obtained. Average edema weight and the inhibition percentage of ear edema by UA were calculated. *$p<0.05$ indicates significant differences compared to untreated group.

3.3.2 Anti-inflammatory effects of UA may be time-dependent

Studies have reported pre-treatment prior to TPA application from 5 minutes to 1 hour (201, 291) For this reason a 30-minute prior to TPA application duration was chosen as a starting point in the UA dose response ear edema assay (3.3.1). To determine if it were possible to decrease this window to 15 minutes instead of 30 minutes for logistical reasons, an ear edema assay was run. 200 nmol/20µL UA was applied either 15 minutes or 30 minutes prior to TPA application. The data demonstrates little to no inhibitory effect by UA applied 15 minutes prior to TPA in comparison to the TPA control (Figure 3). Furthermore, UA applied 30 minutes prior had an inhibitory percentage comparable to the previous study (Figure 3 and Figure 2B).

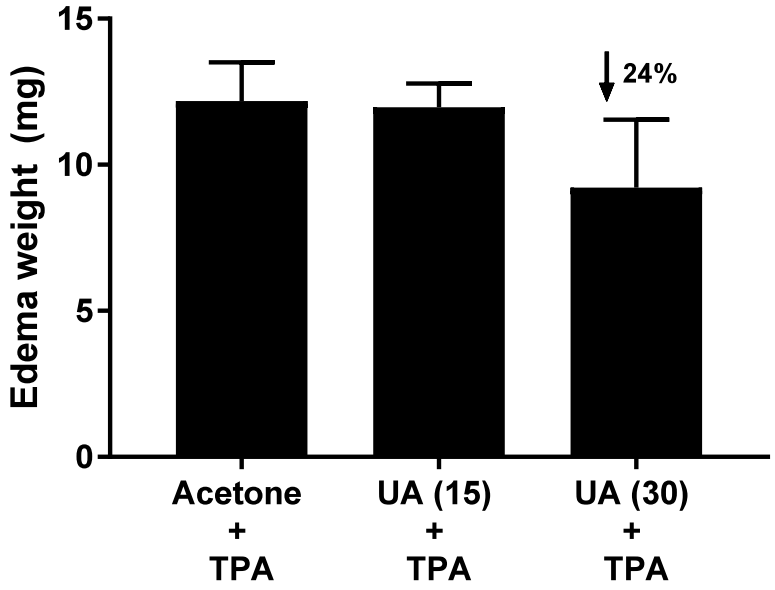


Figure 3. UA attenuates TPA-induced ear edema 30 minutes prior to TPA application but not 15 minutes prior to TPA application. Mice (n=4) were treated with acetone or 200 nmol/20μL of UA 30 minutes (30) or 15 minutes (15) prior to TPA application. Mice were sacrificed 6 hours after application and ear punches were obtained. Average edema weight and the inhibition percentage of ear edema by UA were calculated.

3.3.3 Nrf2 may be required for UA to inhibit TPA-induced inflammation

Because Nrf2 is central to the hypothesis of this research an ear edema assay was conducted evaluating UA's anti-inflammatory effect in Nrf2 (+/+) and Nrf2 (-/-) SKH-1 mice. The data demonstrated UA attenuates TPA-induced ear inflammation in Nrf2 (+/+) mice but not Nrf2 (-/-) mice (Figure 4). Furthermore, TPA-induced inflammation was enhanced in Nrf2 (-/-) mice (Figure 4). These results suggest UA inhibits TPA-induced inflammation and Nrf2 may be required.

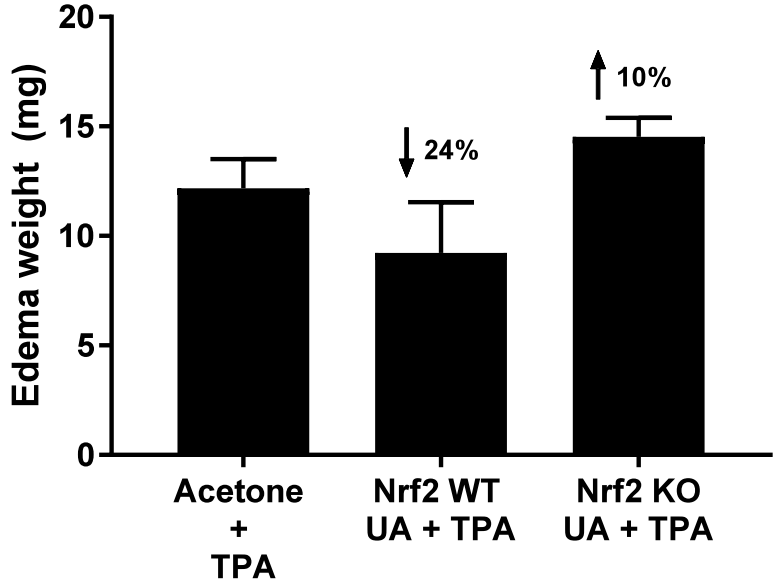


Figure 4. UA attenuates TPA-induced ear edema in Nrf2 (+/+) mice but not in Nrf2 (-/-) mice. Mice (n=4) were treated with acetone or 200 nmol/20μL of UA 30 minutes prior to TPA application. Mice were sacrificed 6 hours after application and ear punches were obtained. Average ear edema weight and the inhibition percentage of the ear edema by UA were calculated. The ratio of the ear punch weight in TPA versus acetone treated skin suggested that Nrf2 protected against TPA-induced increase in ear punch weight.

3.3.4 UA treatment suppresses skin carcinogenesis in a novel B[a]P/TPA model

Topical applications of acetone (vehicle), TPA, and UA (early or late) were applied to the dorsal region of SKH-1 mice twice a week as for 31 weeks post-TPA treatment. The body weight of mice was monitored every two weeks and it was found to increase steadily over the course of the study for all treatments (Figure 5A). The skin condition of the mice was monitored every three days for chemical-induced epidermal hyperplasia and actinic keratosis (265). Topical application of UA alone was not included

in the study. Previous studies in our laboratory using a topical application of 2 μmol/200μL UA in SKH-1 female mice did not demonstrate any toxic or carcinogenic effects. Late UA treatment reduced the percentage of tumor bearing mice, tumor multiplicity (tumor number per mouse), and tumor volume per mouse (Figure 5B, 5C, 5D). Of note, early UA treatment demonstrates comparable results to the B[a]P/TPA group (Figure 5B, 5C, 5D). Pharmacological effects of phytochemicals have been shown to be highly dependent on dose, timing, and duration of treatment (292). It is possible treatment with UA prior to B[a]P initiation resulted in UA acting as a co-carcinogen as previously seen with capsaicin (293). Further studies are needed to explore this observation.

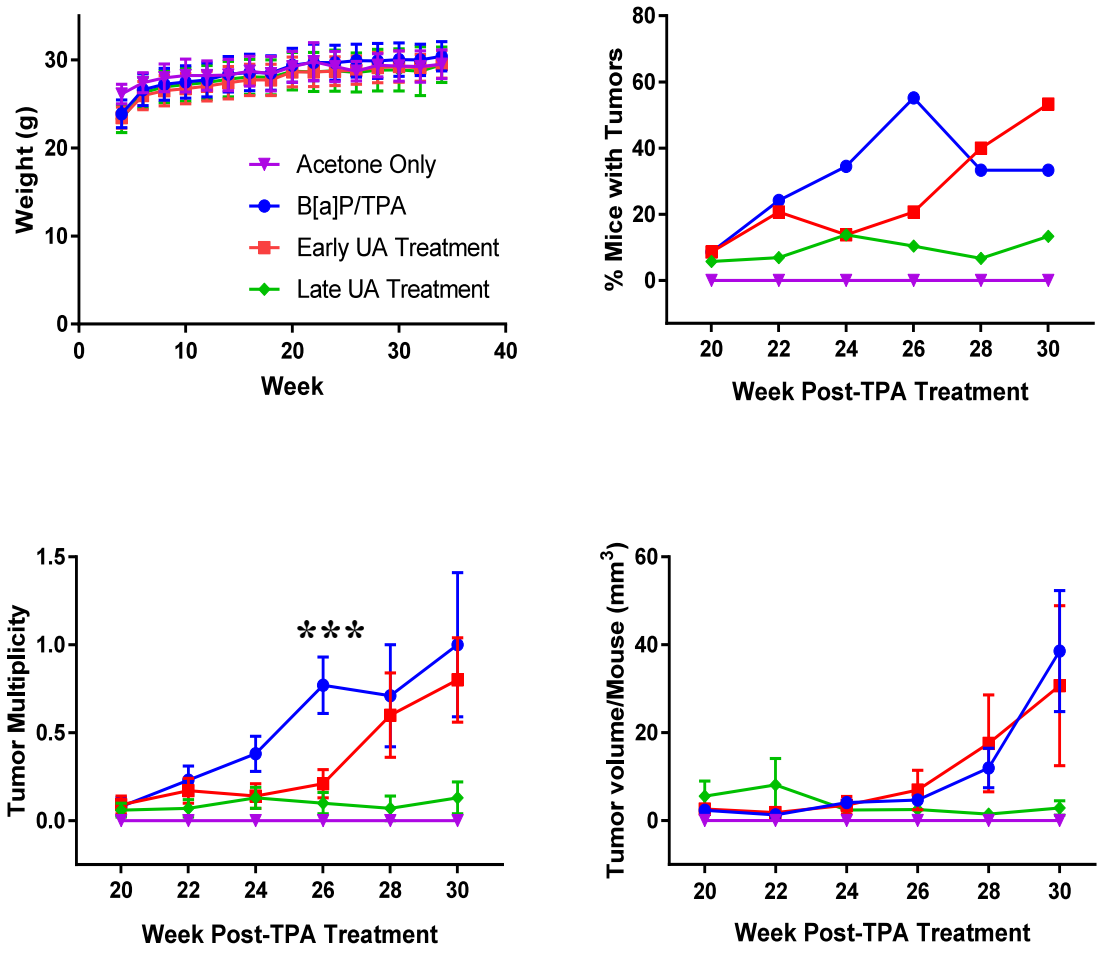
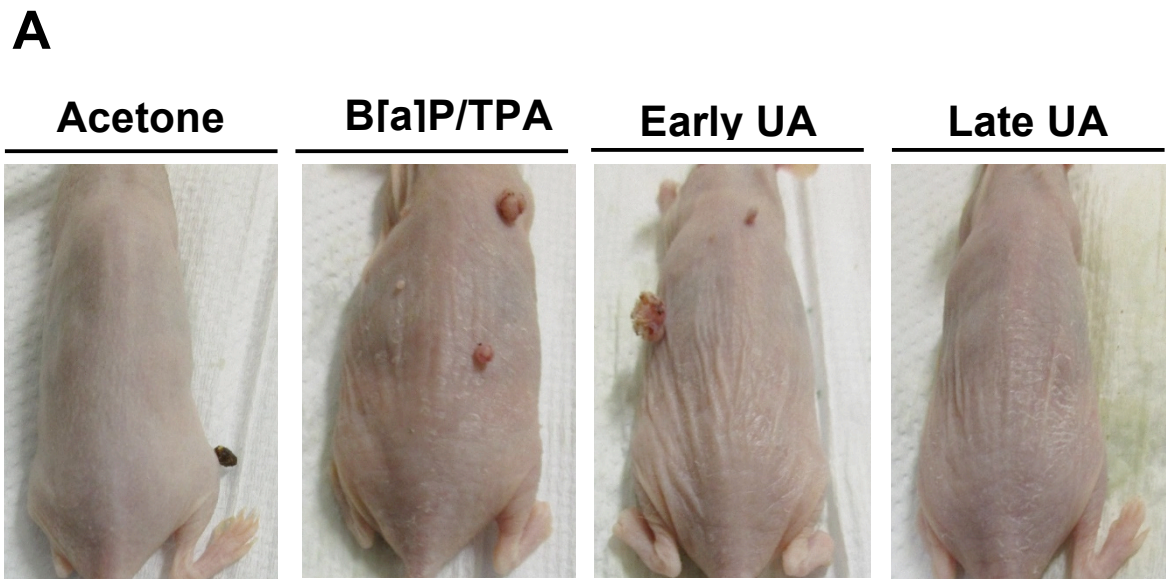


Figure 5. Late UA treatment prevents the progression of skin carcinogenesis. A) Treatment does not affect the body weight of animals. Mice were treated with topical applications of acetone (vehicle), TPA, and UA (early or late) twice a week for 31 weeks post-TPA treatment. Mice were weighed every two weeks and evaluated for any skin abnormalities. B) Percentage of mice with tumors. C) Number of tumors per mouse. P<0.001*** B[a]P vs. Late UA. D) Volume of tumors per mouse.

3.3.5 B[a]P/TPA SKH-1 skin-carcinogenesis model generates squamous cell papillomas

SKH-1 hairless mice are widely used in dermatological research and are often used in UVB-induced skin carcinogenesis studies (294). However, early research in hairless mice reported a poor susceptibility to two-stage chemical carcinogenesis (295). We demonstrate here squamous papillomas can be generated in a B[a]P/TPA SKH-1 skin carcinogenesis model (Figure 6). Papillomas were present in B[a]P/TPA, early UA, and a small fraction of late UA treatment groups (Figure 6A and Figure 5B, 5C, 5D). Whole skin samples were obtained from all mice and processed for histopathological analysis as described in Materials and Methods. Two samples were randomly selected from each group. A representative image of the dorsal region of one of the two mice randomly selected demonstrates the presence of clonal-like growths (Figure 6A). Corresponding H&E images and pathologist consultation confirmed squamous papilloma formation in B[a]P/TPA and early UA groups while only a hyperplastic epidermis is seen in late UA treatment (Figure 6). In a two-stage skin carcinogenesis model a hyperplastic epidermis is expected early in the promotion phase without therapeutic intervention (265). As such, therapeutic intervention with UA (late) suppressed skin carcinogenesis as evidenced in tumor statistics and histopathological analysis (Figure 5 and Figure 6).


A
Acetone
B[a]P/TPA
Early UA
Late UA

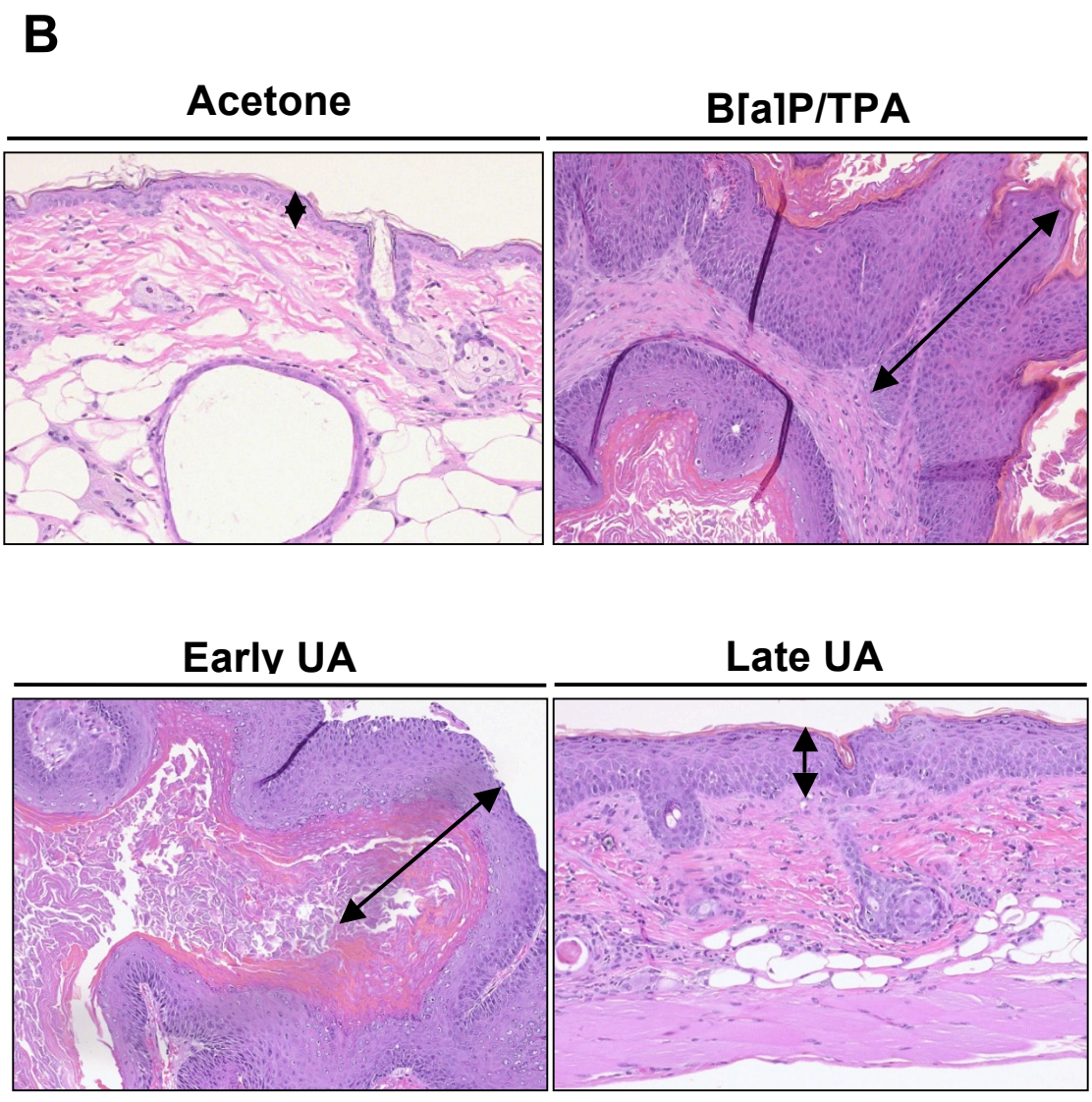

B
Acetone
B[a]P/TPA
Early UA
Late UA

Figure 6. Representative images of each group at 31 weeks post-TPA treatment. A) Dorsal skin images of mice representative of each group. B) H&E images of whole skin samples and tumor samples of animals shown in A. B) Acetone: Normal skin from SKH-1 mice from surface to deep margin had several layers of desquamated keratin layer, 1-2 layers of epidermal cells, dermal extracellular matrix admixed with sebaceous glands, subcuticular adipose and pannicular fat. B) B[a]P/TPA:- Squamous cell papilloma. There was an exophytic neoplasm composed of focal to multifocal proliferation of squamous epithelium arranged in ridges and folds with irregular thickening of the overlying keratin layer. Neoplastic epithelium was well differentiated and uniform in appearance with no evidence of basement membrane invasion. There was orderly differentiation, maturation and keratinization near neoplasms' surface. Inflammation and erosion of the surface of the papilloma was sometimes present. B) Early UA: Squamous cell papilloma. There was an exophytic neoplasm composed of focal to multifocal proliferation of squamous epithelium arranged in ridges and folds with irregular thickening of the overlying keratin layer. Neoplastic epithelium was well differentiated and uniform in appearance with no evidence of basement membrane invasion. There was orderly differentiation, maturation and keratinization near neoplasms' surface. Inflammation and erosion of the surface of the papilloma was sometime present. B) Late UA: Skin with epithelial hyperplasia and slight increases in dermal mononuclear cells. Epithelium was composed of 5-15 layers of epithelial cells with scattered rare vacuolated degenerate cells or pyknotic necrotic cells. The dermal extracellular matrix containing increased numbers of lymphocytes, macrophages and to a lesser extent, neutrophils.

3.4 Discussion

In addition to UV, environmental pollutants such as B[a]P have been shown to promote skin cancer development (124, 271-273). B[a]P is one of the most common environmental carcinogens found as a byproduct from car exhaust and coal burning. The B[a]P/TPA two-stage skin carcinogenesis model has been used to study the potential involvement of epigenetic regulation including DNA methylation and histone modifications (49, 174, 296-300). Epigenetic regulation and global genomic hypermethylation are features associated with the enhancement of many cancers including prostate and colon cancers. Our laboratory has shown Nrf2 is epigenetically silenced during prostate carcinogenesis in TRAMP mice and has also been shown to occur in human prostate cancers (230). A number of studies in our laboratory have shown phytochemicals are able to restore the expression of Nrf2 by the demethylation of the Nrf2 promoter region (207, 301). In Chapter II we demonstrate UA is able to restore the expression of Nrf2 by the demethylation of the Nrf2 promoter region *in vitro*. In this study we've shown UA is able to reduce inflammation in an ear edema assay in and Nrf2 may be required (Figure 4) and late UA treatment is able to suppress skin carcinogenesis in a novel B[a]P/TPA in SKH-1 mice (Figure 6).

The two-stage carcinogenesis model has been explored extensively since the 1920s and a culmination of research has shown that the susceptibility of the model is dependent on mouse strain (302-305). Furred mice have been commonly used for two-stage carcinogenesis model investigation. However, hairless mouse models closely resemble the texture of human skin and have been shown to be more beneficial in skin cancer models (306). Using a hairless mouse model such as SKH-1 offers several

advantages over furred mouse models: 1) Saves time and avoids inflammatory reactions because hair depilation is not required. 2) The start of early carcinogenic response is easily spotted due to their non-pigmented skin. 3) The mice are immunocompetent (euthymic) and react to the development of SCC in a manner comparable to humans. Because the use of an SKH-1 mouse strain in a B[a]P/TPA model was novel, several pilot studies were conducted to determine the feasibility of model used. Firstly, we demonstrated TPA was able to induce inflammation in an ear edema assay using a range of doses that mimicked doses explored in previous two-stage carcinogenesis models. TPA-induced inflammation was demonstrated at all doses (Figure 2A). Taking into account potential toxic effects at the higher doses, 6.8nmol/200μL was selected for the main study. Secondly, we demonstrated UA was able to attenuate TPA-induced inflammation significantly at the highest dose (Figure 2B). Previous studies in our laboratory demonstrated the maximum tolerated dose for use in SKH-1 mice was 2 μmol/200μL. With this in mind, 2 μmol/200μL was selected for use in the main study.

In Chapter I we discuss the importance of dose, time, and model systems in the pharmacological response of phytochemicals. After determining the optimal doses for TPA and UA to use in the main study, we sought to determine the optimal time needed for UA to attenuate TPA-induced inflammation. We demonstrated UA is able to attenuate TPA-induced inflammation at 30-minutes but not at 15-minutes (Figure 3). This suggested, in addition to dose, timing is important in UA treatment. Noting this difference, UA application 30-minutes prior to TPA application was chosen to move forward.

A previous study in our laboratory demonstrated skin carcinogenesis induced in a DMBA/TPA model was enhanced in Nrf2 (-/-) mice in comparison to Nrf2 (+/+) mice and topical treatment of SFN was able to protect against skin carcinogenesis in Nrf2 (+/+) but not Nrf2 (-/-) (54). The same was observed for UA in the TPA-induced inflammation ear edema assay (Figure 4). In the absence of Nrf2 TPA-induced ear edema weight increased 10% despite treatment with UA suggesting Nrf2 is required.

Phytochemicals can prevent the initiation of carcinogenesis via the induction of the cellular defense detoxifying/antioxidant enzymes mediated by Nrf2 [Lee, 2013 #95]. Research has shown treatment with isothiocyanates (ITCs) pre-initiation inhibited cancer development in animal studies of chemically-induced carcinogenesis (307). With this in mind, UA treatment pre-initiation and post-initiation was assessed in the main study (Figure 1). As expected, animal weight increased over the course of the study suggesting treatment applications were not toxic (Figure 5A). UA treatment post-initiation (Late UA) was shown to dramatically suppress carcinogenesis (Figure 5B, 5C, 5D). Of note, UA treatment pre-initiation (Early UA) was comparable to the B[a]P/TPA positive control group suggesting early UA treatment had the opposite effect of what was expected (Figure 5B, 5C, 5D and Figure 6). A similar phenomenon has been observed for capsaicin. While a number of studies have shown capsaicin's chemoprevention potential (293). Other studies suggest it may act as a co-carcinogen or tumor promoter (293). These observations bring us back to the importance of dose, timing, and model systems used to evaluate the chemopreventive effects of dietary phytochemicals. It is possible UA added prior to B[a]P initiation had a co-carcinogenic effect. UA may be acting as a co-carcinogen by positively regulating the aryl hydrocarbon receptor (AHR) [Shin, 2007

#502]. B[a]P bind to AHR which stimulates the expression of CYP1 enzymes [Nebert, 2004 #503]. These enzymes metabolize B[a]P into toxic metabolites responsible for DNA adduct formation and ultimately genetic mutations. These observations warrant further investigation.

3.5 Conclusion

To our knowledge this is the first time a B[a]P/TPA skin carcinogenesis model has been explored in SKH-1 mice for chemopreventive studies. This study demonstrates the B[a]P/TPA SKH-1 model successfully generated squamous papillomas and can be used for future chemoprevention studies (Figure 6). Samples from this study will be further examined for global epigenomic changes through the different stages of skin carcinogenesis and in comparison the global epigenomic changes that arise during UA treatment using Next Generation Sequencing (NGS). This will allow us to determine the role, if any, of epigenetic modulation of Nrf2 in skin carcinogenesis. Ultimately, these studies will contribute to the identification of potential epigenomic biomarkers during skin carcinogenesis that can serve as novel therapeutic targets.

Chapter IV

Significance and Future Prospects

4.1 Significance

4.1.1 Oxidative stress, inflammation, and the Nrf2 pathway in carcinogenesis

Oxidative stress occurs when there is an imbalance between the anti-oxidant defense system and the production of ROS. Although important secondary messengers, important secondary messengers, the activities of ROS can be detrimental to the cell if left unattended. In addition to endogenous production, ROS can be induced through UV-radiation and environmental pollutants. The anti-oxidant stress defense system is responsible for relieving the deleterious effects of ROS. System enzymes such as SODs, catalase, and GPxs directly inactivate ROS. In addition, there are other antioxidant enzymes such as NQO1, UGTs, Txnd, and GSR that facilitate the detoxification of ROS using reduction/conjugation reactions, which facilitate excretion. Other proteins, which provide protection, include HO-1 and HO-2. Nrf2 regulates the genes encoding these enzymes and proteins. Under normal conditions, Nrf2 is sequestered by Keap1 in the cytosol and is degraded by the proteasome. Upon oxidative stress, Nrf2 is released by Keap1 and translocates to the nucleus where it binds the ARE of the antioxidant defense system genes. An excess of ROS can cause an imbalance in the system and induce oxidative stress; a hallmark of cancer (166, 167). The excess oxidative stress results in the activation of pro-inflammatory processes. If left unattended the excess oxidative stress will lead to chronic inflammation and in turn can lead to the initiation of cancer via genetic mutations and epigenetic aberrations. For this reason, the relationship between oxidative stress and inflammation as well as the role of Nrf2 in carcinogenesis is an active area of investigation in chemoprevention by phytochemicals.

4.1.2 Cancer epigenetics and regulation using phytochemicals

DNA methylation and histone modifications have been shown to contribute to the progression of cancer (170) and have been shown to be a hallmark of cancer (173-177). For example, the promoter region of human GSTP1 is hypermethylated in approximately 70%-100% of prostate cancer specimens (178-180). In addition, aberrant epigenetic changes have been associated with the development and progression of skin cancer (49, 181-183). As such, targeting the reversal of DNA methylation and histone modifications presents a novel target for the prevention and treatment of cancer. Chemotherapeutic targeting DNMTs and HDACs have already been approved by the FDA (184). However, they are plagued by adverse events. For this reason, targeting epigenetic modifications using dietary phytochemicals for the prevention or treatment of cancer has become an attractive area of investigation. Dietary phytochemicals have been shown to prevent cancer through epigenetic modulation (185-187).

4.1.3 Anti-cancer and Anti-oxidative effects of Ursolic Acid and the incidence of skin cancer

UA possesses the ability to modulate enzymes such as SOD and GST, regulated by Nrf2, and decrease (ROS) activity (311-313). In addition, a study in HaCaT cells, demonstrated UA's ability to hinder UVA-induced ROS production, lipid peroxidation, MMP-2 expression, and DNA damage (314). Furthermore, UA inhibits B[a]P-and 7,12-DMBA-induced tumor initiating activity *in vitro* and suppresses TPA-induced skin inflammation and tumor promotion in CD-1 and ICR mice (201, 202). UA has been shown to exhibit chemopreventive and anti-cancer effects against a number of cancers *in vitro* and *in vivo* including breast, hepatocellular, gastric, and colorectal (200). However,

the chemopreventive effects of UA have not been extensively explored in skin cancer. As the protective layer of the body, the skin is in constant contact with environmental pollutants and UV-irradiation. Unsurprisingly, skin cancer incidence is rising steadily worldwide and NMSC is the most common cancer in the United States with over 4.0 million new cases diagnosed in 2012 (315). 700,000 were cutaneous SCC which metastasize at a rate of 2-6% (139). Over 5.4 million cases of NMSC are treated annually surmising an annual cost of approximately $5 billion in the treatment of NMSC (316, 317). The development of strategies to reduce the occurrence of chemical induced skin cancer would have a major impact on reducing the economic burden of society.

4.2 Future Prospects

According to the National Center of Health Statistics, cancer was the culprit of nearly 600,000 deaths in 2016 in the United States. It is by far one of the most heterogeneous diseases to treat. Treatment for metastasized cancers remains a challenge despite modern diagnostics and treatment regimens. For this reason, alternative approaches are needed. Chemoprevention using dietary phytochemicals such as UA in the prevention of initiation and/or progression of cancer poses a promising alternative strategy. In this research we have showcased the potential use of UA as a promising chemopreventive agent against skin cancer. The epigenetic modulation of Nrf2 in skin carcinogenesis remains to be explored and will offer novel insight into the global epigenomic response of UA in skin carcinogenesis using NGS.

The epigenetic modulation of Nrf2 and the global epigenomic response of other dietary phytochemicals and synthetic compounds, such as CDDO-Im, have been explored

in our laboratory. Research conducted parallel to early UA studies demonstrated unlike UA, CDDO-Im may not posses the ability to epigenetically modulate Nrf2 (Figure 1).

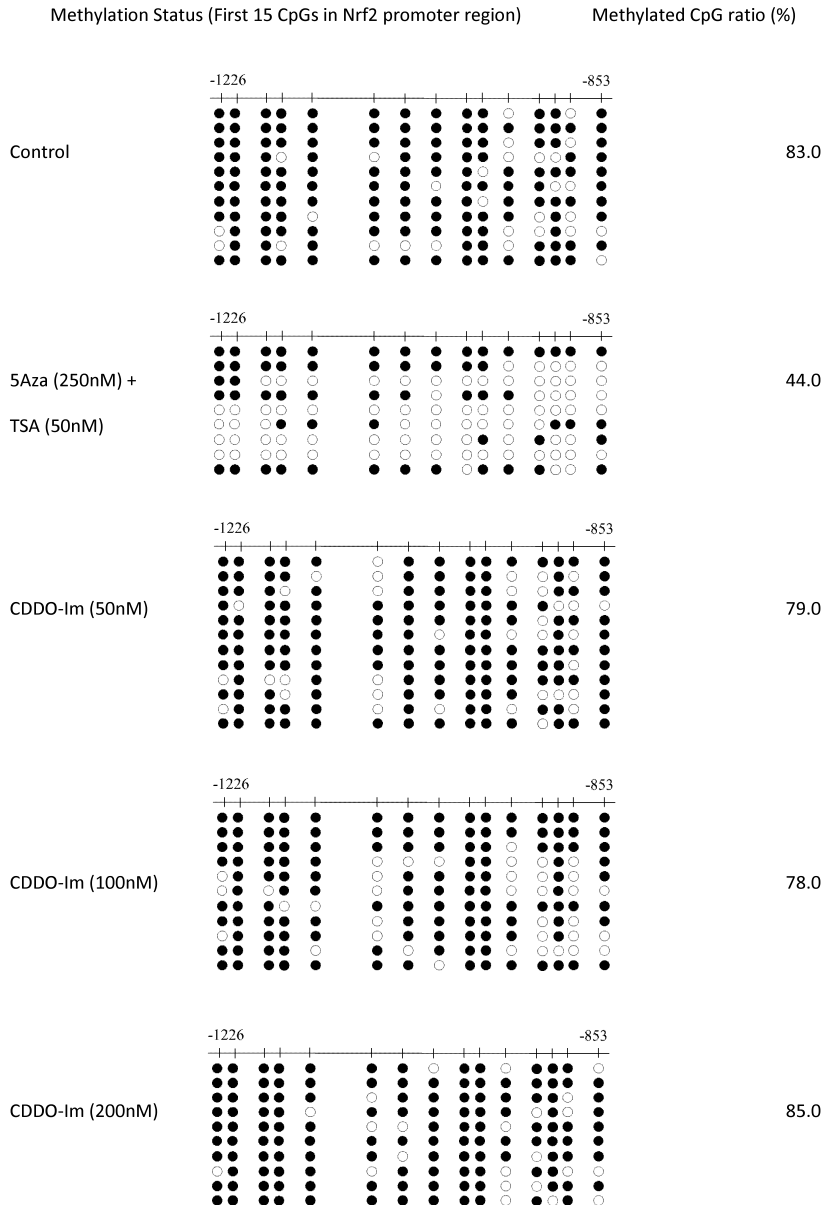


Figure 1. CDDO-Im may not decrease Nrf2 promoter DNA methylation in JB6 P+ cells. The cells were treated with each concentration of CDDO-Im for 5 days, and then the genomic DNA was isolated for bisulfite conversion as previously detailed for UA. The methylation status of the first 15 CpG sites, the region between -1226 and -863 relative to the translational start site, within the promoter of Nrf2, was analyzed. Positive

control cells were treated with 5-aza (250 nM) for 48 h and TSA (50 nM) for 24 h. Ten individual clones were analyzed. The filled and open dots indicate methylated and unmethylated CpG. The data are expressed as a percentage of the total number of methylated cytosines *vs.* total 15 CpGs of three independent experiments

Although preliminary data suggests CDDO-Im does not posses this ability in the model system used, it may not be the case. We have seen with this research and that of others the *in vitro-in vivo* dose response of phytochemicals can vary due in part to the cell line/animal model used, the assay system of the biomarker used for the readout, chemical structure of the functional analog of the phytochemical, and the source of compounds used for the treatment study (292). While the response may vary across different experimental designs, the chemopreventive efficacy appears to remain and demonstrates the therapeutic potential of dietary phytochemicals in cancer prevention and in health in general.

Chapter V

References

1. Estimated new cases and deaths from skin (nonmelanoma) cancer in the United States in 2010: National Cancer Institute (NCI); 2010. Available from: http://www.cancer.gov/cancertopics/types/skin.
2. Sporn MB. Perspective: The big C - for Chemoprevention. Nature. 2011;471(7339):S10-1. doi: 10.1038/471S10a. PubMed PMID: 21430710.
3. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res. 2008;25(9):2097-116. doi: 10.1007/s11095-008-9661-9. PubMed PMID: 18626751; PMCID: PMC2515569.
4. Advances in Cancer Research. Cameron EFaR, editor. New York, NY: Academic Press, INC; 1980. 331 p.
5. Lee JH, Khor TO, Shu L, Su ZY, Fuentes F, Kong AN. Dietary phytochemicals and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression. Pharmacology & therapeutics. 2013;137(2):153-71. doi: 10.1016/j.pharmthera.2012.09.008. PubMed PMID: 23041058; PMCID: 3694988.
6. Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? Carcinogenesis. 2010;31(1):90-9. doi: 10.1093/carcin/bgp231. PubMed PMID: 19793802; PMCID: 2802668.
7. Liby KT, Yore MM, Sporn MB. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. Nature reviews Cancer. 2007;7(5):357-69. Epub 2007/04/21. doi: 10.1038/nrc2129. PubMed PMID: 17446857.
8. Perl A, Hanczko R, Telarico T, Oaks Z, Landas S. Oxidative stress, inflammation and carcinogenesis are controlled through the pentose phosphate pathway by transaldolase. Trends Mol Med. 2011;17(7):395-403. Epub 2011/03/08. doi: S1471-4914(11)00024-4 [pii]

10.1016/j.molmed.2011.01.014. PubMed PMID: 21376665; PMCID: 3116035.
9. Shanmugam MK, Dai X, Kumar AP, Tan BK, Sethi G, Bishayee A. Oleanolic acid and its synthetic derivatives for the prevention and therapy of cancer: preclinical and clinical evidence. Cancer Lett. 2014;346(2):206-16. doi: 10.1016/j.canlet.2014.01.016. PubMed PMID: 24486850; PMCID: PMC4004441.
10. Liu J. Pharmacology of oleanolic acid and ursolic acid. Journal of ethnopharmacology. 1995;49(2):57-68. PubMed PMID: 8847885.
11. Yang L, Shi W, Wang X, Zhou L, Cai Y, Liu H, Wu D. [Effect of ursolic acid on proliferation of T lymphoma cell lines Hut-78 cells and its mechanism]. Zhonghua Xue Ye Xue Za Zhi. 2015;36(2):153-7. doi: 10.3760/cma.j.issn.0253-2727.2015.02.015. PubMed PMID: 25778894.
12. Cho J, Rho O, Junco J, Carbajal S, Siegel D, Slaga TJ, DiGiovanni J. Effect of Combined Treatment with Ursolic Acid and Resveratrol on Skin Tumor Promotion by 12-O-Tetradecanoylphorbol-13-Acetate. Cancer Prev Res (Phila). 2015;8(9):817-25. doi: 10.1158/1940-6207.CAPR-15-0098. PubMed PMID: 26100520; PMCID: PMC4560654.
13. Aguiriano-Moser V, Svejda B, Li ZX, Sturm S, Stuppner H, Ingolic E, Hoger H, Siegl V, Meier-Allard N, Sadjak A, Pfragner R. Ursolic acid from Trailliaedoxa gracilis induces apoptosis in medullary thyroid carcinoma cells. Mol Med Rep. 2015;12(4):5003-11. doi: 10.3892/mmr.2015.4053. PubMed PMID: 26151624; PMCID: PMC4581794.

14. Zhang Y, Kong C, Zeng Y, Wang L, Li Z, Wang H, Xu C, Sun Y. Ursolic acid induces PC-3 cell apoptosis via activation of JNK and inhibition of Akt pathways in vitro. Mol Carcinog. 2010;49(4):374-85. doi: 10.1002/mc.20610. PubMed PMID: 20146252.
15. Kassi E, Papoutsi Z, Pratsinis H, Aligiannis N, Manoussakis M, Moutsatsou P. Ursolic acid, a naturally occurring triterpenoid, demonstrates anticancer activity on human prostate cancer cells. J Cancer Res Clin Oncol. 2007;133(7):493-500. doi: 10.1007/s00432-007-0193-1. PubMed PMID: 17516089.
16. Prasad S, Yadav VR, Sung B, Gupta SC, Tyagi AK, Aggarwal BB. Ursolic acid inhibits the growth of human pancreatic cancer and enhances the antitumor potential of gemcitabine in an orthotopic mouse model through suppression of the inflammatory microenvironment. Oncotarget. 2016;7(11):13182-96. doi: 10.18632/oncotarget.7537. PubMed PMID: 26909608; PMCID: PMC4914350.
17. Gayathri R, Priya DK, Gunassekaran GR, Sakthisekaran D. Ursolic acid attenuates oxidative stress-mediated hepatocellular carcinoma induction by diethylnitrosamine in male Wistar rats. Asian Pac J Cancer Prev. 2009;10(5):933-8. PubMed PMID: 20104993.
18. Kowalczyk MC, Walaszek Z, Kowalczyk P, Kinjo T, Hanausek M, Slaga TJ. Differential effects of several phytochemicals and their derivatives on murine keratinocytes in vitro and in vivo: implications for skin cancer prevention. Carcinogenesis. 2009;30(6):1008-15. doi: 10.1093/carcin/bgp069. PubMed PMID: 19329757; PMCID: PMC2691143.
19. Checker R, Sandur SK, Sharma D, Patwardhan RS, Jayakumar S, Kohli V, Sethi G, Aggarwal BB, Sainis KB. Potent anti-inflammatory activity of ursolic acid, a triterpenoid antioxidant, is mediated through suppression of NF-kappaB, AP-1 and NF-AT. PLoS One. 2012;7(2):e31318. Epub 2012/03/01. doi: 10.1371/journal.pone.0031318. PubMed PMID: 22363615; PMCID: 3282718.
20. Chun J, Lee C, Hwang SW, Im JP, Kim JS. Ursolic acid inhibits nuclear factor-kappaB signaling in intestinal epithelial cells and macrophages, and attenuates experimental colitis in mice. Life Sci. 2014;110(1):23-34. doi: 10.1016/j.lfs.2014.06.018. PubMed PMID: 24992474.
21. Ramos AA, Pereira-Wilson C, Collins AR. Protective effects of ursolic acid and luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells. Mutat Res. 2010;692(1-2):6-11. doi: 10.1016/j.mrfmmm.2010.07.004. PubMed PMID: 20659486.
22. Tsai SJ, Yin MC. Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells. J Food Sci. 2008;73(7):H174-8. doi: 10.1111/j.1750-3841.2008.00864.x. PubMed PMID: 18803714.
23. Wu J, Zhao S, Tang Q, Zheng F, Chen Y, Yang L, Yang X, Li L, Wu W, Hann SS. Activation of SAPK/JNK mediated the inhibition and reciprocal interaction of DNA methyltransferase 1 and EZH2 by ursolic acid in human lung cancer cells. J Exp Clin Cancer Res. 2015;34:99. doi: 10.1186/s13046-015-0215-9. PubMed PMID: 26362062; PMCID: PMC4567809.
24. Chen IH, Lu MC, Du YC, Yen MH, Wu CC, Chen YH, Hung CS, Chen SL, Chang FR, Wu YC. Cytotoxic triterpenoids from the stems of Microtropis japonica.

Journal of natural products. 2009;72(7):1231-6. Epub 2009/06/19. doi: 10.1021/np800694b. PubMed PMID: 19534471.
25. Bonaccorsi I, Altieri F, Sciamanna I, Oricchio E, Grillo C, Contartese G, Galati EM. Endogenous reverse transcriptase as a mediator of ursolic acid's anti-proliferative and differentiating effects in human cancer cell lines. Cancer Lett. 2008;263(1):130-9. doi: 10.1016/j.canlet.2007.12.026. PubMed PMID: 18282657.
26. Zhang T, He YM, Wang JS, Shen J, Xing YY, Xi T. Ursolic acid induces HL60 monocytic differentiation and upregulates C/EBPbeta expression by ERK pathway activation. Anticancer Drugs. 2011;22(2):158-65. doi: 10.1097/CAD.0b013e3283409673. PubMed PMID: 20948428.
27. Zhang J, Wang W, Qian L, Zhang Q, Lai D, Qi C. Ursolic acid inhibits the proliferation of human ovarian cancer stem-like cells through epithelial-mesenchymal transition. Oncology reports. 2015;34(5):2375-84. Epub 2015/09/02. doi: 10.3892/or.2015.4213. PubMed PMID: 26323892.
28. Ramirez-Rodriguez AM, Gonzalez-Ortiz M, Martinez-Abundis E, Acuna Ortega N. Effect of Ursolic Acid on Metabolic Syndrome, Insulin Sensitivity, and Inflammation. Journal of medicinal food. 2017;20(9):882-6. Epub 2017/06/10. doi: 10.1089/jmf.2017.0003. PubMed PMID: 28598231.
29. Cho YH, Lee SY, Kim CM, Kim ND, Choe S, Lee CH, Shin JH. Effect of Loquat Leaf Extract on Muscle Strength, Muscle Mass, and Muscle Function in Healthy Adults: A Randomized, Double-Blinded, and Placebo-Controlled Trial. Evidence-based complementary and alternative medicine : eCAM. 2016;2016:4301621. Epub 2016/12/22. doi: 10.1155/2016/4301621. PubMed PMID: 27999607; PMCID: PMC5143716 paper.
30. Tang L, Zirpoli GR, Guru K, Moysich KB, Zhang Y, Ambrosone CB, McCann SE. Intake of cruciferous vegetables modifies bladder cancer survival. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2010;19(7):1806-11. doi: 10.1158/1055-9965.EPI-10-0008. PubMed PMID: 20551305; PMCID: 2901397.
31. Palmer S. Diet, nutrition, and cancer. Progress in food & nutrition science. 1985;9(3-4):283-341. PubMed PMID: 3010379.
32. Fahey JW, Zalcmann AT, Talalay P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry. 2001;56(1):5-51. PubMed PMID: 11198818.
33. Kliebenstein DJ, Kroymann J, Mitchell-Olds T. The glucosinolate-myrosinase system in an ecological and evolutionary context. Current opinion in plant biology. 2005;8(3):264-71. doi: 10.1016/j.pbi.2005.03.002. PubMed PMID: 15860423.
34. Gupta P, Kim B, Kim SH, Srivastava SK. Molecular targets of isothiocyanates in cancer: recent advances. Molecular nutrition & food research. 2014;58(8):1685-707. doi: 10.1002/mnfr.201300684. PubMed PMID: 24510468; PMCID: 4122603.
35. Keum YS, Jeong WS, Kong AN. Chemopreventive functions of isothiocyanates. Drug news & perspectives. 2005;18(7):445-51. doi: 10.1358/dnp.2005.18.7.939350. PubMed PMID: 16362084.

36. Hayes JD, Kelleher MO, Eggleston IM. The cancer chemopreventive actions of phytochemicals derived from glucosinolates. European journal of nutrition. 2008;47 Suppl 2:73-88. doi: 10.1007/s00394-008-2009-8. PubMed PMID: 18458837.
37. Ji Y, Kuo Y, Morris ME. Pharmacokinetics of dietary phenethyl isothiocyanate in rats. Pharm Res. 2005;22(10):1658-66. doi: 10.1007/s11095-005-7097-z. PubMed PMID: 16180123.
38. Mi L, Wang X, Govind S, Hood BL, Veenstra TD, Conrads TP, Saha DT, Goldman R, Chung FL. The role of protein binding in induction of apoptosis by phenethyl isothiocyanate and sulforaphane in human non-small lung cancer cells. Cancer Res. 2007;67(13):6409-16. doi: 10.1158/0008-5472.CAN-07-0340. PubMed PMID: 17616701.
39. Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, Soteropoulos P, Tolias P, Hart RP, Kong AN. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. The Journal of pharmacology and experimental therapeutics. 2004;310(1):263-71. doi: 10.1124/jpet.103.064261. PubMed PMID: 14988420.
40. Hu R, Khor TO, Shen G, Jeong WS, Hebbar V, Chen C, Xu C, Reddy B, Chada K, Kong AN. Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable. Carcinogenesis. 2006;27(10):2038-46. doi: 10.1093/carcin/bgl049. PubMed PMID: 16675473.
41. Yuan JM, Stepanov I, Murphy SE, Wang R, Allen S, Jensen J, Strayer L, Adams-Haduch J, Upadhyaya P, Le C, Kurzer MS, Nelson HH, Yu MC, Hatsukami D, Hecht SS. Clinical Trial of 2-Phenethyl Isothiocyanate as an Inhibitor of Metabolic Activation of a Tobacco-Specific Lung Carcinogen in Cigarette Smokers. Cancer Prev Res (Phila). 2016;9(5):396-405. Epub 2016/03/10. doi: 10.1158/1940-6207.capr-15-0380. PubMed PMID: 26951845; PMCID: PMC4854759.
42. Cipolla BG, Mandron E, Lefort JM, Coadou Y, Della Negra E, Corbel L, Le Scodan R, Azzouzi AR, Mottet N. Effect of Sulforaphane in Men with Biochemical Recurrence after Radical Prostatectomy. Cancer Prev Res (Phila). 2015;8(8):712-9. doi: 10.1158/1940-6207.CAPR-14-0459. PubMed PMID: 25968598.
43. Zhang Z, Atwell LL, Farris PE, Ho E, Shannon J. Associations between cruciferous vegetable intake and selected biomarkers among women scheduled for breast biopsies. Public health nutrition. 2016;19(7):1288-95. Epub 2015/09/04. doi: 10.1017/s136898001500244x. PubMed PMID: 26329135.
44. Cheung KL, Kong AN. Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. The AAPS journal. 2010;12(1):87-97. doi: 10.1208/s12248-009-9162-8. PubMed PMID: 20013083; PMCID: 2811646.
45. Munday R, Munday CM. Induction of phase II detoxification enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds. Journal of agricultural and food chemistry. 2004;52(7):1867-71. doi: 10.1021/jf030549s. PubMed PMID: 15053522.
46. Bacon JR, Williamson G, Garner RC, Lappin G, Langouet S, Bao Y. Sulforaphane and quercetin modulate PhIP-DNA adduct formation in human HepG2 cells and hepatocytes. Carcinogenesis. 2003;24(12):1903-11. doi: 10.1093/carcin/bgg157. PubMed PMID: 12949046.

47. Dingley KH, Ubick EA, Chiarappa-Zucca ML, Nowell S, Abel S, Ebeler SE, Mitchell AE, Burns SA, Steinberg FM, Clifford AJ. Effect of dietary constituents with chemopreventive potential on adduct formation of a low dose of the heterocyclic amines PhIP and IQ and phase II hepatic enzymes. Nutrition and cancer. 2003;46(2):212-21. doi: 10.1207/S15327914NC4602_15. PubMed PMID: 14690798.
48. Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, Lin W, Reddy B, Chan JY, Kong AN. Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray. Life Sci. 2006;79(20):1944-55. doi: 10.1016/j.lfs.2006.06.019. PubMed PMID: 16828809.
49. Millington GW. Epigenetics and dermatological disease. Pharmacogenomics. 2008;9(12):1835-50. Epub 2008/12/17. doi: 10.2217/14622416.9.12.1835. PubMed PMID: 19072642.
50. Xu C, Yuan X, Pan Z, Shen G, Kim JH, Yu S, Khor TO, Li W, Ma J, Kong AN. Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. Molecular cancer therapeutics. 2006;5(8):1918-26. doi: 10.1158/1535-7163.MCT-05-0497. PubMed PMID: 16928811.
51. Hong F, Freeman ML, Liebler DC. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chemical research in toxicology. 2005;18(12):1917-26. doi: 10.1021/tx0502138. PubMed PMID: 16359182.
52. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(6):3410-5. doi: 10.1073/pnas.051618798. PubMed PMID: 11248092; PMCID: 30667.
53. Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, Cheung WK, Chan JY, Reddy BS, Yang CS, Kong AN. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer Prev Res (Phila). 2008;1(3):187-91. Epub 2009/01/14. doi: 10.1158/1940-6207.capr-08-0028. PubMed PMID: 19138955; PMCID: Pmc3580177.
54. Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, Conney AH, Kong AN. Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res. 2006;66(16):8293-6. doi: 10.1158/0008-5472.CAN-06-0300. PubMed PMID: 16912211.
55. Xu C, Shen G, Chen C, Gelinas C, Kong AN. Suppression of NF-kappaB and NF-kappaB-regulated gene expression by sulforaphane and PEITC through IkappaBalpha, IKK pathway in human prostate cancer PC-3 cells. Oncogene. 2005;24(28):4486-95. doi: 10.1038/sj.onc.1208656. PubMed PMID: 15856023.
56. Jeong WS, Kim IW, Hu R, Kong AN. Modulatory properties of various natural chemopreventive agents on the activation of NF-kappaB signaling pathway. Pharm Res. 2004;21(4):661-70. PubMed PMID: 15139523.
57. Heiss E, Gerhauser C. Time-dependent modulation of thioredoxin reductase activity might contribute to sulforaphane-mediated inhibition of NF-kappaB binding to DNA. Antioxidants & redox signaling. 2005;7(11-12):1601-11. doi: 10.1089/ars.2005.7.1601. PubMed PMID: 16356123.

58. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. The Journal of biological chemistry. 2001;276(34):32008-15. Epub 2001/06/19. doi: 10.1074/jbc.M104794200. PubMed PMID: 11410599.
59. Liao BC, Hsieh CW, Lin YC, Wung BS. The glutaredoxin/glutathione system modulates NF-kappaB activity by glutathionylation of p65 in cinnamaldehyde-treated endothelial cells. Toxicological sciences : an official journal of the Society of Toxicology. 2010;116(1):151-63. doi: 10.1093/toxsci/kfq098. PubMed PMID: 20351055.
60. Bellezza I, Tucci A, Galli F, Grottelli S, Mierla AL, Pilolli F, Minelli A. Inhibition of NF-kappaB nuclear translocation via HO-1 activation underlies alpha-tocopheryl succinate toxicity. The Journal of nutritional biochemistry. 2012;23(12):1583-91. doi: 10.1016/j.jnutbio.2011.10.012. PubMed PMID: 22444871.
61. Wagner AE, Will O, Sturm C, Lipinski S, Rosenstiel P, Rimbach G. DSS-induced acute colitis in C57BL/6 mice is mitigated by sulforaphane pre-treatment. The Journal of nutritional biochemistry. 2013;24(12):2085-91. doi: 10.1016/j.jnutbio.2013.07.009. PubMed PMID: 24231100.
62. Saw CL, Huang MT, Liu Y, Khor TO, Conney AH, Kong AN. Impact of Nrf2 on UVB-induced skin inflammation/photoprotection and photoprotective effect of sulforaphane. Molecular carcinogenesis. 2011;50(6):479-86. doi: 10.1002/mc.20725. PubMed PMID: 21557329.
63. Wong CP, Hsu A, Buchanan A, Palomera-Sanchez Z, Beaver LM, Houseman EA, Williams DE, Dashwood RH, Ho E. Effects of sulforaphane and 3,3'-diindolylmethane on genome-wide promoter methylation in normal prostate epithelial cells and prostate cancer cells. PloS one. 2014;9(1):e86787. doi: 10.1371/journal.pone.0086787. PubMed PMID: 24466240; PMCID: 3899342.
64. Wang LG, Chiao JW. Prostate cancer chemopreventive activity of phenethyl isothiocyanate through epigenetic regulation (review). International journal of oncology. 2010;37(3):533-9. PubMed PMID: 20664922.
65. Fuentes F, Paredes-Gonzalez X, Kong AT. Dietary Glucosinolates Sulforaphane, Phenethyl Isothiocyanate, Indole-3-Carbinol/3,3'-Diindolylmethane: Anti-Oxidative Stress/Inflammation, Nrf2, Epigenetics/Epigenomics and Cancer Chemopreventive Efficacy. Current pharmacology reports. 2015;1(3):179-96. doi: 10.1007/s40495-015-0017-y. PubMed PMID: 26457242; PMCID: 4596548.
66. Zhang C, Su ZY, Khor TO, Shu L, Kong AN. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. Biochem Pharmacol. 2013;85(9):1398-404. doi: 10.1016/j.bcp.2013.02.010. PubMed PMID: 23416117; PMCID: 4123317.
67. Su ZY, Zhang C, Lee JH, Shu L, Wu TY, Khor TO, Conney AH, Lu YP, Kong AN. Requirement and epigenetics reprogramming of Nrf2 in suppression of tumor promoter TPA-induced mouse skin cell transformation by sulforaphane. Cancer Prev Res (Phila). 2014;7(3):319-29. doi: 10.1158/1940-6207.CAPR-13-0313-T. PubMed PMID: 24441674.
68. Ho E, Clarke JD, Dashwood RH. Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. The Journal of nutrition. 2009;139(12):2393-6. doi: 10.3945/jn.109.113332. PubMed PMID: 19812222; PMCID: 2777483.

69. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood). 2007;232(2):227-34. Epub 2007/01/30. doi: 232/2/227 [pii]. PubMed PMID: 17259330; PMCID: 2267876.
70. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. Carcinogenesis. 2006;27(4):811-9. doi: 10.1093/carcin/bgi265. PubMed PMID: 16280330; PMCID: PMC2276576.
71. Wang LG, Liu XM, Fang Y, Dai W, Chiao FB, Puccio GM, Feng J, Liu D, Chiao JW. De-repression of the p21 promoter in prostate cancer cells by an isothiocyanate via inhibition of HDACs and c-Myc. Int J Oncol. 2008;33(2):375-80. PubMed PMID: PMID: 18636159.
72. Liu K, Cang S, Ma Y, Chiao JW. Synergistic effect of paclitaxel and epigenetic agent phenethyl isothiocyanate on growth inhibition, cell cycle arrest and apoptosis in breast cancer cells. Cancer cell international. 2013;13(1):10. doi: 10.1186/1475-2867-13-10. PubMed PMID: 23388416; PMCID: 3637186.
73. Cang S, Ma Y, Chiao JW, Liu D. Phenethyl isothiocyanate and paclitaxel synergistically enhanced apoptosis and alpha-tubulin hyperacetylation in breast cancer cells. Experimental hematology & oncology. 2014;3(1):5. doi: 10.1186/2162-3619-3-5. PubMed PMID: 24495785; PMCID: 3927854.
74. Zhang C, Shu L, Kim H, Khor TO, Wu R, Li W, Kong AN. Phenethyl isothiocyanate (PEITC) suppresses prostate cancer cell invasion epigenetically through regulating microRNA-194. Molecular nutrition & food research. 2016;60(6):1427-36. doi: 10.1002/mnfr.201500918. PubMed PMID: 26820911.
75. Zhu H, Jia Z, Strobl JS, Ehrich M, Misra HP, Li Y. Potent induction of total cellular and mitochondrial antioxidants and phase 2 enzymes by cruciferous sulforaphane in rat aortic smooth muscle cells: cytoprotection against oxidative and electrophilic stress. Cardiovascular toxicology. 2008;8(3):115-25. doi: 10.1007/s12012-008-9020-4. PubMed PMID: 18607771.
76. Huang CS, Lin AH, Liu CT, Tsai CW, Chang IS, Chen HW, Lii CK. Isothiocyanates protect against oxidized LDL-induced endothelial dysfunction by upregulating Nrf2-dependent antioxidation and suppressing NFkappaB activation. Molecular nutrition & food research. 2013;57(11):1918-30. doi: 10.1002/mnfr.201300063. PubMed PMID: 23836589.
77. Hofmann T, Kuhnert A, Schubert A, Gill C, Rowland IR, Pool-Zobel BL, Glei M. Modulation of detoxification enzymes by watercress: in vitro and in vivo investigations in human peripheral blood cells. European journal of nutrition. 2009;48(8):483-91. doi: 10.1007/s00394-009-0039-5. PubMed PMID: 19636603.
78. Ullah MF. Sulforaphane (SFN): An Isothiocyanate in a Cancer Chemoprevention Paradigm. Medicines. 2015;2(3):141-56.
79. Yu S, Khor TO, Cheung KL, Li W, Wu TY, Huang Y, Foster BA, Kan YW, Kong AN. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLoS One. 2010;5(1):e8579. PubMed PMID: PMCID: PMC2799519.
80. Fimognari C, Lenzi M, Cantelli-Forti G, Hrelia P. Induction of differentiation in human promyelocytic cells by the isothiocyanate sulforaphane. In Vivo. 2008;22(3):317-20. PubMed PMID: 18610742.

81. Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, Lindeman GJ, Visvader JE. Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nat Cell Biol. 2007;9(2):201-9. Epub 2006/12/26. doi: 10.1038/ncb1530. PubMed PMID: 17187062.
82. Kouros-Mehr H, Bechis SK, Slorach EM, Littlepage LE, Egeblad M, Ewald AJ, Pai SY, Ho IC, Werb Z. GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model. Cancer Cell. 2008;13(2):141-52. doi: 10.1016/j.ccr.2008.01.011. PubMed PMID: 18242514; PMCID: 2262951.
83. Singh SV, Singh K. Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research. Carcinogenesis. 2012;33(10):1833-42. doi: 10.1093/carcin/bgs216. PubMed PMID: 22739026; PMCID: PMC3529556.
84. McCune K, Mehta R, Thorat MA, Badve S, Nakshatri H. Loss of ERalpha and FOXA1 expression in a progression model of luminal type breast cancer: insights from PyMT transgenic mouse model. Oncology reports. 2010;24(5):1233-9. Epub 2010/09/30. PubMed PMID: 20878115; PMCID: PMC2948410.
85. Dvorankova B, Smetana K, Jr., Chovanec M, Lacina L, Stork J, Plzakova Z, Galovicova M, Gabius HJ. Transient expression of keratin 19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. Int J Mol Med. 2005;16(4):525-31.
86. Gamet-Payrastre L. Signaling pathways and intracellular targets of sulforaphane mediating cell cycle arrest and apoptosis. Curr Cancer Drug Targets. 2006;6(2):135-45. PubMed PMID: 16529543.
87. Huong LD, Shin JA, Choi ES, Cho NP, Kim HM, Leem DH, Cho SD. beta-Phenethyl isothiocyanate induces death receptor 5 to induce apoptosis in human oral cancer cells via p38. Oral Dis. 2012;18(5):513-9. doi: 10.1111/j.1601-0825.2012.01905.x. PubMed PMID: 22309674.
88. Huong le D, Shim JH, Choi KH, Shin JA, Choi ES, Kim HS, Lee SJ, Kim SJ, Cho NP, Cho SD. Effect of beta-phenylethyl isothiocyanate from cruciferous vegetables on growth inhibition and apoptosis of cervical cancer cells through the induction of death receptors 4 and 5. Journal of agricultural and food chemistry. 2011;59(15):8124-31. Epub 2011/06/28. doi: 10.1021/jf2006358. PubMed PMID: 21702500.
89. Gupta P, Adkins C, Lockman P, Srivastava SK. Metastasis of Breast Tumor Cells to Brain Is Suppressed by Phenethyl Isothiocyanate in a Novel Metastasis Model. PloS one. 2013;8(6):e67278. Epub 2013/07/05. doi: 10.1371/journal.pone.0067278. PubMed PMID: 23826254; PMCID: PMC3695065.
90. Chen PY, Lin KC, Lin JP, Tang NY, Yang JS, Lu KW, Chung JG. Phenethyl Isothiocyanate (PEITC) Inhibits the Growth of Human Oral Squamous Carcinoma HSC-3 Cells through G(0)/G(1) Phase Arrest and Mitochondria-Mediated Apoptotic Cell Death. Evidence-based complementary and alternative medicine : eCAM. 2012;2012:718320. Epub 2012/08/25. doi: 10.1155/2012/718320. PubMed PMID: 22919418; PMCID: PMC3418800.
91. Tang NY, Huang YT, Yu CS, Ko YC, Wu SH, Ji BC, Yang JS, Yang JL, Hsia TC, Chen YY, Chung JG. Phenethyl isothiocyanate (PEITC) promotes G2/M phase arrest via p53 expression and induces apoptosis through caspase- and mitochondria-dependent

signaling pathways in human prostate cancer DU 145 cells. Anticancer Res. 2011;31(5):1691-702. Epub 2011/05/28. PubMed PMID: 21617228.
92. Jakubikova J, Cervi D, Ooi M, Kim K, Nahar S, Klippel S, Cholujova D, Leiba M, Daley JF, Delmore J, Negri J, Blotta S, McMillin DW, Hideshima T, Richardson PG, Sedlak J, Anderson KC, Mitsiades CS. Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate, in multiple myeloma. Haematologica. 2011;96(8):1170-9. Epub 2011/06/30. doi: 10.3324/haematol.2010.029363. PubMed PMID: 21712538; PMCID: PMC3148911.
93. Dai MY, Wang Y, Chen C, Li F, Xiao BK, Chen SM, Tao ZZ. Phenethyl isothiocyanate induces apoptosis and inhibits cell proliferation and invasion in Hep-2 laryngeal cancer cells. Oncology reports. 2016;35(5):2657-64. Epub 2016/03/18. doi: 10.3892/or.2016.4689. PubMed PMID: 26986926.
94. Yan H, Zhu Y, Liu B, Wu H, Li Y, Wu X, Zhou Q, Xu K. Mitogen-activated protein kinase mediates the apoptosis of highly metastatic human non-small cell lung cancer cells induced by isothiocyanates. Br J Nutr. 2011;106(12):1779-91. Epub 2011/07/08. doi: 10.1017/s0007114511002315. PubMed PMID: 21733335.
95. Wu X, Zhu Y, Yan H, Liu B, Li Y, Zhou Q, Xu K. Isothiocyanates induce oxidative stress and suppress the metastasis potential of human non-small cell lung cancer cells. BMC Cancer. 2010;10:269. Epub 2010/06/11. doi: 10.1186/1471-2407-10-269. PubMed PMID: 20534110; PMCID: PMC2891640.
96. Li SH, Fu J, Watkins DN, Srivastava RK, Shankar S. Sulforaphane regulates self-renewal of pancreatic cancer stem cells through the modulation of Sonic hedgehog-GLI pathway. Mol Cell Biochem. 2013;373(1-2):217-27. Epub 2012/11/07. doi: 10.1007/s11010-012-1493-6. PubMed PMID: 23129257.
97. Singh SV, Srivastava SK, Choi S, Lew KL, Antosiewicz J, Xiao D, Zeng Y, Watkins SC, Johnson CS, Trump DL, Lee YJ, Xiao H, Herman-Antosiewicz A. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. The Journal of biological chemistry. 2005;280(20):19911-24. Epub 2005/03/15. doi: 10.1074/jbc.M412443200. PubMed PMID: 15764812.
98. Shan Y, Sun C, Zhao X, Wu K, Cassidy A, Bao Y. Effect of sulforaphane on cell growth, G(0)/G(1) phase cell progression and apoptosis in human bladder cancer T24 cells. International journal of oncology. 2006;29(4):883-8. Epub 2006/09/12. PubMed PMID: 16964384.
99. Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J, Terce F. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res. 2000;60(5):1426-33. Epub 2000/03/23. PubMed PMID: 10728709.
100. Suppipat K, Park CS, Shen Y, Zhu X, Lacorazza HD. Sulforaphane induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells. PloS one. 2012;7(12):e51251. Epub 2012/12/20. doi: 10.1371/journal.pone.0051251. PubMed PMID: 23251470; PMCID: PMC3521002.
101. Zuryn A, Litwiniec A, Safiejko-Mroczka B, Klimaszewska-Wisniewska A, Gagat M, Krajewski A, Gackowska L, Grzanka D. The effect of sulforaphane on the cell cycle, apoptosis and expression of cyclin D1 and p21 in the A549 non-small cell lung cancer cell line. International journal of oncology. 2016. Epub 2016/04/02. doi: 10.3892/ijo.2016.3444. PubMed PMID: 27035641.

102. Wang M, Chen S, Wang S, Sun D, Chen J, Li Y, Han W, Yang X, Gao HQ. Effects of phytochemicals sulforaphane on uridine diphosphate-glucuronosyltransferase expression as well as cell-cycle arrest and apoptosis in human colon cancer Caco-2 cells. Chin J Physiol. 2012;55(2):134-44. Epub 2012/05/09. doi: 10.4077/cjp.2012.baa085. PubMed PMID: 22559738.
103. Chen MJ, Tang WY, Hsu CW, Tsai YT, Wu JF, Lin CW, Cheng YM, Hsu YC. Apoptosis Induction in Primary Human Colorectal Cancer Cell Lines and Retarded Tumor Growth in SCID Mice by Sulforaphane. Evidence-based complementary and alternative medicine : eCAM. 2012;2012:415231. Epub 2011/08/02. doi: 10.1155/2012/415231. PubMed PMID: 21804859; PMCID: PMC3139908.
104. Kanematsu S, Uehara N, Miki H, Yoshizawa K, Kawanaka A, Yuri T, Tsubura A. Autophagy inhibition enhances sulforaphane-induced apoptosis in human breast cancer cells. Anticancer Res. 2010;30(9):3381-90. Epub 2010/10/15. PubMed PMID: 20944112.
105. Kim BR, Hu R, Keum YS, Hebbar V, Shen G, Nair SS, Kong AN. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. Cancer Res. 2003;63(21):7520-5. PubMed PMID: 14612554.
106. Yu R, Tan TH, Kong AN. Butylated hydroxyanisole and its metabolite tert-butylhydroquinone differentially regulate mitogen-activated protein kinases. The role of oxidative stress in the activation of mitogen-activated protein kinases by phenolic antioxidants. The Journal of biological chemistry. 1997;272(46):28962-70. Epub 1997/11/20. PubMed PMID: 9360968.
107. Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, Kong AN. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. The Journal of biological chemistry. 1999;274(39):27545-52. Epub 1999/09/17. PubMed PMID: 10488090.
108. Kong AN, Yu R, Lei W, Mandlekar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis. The role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. Restorative neurology and neuroscience. 1998;12(2-3):63-70. Epub 2003/04/03. PubMed PMID: 12671299.
109. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. The American journal of clinical nutrition. 2004;79(5):727-47. PubMed PMID: 15113710.
110. Neveu V, Perez-Jimenez J, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, Scalbert A. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database : the journal of biological databases and curation. 2010;2010:bap024. doi: 10.1093/database/bap024. PubMed PMID: 20428313; PMCID: 2860900.
111. Zhou Y, Zheng J, Li Y, Xu DP, Li S, Chen YM, Li HB. Natural Polyphenols for Prevention and Treatment of Cancer. Nutrients. 2016;8(8). doi: 10.3390/nu8080515. PubMed PMID: 27556486; PMCID: 4997428.
112. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. Phase I clinical trial of curcumin, a chemopreventive

agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 2001;21(4B):2895-900. PubMed PMID: 11712783.
113. Heger M. Drug screening: Don't discount all curcumin trial data. Nature. 2017;543(7643):40. Epub 2017/03/03. doi: 10.1038/543040c. PubMed PMID: 28252078.
114. Panahi Y, Saadat A, Beiraghdar F, Sahebkar A. Adjuvant therapy with bioavailability-boosted curcuminoids suppresses systemic inflammation and improves quality of life in patients with solid tumors: a randomized double-blind placebo-controlled trial. Phytotherapy research : PTR. 2014;28(10):1461-7. Epub 2014/03/22. doi: 10.1002/ptr.5149. PubMed PMID: 24648302.
115. Hejazi J, Rastmanesh R, Taleban FA, Molana SH, Hejazi E, Ehtejab G, Hara N. Effect of Curcumin Supplementation During Radiotherapy on Oxidative Status of Patients with Prostate Cancer: A Double Blinded, Randomized, Placebo-Controlled Study. Nutrition and cancer. 2016;68(1):77-85. Epub 2016/01/16. doi: 10.1080/01635581.2016.1115527. PubMed PMID: 26771294.
116. Cruz-Correa M, Shoskes DA, Sanchez P, Zhao R, Hylind LM, Wexner SD, Giardiello FM. Combination treatment with curcumin and quercetin of adenomas in familial adenomatous polyposis. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2006;4(8):1035-8. doi: 10.1016/j.cgh.2006.03.020. PubMed PMID: 16757216.
117. Carroll RE, Benya RV, Turgeon DK, Vareed S, Neuman M, Rodriguez L, Kakarala M, Carpenter PM, McLaren C, Meyskens FL, Jr., Brenner DE. Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. Cancer Prev Res (Phila). 2011;4(3):354-64. doi: 10.1158/1940-6207.CAPR-10-0098. PubMed PMID: 21372035; PMCID: 4136551.
118. Scapagnini G, Colombrita C, Amadio M, D'Agata V, Arcelli E, Sapienza M, Quattrone A, Calabrese V. Curcumin activates defensive genes and protects neurons against oxidative stress. Antioxidants & redox signaling. 2006;8(3-4):395-403. doi: 10.1089/ars.2006.8.395. PubMed PMID: 16677086.
119. Shishodia S, Chaturvedi MM, Aggarwal BB. Role of curcumin in cancer therapy. Current problems in cancer. 2007;31(4):243-305. doi: 10.1016/j.currproblcancer.2007.04.001. PubMed PMID: 17645940.
120. Woo JH, Kim YH, Choi YJ, Kim DG, Lee KS, Bae JH, Min DS, Chang JS, Jeong YJ, Lee YH, Park JW, Kwon TK. Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. Carcinogenesis. 2003;24(7):1199-208. doi: 10.1093/carcin/bgg082. PubMed PMID: 12807727.
121. Rajasekaran SA. Therapeutic potential of curcumin in gastrointestinal diseases. World journal of gastrointestinal pathophysiology. 2011;2(1):1-14. doi: 10.4291/wjgp.v2.i1.1. PubMed PMID: 21607160; PMCID: 3097964.
122. Basnet P, Skalko-Basnet N. Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. Molecules. 2011;16(6):4567-98. doi: 10.3390/molecules16064567. PubMed PMID: 21642934.
123. Strimpakos AS, Sharma RA. Curcumin: preventive and therapeutic properties in laboratory studies and clinical trials. Antioxidants & redox signaling. 2008;10(3):511-45. doi: 10.1089/ars.2007.1769. PubMed PMID: 18370854.

124. Brinkmann J, Stolpmann K, Trappe S, Otter T, Genkinger D, Bock U, Liebsch M, Henkler F, Hutzler C, Luch A. Metabolically competent human skin models: activation and genotoxicity of benzo[a]pyrene. Toxicol Sci. 2013;131(2):351-9. Epub 2012/11/14. doi: 10.1093/toxsci/kfs316. PubMed PMID: 23148024; PMCID: Pmc3551429.
125. Jancinova V, Perecko T, Nosal R, Mihalova D, Bauerova K, Drabikova K. Pharmacological regulation of neutrophil activity and apoptosis: Contribution to new strategy for modulation of inflammatory processes. Interdisciplinary toxicology. 2011;4(1):11-4. doi: 10.2478/v10102-011-0003-0. PubMed PMID: 21577278; PMCID: 3090048.
126. Zhong F, Chen H, Han L, Jin Y, Wang W. Curcumin attenuates lipopolysaccharide-induced renal inflammation. Biological & pharmaceutical bulletin. 2011;34(2):226-32. PubMed PMID: 21415532.
127. http://www.skincancer.org/publications/sun-and-skin-news/summer-2010-27-2/nonmelanoma-skin-cancer-incidence SCF. Nonmelanoma Skin Cancer Incidence Increases Dramatically. Sun & Skin News Summer2010.
128. Hao F, Kang J, Cao Y, Fan S, Yang H, An Y, Pan Y, Tie L, Li X. Curcumin attenuates palmitate-induced apoptosis in MIN6 pancreatic β-cells through PI3K/Akt/FoxO1 and mitochondrial survival pathways. Apoptosis. 2015;20(11):1420-32.
129. Zhang X, Liang D, Guo L, Liang W, Jiang Y, Li H, Zhao Y, Lu S, Chi ZH. Curcumin protects renal tubular epithelial cells from high glucose-induced epithelial-to-mesenchymal transition through Nrf2-mediated upregulation of heme oxygenase-1. Mol Med Rep. 2015;12(1):1347-55. doi: 10.3892/mmr.2015.3556. PubMed PMID: 25823828.
130. Alinejad B, Ghorbani A, Sadeghnia HR. Effects of combinations of curcumin, linalool, rutin, safranal, and thymoquinone on glucose/serum deprivation-induced cell death. Avicenna journal of phytomedicine. 2013;3(4):321-8.
131. Nazari QA, Kume T, Izuo N, Takada-Takatori Y, Imaizumi A, Hashimoto T, Izumi Y, Akaike A. Neuroprotective effects of curcumin and highly bioavailable curcumin on oxidative stress induced by sodium nitroprusside in rat striatal cell culture. Biological & pharmaceutical bulletin. 2013;36(8):1356-62. PubMed PMID: 23902979.
132. Sakurai R, Villarreal P, Husain S, Liu J, Sakurai T, Tou E, Torday JS, Rehan VK. Curcumin protects the developing lung against long-term hyperoxic injury. Am J Physiol Lung Cell Mol Physiol. 2013;305(4):L301-11. doi: 10.1152/ajplung.00082.2013. PubMed PMID: 23812632; PMCID: PMC3891014.
133. Chen F, Wang H, Xiang X, Yuan J, Chu W, Xue X, Zhu H, Ge H, Zou M, Feng H, Lin J. Curcumin increased the differentiation rate of neurons in neural stem cells via wnt signaling in vitro study. J Surg Res. 2014;192(2):298-304. doi: 10.1016/j.jss.2014.06.026. PubMed PMID: 25033705.
134. Mujoo K, Nikonoff LE, Sharin VG, Bryan NS, Kots AY, Murad F. Curcumin induces differentiation of embryonic stem cells through possible modulation of nitric oxide-cyclic GMP pathway. Protein Cell. 2012;3(7):535-44. doi: 10.1007/s13238-012-2053-2. PubMed PMID: 22773343.
135. Tu SP, Jin H, Shi JD, Zhu LM, Suo Y, Lu G, Liu A, Wang TC, Yang CS. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. Cancer Prev Res (Phila). 2012;5(2):205-15. doi: 10.1158/1940-6207.CAPR-11-0247. PubMed PMID: 22030090; PMCID: PMC3273601.

136. Kim CY, Le TT, Chen C, Cheng JX, Kim KH. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. The Journal of nutritional biochemistry. 2011;22(10):910-20. doi: 10.1016/j.jnutbio.2010.08.003. PubMed PMID: 21189228.
137. Liu H, Liu A, Shi C, Li B. Curcumin suppresses transforming growth factor-beta1-induced cardiac fibroblast differentiation via inhibition of Smad-2 and p38 MAPK signaling pathways. Exp Ther Med. 2016;11(3):998-1004. doi: 10.3892/etm.2016.2969. PubMed PMID: 26998027; PMCID: PMC4774370.
138. Ma J, Ma SY, Ding CH. Curcumin reduces cardiac fibrosis by inhibiting myofibroblast differentiation and decreasing transforming growth factor beta1 and matrix metalloproteinase 9 / tissue inhibitor of metalloproteinase 1. Chin J Integr Med. 2016. doi: 10.1007/s11655-015-2159-5. PubMed PMID: 26956464.
139. http://www.healthguideinfo.com/skin-cancer/p90830/ HC. Metastasis of Squamous Cell Carcinoma.
140. Guo Y, Shu L, Zhang C, Su ZY, Kong AN. Curcumin inhibits anchorage-independent growth of HT29 human colon cancer cells by targeting epigenetic restoration of the tumor suppressor gene DLEC1. Biochemical pharmacology. 2015;94(2):69-78. doi: 10.1016/j.bcp.2015.01.009. PubMed PMID: 25640947; PMCID: 4524742.
141. Khar A, Ali AM, Pardhasaradhi BV, Varalakshmi CH, Anjum R, Kumari AL. Induction of stress response renders human tumor cell lines resistant to curcumin-mediated apoptosis: role of reactive oxygen intermediates. Cell stress & chaperones. 2001;6(4):368-76. PubMed PMID: 11795474; PMCID: 434420.
142. Chan WH, Wu HY, Chang WH. Dosage effects of curcumin on cell death types in a human osteoblast cell line. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2006;44(8):1362-71. doi: 10.1016/j.fct.2006.03.001. PubMed PMID: 16624471.
143. Chen J, Wanming D, Zhang D, Liu Q, Kang J. Water-soluble antioxidants improve the antioxidant and anticancer activity of low concentrations of curcumin in human leukemia cells. Die Pharmazie. 2005;60(1):57-61. PubMed PMID: 15700780.
144. Banerjee A, Kunwar A, Mishra B, Priyadarsini KI. Concentration dependent antioxidant/pro-oxidant activity of curcumin studies from AAPH induced hemolysis of RBCs. Chemico-biological interactions. 2008;174(2):134-9. doi: 10.1016/j.cbi.2008.05.009. PubMed PMID: 18571152.
145. Ali RE, Rattan SI. Curcumin's biphasic hormetic response on proteasome activity and heat-shock protein synthesis in human keratinocytes. Annals of the New York Academy of Sciences. 2006;1067:394-9. doi: 10.1196/annals.1354.056. PubMed PMID: 16804017.
146. Calabrese EJ, Baldwin LA. Hormesis: the dose-response revolution. Annual review of pharmacology and toxicology. 2003;43:175-97. doi: 10.1146/annurev.pharmtox.43.100901.140223. PubMed PMID: 12195028.
147. Calabrese EJ. Hormesis: why it is important to toxicology and toxicologists. Environmental toxicology and chemistry / SETAC. 2008;27(7):1451-74. doi: 10.1897/07-541. PubMed PMID: 18275256.
148. Borriello A, Bencivenga D, Caldarelli I, Tramontano A, Borgia A, Pirozzi AV, Oliva A, Della Ragione F. Resveratrol and cancer treatment: is hormesis a yet unsolved matter? Current pharmaceutical design. 2013;19(30):5384-93. PubMed PMID: 23394084.

149. Bao J, Huang B, Zou L, Chen S, Zhang C, Zhang Y, Chen M, Wan JB, Su H, Wang Y, He C. Hormetic Effect of Berberine Attenuates the Anticancer Activity of Chemotherapeutic Agents. PloS one. 2015;10(9):e0139298. doi: 10.1371/journal.pone.0139298. PubMed PMID: 26421434; PMCID: 4589364.
150. Haddi K, Oliveira EE, Faroni LR, Guedes DC, Miranda NN. Sublethal Exposure to Clove and Cinnamon Essential Oils Induces Hormetic-Like Responses and Disturbs Behavioral and Respiratory Responses in Sitophilus zeamais (Coleoptera: Curculionidae). Journal of economic entomology. 2015;108(6):2815-22. doi: 10.1093/jee/tov255. PubMed PMID: 26318008.
151. Sun B, Ross SM, Trask OJ, Carmichael PL, Dent M, White A, Andersen ME, Clewell RA. Assessing dose-dependent differences in DNA-damage, p53 response and genotoxicity for quercetin and curcumin. Toxicology in vitro : an international journal published in association with BIBRA. 2013;27(6):1877-87. doi: 10.1016/j.tiv.2013.05.015. PubMed PMID: 23764886.
152. Khor TO, Huang Y, Wu TY, Shu L, Lee J, Kong AN. Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation. Biochem Pharmacol. 2011;82(9):1073-8. doi: 10.1016/j.bcp.2011.07.065. PubMed PMID: 21787756.
153. Hager B, Bickenbach JR, Fleckman P. Long-term culture of murine epidermal keratinocytes. J Invest Dermatol. 1999;112(6):971-6.
154. Shu L, Khor TO, Lee JH, Boyanapalli SS, Huang Y, Wu TY, Saw CL, Cheung KL, Kong AN. Epigenetic CpG demethylation of the promoter and reactivation of the expression of Neurog1 by curcumin in prostate LNCaP cells. Aaps J. 2011;13(4):606-14. doi: 10.1208/s12248-011-9300-y. PubMed PMID: 21938566; PMCID: 3231852.
155. Mirza S, Sharma G, Parshad R, Gupta SD, Pandya P, Ralhan R. Expression of DNA methyltransferases in breast cancer patients and to analyze the effect of natural compounds on DNA methyltransferases and associated proteins. Journal of breast cancer. 2013;16(1):23-31. doi: 10.4048/jbc.2013.16.1.23. PubMed PMID: 23593078; PMCID: 3625766.
156. Li W, Pung D, Su ZY, Guo Y, Zhang C, Yang AY, Zheng X, Du ZY, Zhang K, Kong AN. Epigenetics Reactivation of Nrf2 in Prostate TRAMP C1 Cells by Curcumin Analogue FN1. Chemical research in toxicology. 2016;29(4):694-703. doi: 10.1021/acs.chemrestox.6b00016. PubMed PMID: 26991801; PMCID: 4955590.
157. Chiu S, Terpstra KJ, Bureau Y, Hou J, Raheb H, Cernvosky Z, Badmeav V, Copen J, Husni M, Woodbury-Farina M. Liposomal-formulated curcumin [Lipocurc] targeting HDAC (histone deacetylase) prevents apoptosis and improves motor deficits in Park 7 (DJ-1)-knockout rat model of Parkinson's disease: implications for epigenetics-based nanotechnology-driven drug platform. Journal of complementary & integrative medicine. 2013;10. doi: 10.1515/jcim-2013-0020. PubMed PMID: 24200537.
158. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(9):2999-3004. Epub 2004/02/20. doi: 10.1073/pnas.0307323101

0307323101 [pii]. PubMed PMID: 14973191; PMCID: 365734.

159. Saini S, Arora S, Majid S, Shahryari V, Chen Y, Deng G, Yamamura S, Ueno K, Dahiya R. Curcumin modulates microRNA-203-mediated regulation of the Src-Akt axis in bladder cancer. Cancer Prev Res (Phila). 2011;4(10):1698-709. doi: 10.1158/1940-6207.CAPR-11-0267. PubMed PMID: 21836020; PMCID: 3940389.
160. Su ZY, Shu L, Khor TO, Lee JH, Fuentes F, Kong AN. A perspective on dietary phytochemicals and cancer chemoprevention: oxidative stress, nrf2, and epigenomics. Top Curr Chem. 2013;329:133-62. doi: 10.1007/128_2012_340. PubMed PMID: 22836898; PMCID: PMC3924422.
161. Chen C, Kong AN. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. Trends in pharmacological sciences. 2005;26(6):318-26. doi: 10.1016/j.tips.2005.04.004. PubMed PMID: 15925707.
162. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev. 1998;78(2):547-81. PubMed PMID: 9562038.
163. Halliwell BG, J. . Free radicals in biology and medicine. Oxford: Oxford University Press; 1999.
164. Lander HM. An essential role for free radicals and derived species in signal transduction. FASEB J. 1997;11(2):118-24. PubMed PMID: 9039953.
165. Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev. 1994;74(1):139-62. PubMed PMID: 8295932.
166. Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. Biomed Pharmacother. 2004;58(1):39-46. PubMed PMID: 14739060.
167. Cataldi A. Cell responses to oxidative stressors. Current pharmaceutical design. 2010;16(12):1387-95. PubMed PMID: 20166986.
168. Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol. 2009;45(4-5):324-34. doi: 10.1016/j.oraloncology.2008.07.011. PubMed PMID: 18805044; PMCID: PMC2743485.
169. Wolffe AP, Matzke MA. Epigenetics: regulation through repression. Science. 1999;286(5439):481-6. PubMed PMID: 10521337.
170. Esteller M. Epigenetics in cancer. N Engl J Med. 2008;358(11):1148-59. doi: 10.1056/NEJMra072067. PubMed PMID: 18337604.
171. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Perez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nature Genetics. 2005;37(4):391-400. doi: 10.1038/ng1531. PubMed PMID: WOS:000228040000022.
172. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002;3(6):415-28. Epub 2002/06/04. doi: 10.1038/nrg816

nrg816 [pii]. PubMed PMID: 12042769.
173. Nakayama M, Gonzalgo ML, Yegnasubramanian S, Lin X, De Marzo AM, Nelson WG. GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. J Cell Biochem. 2004;91(3):540-52. Epub 2004/02/03. doi: 10.1002/jcb.10740. PubMed PMID: 14755684.

174. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM. Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci. 2007;12:4254-66. Epub 2007/05/09. doi: 2385 [pii]. PubMed PMID: 17485372.
175. Nelson WG, De Marzo AM, Yegnasubramanian S. Epigenetic alterations in human prostate cancers. Endocrinology. 2009;150(9):3991-4002. Epub 2009 Jun 11. PubMed PMID: PMID: 19520778.
176. Jeronimo C, Henrique R. Epigenetic biomarkers in urological tumors: A systematic review. Cancer Lett. 2014;342(2):264-74. doi: 10.1016/j.canlet.2011.12.026. PubMed PMID: 22198482.
177. Sandoval J, Esteller M. Cancer epigenomics: beyond genomics. Curr Opin Genet Dev. 2012;22(1):50-5. Epub 2012/03/10. doi: S0959-437X(12)00019-6 [pii]

10.1016/j.gde.2012.02.008. PubMed PMID: 22402447.
178. Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, Hsieh WS, Isaacs WB, Nelson WG. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci U S A. 1994;91(24):11733-7. Epub 1994/11/22. PubMed PMID: 7972132; PMCID: 45306.
179. Lee WH, Isaacs WB, Bova GS, Nelson WG. CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol Biomarkers Prev. 1997;6(6):443-50. Epub 1997/06/01. PubMed PMID: 9184779.
180. Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA. High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. Prostate. 1999;39(3):166-74. Epub 1999/05/20. doi: 10.1002/(SICI)1097-0045(19990515)39:3<166::AID-PROS4>3.0.CO;2-J [pii]. PubMed PMID: 10334105.
181. van Doorn R, Gruis NA, Willemze R, van der Velden PA, Tensen CP. Aberrant DNA methylation in cutaneous malignancies. Semin Oncol. 2005;32(5):479-87. Epub 2005/10/08. doi: S0093-7754(05)00268-X [pii]

10.1053/j.seminoncol.2005.07.001. PubMed PMID: 16210089.
182. Bachman AN, Curtin GM, Doolittle DJ, Goodman JI. Altered methylation in gene-specific and GC-rich regions of DNA is progressive and nonrandom during promotion of skin tumorigenesis. Toxicol Sci. 2006;91(2):406-18. Epub 2006/03/30. doi: kfj179 [pii]

10.1093/toxsci/kfj179. PubMed PMID: 16569730.
183. Schinke C, Mo Y, Yu Y, Amiri K, Sosman J, Greally J, Verma A. Aberrant DNA methylation in malignant melanoma. Melanoma Res. 2010;20(4):253-65. Epub 2010/04/27. doi: 10.1097/CMR.0b013e328338a35a. PubMed PMID: 20418788; PMCID: 3026062.
184. Helin K, Dhanak D. Chromatin proteins and modifications as drug targets. Nature. 2013;502(7472):480-8. doi: 10.1038/nature12751. PubMed PMID: 24153301.
185. Davis CD, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. Exp Biol Med (Maywood). 2004;229(10):988-95. Epub 2004/11/04. doi: 229/10/988 [pii]. PubMed PMID: 15522834.

186. Fang M, Chen D, Yang CS. Dietary polyphenols may affect DNA methylation. J Nutr. 2007;137(1 Suppl):223S-8S.
187. Yang CS, Fang M, Lambert JD, Yan P, Huang TH. Reversal of hypermethylation and reactivation of genes by dietary polyphenolic compounds. Nutr Rev. 2008;66 Suppl 1:S18-20. Epub 2008/08/21. doi: NURE059 [pii]

10.1111/j.1753-4887.2008.00059.x. PubMed PMID: 18673481; PMCID: 2829855.
188. Rajasekhar VK. Cancer stem cells. Hoboken, New Jersey: John Wiley & Sons; 2014. xxxv, 508 pages, 32 pages of plates p.
189. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature. 2001;411(6835):349-54. doi: 10.1038/35077219. PubMed PMID: 11357142.
190. Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. Stem Cell Rev. 2007;3(1):30-8. PubMed PMID: 17873379.
191. Korkaya H, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J, Clouthier SG, Wicha MS. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. PLoS Biol. 2009;7(6):e1000121. doi: 10.1371/journal.pbio.1000121. PubMed PMID: 19492080; PMCID: 2683567.
192. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res. 2005;7(3):86-95. doi: 10.1186/bcr1021. PubMed PMID: 15987436; PMCID: 1143566.
193. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7. PubMed PMID: 9212098.
194. Dawood S, Austin L, Cristofanilli M. Cancer stem cells: implications for cancer therapy. Oncology (Williston Park). 2014;28(12):1101-7, 10. PubMed PMID: 25510809.
195. Katoh M. Canonical and non-canonical WNT signaling in cancer stem cells and their niches: Cellular heterogeneity, omics reprogramming, targeted therapy and tumor plasticity (Review). International journal of oncology. 2017;51(5):1357-69. doi: 10.3892/ijo.2017.4129. PubMed PMID: 29048660; PMCID: PMC5642388.
196. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov. 2009;8(10):806-23. doi: 10.1038/nrd2137. PubMed PMID: 19794444.
197. Kawasaki BT, Hurt EM, Mistree T, Farrar WL. Targeting cancer stem cells with phytochemicals. Mol Interv. 2008;8(4):174-84. doi: 10.1124/mi.8.4.9. PubMed PMID: 18829843.
198. Ichim G, Tait SW. A fate worse than death: apoptosis as an oncogenic process. Nature reviews Cancer. 2016;16(8):539-48. doi: 10.1038/nrc.2016.58. PubMed PMID: 27364482.
199. Ikeda Y, Murakami A, Ohigashi H. Ursolic acid: an anti- and pro-inflammatory triterpenoid. Molecular nutrition & food research. 2008;52(1):26-42. doi: 10.1002/mnfr.200700389. PubMed PMID: 18203131.
200. Shanmugam MK, Dai X, Kumar AP, Tan BK, Sethi G, Bishayee A. Ursolic acid in cancer prevention and treatment: molecular targets, pharmacokinetics and clinical studies. Biochemical pharmacology. 2013;85(11):1579-87. Epub 2013/03/19. doi: 10.1016/j.bcp.2013.03.006. PubMed PMID: 23499879.

201. Huang MT, Ho CT, Wang ZY, Ferraro T, Lou YR, Stauber K, Ma W, Georgiadis C, Laskin JD, Conney AH. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res. 1994;54(3):701-8. PubMed PMID: 8306331.
202. Tokuda H, Ohigashi H, Koshimizu K, Ito Y. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Cancer Lett. 1986;33(3):279-85. Epub 1986/12/01. PubMed PMID: 3802058.
203. Harmand PO, Duval R, Delage C, Simon A. Ursolic acid induces apoptosis through mitochondrial intrinsic pathway and caspase-3 activation in M4Beu melanoma cells. International journal of cancer Journal international du cancer. 2005;114(1):1-11. Epub 2004/11/04. doi: 10.1002/ijc.20588. PubMed PMID: 15523687.
204. Soo Lee Y, Jin D-Q, Beak S-M, Lee E-S, Kim J-A. Inhibition of ultraviolet-A-modulated signaling pathways by asiatic acid and ursolic acid in HaCaT human keratinocytes. European Journal of Pharmacology. 2003;476(3):173-8. doi: 10.1016/s0014-2999(03)02177-0.
205. Li L, Zhang X, Cui L, Wang L, Liu H, Ji H, Du Y. Ursolic acid promotes the neuroprotection by activating Nrf2 pathway after cerebral ischemia in mice. Brain research. 2013;1497:32-9. Epub 2013/01/02. doi: 10.1016/j.brainres.2012.12.032. PubMed PMID: 23276496.
206. Ma JQ, Ding J, Zhang L, Liu CM. Protective effects of ursolic acid in an experimental model of liver fibrosis through Nrf2/ARE pathway. Clinics and research in hepatology and gastroenterology. 2014. doi: 10.1016/j.clinre.2014.09.007. PubMed PMID: 25459994.
207. Paredes-Gonzalez X, Fuentes F, Su ZY, Kong AN. Apigenin reactivates Nrf2 anti-oxidative stress signaling in mouse skin epidermal JB6 P + cells through epigenetics modifications. The AAPS journal. 2014;16(4):727-35. Epub 2014/05/17. doi: 10.1208/s12248-014-9613-8. PubMed PMID: 24830944; PMCID: Pmc4070251.
208. Wu TY, Khor TO, Su ZY, Saw CL, Shu L, Cheung KL, Huang Y, Yu S, Kong AN. Epigenetic modifications of Nrf2 by 3,3'-diindolylmethane in vitro in TRAMP C1 cell line and in vivo TRAMP prostate tumors. The AAPS journal. 2013;15(3):864-74. Epub 2013/05/10. doi: 10.1208/s12248-013-9493-3. PubMed PMID: 23658110; PMCID: Pmc3691436.
209. Huang Y, Khor TO, Shu L, Saw CL, Wu TY, Suh N, Yang CS, Kong AN. A gamma-tocopherol-rich mixture of tocopherols maintains Nrf2 expression in prostate tumors of TRAMP mice via epigenetic inhibition of CpG methylation. J Nutr. 2012;142(5):818-23. doi: 10.3945/jn.111.153114. PubMed PMID: 22457388; PMCID: 3327740.
210. Wang L, Zhang C, Guo Y, Su ZY, Yang Y, Shu L, Kong AN. Blocking of JB6 Cell Transformation by Tanshinone IIA: Epigenetic Reactivation of Nrf2 Antioxidative Stress Pathway. Aaps J. 2014;16(6):1214-25. Epub 2014/10/03. doi: 10.1208/s12248-014-9666-8. PubMed PMID: 25274607.
211. Su ZY, Khor TO, Shu L, Lee JH, Saw CL, Wu TY, Huang Y, Suh N, Yang CS, Conney AH, Wu Q, Kong AN. Epigenetic reactivation of Nrf2 in murine prostate cancer TRAMP C1 cells by natural phytochemicals Z-ligustilide and Radix angelica sinensis via promoter CpG demethylation. Chemical research in toxicology. 2013;26(3):477-85. doi: 10.1021/tx300524p. PubMed PMID: 23441843.

212. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: a cancer journal for clinicians. 2014;64(1):9-29. doi: 10.3322/caac.21208. PubMed PMID: 24399786.
213. Leiter U, Eigentler T, Garbe C. Epidemiology of skin cancer. Advances in experimental medicine and biology. 2014;810:120-40. Epub 2014/09/11. PubMed PMID: 25207363.
214. Chun KS, Kundu J, Kundu JK, Surh YJ. Targeting Nrf2-Keap1 signaling for chemoprevention of skin carcinogenesis with bioactive phytochemicals. Toxicol Lett. 2014;229(1):73-84. doi: 10.1016/j.toxlet.2014.05.018. PubMed PMID: 24875534.
215. Kansanen E, Kuosmanen SM, Leinonen H, Levonen AL. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox biology. 2013;1(1):45-9. doi: 10.1016/j.redox.2012.10.001. PubMed PMID: 24024136; PMCID: 3757665.
216. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. The Journal of experimental medicine. 2005;202(1):47-59. doi: 10.1084/jem.20050538. PubMed PMID: 15998787; PMCID: 2212893.
217. Calkins MJ, Johnson DA, Townsend JA, Vargas MR, Dowell JA, Williamson TP, Kraft AD, Lee JM, Li J, Johnson JA. The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. Antioxidants & redox signaling. 2009;11(3):497-508. doi: 10.1089/ARS.2008.2242. PubMed PMID: 18717629; PMCID: 2933570.
218. Funes JM, Henderson S, Kaufman R, Flanagan JM, Robson M, Pedley B, Moncada S, Boshoff C. Oncogenic transformation of mesenchymal stem cells decreases Nrf2 expression favoring in vivo tumor growth and poorer survival. Molecular cancer. 2014;13:20. doi: 10.1186/1476-4598-13-20. PubMed PMID: 24491031; PMCID: 4015761.
219. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nature reviews Cancer. 2006;6(2):107-16. Epub 2006/02/24. doi: 10.1038/nrc1799. PubMed PMID: 16491070.
220. Tsai HC, Baylin SB. Cancer epigenetics: linking basic biology to clinical medicine. Cell research. 2011;21(3):502-17. doi: 10.1038/cr.2011.24. PubMed PMID: 21321605; PMCID: 3193419.
221. Saha K, Hornyak TJ, Eckert RL. Epigenetic cancer prevention mechanisms in skin cancer. The AAPS journal. 2013;15(4):1064-71. Epub 2013/08/02. doi: 10.1208/s12248-013-9513-3. PubMed PMID: 23904153; PMCID: Pmc3787232.
222. Shankar S, Kumar D, Srivastava RK. Epigenetic modifications by dietary phytochemicals: implications for personalized nutrition. Pharmacology & therapeutics. 2013;138(1):1-17. Epub 2012/11/20. doi: 10.1016/j.pharmthera.2012.11.002. PubMed PMID: 23159372; PMCID: Pmc4153856.
223. Hatzimichael E, Crook T. Cancer epigenetics: new therapies and new challenges. Journal of drug delivery. 2013;2013:529312. Epub 2013/03/28. doi: 10.1155/2013/529312. PubMed PMID: 23533770; PMCID: Pmc3600296.
224. Vanden Berghe W. Epigenetic impact of dietary polyphenols in cancer chemoprevention: lifelong remodeling of our epigenomes. Pharmacological research : the official journal of the Italian Pharmacological Society. 2012;65(6):565-76. doi: 10.1016/j.phrs.2012.03.007. PubMed PMID: 22465217.

225. Miceli M, Bontempo P, Nebbioso A, Altucci L. Natural compounds in epigenetics: a current view. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2014;73:71-83. doi: 10.1016/j.fct.2014.08.005. PubMed PMID: 25139119.
226. Schnekenburger M, Dicato M, Diederich M. Plant-derived epigenetic modulators for cancer treatment and prevention. Biotechnology advances. 2014;32(6):1123-32. doi: 10.1016/j.biotechadv.2014.03.009. PubMed PMID: 24699435.
227. Vaid M, Prasad R, Singh T, Jones V, Katiyar SK. Grape seed proanthocyanidins reactivate silenced tumor suppressor genes in human skin cancer cells by targeting epigenetic regulators. Toxicology and applied pharmacology. 2012;263(1):122-30. doi: 10.1016/j.taap.2012.06.013. PubMed PMID: 22749965; PMCID: 3407315.
228. Wang LS, Kuo CT, Cho SJ, Seguin C, Siddiqui J, Stoner K, Weng YI, Huang TH, Tichelaar J, Yearsley M, Stoner GD, Huang YW. Black raspberry-derived anthocyanins demethylate tumor suppressor genes through the inhibition of DNMT1 and DNMT3B in colon cancer cells. Nutrition and cancer. 2013;65(1):118-25. doi: 10.1080/01635581.2013.741759. PubMed PMID: 23368921; PMCID: PMC3570951.
229. Dhar A, Young MR, Colburn NH. The role of AP-1, NF-kappaB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. Mol Cell Biochem. 2002;234-235(1-2):185-93. Epub 2002/08/07. PubMed PMID: 12162432.
230. Yu S, Khor TO, Cheung KL, Li W, Wu TY, Huang Y, Foster BA, Kan YW, Kong AN. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLoS One. 2010;5(1):e8579. doi: 10.1371/journal.pone.0008579. PubMed PMID: 20062804; PMCID: PMC2799519.
231. Bickers DR, Athar M. Oxidative stress in the pathogenesis of skin disease. J Invest Dermatol. 2006;126(12):2565-75. doi: 10.1038/sj.jid.5700340. PubMed PMID: 17108903.
232. Yang HS, Knies JL, Stark C, Colburn NH. Pdcd4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation. Oncogene. 2003;22(24):3712-20. Epub 2003/06/13. doi: 10.1038/sj.onc.1206433. PubMed PMID: 12802278.
233. Bode AM, Ma WY, Surh YJ, Dong Z. Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. Cancer Res. 2001;61(3):850-3. Epub 2001/02/28. PubMed PMID: 11221868.
234. Dong Z, Ma W, Huang C, Yang CS. Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. Cancer Res. 1997;57(19):4414-9.
235. Lu YP, Chang RL, Lou YR, Huang MT, Newmark HL, Reuhl KR, Conney AH. Effect of curcumin on 12-O-tetradecanoylphorbol-13-acetate- and ultraviolet B light-induced expression of c-Jun and c-Fos in JB6 cells and in mouse epidermis. Carcinogenesis. 1994;15(10):2363-70. Epub 1994/10/01. PubMed PMID: 7955078.
236. Kowalczyk MC, Junco JJ, Kowalczyk P, Tolstykh O, Hanausek M, Slaga TJ, Walaszek Z. Effects of combined phytochemicals on skin tumorigenesis in SENCAR mice. International journal of oncology. 2013;43(3):911-8. Epub 2013/07/10. doi: 10.3892/ijo.2013.2005. PubMed PMID: 23835587; PMCID: Pmc3787890.
237. Shin JW, Ohnishi K, Murakami A, Lee JS, Kundu JK, Na HK, Ohigashi H, Surh YJ. Zerumbone induces heme oxygenase-1 expression in mouse skin and cultured murine

epidermal cells through activation of Nrf2. Cancer Prev Res (Phila). 2011;4(6):860-70. Epub 2011/03/04. doi: 10.1158/1940-6207.capr-10-0354. PubMed PMID: 21367956.
238. Shih YH, Chein YC, Wang JY, Fu YS. Ursolic acid protects hippocampal neurons against kainate-induced excitotoxicity in rats. Neuroscience letters. 2004;362(2):136-40. Epub 2004/06/15. doi: 10.1016/j.neulet.2004.03.011. PubMed PMID: 15193771.
239. Saravanan R, Pugalendi V. Impact of ursolic acid on chronic ethanol-induced oxidative stress in the rat heart. Pharmacological reports : PR. 2006;58(1):41-7. Epub 2006/03/15. PubMed PMID: 16531629.
240. Senthil S, Chandramohan G, Pugalendi KV. Isomers (oleanolic and ursolic acids) differ in their protective effect against isoproterenol-induced myocardial ischemia in rats. International journal of cardiology. 2007;119(1):131-3. Epub 2006/10/21. doi: 10.1016/j.ijcard.2006.07.108. PubMed PMID: 17052790.
241. Saikali M, Ghantous A, Halawi R, Talhouk SN, Saliba NA, Darwiche N. Sesquiterpene lactones isolated from indigenous Middle Eastern plants inhibit tumor promoter-induced transformation of JB6 cells. BMC complementary and alternative medicine. 2012;12:89. Epub 2012/07/11. doi: 10.1186/1472-6882-12-89. PubMed PMID: 22776414; PMCID: Pmc3439278.
242. Bryan HK, Olayanju A, Goldring CE, Park BK. The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. Biochemical pharmacology. 2013;85(6):705-17. doi: 10.1016/j.bcp.2012.11.016. PubMed PMID: 23219527.
243. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes & development. 2013;27(20):2179-91. doi: 10.1101/gad.225680.113. PubMed PMID: 24142871; PMCID: 3814639.
244. Liby KT, Sporn MB. Synthetic oleanane triterpenoids: multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease. Pharmacological reviews. 2012;64(4):972-1003. doi: 10.1124/pr.111.004846. PubMed PMID: 22966038; PMCID: 3462991.
245. Zhang DD, Hannink M. Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress. Molecular and Cellular Biology. 2003;23(22):8137-51. doi: 10.1128/mcb.23.22.8137-8151.2003.
246. Hur W, Gray NS. Small molecule modulators of antioxidant response pathway. Current opinion in chemical biology. 2011;15(1):162-73. doi: 10.1016/j.cbpa.2010.12.009. PubMed PMID: 21195017.
247. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, Talalay P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(18):11908-13. Epub 2002/08/24. doi: 10.1073/pnas.172398899. PubMed PMID: 12193649; PMCID: Pmc129367.
248. Kang KA, Piao MJ, Kim KC, Kang HK, Chang WY, Park IC, Keum YS, Surh YJ, Hyun JW. Epigenetic modification of Nrf2 in 5-fluorouracil-resistant colon cancer cells: involvement of TET-dependent DNA demethylation. Cell death & disease. 2014;5:e1183. doi: 10.1038/cddis.2014.149. PubMed PMID: 24743738; PMCID: 4001304.

249. Pathak AK, Bhutani M, Nair AS, Ahn KS, Chakraborty A, Kadara H, Guha S, Sethi G, Aggarwal BB. Ursolic acid inhibits STAT3 activation pathway leading to suppression of proliferation and chemosensitization of human multiple myeloma cells. Molecular cancer research : MCR. 2007;5(9):943-55. doi: 10.1158/1541-7786.MCR-06-0348. PubMed PMID: 17855663.
250. Nandakumar V, Vaid M, Tollefsbol TO, Katiyar SK. Aberrant DNA hypermethylation patterns lead to transcriptional silencing of tumor suppressor genes in UVB-exposed skin and UVB-induced skin tumors of mice. Carcinogenesis. 2011;32(4):597-604. Epub 2010/12/28. doi: 10.1093/carcin/bgq282. PubMed PMID: 21186298; PMCID: Pmc3066413.
251. Nguyen T, Kuo C, Nicholl MB, Sim MS, Turner RR, Morton DL, Hoon DS. Downregulation of microRNA-29c is associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma. Epigenetics : official journal of the DNA Methylation Society. 2011;6(3):388-94. Epub 2010/11/18. PubMed PMID: 21081840; PMCID: Pmc3063331.
252. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, Baylin SB, Vogelstein B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature. 2002;416(6880):552-6. Epub 2002/04/05. doi: 10.1038/416552a. PubMed PMID: 11932749.
253. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov. 2006;5(9):769-84. doi: 10.1038/nrd2133. PubMed PMID: 16955068.
254. Dickinson SE, Rusche JJ, Bec SL, Horn DJ, Janda J, Rim SH, Smith CL, Bowden GT. The effect of sulforaphane on histone deacetylase activity in keratinocytes: Differences between in vitro and in vivo analyses. Mol Carcinog. 2014. doi: 10.1002/mc.22224. PubMed PMID: 25307283.
255. Soltani-Arabshahi R, Tristani-Firouzi P. Chemoprevention of nonmelanoma skin cancer. Facial Plast Surg. 2013;29(5):373-83. doi: 10.1055/s-0033-1353377. PubMed PMID: 24037930.
256. Tang JY, So PL, Epstein EH, Jr. Novel Hedgehog pathway targets against basal cell carcinoma. Toxicology and applied pharmacology. 2007;224(3):257-64. doi: 10.1016/j.taap.2006.12.011. PubMed PMID: 17276471; PMCID: PMC2719777.
257. Kasper M, Jaks V, Hohl D, Toftgard R. Basal cell carcinoma - molecular biology and potential new therapies. J Clin Invest. 2012;122(2):455-63. doi: 10.1172/JCI58779. PubMed PMID: 22293184; PMCID: PMC3266783.
258. Prado R, Francis SO, Mason MN, Wing G, Gamble RG, Dellavalle R. Nonmelanoma skin cancer chemoprevention. Dermatol Surg. 2011;37(11):1566-78. doi: 10.1111/j.1524-4725.2011.02108.x. PubMed PMID: 21895847.
259. Stratton SP. Prevention of non-melanoma skin cancer. Curr Oncol Rep. 2001;3(4):295-300. PubMed PMID: 11389812.
260. Campbell RM, DiGiovanna JJ. Skin cancer chemoprevention with systemic retinoids: an adjunct in the management of selected high-risk patients. Dermatol Ther. 2006;19(5):306-14. doi: 10.1111/j.1529-8019.2006.00088.x. PubMed PMID: 17014486.
261. Karagas MR, Waterboer T, Li Z, Nelson HH, Michael KM, Bavinck JN, Perry AE, Spencer SK, Daling J, Green AC, Pawlita M, New Hampshire Skin Cancer Study G. Genus beta human papillomaviruses and incidence of basal cell and squamous cell

carcinomas of skin: population based case-control study. BMJ. 2010;341:c2986. doi: 10.1136/bmj.c2986. PubMed PMID: 20616098; PMCID: PMC2900549.
262. Gilchrest BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. N Engl J Med. 1999;340(17):1341-8. doi: 10.1056/NEJM199904293401707. PubMed PMID: 10219070.
263. Yuspa SH. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis--thirty-third G. H. A. Clowes Memorial Award Lecture. Cancer Res. 1994;54(5):1178-89. PubMed PMID: 8118803.
264. Encyclopedia of Cancer. In: Schwab M, editor. Skin Carcinogenesis. 4 ed. Springer-Verlag Berlin Heidelberg: Berlin Heidelberg; 2017.
265. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. Nat Protoc. 2009;4(9):1350-62. doi: 10.1038/nprot.2009.120. PubMed PMID: 19713956; PMCID: PMC3213400.
266. Tilley C, Deep G, Agarwal R. Chemopreventive opportunities to control basal cell carcinoma: Current perspectives. Molecular carcinogenesis. 2015;54(9):688-97. doi: 10.1002/mc.22348. PubMed PMID: 26053157.
267. Kipling MD, Waldron HA. Percivall Pott and cancer scroti. Br J Ind Med. 1975;32(3):244-6. PubMed PMID: 1098690; PMCID: PMC1008067.
268. Conney AH. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. Cancer Res. 1982;42(12):4875-917. PubMed PMID: 6814745.
269. Yuspa SH. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J Dermatol Sci. 1998;17(1):1-7. PubMed PMID: 9651822.
270. Indra AK, Castaneda E, Antal MC, Jiang M, Messaddeq N, Meng X, Loehr CV, Gariglio P, Kato S, Wahli W, Desvergne B, Metzger D, Chambon P. Malignant transformation of DMBA/TPA-induced papillomas and nevi in the skin of mice selectively lacking retinoid-X-receptor alpha in epidermal keratinocytes. J Invest Dermatol. 2007;127(5):1250-60. doi: 10.1038/sj.jid.5700672. PubMed PMID: 17301838.
271. Man'cheva TA, Demidov DV, Plotnikova NA, Kharitonova TV, Pashkevich IV, Anisimov VN. Melatonin and metformin inhibit skin carcinogenesis and lipid peroxidation induced by benz(a)pyrene in female mice. Bulletin of experimental biology and medicine. 2011;151(3):363-5. Epub 2012/03/28. PubMed PMID: 22451888.
272. Das S, Das J, Samadder A, Paul A, Khuda-Bukhsh AR. Efficacy of PLGA-loaded apigenin nanoparticles in Benzo[a]pyrene and ultraviolet-B induced skin cancer of mice: mitochondria mediated apoptotic signalling cascades. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2013;62:670-80. Epub 2013/10/15. doi: 10.1016/j.fct.2013.09.037. PubMed PMID: 24120900.
273. Bassi DE, Cenna J, Zhang J, Cukierman E, Klein-Szanto AJ. Enhanced aggressiveness of benzopyrene-induced squamous carcinomas in transgenic mice overexpressing the proprotein convertase PACE4 (PCSK6). Molecular carcinogenesis. 2015;54(10):1122-31. Epub 2014/05/23. doi: 10.1002/mc.22183. PubMed PMID: 24845697; PMCID: Pmc4240754.

274. Slaga TJ. SENCAR mouse skin tumorigenesis model versus other strains and stocks of mice. Environmental health perspectives. 1986;68:27-32. PubMed PMID: 3096709; PMCID: 1474258.
275. Lei W, Yu R, Mandlekar S, Kong AN. Induction of apoptosis and activation of interleukin 1beta-converting enzyme/Ced-3 protease (caspase-3) and c-Jun NH2-terminal kinase 1 by benzo(a)pyrene. Cancer Res. 1998;58(10):2102-6. Epub 1998/05/30. PubMed PMID: 9605752.
276. Miller ML, Vasunia K, Talaska G, Andringa A, de Boer J, Dixon K. The tumor promoter TPA enhances benzo[a]pyrene and benzo[a]pyrene diolepoxide mutagenesis in Big Blue mouse skin. Environmental and molecular mutagenesis. 2000;35(4):319-27. PubMed PMID: 10861950.
277. Iskander K, Paquet M, Brayton C, Jaiswal AK. Deficiency of NRH:quinone oxidoreductase 2 increases susceptibility to 7,12-dimethylbenz(a)anthracene and benzo(a)pyrene-induced skin carcinogenesis. Cancer Res. 2004;64(17):5925-8. doi: 10.1158/0008-5472.CAN-04-0763. PubMed PMID: 15342368.
278. Siddens LK, Larkin A, Krueger SK, Bradfield CA, Waters KM, Tilton SC, Pereira CB, Lohr CV, Arlt VM, Phillips DH, Williams DE, Baird WM. Polycyclic aromatic hydrocarbons as skin carcinogens: comparison of benzo[a]pyrene, dibenzo[def,p]chrysene and three environmental mixtures in the FVB/N mouse. Toxicology and applied pharmacology. 2012;264(3):377-86. doi: 10.1016/j.taap.2012.08.014. PubMed PMID: 22935520; PMCID: 3483092.
279. Woodworth CD, Michael E, Smith L, Vijayachandra K, Glick A, Hennings H, Yuspa SH. Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte. Carcinogenesis. 2004;25(9):1771-8. doi: 10.1093/carcin/bgh170. PubMed PMID: 15105299.
280. Gimenez-Conti IB, Bianchi AB, Fischer SM, Reiners JJ, Jr., Conti CJ, Slaga TJ. Dissociation of sensitivities to tumor promotion and progression in outbred and inbred SENCAR mice. Cancer Res. 1992;52(12):3432-5. PubMed PMID: 1375869.
281. Hennings H, Glick AB, Lowry DT, Krsmanovic LS, Sly LM, Yuspa SH. FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. Carcinogenesis. 1993;14(11):2353-8. PubMed PMID: 8242866.
282. Ewing MW, Conti CJ, Kruszewski FH, Slaga TJ, DiGiovanni J. Tumor progression in Sencar mouse skin as a function of initiator dose and promoter dose, duration, and type. Cancer Res. 1988;48(24 Pt 1):7048-54. PubMed PMID: 3142681.
283. DuBowski A, Johnston DA, Rupp T, Beltran L, Conti CJ, DiGiovanni J. Papillomas at high risk for malignant progression arising both early and late during two-stage carcinogenesis in SENCAR mice. Carcinogenesis. 1998;19(6):1141-7. PubMed PMID: 9667755.
284. Yang AY, Lee JH, Shu L, Zhang C, Su ZY, Lu Y, Huang MT, Ramirez C, Pung D, Huang Y, Verzi M, Hart RP, Kong AN. Genome-wide analysis of DNA methylation in UVB- and DMBA/TPA-induced mouse skin cancer models. Life Sci. 2014;113(1-2):45-54. doi: 10.1016/j.lfs.2014.07.031. PubMed PMID: 25093921.
285. Fenaux P, Ades L. Review of azacitidine trials in Intermediate-2-and High-risk myelodysplastic syndromes. Leuk Res. 2009;33 Suppl 2:S7-11. Epub 2009/12/17. doi: S0145-2126(09)70227-9 [pii]

10.1016/S0145-2126(09)70227-9. PubMed PMID: 20004796.

286. Golabek K, Strzelczyk JK, Wiczkowski A, Michalski M. Potential use of histone deacetylase inhibitors in cancer therapy. Contemp Oncol (Pozn). 2015;19(6):436-40. doi: 10.5114/wo.2015.51824. PubMed PMID: 26843838; PMCID: PMC4731444.
287. Kundu JK, Shin YK, Surh YJ. Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin in vivo: NF-kappaB and AP-1 as prime targets. Biochem Pharmacol. 2006;72(11):1506-15. doi: 10.1016/j.bcp.2006.08.005. PubMed PMID: 16999939.
288. Wei SJ, Chang RL, Merkler KA, Gwynne M, Cui XX, Murthy B, Huang MT, Xie JG, Lu YP, Lou YR, Jerina DM, Conney AH. Dose-dependent mutation profile in the c-Ha-ras proto-oncogene of skin tumors in mice initiated with benzo[a]pyrene. Carcinogenesis. 1999;20(9):1689-96. PubMed PMID: 10469612.
289. Balmain A, Harris CC. Carcinogenesis in mouse and human cells: parallels and paradoxes. Carcinogenesis. 2000;21(3):371-7. Epub 2000/02/26. PubMed PMID: 10688857.
290. Knatko EV, Higgins M, Fahey JW, Dinkova-Kostova AT. Loss of Nrf2 abrogates the protective effect of Keap1 downregulation in a preclinical model of cutaneous squamous cell carcinoma. Sci Rep. 2016;6:25804. doi: 10.1038/srep25804. PubMed PMID: 27216826; PMCID: PMC4877584.
291. Joung EJ, Lee MS, Choi JW, Kim JS, Shin T, Jung BM, Yoon NY, Lim CW, Kim JI, Kim HR. Anti-inflammatory effect of ethanolic extract from Myagropsis myagroides on murine macrophages and mouse ear edema. BMC complementary and alternative medicine. 2012;12:171. doi: 10.1186/1472-6882-12-171. PubMed PMID: 23031211; PMCID: PMC3517429.
292. Ramirez CN, Li W, Zhang C, Wu R, Su S, Wang C, Gao L, Yin R, Kong AN. In Vitro-In Vivo Dose Response of Ursolic Acid, Sulforaphane, PEITC, and Curcumin in Cancer Prevention. Aaps J. 2017;20(1):19. doi: 10.1208/s12248-017-0177-2. PubMed PMID: 29264822; PMCID: PMC6021020.
293. Surh YJ, Lee SS. Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. Life Sci. 1995;56(22):1845-55. PubMed PMID: 7746093.
294. Thomas G, Tuk B, Song JY, Truong H, Gerritsen HC, de Gruijl FR, Sterenborg HJ. Studying skin tumourigenesis and progression in immunocompetent hairless SKH1-hr mice using chronic 7,12-dimethylbenz(a)anthracene topical applications to develop a useful experimental skin cancer model. Lab Anim. 2017;51(1):24-35. doi: 10.1177/0023677216637305. PubMed PMID: 26946120.
295. Giovanella BC, Liegel J, Heidelberger C. The refractoriness of the skin of hairless mice to chemical carcinogenesis. Cancer Res. 1970;30(10):2590-7. PubMed PMID: 5474182.
296. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Perez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet. 2005;37(4):391-400. doi: 10.1038/ng1531. PubMed PMID: 15765097.
297. Esteller M, Almouzni G. How epigenetics integrates nuclear functions. Workshop on epigenetics and chromatin: transcriptional regulation and beyond. EMBO Rep.

2005;6(7):624-8. doi: 10.1038/sj.embor.7400456. PubMed PMID: 15976819; PMCID: PMC1369115.
298. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S. DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet. 2006;38(12):1378-85. PubMed PMID: PMID: 17072317.
299. Lopez J, Percharde M, Coley HM, Webb A, Crook T. The context and potential of epigenetics in oncology. Br J Cancer. 2009;100(4):571-7. PubMed PMID: PMID: 19223907.
300. Vanden Berghe W, Haegeman G. Epigenetic remedies by dietary phytochemicals against inflammatory skin disorders: myth or reality? Curr Drug Metab. 2010;11(5):436-50. PubMed PMID: 20540697.
301. Wang T, Liu Q, Li X, Wang X, Li J, Zhu X, Sun ZS, Wu J. RRBS-analyser: a comprehensive web server for reduced representation bisulfite sequencing data analysis. Human mutation. 2013;34(12):1606-10. Epub 2013/10/10. doi: 10.1002/humu.22444. PubMed PMID: 24106010.
302. DiGiovanni J, Rho O, Xian W, Beltran L. Role of the epidermal growth factor receptor and transforming growth factor alpha in mouse skin carcinogenesis. Prog Clin Biol Res. 1994;387:113-38. PubMed PMID: 7972243.
303. Imamoto A, Wang XJ, Fujiki H, Walker SE, Beltran LM, DiGiovanni J. Comparison of 12-O-tetradecanoylphorbol-13-acetate and teleocidin for induction of epidermal hyperplasia, activation of epidermal PKC isozymes and skin tumor promotion in SENCAR and C57BL/6 mice. Carcinogenesis. 1993;14(4):719-24. PubMed PMID: 8472338.
304. DiGiovanni J, Walker SC, Beltran L, Naito M, Eastin WC, Jr. Evidence for a common genetic pathway controlling susceptibility to mouse skin tumor promotion by diverse classes of promoting agents. Cancer Res. 1991;51(5):1398-405. PubMed PMID: 1900038.
305. Naito M, DiGiovanni J. Genetic background and development of skin tumors. Carcinog Compr Surv. 1989;11:187-212. PubMed PMID: 2493334.
306. Benavides F, Oberyszyn TM, VanBuskirk AM, Reeve VE, Kusewitt DF. The hairless mouse in skin research. J Dermatol Sci. 2009;53(1):10-8. doi: 10.1016/j.jdermsci.2008.08.012. PubMed PMID: 18938063; PMCID: PMC2646590.
307. Kuroiwa Y, Nishikawa A, Kitamura Y, Kanki K, Ishii Y, Umemura T, Hirose M. Protective effects of benzyl isothiocyanate and sulforaphane but not resveratrol against initiation of pancreatic carcinogenesis in hamsters. Cancer letters. 2006;241(2):275-80. doi: 10.1016/j.canlet.2005.10.028. PubMed PMID: 16386831.
308. Esteller M, Almouzni G. How epigenetics integrates nuclear functions. Workshop on epigenetics and chromatin: transcriptional regulation and beyond. EMBO Rep. 2005;6(7):624-8. Epub 2005/06/25. doi: 10.1038/sj.embor.7400456. PubMed PMID: 15976819; PMCID: 1369115.
309. Shanmugam MK, Ong TH, Kumar AP, Lun CK, Ho PC, Wong PT, Hui KM, Sethi G. Ursolic acid inhibits the initiation, progression of prostate cancer and prolongs the survival of TRAMP mice by modulating pro-inflammatory pathways. PloS one.

2012;7(3):e32476. doi: 10.1371/journal.pone.0032476. PubMed PMID: 22427843; PMCID: PMC3299664.
310. Dinkova-Kostova AT, Liby KT, Stephenson KK, Holtzclaw WD, Gao X, Suh N, Williams C, Risingsong R, Honda T, Gribble GW, Sporn MB, Talalay P. Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. Proc Natl Acad Sci U S A. 2005;102(12):4584-9. Epub 2005/03/16. doi: 10.1073/pnas.0500815102. PubMed PMID: 15767573; PMCID: 555528.
311. Laszczyk MN. Pentacyclic triterpenes of the lupane, oleanane and ursane group as tools in cancer therapy. Planta Med. 2009;75(15):1549-60. doi: 10.1055/s-0029-1186102. PubMed PMID: 19742422.
312. Nakamura Y, Colburn NH, Gindhart TD. Role of reactive oxygen in tumor promotion: implication of superoxide anion in promotion of neoplastic transformation in JB-6 cells by TPA. Carcinogenesis. 1985;6(2):229-35. Epub 1985/02/01. PubMed PMID: 2982513.
313. Amstad PA, Liu H, Ichimiya M, Berezesky IK, Trump BF. Manganese superoxide dismutase expression inhibits soft agar growth in JB6 clone41 mouse epidermal cells. Carcinogenesis. 1997;18(3):479-84. Epub 1997/03/01. PubMed PMID: 9067545.
314. Soo Lee Y, Jin DQ, Beak SM, Lee ES, Kim JA. Inhibition of ultraviolet-A-modulated signaling pathways by asiatic acid and ursolic acid in HaCaT human keratinocytes. Eur J Pharmacol. 2003;476(3):173-8. PubMed PMID: 12969763.
315. Rogers HW, Weinstock MA, Feldman SR, Coldiron BM. Incidence Estimate of Nonmelanoma Skin Cancer (Keratinocyte Carcinomas) in the U.S. Population, 2012. JAMA Dermatol. 2015;151(10):1081-6. doi: 10.1001/jamadermatol.2015.1187. PubMed PMID: 25928283.
316. Rogers HW, Weinstock MA, Feldman SR, Coldiron BM. Incidence Estimate of Nonmelanoma Skin Cancer (Keratinocyte Carcinomas) in the US Population, 2012. JAMA Dermatol. 2015;151(10):1081-6. doi: 10.1001/jamadermatol.2015.1187. PubMed PMID: 25928283.
317. Guy GP, Jr., Machlin SR, Ekwueme DU, Yabroff KR. Prevalence and costs of skin cancer treatment in the U.S., 2002-2006 and 2007-2011. Am J Prev Med. 2015;48(2):183-7. doi: 10.1016/j.amepre.2014.08.036. PubMed PMID: 25442229; PMCID: 4603424.