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The Effects of the Combination of Ursolic Acid and Curcumin Administered Topically and in the Diet on Skin Tumor Promotion by TPA

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by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2018

Acknowledgements

First and foremost I would like to thank my mentor Dr. John DiGiovanni for all of his support and guidance during my graduate career. He continually pushed me to do my best work and never settled for less. I have learned a lot as a scientist while in his lab and I am forever grateful that he accepted me to work in his lab.

I thank my committee members Dr. Karen Vasquez, Dr. Dawit Kidane and Dr. Thomas J. Slaga for taking time out of their busy schedules to serve on my committee and for their guidance throughout my Ph.D.

I would also like to thank the DiGiovanni lab members past and present for their moral support and help with my experiments. Dr. Jiyoon Cho was a senior graduate student when I first started in the lab and taught me all of the skills I have used in my project, from handling and treating the mice to collecting tissues and running beautiful Western blots. Dr. Okkyung Rho provided tremendous help throughout my project, particularly with my topical tumor experiment. Dr. Jaya Srivastava taught me various techniques with incredible patience and thoroughness and offered me moral support and advice throughout my graduate career. Katie Na was an undergraduate student who helped immensely with my diet study tumor experiment. The DiGiovanni lab members have been extremely supportive, especially when my experiments were not working, and make the lab a fun place to work. Thank you Jeremy Cheng, Sabin Kshattry, Fernando Eguiarte-Solomon, Carly Wilder, Steve Carbajal, Achinto Saha, Zhao Chen and Songyeon Ahn. I will greatly miss all of you.

Lastly I would like to thank my friends and family. Without your support over the years I would not be where I am today.

The Effects of the Combination of Ursolic Acid and Curcumin Administered Topically and in the Diet on Skin Tumor Promotion by TPA

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The University of Texas at Austin, 2018

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Prevention remains an important strategy to reduce the burden of cancer. One approach to prevent cancer is the use of phytochemicals in various combinations as safe and effective cancer preventative agents. The purpose of this study was to examine the effects of the topical combination of ursolic acid (UA) and curcumin (Curc) for potential combinatorial inhibition of skin tumor promotion using the mouse two-stage skin carcinogenesis model. Furthermore, UA, Curc and resveratrol (Res) were evaluated for their ability to inhibit skin tumor promotion when given in the diet alone and in combination.

In short-term experiments, the combination of UA and Curc pretreated topically inhibited TPA-induced activation of epidermal EGFR, p70S6K, NF-κB p50, Src, c-Jun, Rb, c-Fos and IκBα. Levels of c-Fos, c-Jun and Cox-2 were also significantly reduced by the combination. The alterations in these signaling pathways by the combination of UA and Curc were associated with decreased epidermal proliferation as assessed by measuring BrdU incorporation. Significant effects were also seen with the combination on epidermal

and inflammatory gene expression and dermal inflammation with the greatest effects on expression of IL-1β, IL-6, IL-22 and CXCL12. Furthermore, results from skin tumor experiments demonstrated that the combination of UA and Curc given topically significantly inhibited mouse skin tumor promotion by TPA to a greater extent than the individual compounds given alone.

Initial short term experiments suggested UA, Curc and Res given in the diet alone or in combination inhibited TPA-induced EGFR, c-Jun, NF-κB p50, Cox-2 and Rb. Furthermore, Curc and Res, given alone and in combination with UA, inhibited TPA-induced hyperproliferation. However, in the two-stage skin carcinogenesis model none of the compounds given in the diet alone or in combination inhibited tumor multiplicity or tumor incidence at the dose give. Res alone did significantly inhibit tumor size and weight to levels comparable with Met, indicating it is inhibiting tumor growth, but not initial tumor development.

These results demonstrate the potential cancer chemopreventive activity and mechanism(s) for the combination of topically applied UA and Curc.

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Chapter 1: Introduction and Background

1.1 CHEMOPREVENTION

Despite the many advances that have been made in understanding cancer and developing new therapies, cancer is still the second leading cause of death in the US and accounts for about one in four deaths. Furthermore, approximately two out of five people will develop cancer in their lifetime, and in many cases cancer can be preventable (1). Aside from lifestyle choices such as avoiding tobacco products and maintaining a lean bodyweight, chemoprevention remains a strategy to reduce cancer risk, especially in high-risk populations. Chemoprevention is the use of natural, synthetic, or biological agents to reverse, suppress, or prevent the initial development of carcinogenesis or the progression to metastatic cancer (2).

Epidemiological and meta-analysis studies have been conducted exploring consumption of fruits and vegetables as chemopreventive measures. For example, in breast cancer, one meta-analysis study found high consumption of vegetables was associated with a reduced relative risk, another looked specifically at cruciferous vegetable intake and found an inverse correlation and an epidemiological study demonstrated that eating grapes correlated with a decreased risk of breast cancer risk (3-5). A case-control study investigating the relative risk of prostate cancer found a reduced risk with consumption of yellow or orange vegetables, particularly corn and carrots, cruciferous vegetables or legumes (6). Another epidemiological study found an inverse correlation between high consumption of fruit, dark-green vegetables, deep-yellow vegetables or onions and garlic and risk of colorectal adenoma, which can eventually develop into colorectal carcinomas

(7). Overall these studies and others demonstrate a high consumption of fruits and vegetables can decrease the relative risk of developing certain cancers and is thought to be attributed, at least in part, to their naturally occurring phytochemicals (8).

Many studies to date have examined phytochemicals as chemopreventive agents due to their ability to act on a broad spectrum of signaling pathways and their relatively low toxicity (8-11). Phytochemicals have been shown to inhibit all stages of cancer development including initiation, promotion and progression. Many phytochemicals, such as sulforaphane, resveratrol and various flavonoids, can prevent initiation by inhibiting enzymes involved in phase I metabolism of pro-carcinogens into carcinogens, namely cytochrome P450 (CYP450) enzymes, and activating detoxifying phase II enzymes such as glutathione reductase, glucuronosyltransferase and thioredoxin reductase (12-15). During promotion and progression, various phytochemicals can impede oncogenic transcription factors, growth and proliferation signaling cascades, inflammation and metastasis by inhibiting targets such as Forkhead Box M1 (FOXM1), Wnt/β-catenin, Stat3, NF-κB, PI3K/Akt/mTOR, EGFR, MAPKs, androgen receptors and estrogen receptors (10,16-19). Furthermore, phytochemicals such as EGCG, genistein and sulforaphane have been shown to provide cancer preventive effects through epigenetic regulation by altering expression of DNA methyltransferases and histone deacetylases (20,21).

1.2 Two-Stage Skin Carcinogenesis Model

The two-stage skin carcinogenesis model is a well-established model that mimics the multistage nature of many human cancers and allows us to examine the various stages

of cancer development from initiation, to promotion and finally progression (22). Furthermore, we can use this model to see how phytochemicals affect these different stages of cancer and whether they would be effective as chemopreventive agents.

In this model, a sub-carcinogenic dose of an initiating agent, such as 7,12-dimethylbenz[a]anthracene (DMBA), is first applied topically to the backs of the mice (22). During this initiation step, a mutation occurs primarily in the *Hras* gene, often causing an A to T transversion in codon 61 (22,23). This mutation persists for the lifetime of the animal, but does not cause tumorigenesis alone (22). The keratinocyte stem cells are thought to be the main target of DMBA initiation (24).

The next stage is promotion, in which a promoting agent, such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is repeatedly applied topically (22). This promotion phase is reversible if treatment is not continued and results in hyperplasia, hyperproliferation, increased DNA synthesis and an increase in inflammation (22). This ultimately results in clonal outgrowths called papillomas (22). TPA activates protein kinase C (PKC), which is thought to mediate some of its effects by activating ornithine decarboxylase (ODC) and epidermal growth factor receptor (EGFR) (25). The promotion stage is the most amendable to chemoprevention because it reversible and occurs over a long period of time.

During the progression stage, which can occur independently of continued treatment with a tumor promoter, there is an accumulation of additional genetic changes and dysplasia, and some of the papillomas can convert to squamous cell carcinoma (SCC) (22). Ultimately the SCCs can invade the basement membrane and metastasize (22).

1.2.1 Skin Tumor Promotion by TPA

TPA treatment causes an increase in edema, inflammation and proliferation and alters gene expression and enzyme activities in signaling pathways involved in tumorigenesis (22,25,26). With a single treatment of TPA, leukocytes, macrophages and neutrophils begin to infiltrate into the dermis, and the number of dendritic cells increases, contributing to an inflammatory response (25-27). The expression of several chemokines and cytokines are also upregulated. For example, CXCL2, Cox-2 and TNF-α are upregulated during TPA treatment and a thought to be mediated, at least in part, via activation of PKCα (28). The role of TNF-α seems to be essential in TPA-induced inflammation and in DMBA/TPA-induced carcinogenesis. TNF-α knockout mice were resistant to tumor development in the two-stage carcinogenesis model and TPA-induced proliferation. Moreover, the TNF-α knockout mice had decreased neutrophil and eosinophil infiltration into the dermis (27).

As shown in **Figure 1.1**, TPA binds to and activates PKC, which is thought to mediate many of the effects during skin tumor promotion (25). TPA activation of PKC induces epidermal ODC, the rate limiting enzyme in polyamine synthesis. This increases the levels of putrescine and spermidine and allows for increased DNA synthesis (25,29,30). PKC also activates the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway through phosphorylation of Raf, leading to increased cellular proliferation (26,31-33). In addition, PKC phosphorylates and activates c-Jun N-terminal kinase (JNK) (34). In turn JNK can increase levels of c-Jun and c-Fos, by phosphorylating and activating transcription factors responsible for their transcription, as well as phosphorylate and activate c-Jun (c-

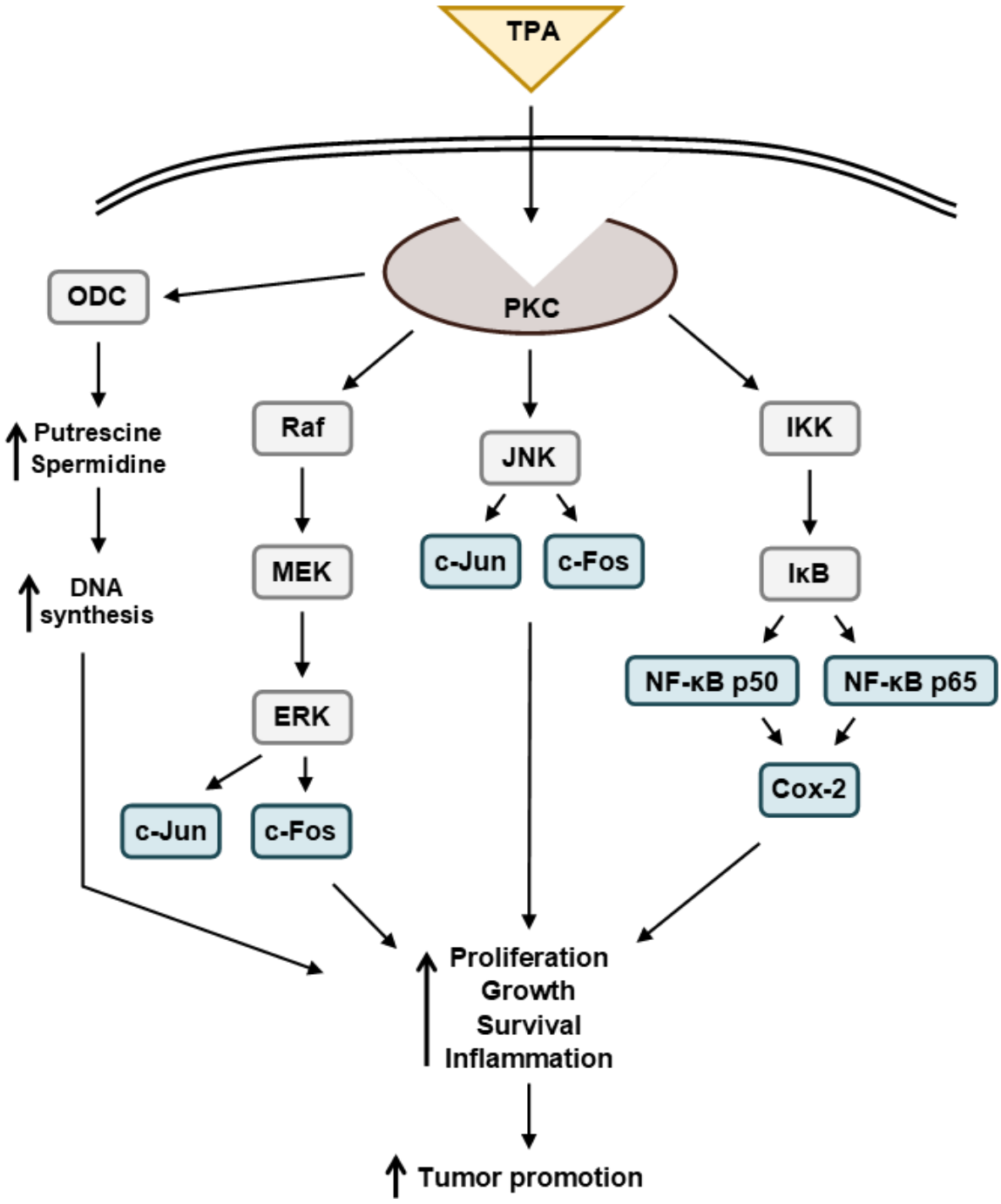

Figure 1.1: TPA activates PKC to mediate tumor promoting effects

Jun can also activate transcription of itself in partner with ATF2) (35). Finally, PKC has also been shown to activate NF-κB signaling by phosphorylating IκB Kinase β (IKKβ) (36).

EGFR is a receptor tyrosine kinase that is overexpressed in many human cancers, including glioblastomas, lung cancer, esophageal cancer and breast cancer (37-40). Furthermore, EGFR is known to play a critical role in skin tumor promotion by TPA. EGFR ligands such as heparin-binding epidermal growth factor (HB-EGF), amphiregulin (AR) and transforming growth factor-alpha (TGFα) have been shown to be upregulated by TPA in the two-stage skin carcinogenesis model, leading to an increase in phosphorylation and activation of EGFR (**Figure 1.2**) (41-43). Moreover, transgenic mice expressing a dominant negative form of EGFR in the basal layer of the epidermis were resistant to tumor growth (44). EGFR mediates its tumor promoting activity by activating a signaling cascade of pathways such the Ras/Raf/MAPK, PI3K/Akt/mTOR and STAT3 pathways, leading to cell proliferation, survival tumor growth (45,46).

IFG-1R is another important growth factor signaling pathway that gets activated during skin tumor promotion by TPA (47). IGF-1 signaling is upregulated in many human cancers, including lung cancer, colorectal cancer, pancreatic cancer and breast cancer, and plays an important role in cell proliferation and inhibition of apoptosis (48). Notably, one of the signaling cascades this activates is the PI3K/Akt/mTOR pathway (49). mTORC1 is a highly conserved serine/threonine kinase that regulates cell growth, proliferation, protein translation, and autophagy via serine/threonine phosphorylation on downstream targets, such as p70S6K, 4EBP1 and ULK1 (50,51).

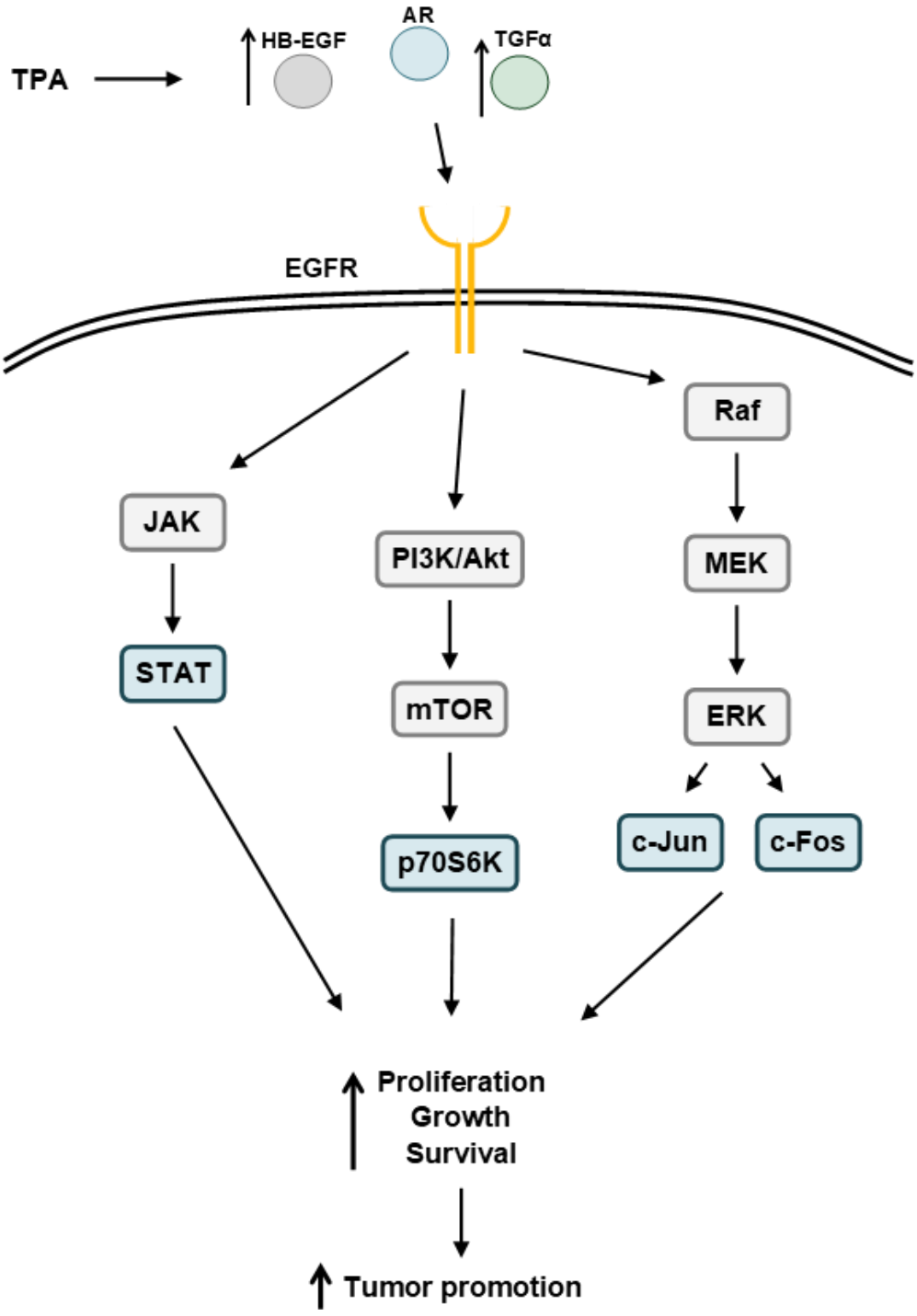

Figure 1.2: TPA activates growth factor signaling pathways

AP-1 is a transcription factor composed of Jun and Fos family members and plays a role in various processes during tumorigenesis, including differentiation, proliferation, and transformation (52). It can activate transcription of proteins involved in these processes such as keratinocyte growth factor, cyclin D1 and MMP-9 and inhibit transcription of p53 (53-57). AP-1 is highly involved in keratinocyte differentiation and proliferation (58). It also plays an important role in skin tumor promotion by TPA. In a mouse model using an AP-1 inhibitor, mice developed significantly less tumors in the two-stage skin carcinogenesis model (59).

NF-κB signaling plays an essential role in inflammation and cancer. Ligands such as IL-1β and TNFα can active Toll-like receptors (TLRs), Interleukin-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors, leading to the phosphorylation of IκB kinase (IKK)(60). Additionally, as mentioned above, PKC can directly phosphorylate IKK (36). IKK then phosphorylates inhibitor of κB (IκB), targeting it for proteosomal degradation, allowing NF-κB p65 and p50 to enter the nucleus and activate transcription of genes (60). NF-κB signaling is highly involved in skin tumor promotion by TPA. Mice with an epidermal keratinocyte-specific deletion of NF-κB p65 developed significantly less tumors in the two-stage skin carcinogenesis assay using DMBA and TPA. Furthermore the absence of p65 in the epidermis inhibited TPA-induced hyperplasia and hyperproliferation (61). In addition, mice with a deletion of Cox-2, whose transcription is activated by NF-κB, were resistant to the two-stage skin carcinogenesis model. Moreover, Cox-2 deficient mice had reduced TPA-induced hyperplasia and hyperproliferation (62). Mice overexpressing Cox-2 developed papillomas after a single

treatment of a subcarcinogenic dose of DMBA and did not require a tumor promoting agent to be applied (63). These studies highlight the importance of NF-κB, particularly Cox-2, signaling during the tumor promotion phase.

1.3 URSOLIC ACID

UA is a pentacyclic triterpenoid found in plants and herbs such as *Perilla frutescens*, rosemary, cranberries and the peels of apples (64,65). UA, like other pentacyclic triterpenoids, has been shown to have many beneficial effects such as anti-inflammatory, antioxidant, anti-proliferative, anti-cancer, and antimicrobial effects (65,66). It has been shown in mouse models to inhibit various types of cancer including non-melanoma skin cancer, breast cancer, prostate cancer, pancreatic cancer and leukemia (67-74).

It has been demonstrated that UA can inhibit the PI3K/Akt/mTORC pathway. In vitro, UA was shown to decrease total levels of PI3K and phosphorylation of Akt in breast cancer cell lines T47D, MCF-7 and MDA-MB-231 and in pancreatic cell line MIA PaCa-2 (74,75). UA also increased phosphorylation of AMPK and decreased phosphorylation of mTOR and Akt in HepG2 liver cancer cells (76). In a colorectal cancer xenograft model, UA decreased phosphorylation of Akt and p70S6K in the tumor tissue (77).

UA can also inhibit NF-κB signaling. UA decreased total levels of IKKα and IκBα and decreased phosphorylation of NF-κB p65 in T47D, MCF-7 and MDA-MB-231 breast cancer cells. Furthermore, this inhibition of NF-κB signaling was thought to contribute to a reduction of levels of TNF-α, IL-6, IL-1β, IL-18 and IFN-γ (75). In a dextran sulfate

sodium induced colitis mouse model, UA protected against colitis, in part, by decreasing levels of nuclear NF-κB p65 (78). UA also inhibited carbon tetrachloride induced nephrotoxicity and hepatotoxicity in mice and decreased levels of nuclear NF-κB p65 (79,80).

Another mechanism by which UA provides anti-tumor effects is by inducing apoptosis. UA has been shown to induce apoptosis in a number of cell lines, including prostate cancer, pancreatic cancer, leukemia and breast cancer (70,72-75,81). Shanmugam et al. found UA increased DNA-fragmentation and annexin V staining in DU145 and LNCaP cells. Furthermore, when DU145 cells were injected in male nude mice, UA administered i.p increased caspase 3 expression in the prostate tumor tissue (70). The same group found that UA given in the diet in a transgenic adenocarcinoma of mouse prostate model increased caspase 3 expression in the dorsolateral prostate, which was thought to mediate some of UA's anti-tumor activity (71).

1.3.1 UA Activity in the Skin

UA inhibits TPA induced inflammation of the skin (64). Furthermore, UA has been shown to inhibit skin tumor promotion by TPA. Specifically, UA applied topically prior to TPA treatment significantly reduced the number of papillomas during the two-stage skin carcinogenesis protocol when initiated with DMBA (67,68,82,83). Our lab has shown that in short term experiments when UA was treated prior to TPA twice weekly for two weeks, UA significantly inhibited TPA-induced proliferation and hyperplasia by inhibiting various signaling pathways that are upregulated by TPA. UA decreased TPA-induced

phosphorylation of NF-κB p65, Akt and JNK 1/2 as well as levels of Cox 2. In addition, UA inhibited TPA-induced infiltration of mast cells and $CD45^+$ cells (67,68). Finally, UA decreased TPA-induced gene expression of inflammatory markers such as Cox-2, CXCL2 and IL-6 (67,68,83).

1.4 CURCUMIN

Curcumin (Curc), a polyphenol found in turmeric, has been shown to possess anti-inflammatory, anti-proliferative and antioxidant activity, which are thought to play major roles in curcumin's chemoprevention action (84). Curc has been shown in a number of mouse models to inhibit tumorigenesis, including non-melanoma skin cancer, colorectal cancer, lung cancer and glioma (85-88).

Curc has been shown to inhibit NF-κB signaling in numerous instances, which contributes to its anti-tumor activity. In early studies, Curc was shown to inhibit NF-κB binding activity in ML-1a cells (human myelomonoblastic leukemia cells) and HT-29 cells (human colonic epithelial cells) (89,90). More recent studies have continued to demonstrate this. For example, in human colon SW480 and LoVo cells, Curc inhibited NF-κB binding activity, which was thought to contribute to decreasing levels of matrix metalloproteinase-9 (MMP9) and urokinase-type plasminogen activator (uPA) (91). In human pancreatic cancer BxPC-3 and Panc-1 cells, Curc inhibited hydrogen peroxide induced phosphorylation of NF-κB and levels of MMP2 and MMP9 (92). Curc also inhibited NF-κB activity and decreased level of MMP-9 in human osteoclastoma cells (93).

Another well-known target of Curc is Stat3. Bharti et al. were one of the first groups

to demonstrate this and showed that Curc inhibited constitutive phosphorylation of Stat3 and translocation of Stat3 to the nucleus in human multiple myeloma cell line U266 (94). More recently, our group has shown that Curc inhibits phosphorylation of Stat3 in mouse prostate cancer HMVP2 cells (95). In a human non-small cell lung cancer ectopic xenograft model, Curc treatment decreased tumor growth and phosphorylation of Stat3 in the tumor tissue (96).

Curc has also been shown to induce apoptosis in a variety of cells. Curc induced caspase-3 activity in human osteoclastoma cells (93). In HT-29 colon adenocarcinoma cells, Curc increased the number of apoptotic cells, which was thought to be induced by an increase in reactive oxygen species (ROS) (97). Curc also decreased levels of anti-apoptotic protein Bcl2 and increased levels of apoptotic proteins Bax, caspase 8, cleaved caspase 9 and cleaved caspase 3 in breast cancer stem cells from SUM159 and MCF7 tumor spheres (98). In melanoma cell lines, Curc increased apoptosis in a manner independent of p53 by activating the Fas death receptor (99).

1.4.1 Curcumin Activity in the Skin

Curc has been shown to have anti-inflammatory effects in the skin and to inhibit tumor development in the two-stage skin carcinogenesis model (85,100). In one study, Curc was shown to inhibit skin tumor promotion by TPA by a strong anti-oxidant effect as shown by a significant reduction in the formation of the oxidized DNA base 5-hydroxymethyl-29-deoxyuridine and the production of hydrogen peroxide. Curc also inhibited DNA synthesis in this study (85). Furthermore, Curc has been shown to inhibit Cox activity as shown by a decrease in metabolism of arachidonic acid to prostaglandin E_2

(PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (100). In another study, Curc decreased TPA-induced mRNA levels of c-Jun, c-Fos and c-Myc (101).

1.5 RESVERATROL

Resveratrol (Res) is a polyphenol phytoalexin found in many plants, including grapes, berries, plums and peanuts (102). Like UA and Curc, Res also has anti-inflammatory, antioxidant and anti-proliferative effects (102). Res has been shown to inhibit tumorigenesis in many rodent models, such as skin, colon, breast, liver, pancreatic and prostate cancer (103-108). Initial studies demonstrated the chemopreventive effects of Res due to its ability to inhibit Cox-1 and Cox-2 activity, inflammation and free radical formation. Subsequently, Res was able to inhibit skin tumor promotion by TPA (103). Furthermore, our lab has shown Res inhibited TPA-induced phosphorylation of NF-κB p65 and Akt and others have shown Res inhibited TPA-induced AP-1 activity (67,109). In squamous cell carcinoma cells Ca3/7, Res protected against oxidative DNA damage and inhibited activity of the cytochrome P450 (CYP) enzymes CYP1A1 and CYP1B1 (13).

1.6 METFORMIN

Metformin (Met) is a common diabetes medication and has been shown in retrospective studies to reduce cancer incidence in type II diabetic patients (110). Additionally, several mouse model studies have corroborated the association of metformin and cancer prevention (111-114). Metformin activates AMPK and as a result leads to reduced gluconeogenesis (115). AMPK negatively regulates the PI3K/Akt/mTORC1 pathway (116). As mentioned above, this pathway regulates cell growth, proliferation,

protein translation, and autophagy (50,51). Our lab has previously shown that Met inhibits skin tumor promotion by TPA (111). In this study, Met significantly decreased TPA-induced serum insulin levels in obese mice and reduced epidermal hyperproliferation and hyperplasia. Furthermore, Met inhibited the PI3K/Akt/mTOR pathway by activating AMPK and producing a subsequent decrease in phosphorylation of p70S6K and S6 ribosomal and increase in levels of the tumor suppressor Pdcd4 (111).

Chapter 2: Materials and Methods

2.1 Animals and Diets

Female ICR (CD-1) (Envigo, Indianapolis, IN) were fed ad libitum and group housed for all experiments. For all topical experiments, mice were maintained on a regular chow diet. For the diet study short term experiments, mice were maintained on an AIN-76A diet with or without UA (2 g/kg), Res (5 g/kg) and Curc (10 g/kg). For the diet study two-stage skin carcinogenesis experiments, mice were maintained on an AIN-93M diet with or without UA (2 g/kg), Res (5 g/kg) and Curc (10 g/kg). All animal experiments were performed according to protocols approved by The University of Texas at Austin Institutional Animal Care and Use Committee.

2.2 Chemicals

For the topical experiments, UA (90%) was purchased from Sabinsa (East Windsor, NJ) and Curc (≥65%) was purchased from Sigma Aldrich (St. Louis, MO). UA (98%) and Curc (95%) used for incorporation into the diets were purchased from Standford Chemicals (Irvine, CA). Res (99%) for incorporation into the diets was purchased from Mega Resveratrol (Danbury, CT). DMBA (≥95%) and 5-Bromo-2′-deoxyuridine (BrdU) (≥99%) were purchased from Sigma Aldrich (St. Louis, MO). TPA (>99.5%) was purchased from LC Laboratories (Woburn, MA).

2.3 TWO-STAGE SKIN CARCINOGENESIS

2.3.1 Topical experiment

As shown in **Figure 2.1**, female ICR mice 7 weeks old (n = 30/group) were shaved on the dorsal skin and then two days later initiated topically with 25 nmol DMBA. Two weeks after initiation with DMBA, mice were pretreated topically with acetone (Ace) vehicle, UA (1 μmol), Curc (2 μmol) or a combination of UA (1 μmol) and Curc (2 μmol) prior to TPA (6.8 nmol) treatment (UA was applied 15 minutes prior and Curc was applied 30 minutes prior to TPA). All treatments were given twice-weekly for the duration of the experiment (25 weeks). Bodyweight and tumor incidence (percent of mice with papillomas) were measured once a week, and tumor multiplicity (average number of papillomas per mouse) was measured every other week. Tumor size was measured using digital calipers at the termination of the experiment. Treatment groups for this experiment are shown in **Table 2.1**.

2.3.2 Diet study

As shown in **Figure 2.2**, female ICR mice, 6 weeks of age (n = 30/group), were initiated with 25 nmol DMBA. Two weeks after initiation with DMBA, mice were started on diets containing UA (2 g/kg diet), Res (5 g/kg diet), Curc (10 g/kg diet), UA (2 g/kg) + Res (5 g/kg), UA (2 g/kg) + Curc (10 g/kg) or Res (5 g/kg) + Curc (10 g/kg) and/or given metformin in the drinking water (250 mg/kg bw). TPA (6.8 nmol) treatment began four weeks after the experimental diets were started and was treated topically twice-weekly for

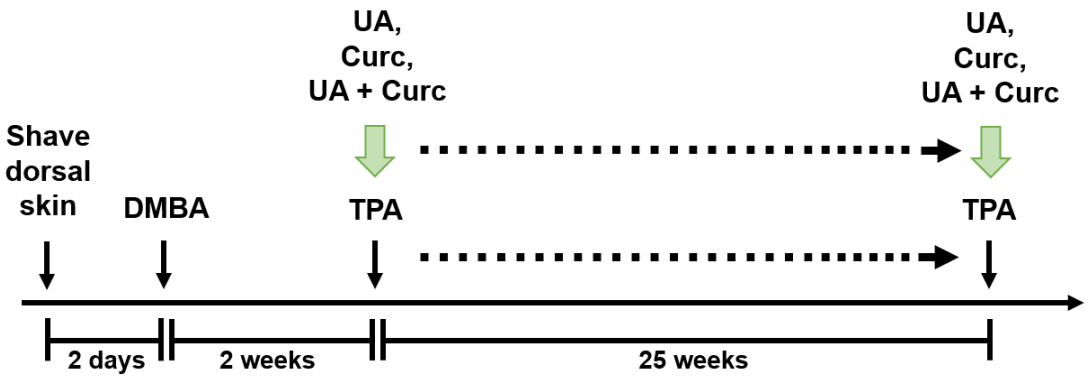

Figure 2.1: Topical two-stage skin carcinogenesis protocol

Group	Initiation	1st Pretreatment (-30 min)	2nd Pretreatment (-15 min)	Treatment
Ace	DMBA (25 nmol)	Ace (200 µL)	Ace (200 µL)	Ace (200 µL)
UA (2) + Ace	DMBA (25 nmol)	Ace (200 µL)	UA (2 µmol)	Ace (200 µL)
Curc (2) + Ace	DMBA (25 nmol)	Curc (2 µmol)	Ace (200 µL)	Ace (200 µL)
UA (2) + Curc (2) + Ace	DMBA (25 nmol)	Curc (2 µmol)	UA (2 µmol)	Ace (200 µL)
TPA	DMBA (25 nmol)	Ace (200 µL)	Ace (200 µL)	TPA (6.8 nmol)
UA (1) + TPA	DMBA (25 nmol)	Ace (200 µL)	UA (1 µmol)	TPA (6.8 nmol)
UA (2) + TPA	DMBA (25 nmol)	Ace (200 µL)	UA (2 µmol)	TPA (6.8 nmol)
Curc (1) + TPA	DMBA (25 nmol)	Curc (1 µmol)	Ace (200 µL)	TPA (6.8 nmol)
Curc (2) + TPA	DMBA (25 nmol)	Curc (2 µmol)	Ace (200 µL)	TPA (6.8 nmol)
UA (1) + Curc (1) + TPA	DMBA (25 nmol)	Curc (1 µmol)	UA (1 µmol)	TPA (6.8 nmol)
UA (2) + Curc (1) + TPA	DMBA (25 nmol)	Curc (1 µmol)	UA (2 µmol)	TPA (6.8 nmol)
UA (1) + Curc (2) + TPA	DMBA (25 nmol)	Curc (2 µmol)	UA (1 µmol)	TPA (6.8 nmol)
UA (2) + Curc (2) + TPA	DMBA (25 nmol)	Curc (2 µmol)	UA (2 µmol)	TPA (6.8 nmol)

Table 2.1: Treatment groups used for topical two-stage skin carcinogenesis assay

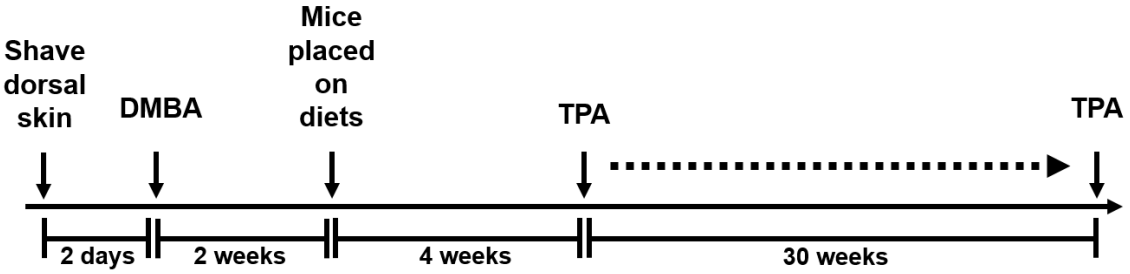

Figure 2.2: Diet study two-stage skin carcinogenesis protocol

Group	Initiation	Diet/Water	Treatment
AIN-93M	DMBA (25 nmol)	AIN-93M [Control diet]	Ace (200 µL)
UA	DMBA (25 nmol)	UA (2 g/kg)	Ace (200 µL)
Res	DMBA (25 nmol)	Res (5 g/kg)	Ace (200 µL)
Curc	DMBA (25 nmol)	Curc (10 g/kg)	Ace (200 µL)
Met	DMBA (25 nmol)	AIN-93M + Met (250 mg/kg bw)	Ace (200 µL)
Met + UA	DMBA (25 nmol)	Met (250 mg/kg bw) + UA (2 g/kg)	Ace (200 µL)
Met + Res	DMBA (25 nmol)	Met (250 mg/kg bw) + Res (5 g/kg)	Ace (200 µL)
Met + Curc	DMBA (25 nmol)	Met (250 mg/kg bw) + Curc (10 g/kg)	Ace (200 µL)
UA + Res	DMBA (25 nmol)	UA (2 g/kg) + Res (5 g/kg)	Ace (200 µL)
UA + Curc	DMBA (25 nmol)	UA (2 g/kg)+ Curc (10 g/kg)	Ace (200 µL)
Res + Curc	DMBA (25 nmol)	Res (5 g/kg) + Curc (10 g/kg)	Ace (200 µL)
AIN-93M + TPA	DMBA (25 nmol)	AIN-93M [Control diet]	TPA (6.8 nmol)
UA + TPA	DMBA (25 nmol)	UA (2 g/kg)	TPA (6.8 nmol)
Res + TPA	DMBA (25 nmol)	Res (5 g/kg)	TPA (6.8 nmol)
Curc + TPA	DMBA (25 nmol)	Curc (10 g/kg)	TPA (6.8 nmol)
Met + TPA	DMBA (25 nmol)	AIN-93M + Met (250 mg/kg bw)	TPA (6.8 nmol)
Met + UA + TPA	DMBA (25 nmol)	Met (250 mg/kg bw) + UA (2 g/kg)	TPA (6.8 nmol)
Met + Res + TPA	DMBA (25 nmol)	Met (250 mg/kg bw) + Res (5 g/kg)	TPA (6.8 nmol)
Met + Curc + TPA	DMBA (25 nmol)	Met (250 mg/kg bw) + Curc (10 g/kg)	TPA (6.8 nmol)
UA + Res + TPA	DMBA (25 nmol)	UA (2 g/kg) + Res (5 g/kg)	TPA (6.8 nmol)
UA + Curc + TPA	DMBA (25 nmol)	UA (2 g/kg) + Curc (10 g/kg)	TPA (6.8 nmol)
Res + Curc	DMBA (25 nmol)	Res (5 g/kg) + Curc (10 g/kg)	TPA (6.8 nmol)

Table 2.2: Treatment groups used for diet study two-stage skin carcinogenesis assay

the duration of the experiment (30 weeks). Bodyweight, tumor incidence, tumor multiplicity and tumor size were measured as described in the topical experiment. Treatment groups used for this experiment are shown in **Table 2.2**.

2.4 SHORT TERM TREATMENT PROTOCOL

2.4.1 Topical experiment

As shown in **Figure 2.3**, the dorsal skin of female ICR mice (7–9 weeks of age, n = 4-5/group) was shaved and then treated two days later with either Ace vehicle, UA (1 μmol), Curc (2 μmol) or a combination of UA (1 μmol) + Curc (2 μmol) prior to TPA (6.8 nmol) treatment (UA was applied 15 minutes prior and Curc was applied 30 minutes prior to TPA). All treatments were given twice weekly for two weeks. The treatment groups used for these experiments are shown in **Table 2.3**

2.4.2 Diet study

As shown in **Figure 2.4**, six week old, female, ICR mice were received and placed on AIN-76A diet for equilibration. One week later, mice were placed on diets containing UA (2 g/kg diet), Res (5 g/kg diet), Curc (10 g/kg diet), the combination of Curc (10 g/kg) and UA (2 g/kg), the combination of Res (5 g/kg) and UA (2 g/kg) or remained on the control diet (AIN-76A). One group received Met in the drinking water for comparison (250 mg/kg bw per day). Four weeks later the dorsal skin of the mice was shaved two days prior to the first TPA treatment. TPA (6.8 nmol) was applied topically twice weekly for two weeks and Ace (200 μL) was used as the vehicle control. Treatment groups used for these

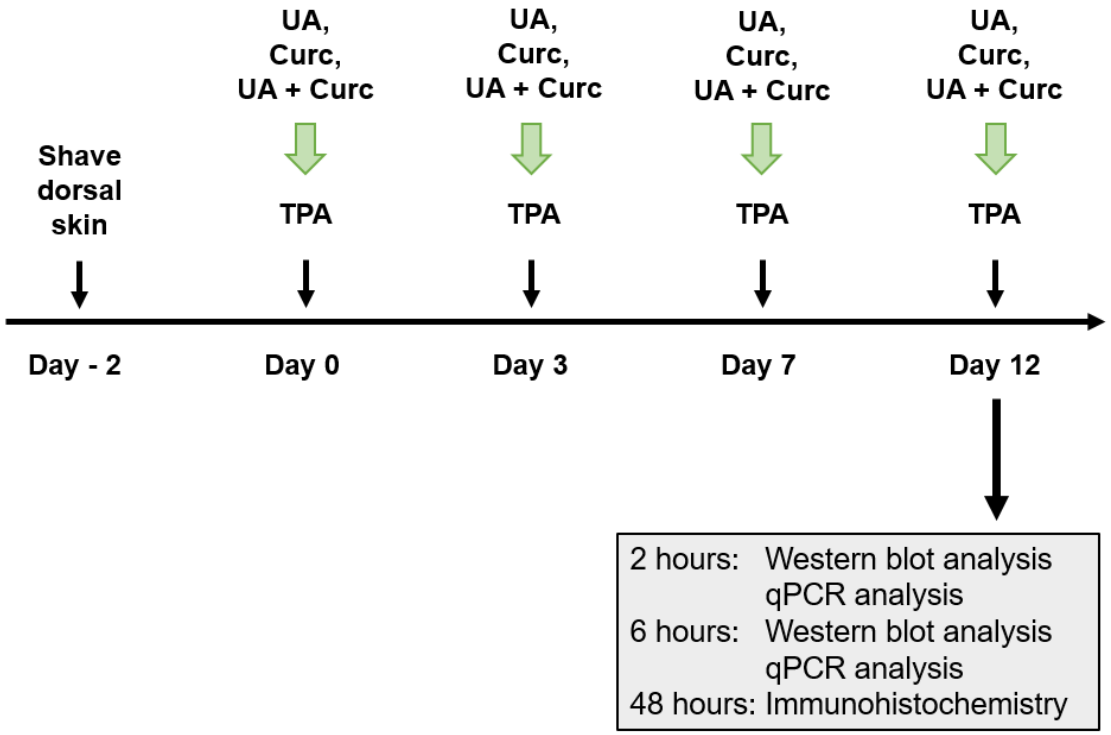

Figure 2.3: Short term topical experiment protocol

Group	1st Pretreatment (-30 min)	2nd Pretreatment (-15 min)	Treatment
Ace	Ace (200 µL)	Ace (200 µL)	Ace (200 µL)
TPA	Ace (200 µL)	Ace (200 µL)	TPA (6.8 nmol)
UA (1 µmol) + TPA	Ace (200 µL)	UA (1 µmol)	TPA (6.8 nmol)
Curc (2 µmol) + TPA	Curc (2 µmol)	Ace (200 µL)	TPA (6.8 nmol)
Curc (2 µmol) + UA (1 µmol) + TPA	Curc (2 µmol)	UA (1 µmol)	TPA (6.8 nmol)

Table 2.3: Treatment groups for short term topical experiment

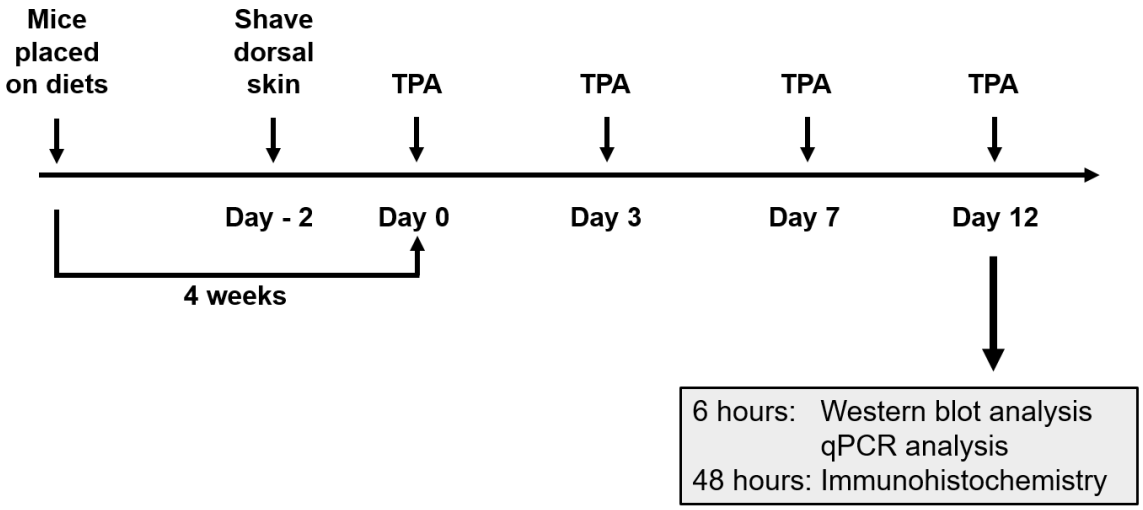

Figure 2.4: Short term diet study protocol

Group	Diet/Water	Treatment
AIN-76A	AIN-76A [Control diet]	Ace (200 µL)
UA	UA (2 g/kg)	Ace (200 µL)
Res	Res (5 g/kg)	Ace (200 µL)
Curc	Curc (10 g/kg)	Ace (200 µL)
UA + Res	UA (2 g/kg) + Res (5 g/kg)	Ace (200 µL)
UA + Curc	UA (2 g/kg)+ Curc (10 g/kg)	Ace (200 µL)
Met	AIN-76A + Met (250 mg/kg bw)	Ace (200 µL)
AIN-76A + TPA	AIN-76A [Control diet]	TPA (6.8 nmol)
UA + TPA	UA (2 g/kg)	TPA (6.8 nmol)
Res + TPA	Res (5 g/kg)	TPA (6.8 nmol)
Curc + TPA	Curc (10 g/kg)	TPA (6.8 nmol)
UA + Res + TPA	UA (2 g/kg) + Res (5 g/kg)	TPA (6.8 nmol)
UA + Curc + TPA	UA (2 g/kg)+ Curc (10 g/kg)	TPA (6.8 nmol)
Met + TPA	AIN-76A + Met (250 mg/kg bw)	TPA (6.8 nmol)

Table 2.4: Treatment groups used for short term diet study

experiments are shown in **Table 2.4**.

2.5 HISTOLOGICAL ANALYSES

Mice were treated as described in the short term protocols (**Figures 2.3** and **2.4, Tables 2.3** and **2.4**). BrdU (100 μg/g bw) was dissolved in PBS and injected i.p. to mice 30 minutes prior to sacrifice. 48 hours after the last TPA treatment, the dorsal skin was fixed in 10% formalin-buffered solution, embedded in paraffin and sectioned for BrdU (Abcam, Cambridge, MA) or toluidine blue O (Fisher Scientific, Pittsburgh, PA) staining. The labeling index (LI) of the BrdU-stained sections was measured as previously described (117). Epidermal thickness was measured by taking the average of 20 measurements per skin section for each mouse.

2.6 WESTERN BLOT ANALYSES

Mice were treated as described in the short term treatment protocols (**Figures 2.3** and **2.4, Tables 2.3** and **2.4**) and sacrificed two or six hours after the last TPA treatment. Epidermal tissue was collected and western blot analyses were performed using epidermal protein lysates as previously described (118). For some experiments, the nuclear fraction was extracted using Thermo Fisher (Waltham, MA) NE-PER™ Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol. Antibodies against the following proteins were purchased from Cell Signaling Technology (Danvers, MA): p-c-Jun^{S73} (#9164), c-Jun (#9165S), c-Fos (#2250), p-Rb^{S780} (#9307), p-Src^{Y416} (#2101), Src (#2109), p-p70S6K^{T389} (#9234), p70S6K (#9202), Vinculin (#13901), GAPDH (#2118), p-Stat3^{Y705} (# 9145), p-Stat3^{S727} (#9134) and Stat3 (#9139). Antibodies against p-IκBα$^{S32/36}$

(sc-101713), IκBα (sc-847), p-p50^{S337} (sc-101744) and p50 (sc-8414) were purchased from Santa Cruz Biotechnology (Dallas, TX). Cox-2 (160126) was purchased from Cayman Chemical (Ann Arbor, MI), EGFR (06-847) was purchased from Millipore (Burlington, MA), p-EGFR^{Y1086} (ab5650) was purchased from AbCam (Cambridge, MA) and p27 (610242) was purchased from BD Biosciences (Bradford, MA).

2.7 qRT-PCR Analyses

Mice were treated as described in the short-term protocol and sacrificed six hours after the last treatment. Epidermal RNA samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was then prepared using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Grand Island, NY) according to the manufacturer's protocol. For qRT-PCR analysis, 2 µL of cDNA was mixed with 5 µL of 2X iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA), 1 µL of 10 µM forward primers, 1 µL of 10 µM reverse primers and 1 µL of RNase-free water for a total volume of 10 µL. qRT-PCR reactions were performed and analyzed on a Viia 7 (Applied Biosystems, Carlsbad, CA) using the comparative CT method and normalized to GAPDH.

2.8 miR-21 Analysis

Mice were treated as described in the short-term protocol and sacrificed six hours after the last treatment. Epidermal RNA samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was then prepared using the TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems,

Grand Island, NY) according to the manufacturer's protocol. qRT-PCR reactions were performed using the TaqMan Advanced miRNA Assay (mmu-miR-21a-5p) according to the manufacturer's protocol . Reactions were analyzed on a Viia 7 using the comparative CT method and normalized to miR-16-5p (has-miR-16-5p).

2.9 STATISTICAL ANALYSIS

The Mann–Whitney U test was used for analysis of tumor multiplicity, tumor size, LI, epidermal thickness, quantitation of Western blots, gene expression and mast cell infiltration. The Mantel–Cox test was used for analysis of tumor latency. Fisher's exact test was used for analysis of tumor incidence. Significance in all cases was set at $p \leq 0.05$.

Chapter 3: Inhibition of Skin Tumor Promotion by TPA with the Topical Combination of UA + Curc

3.1 INTRODUCTION

Despite the many advances that have been made in understanding cancer and developing new therapies, cancer is still the second leading cause of death in the US and accounts for about one in four deaths. Furthermore, approximately two out of five people will develop cancer in their lifetime, and in many cases cancer can be preventable (1). Aside from lifestyle choices such as avoiding tobacco products and maintaining a lean bodyweight, chemoprevention remains a strategy to reduce cancer risk, especially in high-risk populations. Chemoprevention is the use of natural, synthetic, or biological agents to reverse, suppress or prevent the initial development of carcinogenesis or the progression to metastatic cancer (2). Phytochemicals have been widely studied as potential cancer chemopreventive agents given their abundance and overall relatively low toxicity (8-11).

UA and Curc are two examples of phytochemicals that have been shown to inhibit tumor development as single agents in a two-stage skin carcinogenesis model, especially during the tumor promotion stage (67,68,85). UA is a pentacyclic triterpenoid found in plants and herbs such as *Perilla frutescens* (Japanese basil), rosemary, cranberries and the peels of apples (64,65). UA has been shown to possess many beneficial cancer prevention properties including anti-inflammatory, antioxidant and anti-proliferative activities (64,65). Curc, a polyphenol found in turmeric, has been shown to possess anti-inflammatory and anti-oxidant activities, which are thought to play major roles in its

chemopreventive action (84). UA and Curc have low toxicity and are well tolerated in patients, making them ideal candidates as cancer prevention agents (119,120).

A number of combinations of phytochemicals have now been tested and show increased anti-tumor activity when combined than when given as individual compounds. For example, our lab has previously shown that the combination of UA + Res inhibits skin tumor promotion to a greater extent than the compounds alone (67). Another study from our laboratory examined the combinations of UA + Curc, UA + Res and Res + Curc given in the diet and demonstrated a synergistic inhibition of tumor growth in an allograft prostate model (95). Xu et al. showed the combination of dietary Curc + green tea catechins provided greater inhibition of dimethylhydrazine-induced colon carcinogenesis than the individual compounds (121). These and many other studies demonstrate the greater potential for combinations of phytochemicals to be more effective as chemopreventive agents.

For the current study, we applied UA and Curc individually as well as in combination topically during the tumor promotion phase before each TPA treatment to determine if the combination was more effective at inhibiting skin tumor development than the individual compounds. The results demonstrate that the combination of UA + Curc inhibited skin tumor promotion by TPA to a greater extent than either of the compounds alone, producing a significant combinatorial chemopreventive effect. The greater chemopreventive effect of the combination was associated with greater inhibition of both growth factor and inflammatory signaling pathways.

3.2 RESULTS

3.2.1 Effect of UA, Curc and UA + Curc on skin tumor promotion by TPA

A two-stage skin carcinogenesis assay was performed to examine the effects of UA, Curc, and the combination of UA + Curc on skin tumor promotion by TPA. We found that pretreatment with UA, Curc, and the combination significantly decreased final tumor multiplicity by 35%, 63%, and 75%, respectively, as shown in Fig. 1B ($p < 0.05$, Mann-Whitney *U* test). In addition, the combination significantly reduced tumor multiplicity compared to the UA alone treated group ($p < 0.05$, Mann-Whitney *U* test). The final tumor incidence was reduced from 97% in the TPA treated group to 86% and 72% in the Curc and combination treated groups, respectively (**Figure 3.1A**). The reduction in tumor incidence by the combination of UA and Curc was significant compared to the TPA and UA + TPA groups ($p < 0.05$, Fisher's exact test). **Figure 3.1C** shows that pretreatment with UA or Curc delayed the onset of tumor development compared to TPA ($p < 0.05$; Mantel-Cox test). Furthermore, the combination significantly delayed the onset of tumor development to a greater extent than both UA and Curc alone ($p < 0.05$; Mantel-Cox test). Finally, the combination of UA + Curc significantly decreased tumor size and weight compared to TPA only, UA + TPA, and Curc + TPA treated groups (**Figure 3.1E, F**) ($p < 0.05$, Mann-Whitney *U* test). No apparent toxicity was observed in any of the phytochemical treated groups, as there was no significant difference in bodyweight between the different treatments (**Figure 3.1D**). Overall, these results show that the combination of UA + Curc provided greater inhibition of skin tumor promotion by TPA

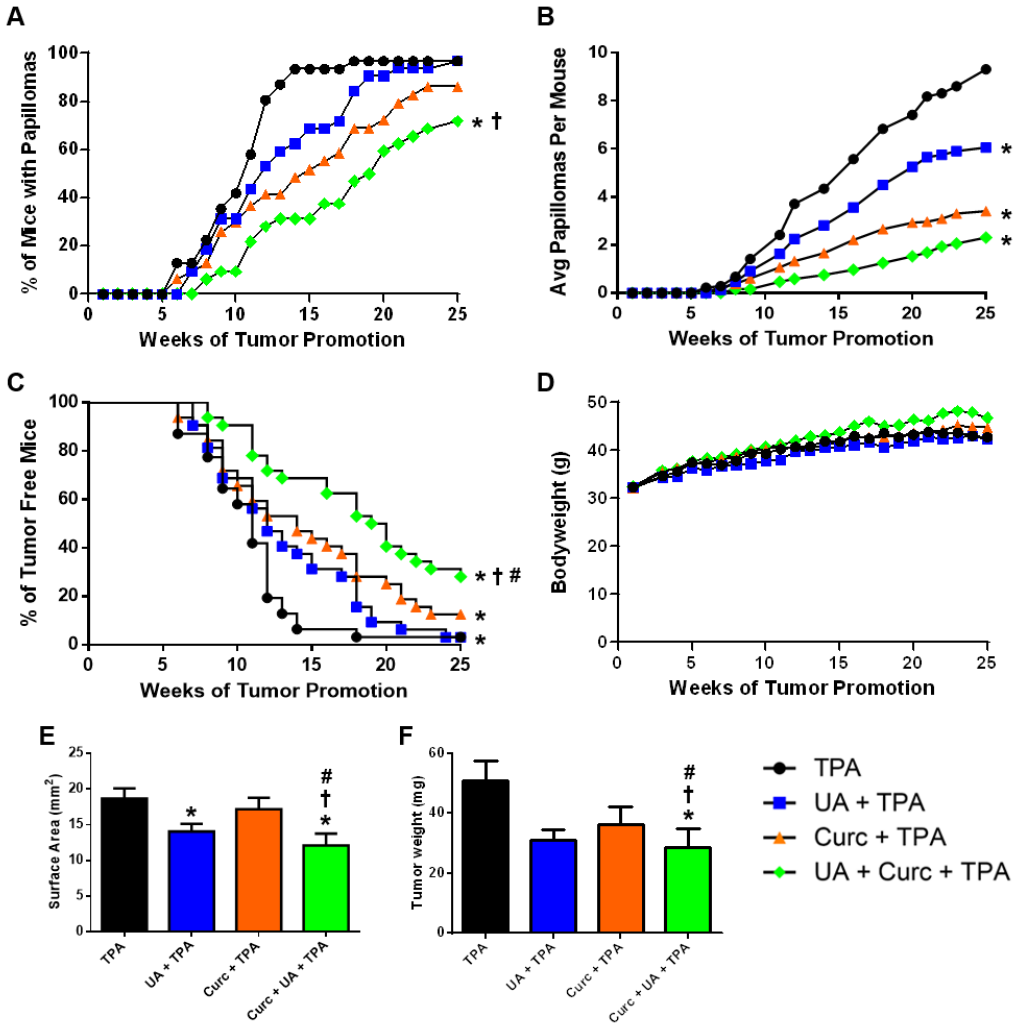

Figure 3.1: Effect of UA and Curc on skin tumor promotion by TPA

Female ICR mice 7 weeks old (n = 30/group) were initiated with 25 nmol DMBA. Two weeks after initiation with DMBA, mice were pretreated with Ace vehicle, UA (1 µmol), Curc (2 µmol) or the combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. All treatments were given twice-weekly for the remainder of the experiment (25 weeks). **A**. Tumor multiplicity. **B**. Tumor incidence. **C**. Tumor latency shown using a Kaplan Meier curve of tumor free mice. **D**. Average bodyweight (g) per mouse. **E**. Average surface area of tumors (mm^2). **F**. Average tumor weight (mg). * Significant when compared to Ace + TPA group; †, Significant when compared to UA + TPA; #, Significant when compared to Curc + TPA; ($p < 0.05$). Mann-Whitney U test was used for analysis of tumor multiplicity, tumor size and tumor weight, Fisher's exact test was used for analysis of tumor incidence, and Mantel-Cox test was used for analysis of tumor latency.

than either of the compounds alone.

3.2.2 Effect of UA, Curc and UA + Curc on TPA-induced epidermal hyperproliferation

UA, Curc and the combination significantly decreased TPA induced epidermal hyperproliferation as shown in **Figure 3.2**. Pretreatment with UA or Curc alone significantly reduced LI from 15.20% in the TPA only group to 11.03% and 11.07%, respectively, and epidermal thickness from 44.81 μm in the TPA only group to 34.69 μm and 33.45 μm ($p \leq 0.05$; Mann-Whitney *U* test)(**Figure 3.2B,C**). The combination of UA + Curc further reduced LI to 7.02% and epidermal thickness to 25.86 μm, which was significantly lower than the TPA only, UA + TPA and Curc + TPA groups ($p \leq 0.05$; Mann-Whitney *U* test). These results demonstrate that the combination of UA + Curc was more effective than either of the compounds given alone at reducing TPA-induced epidermal cell proliferation.

3.2.3 Effect of UA, Curc and UA + Curc on TPA-induced epidermal signaling pathways

TPA induces activation of a number of signaling pathways that are required for the promotion of skin tumors in the two-stage model (22). Using the short-term protocol, we investigated which signaling pathways were most impacted by the combination of UA + Curc. As presented in **Figure 3.3**, TPA induced increased phosphorylation of EGFR, Src, p70S6K, c- Jun, IκBα, NF-κB p50 and Rb as well as increased total protein levels of c-Jun, c-Fos, NF-κB p50 and Cox-2. TPA treatment also led to decreased levels of epidermal p27.

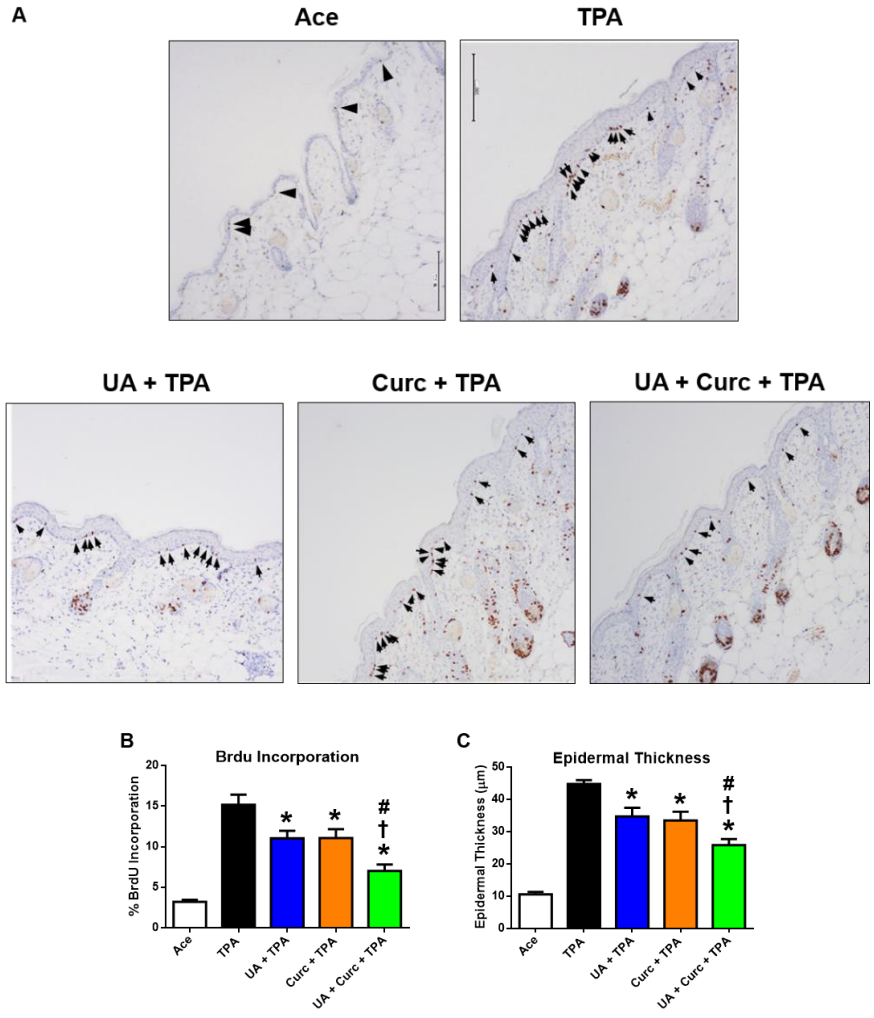

Figure 3.2: Effect of UA, Curc and the combination of UA + Curc on TPA-induced epidermal hyperproliferation and hyperplasia

The dorsal skin of female ICR mice (7–9 weeks of age; n = 8/group) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol) or a combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. All treatments were given twice-weekly for two weeks. Forty-eight hours after the last TPA treatment, dorsal skin was fixed in 10% formalin-buffered solution, embedded in paraffin and sectioned for BrdU-staining. **A**. Representative BrdU-stained sections of dorsal skin. Arrows indicate BrdU stained cells in the epidermis. **B**. Quantitative evaluation (mean ± SEM) of epidermal hyperproliferation (BrdU incorporation). **C**. Quantitative evaluation (mean ± SEM) of hyperplasia (epidermal thickness). *, Significant when compared to TPA group; †, Significant when compared to UA + TPA; #, Significant when compared to Curc + TPA; ($p \leq 0.05$, Mann-Whitney U test).

Pretreatment with Curc alone significantly decreased phosphorylation of p70S6K and NF-κB p50, decreased total levels of NF-κB p50 and increased levels of p27 compared to the TPA only group. Pretreatment with UA alone significantly decreased phosphorylation of IκBα and NF-κB p50 and increased levels of p27 compared to the TPA only group. Notably, when both compounds were given together before TPA treatment, the combination significantly decreased phosphorylation of EGFR, Src, p70S6K, c-Jun, IκBα, NF-κB p50 and Rb compared to the TPA only group. The combination also significantly reduced protein levels of c-Jun, c-Fos NF-κB p50 and Cox-2 compared to the TPA only group. When compared to the individual compounds, the most significant effects of the combination were seen on phosphorylation of EGFR, Src, c-Jun, IκBα and Rb and on total levels of c-Jun, c-Fos and Cox-2. Thus, the combination significantly inhibited a much broader range of target pathways known to play an important role in skin tumor promotion.

Since NF-κB, c-Jun and c-Fos are transcription factors, further experiments were performed to examine the status of their nuclear localization following treatment with the combination. Indeed we found, particularly with the combination of UA + Curc, that the nuclear levels of c-Fos, c-Jun, p-c-Jun^{Ser73}, NF-κB p50 and p-NF-κB p50^{Ser336} were all decreased (**Figure 3.4**). Finally, although the individual compounds showed no effect on phosphorylation of Stat3, the combination of UA + Curc significantly reduced tyrosine phosphorylation of Stat3 compared to TPA at the two hour time point (**Figure 3.5**).

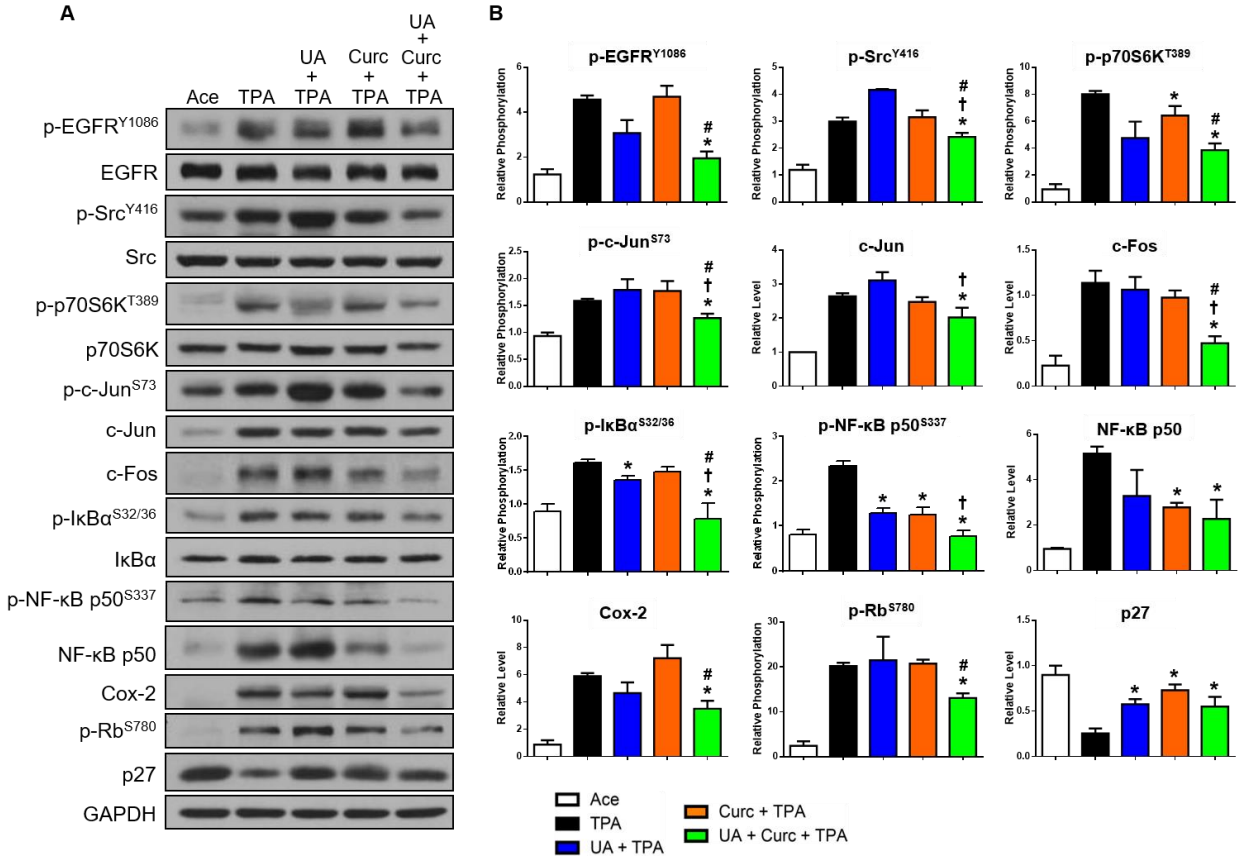

Figure 3.3: Effect of UA, Curc and the combination of UA + Curc on TPA-induced signaling pathways

The dorsal skin of female ICR mice (7–9 weeks of age; n = 4-5/group) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol) or a combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. Six hours after the last TPA treatment, epidermal lysates were prepared for Western blot analyses (pooled groups). A, Representative Western blots. B, Quantitation of Western blots (mean ± SEM; average of at least 3 independent experiments). *, Significant when compared to TPA group; †, Significant when compared to UA + TPA; #, Significant when compared to Curc + TPA; ($p \leq 0.05$, Mann-Whitney U test).

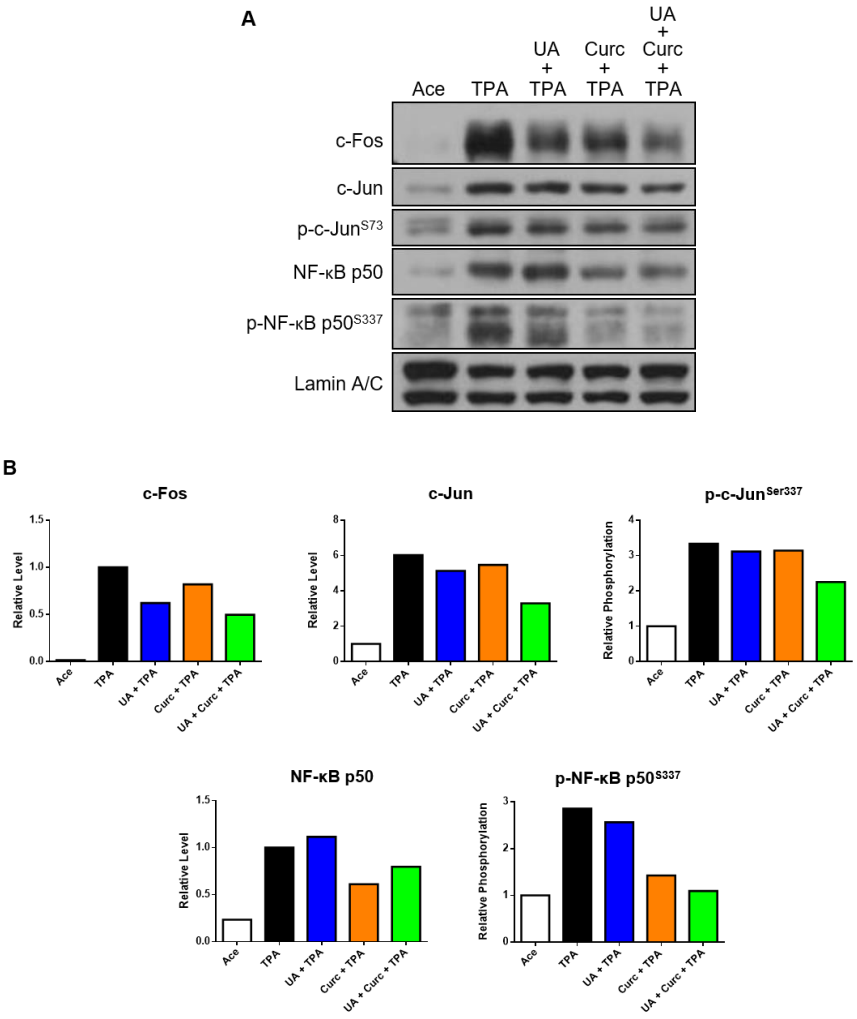

Figure 3.4: Effect of the combination of UA + Curc on TPA-induced signaling in the nucleus

The dorsal skin of female ICR mice (7–9 weeks of age) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol) or a combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. Six hours after the last TPA treatment, the nuclear fractions from epidermal lysates were prepared for Western blot analyses. **A**. Representative Western Blots. **B**. Quantitation of Western blots. Graph represent mean (average of at technical replicates).

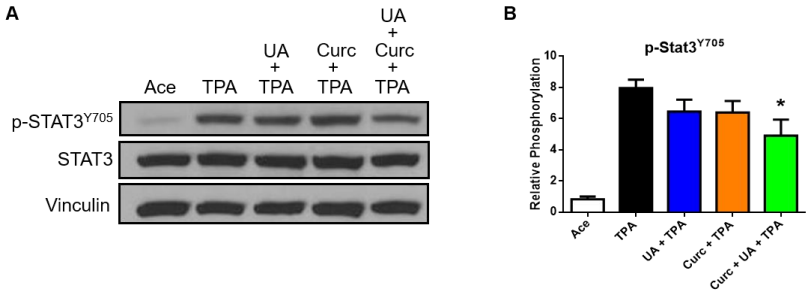

Figure 3.5: Effect of the combination of UA + Curc on TPA-induced Stat3 signaling

The dorsal skin of female ICR mice (7–9 weeks of age) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol), or a combination of UA (1 µmol) and Curc (2 µmol) prior to TPA (6.8 nmol) treatment. Two hours after the last TPA treatment, epidermal lysates were prepared for Western blot analysis (pooled groups). A, Representative Western blots. B, Quantitation of Western blots. *, Significant when compared to TPA group ($p \leq 0.05$, Mann-Whitney U test).

3.2.4 Effect of UA, Curc and UA + Curc on infiltration of mast cells and inflammatory gene expression

The effects of UA, Curc and the combination on inflammation were investigated using the short-term protocol. **Figure 3.6** shows that TPA increased levels of mRNAs in the epidermis for a number of inflammatory markers genes including IL-1β, IL-6, IL-19, IL-22, CXCL2 and Cox-2. In addition, TPA treatment increased levels of VEGFA, an angiogenesis factor. Although at the dose given, UA alone did not provide significant inhibition of most of the inflammatory genes analyzed, it did significantly reduce levels of Cox-2 mRNA ($p \leq 0.05$). Curc treatment alone provided more inhibition and significantly decreased the expression of IL-6, IL-19, Cox-2 and VEGFA ($p \leq 0.05$). The combination of UA + Curc was more effective than the individual compounds alone and inhibited the expression of IL-1β, IL-6, IL-19, IL-22, CXCL2, Cox-2 and VEGFA ($p \leq 0.05$). Furthermore, the combination provided significantly more inhibition than UA alone for IL-19, Cox-2 and VEGFA and was significantly better than both UA and Curc alone for reducing IL-1β, IL-6, IL-22 and CXCL2 mRNAs ($p \leq 0.05$). The infiltration of mast cells into the dermis was also evaluated (**Figure 3.7**). Pretreatment with both UA and Curc alone significantly inhibited the infiltration of mast cells into the dermis ($p \leq 0.05$). Moreover, the combination of UA + Curc provided further inhibition of mast cell infiltration that was significantly reduced compared to the TPA treated group and compared to the effects of UA or Curc alone ($p \leq 0.05$). These experiments show that both UA and Curc alone significantly reduced TPA-induced inflammation but that the combination provided a greater overall inhibition of TPA-induced skin inflammation.

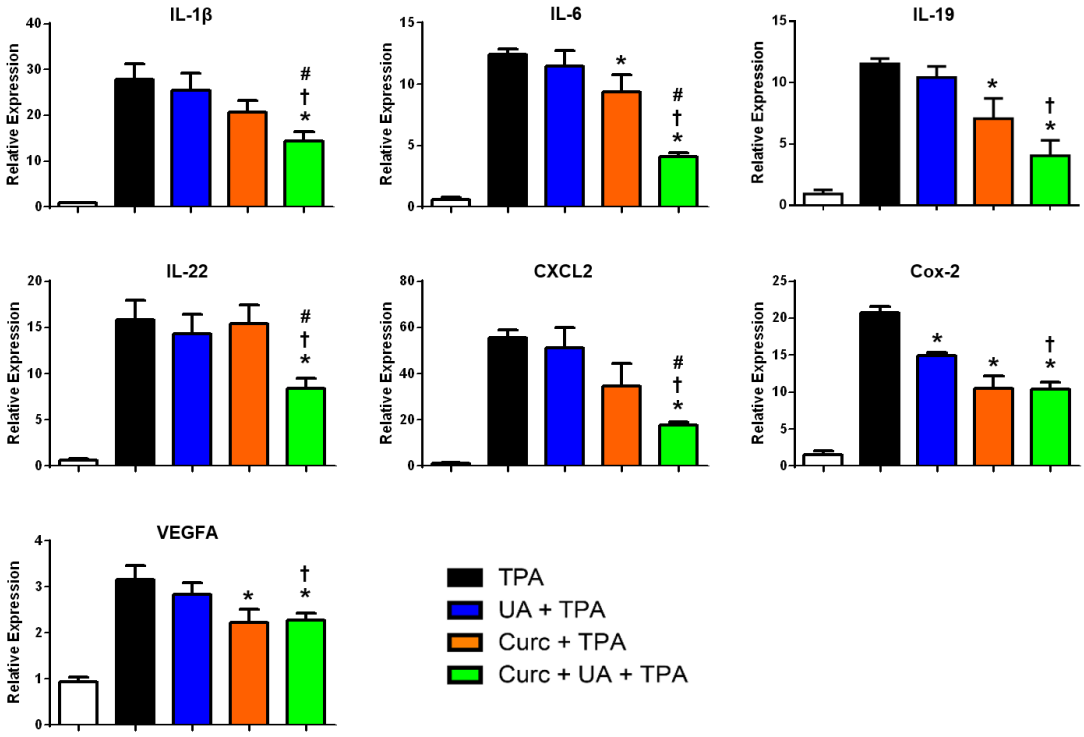

Figure 3.6: Effects of UA, Curc and the combination of UA + Curc on TPA-induced gene expression

The dorsal skin of female ICR mice (7–9 weeks of age; n = 4-5/group) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol) or a combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. All treatments were given twice-weekly for two weeks. Six hours after the last TPA treatment, epidermal lysates were prepared for RNA isolation. Graphs show qRT-PCR analyses of gene expression of IL-1β, IL-6, IL-19, IL-22, CXCL2, Cox-2 and VEGFA. Graphs represent mean ± SEM (average of at least 3 independent experiments). *, Significant when compared to TPA group; †, Significant when compared to UA + TPA; #, Significant when compared to Curc + TPA; ($p \leq 0.05$, Mann-Whitney U test).

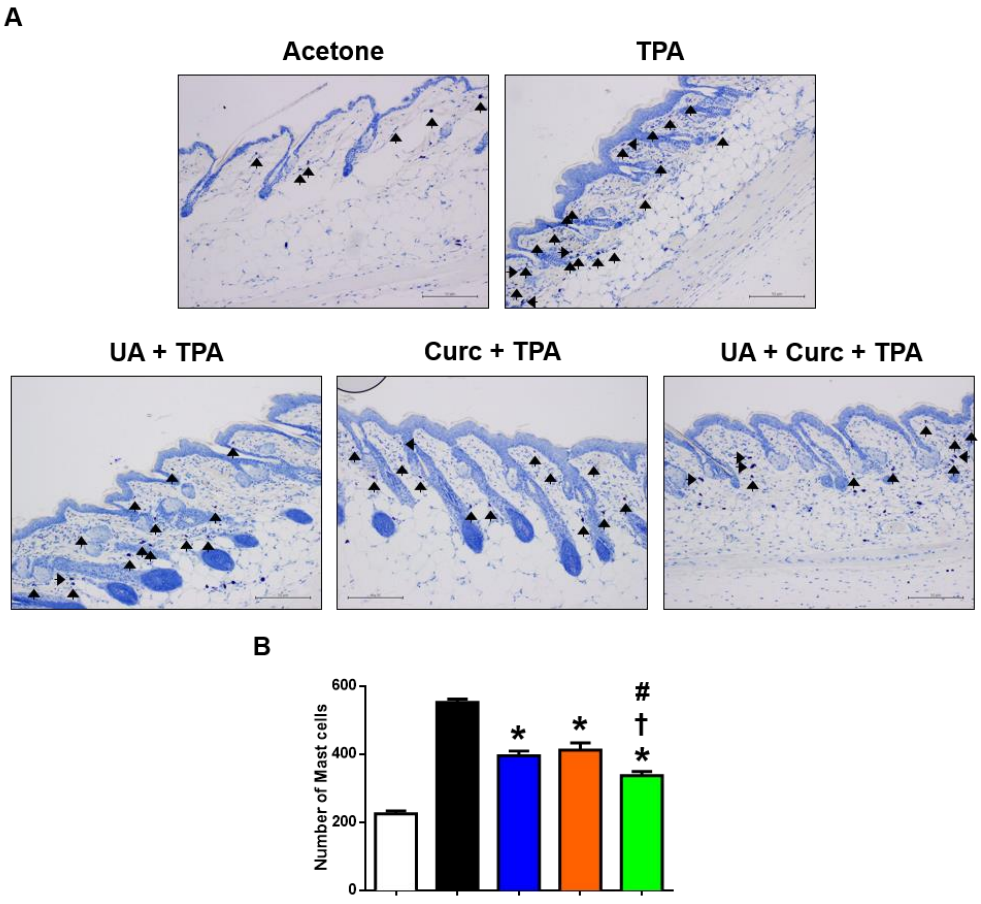

Figure 3.7: Effect of UA, Curc and the combination of UA + Curc on TPA-induced infiltration of mast cells.

The dorsal skin of female ICR mice (7–9 weeks of age; n = 3/group) was shaved and then two days later treated with either acetone vehicle, UA (1 µmol), Curc (2 µmol) or a combination of UA (1 µmol) + Curc (2 µmol) prior to TPA (6.8 nmol) treatment. All treatments were given twice-weekly for two weeks. Forty-eight hours after the last TPA treatment, dorsal skin was fixed in 10% formalin-buffered solution, embedded in paraffin and sectioned for toluidine blue O staining. A, Representative toluidine blue O stained sections of dorsal skin. Arrows indicate mast cells. B, Quantitative evaluation (mean ± SEM) of mast cell infiltration in dermis. *, Significant when compared to TPA group; †, Significant when compared to UA + TPA; #, Significant when compared to Curc + TPA; ($p \leq 0.05$, Mann-Whitney U test).

3.2.5 The combination of UA and Curc inhibit expression of miR-21

UA and Curc were also evaluated for their effects on expression of miR-21. miR-21 is a microRNA upregulated in many cancers, including breast, colon, lung, pancreas, prostate, and stomach, and has been shown to be involved in the two-stage skin carcinogenesis mouse model (122,123). As shown in **Figure 3.8**, TPA treatment induced a significant increase in miR21 levels. At the doses used in this experiment, the individual compounds did not provide any inhibition whereas the combination significantly reduced the levels of miR21a-5p.

3.3 DISCUSSION

In this study, we have demonstrated that the combination of topically applied UA + Curc inhibited skin tumor promotion by TPA to a greater extent than the individual compounds given alone. The most prominent effects with the combination compared to the individual compounds were seen on tumor free survival, tumor size and tumor weight. Additional investigation corroborated these findings and showed that the combination significantly inhibited TPA-induced epidermal hyperproliferation and hyperplasia compared to the compounds alone. Furthermore, the combination inhibited a broad range of signaling pathways involved in cell growth, proliferation and inflammation. For example, the combination significantly reduced phosphorylation of EGFR, Src, p70S6K, c-Jun, Iκ-Bα, NF-κB p50, Stat3 and Rb as well as protein levels of c-Jun, c-Fos and Cox-2 compared to the individual compounds alone. UA + Curc also significantly inhibited

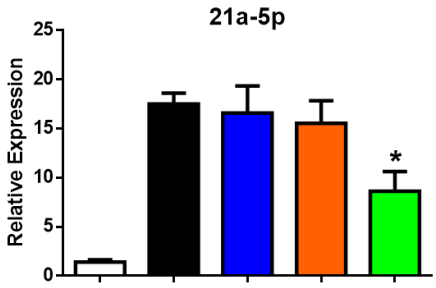

Figure 3.8: The effect of the combination of UA + Curc on TPA-induced expression of miR-21

The dorsal skin of female ICR mice (7–9 weeks of age) was shaved and then two days later treated with either acetone vehicle, UA (1 μmol), Curc (2 μmol) or a combination of UA (1 μmol) and Curc (2 μmol) prior to TPA (6.8 nmol) treatment. Six hours after the last TPA treatment, epidermal lysates were prepared for qPCR analysis. Graph show qRT-PCR analysis of gene expression of miR-21a-5p. Graph represent mean ± SEM (average of at 4 independent experiments). *, Significant when compared to TPA group ($p \leq 0.05$, Mann-Whitney U test).

TPA-induced expression of miR-21. mRNA levels of inflammatory genes IL-1β, IL-6, IL-22 and CXCL2 were also significantly decreased by the combination compared to the compounds alone. Finally, the combination significantly inhibited mast cell infiltration into the dermis compared to the compounds alone. Collectively, these data demonstrate a strong anti-proliferative and anti-inflammatory effect with the combination of UA + Curc that correlated with its greater effects on tumor promotion compared to the individual compounds.

As mentioned in the introduction, a combination of UA + Curc given in the diet was shown to synergistically inhibit growth of mouse prostate cancer cells (HMVP2 cells) in a tumor allograft model (95). In these earlier studies, while not all of the same pathways examined in the current study were investigated, the combination of UA + Curc inhibited phosphorylation of Src, Stat3 and p70S6K in the HMVP2 tumor cells to a greater extent than either compound alone. The combination also increased apoptosis more than the individual compounds alone in both HMVP2 and DU145 cells as shown by an increase in annexin V staining and cleaved PARP (95). In the current study, there was no strong indication of an increase in apoptosis by the combination of UA + Curc at the 2 and 6 hour time points with the doses given (data not shown), indicating that in the skin model the inhibitory activity seemed to be due more to an inhibition of proliferation and inflammation. Nevertheless, inhibition of some of the same pathways in epidermis with the combination of UA + Curc after topical application supports the importance of these mechanisms and provides evidence that this combination could be effective at preventing other cancers as well.

As noted in the Introduction, previous work from our laboratory showed that the combination of UA + Res significantly inhibited skin tumor promotion by TPA to a greater extent than UA or Res alone (67). In the present study a lower dose of UA was used (1 μmol vs. 2 μmol in previous study). Similar effects with the combination of UA + Curc and the combination of UA + Res were seen on decreasing phosphorylation EGFR, Src and c-Jun. Although UA + Res seemed to provide more inhibition of NF-κB p65 and UA + Curc provided more inhibition of NF-κB p50, both combinations effectively inhibited NF-κB signaling and decreased levels of one of its transcriptional targets Cox-2 as well as gene expression of IL-1β and Cox-2. One difference seen was that the combination of UA + Res inhibited phosphorylation of JNK 1/2 and p38, which was not seen with the combination of UA + Curc. Also, the combination of UA + Curc decreased phosphorylation of Rb and increased levels of p27, whereas the combination of UA + Res increased levels of p21. Although some differences were present in the mechanism of how the combinations were acting, both UA + Curc and UA + Res effectively inhibited skin tumor promotion by TPA to a greater extent than their respective compounds alone. These findings highlight the importance of inhibiting EGFR, Src, c-Jun and NF-κB during skin tumor promotion by TPA.

AP-1 is a transcription factor composed of Jun and Fos family members and is known to be important for skin carcinogenesis and skin tumor promotion (59). Furthermore, AP-1 is involved in a number of processes including differentiation, proliferation, and transformation (52). In this study, the combination of UA + Curc significantly inhibited phosphorylation of c-Jun as well as total levels of c-Jun and c-Fos

induced by TPA treatment. Thus, reduction in AP-1 activity appears to be an important part of the inhibitory effects of the combination on skin tumor promotion. A similar effect was also seen in our previous studies with a combination of UA + Res where AP-1 activity was significantly reduced (67). The reduction in phosphorylation of c-Jun as well as total levels of c-Jun and c-Fos induced by TPA treatment seen with the combination correlated with a decrease in epidermal proliferation as seen by the reduction in BrdU incorporation (**Figure 3.2**).

Inflammation is a critical component of tumorigenesis, in part by creating a tumor promoting microenvironment conducive to tumor growth (124). NF- κB plays a major role in inflammatory signaling and tumor development, and this pathway is inhibited by Curc (125,126). In the present study, the combination of UA + Curc significantly inhibited phosphorylation of IκBα and NF-κB p50 as well as total levels of NF-κB p50. This inhibition of NF-κB signaling likely contributed to the decrease in protein levels of Cox-2 as well as reduced expression of inflammatory genes such as Cox-2, IL-1β and IL-6, as NF-κB is known to induce transcription of these genes (127-129). Both NF-κB and Cox-2 signaling pathways play critical roles in skin tumor promotion by TPA and other tumor promoters (26,61,62).

As mentioned above miR-21 is a microRNA upregulated in many cancers and is involved in the two-stage skin carcinogenesis mouse model (122,123). It negatively regulates targets such as the tumor suppressors Pdcd4, Sprouty 1 or Sprouty 2 (130-132). However, in our experiments we did not see a significant increase in Pdcd4, Sprouty 1 or Sprouty 2 (data not shown) by the combination of UA + Curc. Further experiments will

need to be done to elucidate whether the inhibition of miR-21 by UA + Curc is an important mechanism of its inhibitory action and whether Pdcd4, Sprouty 1 or Sprouty 2 are increased by UA + Curc at later timepoints.

Phytochemicals are ideal candidates for chemoprevention because they are relatively safe, act on a broad spectrum of signaling pathways and are inexpensive (8). In general, it is believed that combinations of phytochemicals will provide greater effects than single compounds. There are now a number of studies that demonstrate this idea. For example, as mentioned above, the combination of UA + Curc synergistically inhibited tumor growth of prostate cancer cells in an allograft model (95). In the same study, the combinations of UA + Res and Curc + Res also produced synergistic inhibition of tumor growth (95). Another previous study in our lab, mentioned above, showed the combination of UA + Res inhibited skin tumor promotion by TPA to a greater extent than either compound alone (67). Jin *et al* showed the combination of curcumin and epigallocatechin-3-gallate (EGCG) suppressed colorectal carcinoma PDX tumor growth to a greater extent than either compound alone (133). Another example is the combination of luteolin and EGCG that inhibited xenograft tumor growth of a head and neck squamous cell carcinoma cell line to a greater extent than either compound alone (134). Generally, because certain combinations are more effective than their respective individual compounds, they can be expected to provide greater chemopreventative activity and at lower doses.

In conclusion, the current study demonstrates that the combination of UA + Curc given topically provides greater inhibition of skin tumor promotion by TPA than either compound given alone. Mechanistically, this was associated with significant anti-

inflammatory and anti-proliferative effects. These results reveal the potential for the combination of UA + Curc to be used for chemopreventative strategies for a number of cancers, including non-melanoma skin cancer.

Chapter 4: Evaluating the effects of UA, Curc, Res and the Combination in the Diet on Skin Tumor Promotion by TPA

4.1 INTRODUCTION

UA, Res and Curc have all been shown to inhibit skin tumor promotion by TPA when applied topically (82,85,103). Furthermore, when given in the diet these compounds have shown to inhibit tumor development in various mouse models. For example, UA in the diet inhibited tumorigenesis in the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model and a postmenopausal breast cancer mouse model (69,71). Res in the diet inhibited tumorigenesis in a sporadic model of colorectal cancer, DMBA-induced mammary carcinogenesis and a two-stage model of rat hepatocarcinogenesis (104,105,107). Curc given in the diet inhibited tumor development in K-ras-induced lung cancer in mice (87). However, the ability of UA, Res and Curc to inhibit tumor development in the two-stage skin carcinogenesis when administered in the diet has not been evaluated to our knowledge.

We receive a plethora of phytochemicals from many fruits and vegetables in our diets, not just a single phytochemical alone. It is believed that receiving these combinations of phytochemicals provides us with a stronger chemopreventive effect than a single agent alone. In Chapter 3 I showed that the topical administration of the combination of UA and Curc provided greater inhibition of skin tumor promotion by TPA than the compounds alone. Our lab has also shown this to be true for the combination of UA and Res (67). Furthermore, we showed that the combinations of UA + Curc, UA + Res and Res + Curc

when given in the diet inhibited tumor growth in a xenograft prostate mouse model to a greater extent than the compounds alone (95).

In this study we wanted to investigate whether dietary administration of UA, Res and Curc as single agents inhibit skin tumor promotion by TPA. In addition we wanted to examine whether combinations of these phytochemicals in the diet provide further inhibition of skin tumor promotion. Finally, we analyzed whether the compounds in the diet combined with Met in the drinking water provided greater inhibition of skin tumor promotion.

4.2 RESULTS

Initial short term experiments suggested UA, Res, Curc and their combinations in the diet inhibited signaling pathways involved in tumorigenesis (**Figures 4.1, 4.2**). UA, Res, Curc, UA + Res and UA + Curc appeared to decrease phosphorylation of EGFR. The combination of UA + Res decreased phosphorylation of c-Jun and Res alone decreased total levels of c-Jun. Dietary administration also seemed to downregulate NF-κB signaling. The combinations of UA + Res and UA + Curc decreased both total levels and phosphorylation of NF-κB p50. Furthermore, the combination of UA + Curc decreased levels of the transcriptional target of NF-κB, Cox-2. Finally, Curc, UA + Res and UA + Curc inhibited phosphorylation of Rb (**Figure 4.2**). The decreases in these signaling pathways correlated with a reduction of TPA-induced epidermal hyperproliferation by Res, Curc, UA + Res and UA + Curc (**Figure 4.3**).

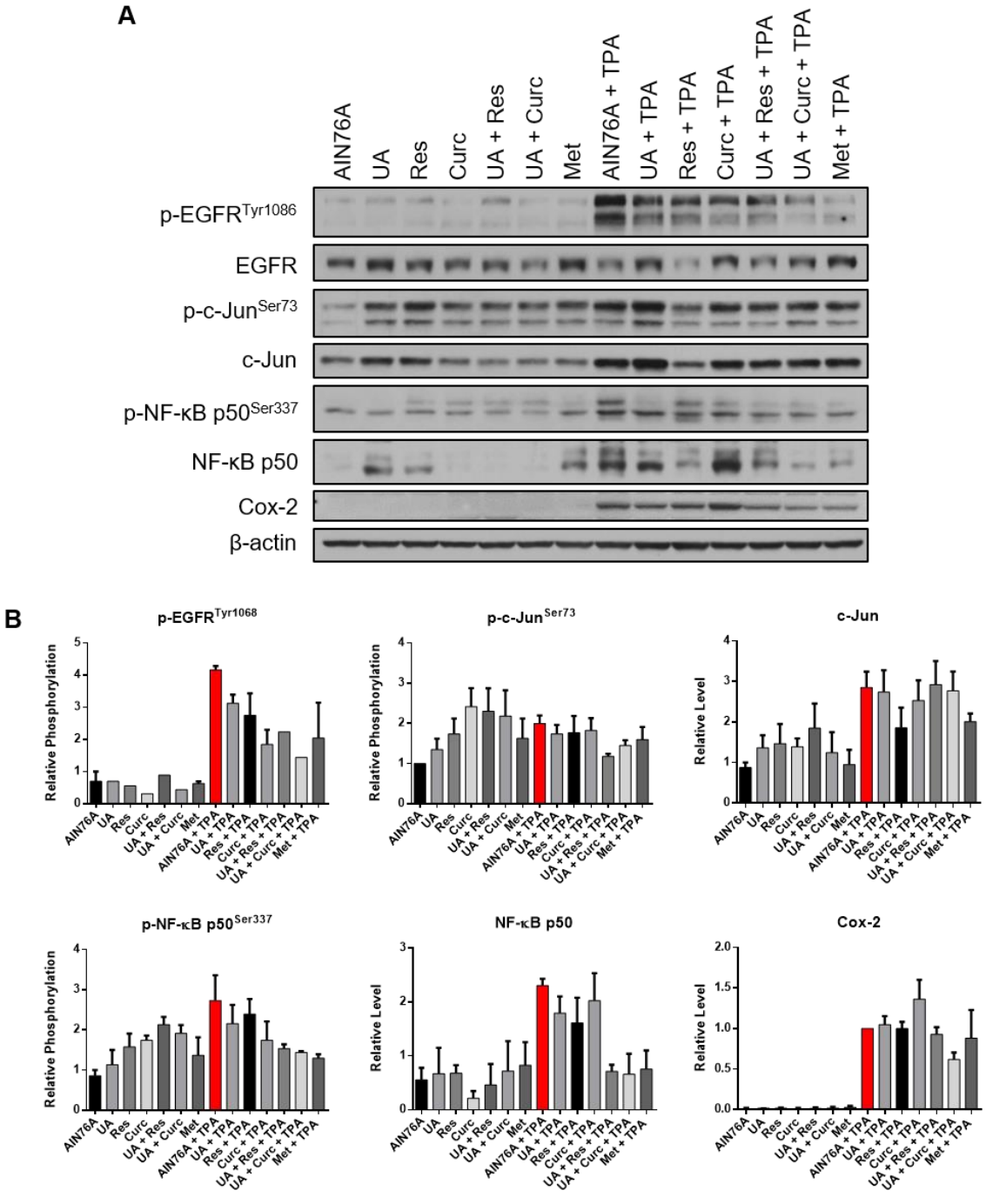

Figure 4.1: Effects of UA, Curc and Res in the diet on EGFR, c-Jun, NF-κB and Cox-2

Mice were treated according to the short term diet study protocol. **A**. Representative Western blots. **B**. Quantitation of Western blots.

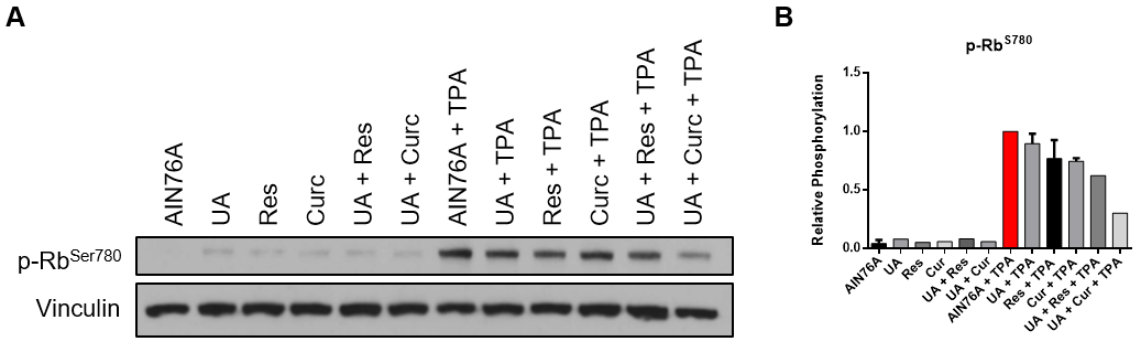

Figure 4.2: Effect of UA, Curc and Res in the diet on phosphorylation of Rb

Mice were treated according to the short term treatment protocol. **A**. Representative Western blots. **B**. Quantitation of Western blots.

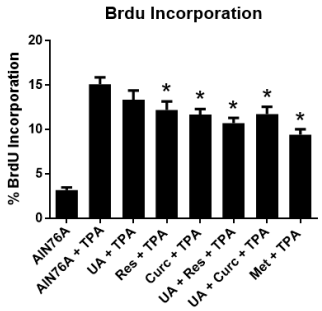

Figure 4.3: Effect of UA, Curc and Res in the diet on TPA-induced hyperproliferation

Mice were treated according to the short term protocol. Quantitative evaluation (mean ± SEM) of epidermal hyperproliferation (BrdU incorporation). *, Significantly different from AIN76A + TPA group ($p < 0.05$).

Based on these preliminary findings a two-stage skin carcinogenesis assay was performed with these compounds in the diet. Contrary to our hypothesis, there were no significant differences in tumor incidence, tumor multiplicity or onset of tumor development compared to the TPA group (**Figure 4.4**). Res alone in the diet did however significantly reduce tumor size and weight compared to the TPA group (**Figure 4.4 C, D**). Furthermore, the combinations (UA + Res, UA + Curc, Res + Curc) provided no further inhibition than the compounds alone (**Figure 4.5**). UA, Res and Curc were also investigated in combination with Met, but again we did not see any further inhibition with the combinations (**Figure 4.6**).

4.3 DISCUSSION

In preliminary short term experiments, UA, Res, Curc and the combinations administered in the diet reduced phosphorylation of EGFR, c-Jun, NF-κB p50 and Rb and total levels of c-Jun, NF-κB p50 and Cox-2. However, at the doses given, the compounds in the diet did not significantly reduce tumor incidence or tumor multiplicity or delay the onset of tumor development and the combinations did not provide any further inhibition than the compounds alone. On the other hand, Res in the diet did significantly reduce tumor size and tumor weight, indicating it is providing some inhibition on skin tumor promotion by TPA.

It could be that the doses given were not high enough to produce an anti-tumor effect in the skin. Also, because the compounds were given in the diet, metabolism, absorption, distribution to the skin and clearance become an issue. Curc is known to be

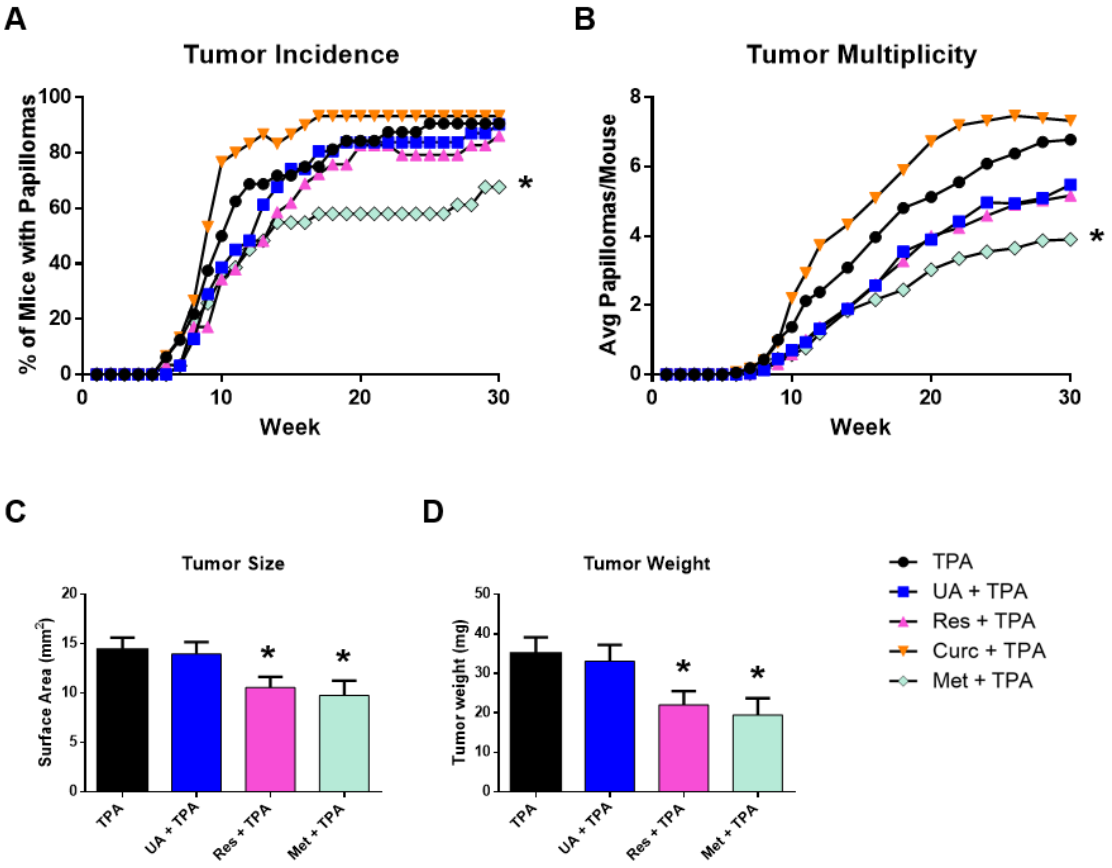

Figure 4.4: Effect of UA, Curc and Res as single agents in the diet on skin tumor promotion by TPA

Mice were treated according to the diet study two-stage skin carcinogenesis assay. **A**. Tumor incidence. **B.** Tumor multiplicity. **C**. Tumor latency shown using a Kaplan Meier curve of tumor free mice. D, Average bodyweight (g) per mouse. E, Average surface area of tumors (mm2). F, Average tumor weight (mg). * Significant when compared to TPA group. Mann-Whitney U test was used for analysis of tumor multiplicity, tumor size and tumor weight, and Fisher's exact test was used for analysis of tumor incidence.

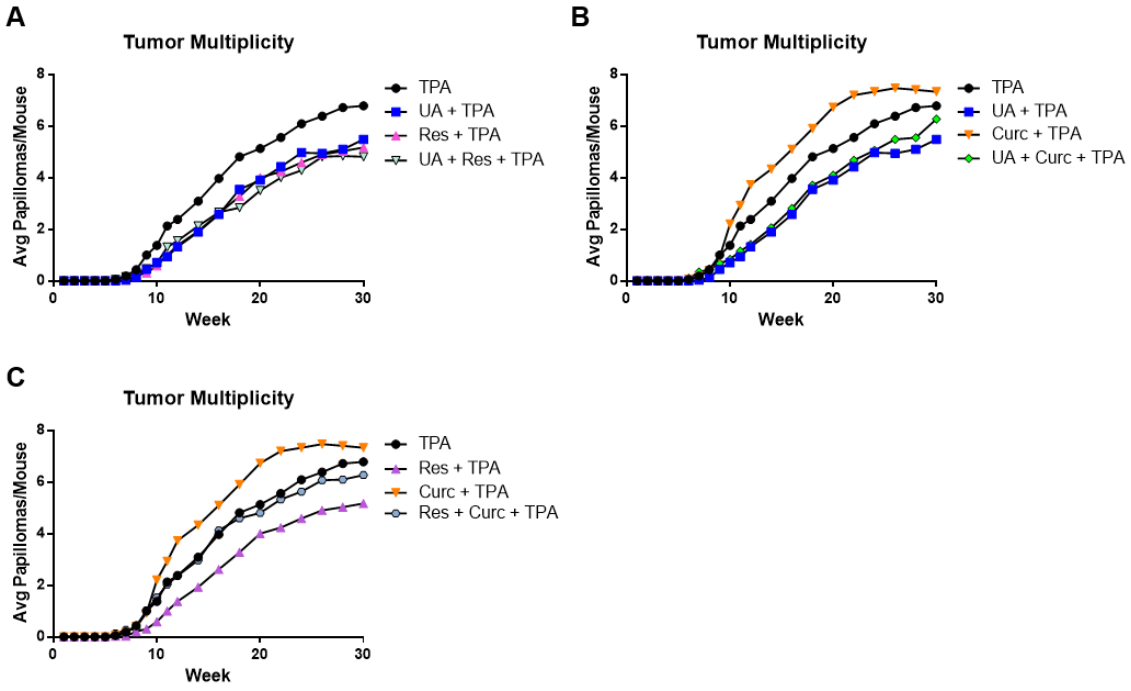

Figure 4.5: Effects of combinations of UA, Curc and Res on skin tumor promotion by TPA

Mice were treated according to the diet study skin carcinogenesis assay. **A**. Tumor multiplicity for the combination of UA + Res given in the diet. **B**. Tumor multiplicity for the combination of UA + Curc given in the diet. **C**. Tumor multiplicity for the combination of Res + Curc given in the diet.

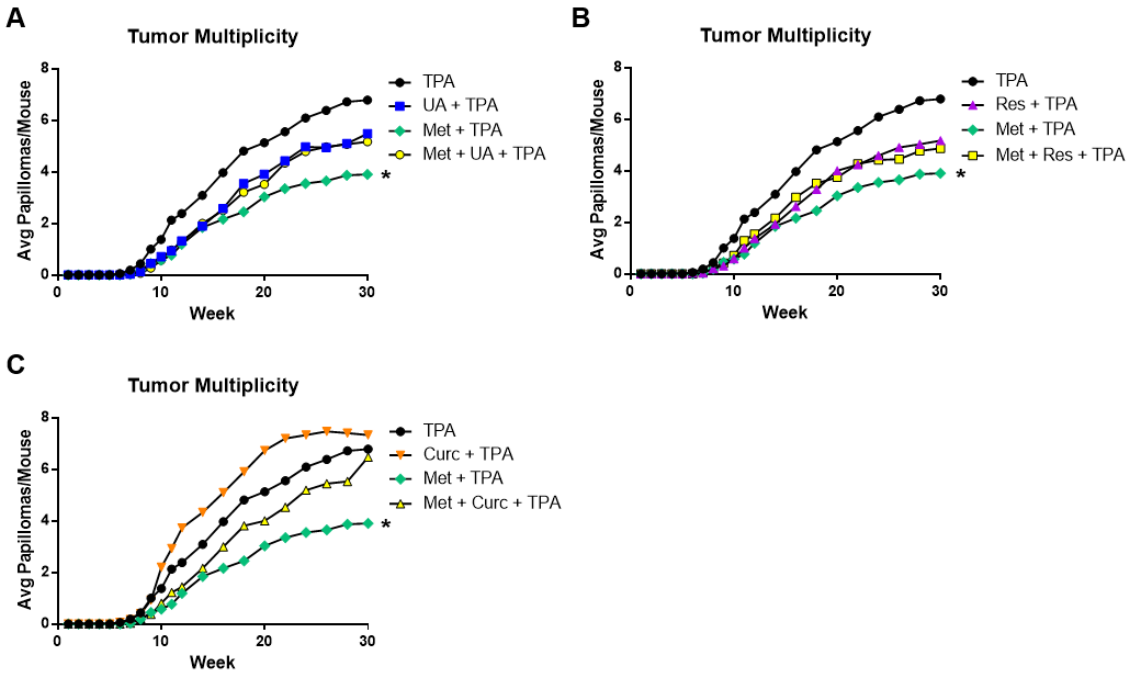

Figure 4.6: Effects of UA, Curc or Res in combination with Met on skin tumor promotion by TPA

Mice were treated according to the diet study skin carcinogenesis assay. **A**. Tumor multiplicity for the combination of Met given in the drinking water and UA given in the diet. **B**. Tumor multiplicity for the combination of Met given in the drinking water and Res given in the diet. **C**. Tumor multiplicity for the combination of Met given in the drinking water and Curc given in the diet. * Significant when compared to TPA group (Mann-Whitney U, $p < 0.05$).

poorly absorbed and metabolized quickly, requiring high doses to be detected in the serum (135). For example, in one clinical trial, patients receiving 2.2 g of Curc per day did not have any detectable levels of Curc in the blood (119). UA is hydrophobic, and therefore not very well absorbed, and has limited bioavailability (136). Res is fairly well absorbed, however its metabolism leads to poor bioavailability (137).

Many different strategies have been employed to increase the bioavailability of UA, Curc and Res. For example, studies have been done to package UA, Curc and Res in nanoparticles and liposomes (138-141). Furthermore, several synthetic analogs have been developed to help improve Curc's solubility and inhibit its rapid metabolism (142). Another tactic has been to add other compounds to improve bioavailability. Curc has been co-encapsulated with piperine, which is thought to improve Curc's absorption (143). Res has also been co-administered with piperine, which increased the bioavailability of Res by delaying its glucuronidation and therefore slowing its metabolism (144).

Overall these experiments did not demonstrate that UA or Curc at the doses given in the diet inhibit skin tumor promotion by TPA. Furthermore, UA, Curc and Res given in various combinations, or in combination with Met, did not provide additional inhibition of skin tumor promotion. Res in the diet did significantly inhibit tumor size and weight to levels that were comparable with Met, signifying it is providing some growth inhibition of tumorigenesis, but is not inhibiting the initial tumor development. Further experiments will need to be done to investigate why UA, Curc and Res given in the diet did not significantly inhibit skin tumor promotion by TPA.

Chapter 5: Summary, significance and future directions

Overall I found that the combination of topically applied UA + Curc significantly inhibited skin tumor promotion by TPA to a greater extent that either compound alone. Notably, the combination significantly delayed the onset of tumor development and inhibited tumor size and weight significantly more than either UA or Curc given as individual compounds. UA + Curc inhibited skin tumor development by acting by a strong anti-proliferative and anti-inflammatory mechanism. EGFR, c-Fos, c-Jun, Src, NF-κB and Stat3 seem to be the most important targets for the inhibitory effects of this combination. Furthermore, the inhibition of NF-kB led to a reduced expression of inflammatory cytokines from the keratinocytes which play an important role in dermal inflammation induced by TPA during the process of tumor promotion. This study highlights the potential of the combination of UA + Curc to be used in chemopreventive strategies.

We next examined the effects of UA, Curc and Res given in the diet on skin tumor promotion by TPA as a more relevant model of how humans would be exposed to these compounds. Contrary to our preliminary results which suggested UA, Curc and Res in the diet were inhibiting EGFR, c-Jun, NF-κB p50 and Rb, we found that these compounds did not inhibit skin tumor promotion by TPA at the doses given when administered in the diet. Moreover, combinations of the phytochemicals did not produce further inhibition and combining the phytochemicals with Met did not provide any further inhibition. Res did significantly reduce tumor size and weight to an extent similar to Met, suggesting it is providing some growth inhibition of the tumors, but not the initial onset of tumor development. Further experiments discussed below will need to be done to investigate why

UA, Curc and Res did not inhibit skin tumor promotion by TPA when administered in the diet.

5.1 FUTURE DIRECTIONS

The transcription factors c-Fos, c-Jun, NF-κB and Stat3 seem to play an important role in the anti-tumor activity of the combination of topically applied UA + Curc. AP-1, NF-κB and Stat3 have been shown to play critical roles in skin tumor promotion by TPA (59,61,117). It would be valuable to further examine the importance of these transcription factors in the inhibitory effect of UA + Curc. Both overexpressing and knocking out or inhibiting c-Fos, c-Jun, NF-κB or Stat3 would further reveal the mechanism by which UA + Curc is inhibiting tumorigenesis.

Although we saw a significant decrease in miR-21 expression with the combination of UA + Curc, we did not see a significant increase in targets known to be inhibited by miR-21 such as the tumor suppressors Pdcd4, Sprouty 1 or Sprouty 2 (130-132). Additional experiments will need to be done to investigate these effects on miR-21 and downstream targets. It could be that the 6 hour timepoint was not the ideal time to see the increase of Pdcd4, Sprouty 1 or Sprouty 2. A time course experiment would allow us to investigate whether the inhibition of miR-21 is an important mechanism of the inhibitory action of UA + Curc and whether this inhibition produces a subsequent increase in Pdcd4, Sprouty 1 or Sprouty 2.

Further experiments with the diet studies need to be done to investigate why UA, Curc and Res did not inhibit skin tumor promotion by TPA when given in the diet. One

question that needs to be addressed is whether these compounds are bioavailable and reaching the skin at the doses given. Serum and tissues, including skin, kidney, liver, pancreas and lung were collected after 4, 19 and 31 weeks on the diets. It would be of interest to examine the levels of UA, Curc and Res both in the serum and skin. If the levels of these phytochemicals are low in the serum and skin, other options would need to be examined to increase their bioavailability. For example, we could explore packaging them in nanoparticles, using synthetic analogs or co-administering with other compounds to increase their absorption.

Finally, the dose of Met used in the diet studies was very effective alone and we did not see any further inhibition of tumor development when combined with UA, Curc or Res. It would be beneficial to test lower doses of Met to allow us to see potential combinatorial effects when combined with UA, Curc or Res.

These studies would allow us to further explore the potential of UA, Curc and Res as chemopreventive agents in humans for NMSC as well as other cancers.

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