ABSTRACT

APIGENIN PREVENTS CHEMICALLY-INDUCED SKIN TUMOR FORMATION AND INHIBITS COX-2 EXPRESSION AND ACTIVITY IN SKH-1 MOUSE EPIDERMIS

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The most common type of cancer in the United States is non-melanoma skin cancer (NMSC), with an estimated two million new cases diagnosed each year. Numerous malignancies including NMSC, colon, breast, and prostate cancer overexpress cyclooxygenase-2 (COX-2). COX-2 catalyzes the synthesis of prostaglandins including prostaglandin E2 (PGE_2) which activates E-type prostaglandin (EP) receptors and promotes tumor development when overexpressed. Pharmacological inhibition or genetic deletion of COX-2 significantly decreases skin carcinogenesis mediated by the two-stage chemical carcinogenesis model using 7, 12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or by UVB-light. Apigenin is a non-mutagenic bioflavonoid present in various foods. Previous work in our lab has shown that apigenin blocks TPA- and UV-light induced COX-2 expression in vitro and apigenin also inhibits skin carcinogenesis; however, the mechanism by which apigenin blocks tumor formation is unclear. These observations suggest that apigenin prevents chemically-induced tumor formation through the

inhibition of the COX-2/PGE_2 pathway. In specific aim 1, our goal was to determine the acute effect of apigenin on TPA-induced COX-2 expression, PGE_2 production, and EP receptor expression *in vivo* to gain a better understanding of the molecular mechanisms that may be responsible for its antitumor activity. Female SKH-1 hairless mice were pretreated with apigenin or vehicle one hour before exposure to TPA. Animals were euthanized 8 hours later and the epidermis isolated, pooled, and analyzed for COX-2 expression, PGE_2 production, and EP receptor expression. Apigenin significantly inhibited COX-2 expression, PGE_2 production, and EP1 receptor expression in the epidermis. In specific aim 2, our goal was to determine if apigenin-mediated blockade of chemically-induced skin Tumorigenesis occurs as a result of the inhibition of COX-2 expression, PGE_2 production, and EP receptor expression. Skin carcinogenesis was induced in animals using the two-stage chemical carcinogenesis model. Female SKH-1 mice were exposed to a single topical dose of DMBA (tumor initiating agent) followed by biweekly applications of TPA (tumor promoting agent) for 25 weeks. Animals were treated one hour prior to TPA exposure with apigenin or vehicle. At the end of the study, the number of tumors and the size of each tumor were determined. Tumors and surrounding epidermal tissue were then harvested, classified, and subjected to immunohistochemical analysis. Apigenin caused a dose-dependent decrease in tumor multiplicity and incidence in animals exposed to DMBA/TPA. Apigenin significantly inhibited the chemically-mediated increase in COX-2 expression, PGE_2 production, and EP receptor expression in skin surrounding the tumors whereas all tumors overexpressed COX-2. In addition, apigenin increased terminal differentiation and decreased proliferation in the surrounding epidermis of tumor bearing animals.

Collectively, our findings demonstrate that apigenin suppresses chemically-induced tumor formation and also inhibits COX-2 expression, PGE_2 production, and EP receptor expression in the surrounding epidermis. Inhibition of the COX-2/PGE_2 pathway by apigenin may be responsible for its antitumor activity thus supporting the development of apigenin or apigenin-derivatives as chemopreventive agents for NMSC.

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Presented in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Pharmacology and Toxicology

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DEDICATION:

I would like to take this opportunity to thank my loving family for all of their support; Martin, Elizabeth, Rob, Monica, Sarah, RJ, and Micah. Furthermore, I would like to thank Allison Pittman, Bella Disco Razzmatazz and their family for everything they have done for me throughout the years.

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TPA	12-O-tetradecanoylphorbol-13-acetate
DMBA	7, 12-dimethylbenz[a]anthracene
AA	Arachidonic Acid
BCC	Basal Cell Carcinoma
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
DAB	Diaminobenzidine
DMSO	Dimethyl sulfoxide
EP	E-type prostaglandin receptor
FI	Fold Induction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
K1	Keratin-1
K10	Keratin-10
mPGES-1	microsomal Prostaglandin Synthase-1
mPGES-2	microsomal Prostaglandin Synthase-2
NMSC	Non-melanoma Skin Cancer
NSAID	Non-steroidal anti-inflammatory drugs

OD	Optical densitometry
PKB/Akt	Protein kinase B
PG	Prostaglandin
EP1	Prostaglandin E receptor subtype 1
EP2	Prostaglandin E receptor subtype 2
EP3	Prostaglandin E receptor subtype 3
EP4	Prostaglandin E receptor subtype 4
PGES-1	Prostaglandin Synthase-1
PGES-2	Prostaglandin Synthase-2
PKC	Protein kinase C
SCC	Squamous Cell Carcinoma
UVB	Ultraviolet light-B
XP	Xeroderma pigmentosa

Chapter 1

Introduction

1.1 Epidemiology of skin cancer

Skin cancer is a major public health concern throughout the world and represents more than half of all cancers diagnosed in the United States. The most common type of skin cancer is non-melanoma skin cancer (NMSC) with an estimated two million new cases diagnosed each year (Rogers, et al., 2010; Siegel, et al., 2011). One out of every five Americans will develop some form of skin cancer throughout their lifetime (Robinson, 2005). Data analysis from a research survey conducted by Medicare revealed that between the years of 1992 and 2006 procedures and treatments associated with NMSC have increased over 77% and are among the most costly of all cancers (Stern, 2010; Housman, et al., 2003). In a study conducted in 2004, total direct costs of NMSC in the United States were approximately $1.5 billion (Bickers, et al., 2006). Thus, identification of molecular mechanisms of tumor formation and development of novel chemotherapeutic agents is a primary focus in the battle against skin cancer.

1.2 *The skin*

The skin is the largest organ of the human body and serves as the first line of defense against a wide array of factors including physical, microbial, and chemical assaults (Jablonski and Chaplin, 2002; Jensen and Proksch, 2009). Additionally, the skin regulates body temperature, provides insulation, produces energy, and synthesizes

vitamin D (Jablonski and Chaplin, 2002; Jensen and Proksch, 2009). The skin is composed of three distinct layers: the epidermis, dermis, and hypodermis/subcutaneous layer (Figure 1).

The epidermis is the most superficial layer and is primarily composed of keratinocytes but also houses melanocytes, Langerhans cells, and Merkel cells. In humans, the epidermis can be further subdivided into stratified layers each corresponding to different stages of differentiation (Wysocki, 1999; Jablonski and Chaplin, 2002; Jensen and Proksch, 2009). These layers include the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and the stratum corneum (Figure 2). The stratum basale or the basal layer is the innermost layer and is primarily composed of proliferating keratinocytes and is the site where mitosis takes place (Eckert and Rorke, 1989). Within the basal layer, terminal differentiation takes place as keratinocytes become anucleated (corneocytes) and migrate into the next layer, the stratum spinosum. In the stratum spinosum cells synthesize cytokeratins that interact with desmosomes to collaborate and provide structural support. The next layer is the stratum granulosum where squamous cells reside and appear granular due to small basophilic granules present within the cytoplasm. The stratum lucidum is a clear translucent layer only appearing in the palms and soles in humans. The stratum corneum is the outer most layer and serves as a physical barrier against chemical and infectious agents and also prevents dehydration. In addition, as the dead cells slough off, they are continually replaced as cells terminally differentiate from the basal layer (Eckert and Rorke, 1989;Wysocki, 1999; Jablonski and Chaplin, 2002; Jensen and Proksch, 2009).

Directly below the epidermis lies the dermis, which provides strength and elasticity to the skin. The dermis is composed of collagen, elastin, and proteoglycans and consists of various cell types including fibroblasts, macrophages, and adipocytes (Jensen and Proksch, 2009). The dermis is connected to the epidermis through the basement membrane. In addition, the dermis is vascularized which provides nourishment and waste removal for both the dermal and epidermal layer. The bottom most layer is the hypodermis. This layer is made up of subcutaneous fat and connective tissue and is responsible for binding the dermis to tissue/muscle (Wysocki, 1999)

1.3 Skin cancer

There are two major categories of skin cancer: melanoma and non-melanoma skin cancer. Melanoma accounts for a small percentage of skin cancer cases in the United States; however, the majority of deaths related to skin cancer are due to melanoma. There are various forms of non-melanoma skin cancer with the two major types being basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC is the most common form of NMSC and accounts for approximately 80% of all NMSC cases (Rogers, et al., 2010; Siegel, et al., 2011). BCC develops within the basal layer of the epidermis and carries the potential for local destruction of tissue. BCCs characteristically occur on sun-exposed areas of the skin, develop at a slow rate, rarely metastasize, and generally appear as a shiny, pearly-white nodule (McGuire, et al., 2009). On the other hand, SCCs are invasive tumors that have the potential to metastasize. SCCs develop from squamous cells within the epidermis of the skin and appear as scaly or ulcerated lesions with raised edges and are asymmetric in shape

(Stulberg, et al., 2004; McGuire, et al., 2009). SCCs account for approximately 20% of NMSC cases (Rogers, et al., 2010; Siegel, et al., 2011).

1.4 Skin carcinogenesis

Skin tumor development is a multistage molecular process that occurs as a result of various cellular, molecular, and biochemical changes. There are a wide variety of agents that cause skin cancer including: industrial and environmental chemicals; viruses; and ultraviolet radiation (UV). It is generally accepted that UV radiation is the major cause of skin cancer in humans (Afaq, et al., 2005). Skin tumors develop as a consequence of acquiring mutations in genes that regulate cell proliferation, terminal differentiation, and apoptosis. These genetic alterations ultimately enhance tumor cell growth and survival (Fischer, et al., 1989; Sarasin, 1999). Various animal models are available to evaluate the molecular and cellular events that promote or prevent cancer. The most commonly used is the two-stage chemical carcinogenesis animal model developed in the late 1940s.

The chemical carcinogenesis model involves three distinct stages; initiation, promotion, and progression (Figure 3) (Yuspa, et al., 1976). Tumor initiation is an irreversible step achieved by a single cutaneous exposure to an agent that causes genetic mutations in genes encoding proto-oncogenes (i.e. H-*ras*) or tumor suppressor proteins (i.e. p53) (Barrett, 1993). There are several tumor initiating agents (Table 1) with the most frequently used being 7, 12 - dimethylbenz[*a*]anthracene (DMBA). DMBA is a polycyclic aromatic hydrocarbon that is metabolized by the cytochrome P450 (CYP1B1) enzyme to its active form (DiGiovanni, 1992). Once activated, DMBA

initiates an adenine (A) to thymine (T) transversion mutation in codon 61 of the H-*ras* gene. This irreversible mutation can lead to cellular progression to a precancerous lesion following exposure to a tumor promoter (DiGiovanni, 1992; Hennings, et al., 1993).

Tumor promotion is a reversible process that involves inducing cellular proliferation and altering signal transduction in initiated cells, ultimately leading to the formation of pre-malignant and malignant lesions. These lesions are achieved by repeated applications of a nonmutagenic pro-inflammatory agent (Yuspa, et al., 1976; Goel, et al., 2007). The most commonly used experimental tumor promoting agent is the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Other tumor promoting agents include phenol, anthralin, and benzoyl peroxide (Table 1) (Yuspa, et al., 1976). TPA is a potent pro-inflammatory agent derived from the croton plant that activates the protein kinase C (PKC) signal transduction pathway and increases reactive oxygen species (ROS) production in keratinocytes (reviewed in Rundhaug and Fischer, 2010). TPA is also known to induce epidermal hyperplasia and edema, increase keratinocyte proliferation, and induce epidermal ornithine decarboxylase which is necessary for polyamine biosynthesis (Verma and Boutwell, 1980; Byus and Weiner, 1982; Slaga, 1983; O'Brien, et al., 1997; Vargo, et al., 2006; Hara, et al., 2010). As a result, promoting agents induce clonal expansion of the initiated cells and formation of a premalignant papilloma; however, only a small percentage of papillomas that develop will progress to a malignant tumor.

The final step in skin cancer formation is tumor progression. Tumor progression is characterized by enhanced cellular division and the accumulation of additional

genetic changes in the affected cells. It is during this stage that a benign papilloma is converted to a malignant carcinoma with the ability to metastasize (Yuspa, et al., 1976; DiGiovanni, 1992).

An alternative model for analyzing skin carcinogenesis in animals is the UV light-induced photocarcinogenesis protocol. UV radiation can be subdivided into three different spectrums corresponding to their wavelength; UVA (400 – 315 nm), UVB (315 – 280), and UVC (280 – 100 nm). Of the three, UVB is primarily responsible for skin cancer formation. UV light is a complete carcinogen because it causes both tumor initiation and promotion. One potential downfall is that the different stages of tumor development cannot be studied individually as in the two-stage chemical carcinogenesis protocol. Acute cutaneous exposure to UV-light can trigger an inflammatory response and lead to erythema (sunburn). Chronic exposure to UV, more specifically UVB, induces DNA damage through the formation of cyclobutyl pyrimidine dimers and pyrimidine (6-4) pyrimidinone photoproducts. These types of DNA mutations occur commonly in genes encoding tumor suppressor proteins such as p53 and ultimately lead to tumor development (Sarasin, 1999; Afaq, et al., 2005).

1.5 The cyclooxygenase inflammatory cascade

A common characteristic of both chemically- and UV-induced skin carcinogenesis is the induction of the cyclooxygenase (COX) cascade (Figure 4). Chemical and UV exposure increases the phosphorylation of cytosolic phospholipase A_2 which catalyzes the release of arachidonic acid from membrane phospholipids. Free

arachidonic acid is then metabolized to prostaglandin H_2 (PGH_2) by prostaglandin H synthase, commonly known as COX. COX exists as two different isoforms, COX-1 and COX-2. COX-1 is constitutively expressed whereas COX-2 is inducible. PGH_2 is then metabolized by prostaglandin synthases to PGE_2, PGD_2, PGF_2, prostacyclin (PGI_2) and thromboxane A2. Prostaglandins play a critical role in various physiological functions including wound healing, dilation/constriction of vascular smooth muscle, induction of labor, and blood clotting. Furthermore, prostaglandins such as PGE_2 play an important role in various pathological processes including cancer development. PGE_2 is produced by the metabolism of PGH_2 by cytosolic PGES (cPGES), microsomal PGE Synthase-1 (mPGES-1), and microsomal PGE Synthase-2 (mPGES-2). PGE_2 has been shown to promote abnormal cell growth and proliferation, angiogenesis, and tumor metastasis (reviewed in Greenhough, et al., 2009).

1.6 The role of COX-2/PGE_2 signaling in skin carcinogenesis

COX-1 is a house keeping gene expressed in most tissues and regulates gastric- and renal-homeostasis. COX-2 on the other hand is an immediate-early gene product expressed in response to cytokines, growth factors, and various other stress-inducing factors. There are various reports showing that the overexpression of COX-2 is involved in the promotion of several types of epithelial cell-derived cancers including colon, lung, breast, prostate, and skin (Eberhart, et al., 1994; Wolff, et al., 1998; Gupta, et al., 2000; Kagoura, et al., 2001; Shim, et al., 2003). These reports have led to both genetic and pharmacological approaches to validate the role of COX-2 in skin

carcinogenesis (Oshima, et al., 1996; Muller-Decker, et al., 2002; Muller-Decker and Furstenberger, 2007; Rundhaug, et al., 2007b). For instance, Tiano et al. utilized the two-stage chemical carcinogenesis protocol in COX-1 and COX-2 knockout animals. COX-1 -/- mice displayed an approximate 30% decrease in tumor formation and COX-2 -/- mice displayed an approximate 60% decrease in tumor formation in comparison to wild-type animals (Figure 5) (Tiano, et al., 2002). COX deficient mice also displayed a decrease in proliferating keratinocytes and an increase in both keratin-1 (K1) and keratin-10 (K10), both early markers of keratinocyte differentiation, suggesting that inhibition of COX induces terminal differentiation. In another study, Muller-Decker et al. demonstrated that transgenic mice that overexpress COX-2 develop more tumors than their wild-type counterparts when exposed to DMBA only (Muller-Decker, et al., 2002). These and many other reports highlight the importance of COX-2 in tumor development.

In addition to using genetic approaches to understand the role of COX-2 in tumor development, pharmacological agents have also been employed. For example, a clinical study reported that the selective COX-2 inhibitor, celecoxib, decreased the number of colorectal polyps formed in individuals with familial adenomatous polyposis disorder (Steinbach, et al., 2000). Furthermore, diclofenac, a non-selective COX inhibitor, is an approved treatment for patients displaying actinic keratosis (small pre-malignant lesion that has the potential to transform into an invasive NMSC) (Marks, et al., 1988; Berman, et al., 2009). In an *in vivo* animal study, Oberyszyn et al. showed that topical application of celecoxib prevented UVB-induced skin tumorigenesis (Oberyszyn, et al., 2001).

Other studies show that the cyclooxygenase metabolic product, PGE_2, is also increased in various animal and human tumors (Furstenberger, et al., 1989; Tiano, et al., 2002). Ansari et al. reported that endogenous and exogenous PGE_2 is sufficient to induce cell proliferation both *in vivo* and *in vitro*, suggesting that PGE_2 can enhance tumor promotion (Ansari, et al., 2008). PGE_2 has also been shown to increase angiogenesis, tumor invasiveness, and metastasis (reviewed in Greenhough, et al., 2009). Given the adverse effects associated with COX-2 inhibitors, several groups proposed that inhibiting the synthesis of PGE_2 by targeting its synthetic enzymes would be an effective approach to prevent cancer development. Experimental observations revealed that out of the three PGES enzymes, only mPGES-1 regulates carcinogenesis (Kamei, et al., 2003; Kamei, et al., 2010). Other studies show that mPGES-1 is overexpressed in colorectal adenomas and that genetic deletion of mPGES-1 reduces the number of preneoplastic aberrant crypt foci in mice (Yoshimatsu, et al., 2001; Nakanishi, et al., 2008).

Investigators have more recently identified an alternative novel therapeutic target; the E-type prostaglandin (EP) receptor. Once PGE_2 is produced, it is then exported out of the cell by a multidrug resistance-associated protein, MRP4, where it can act either in an autocrine or paracrine fashion to activate any of four EP receptors (EP1, EP2, EP3, and/or EP4). The EP receptors are G-protein coupled receptors that contain seven transmembrane-spanning α helices, an extracellular N terminus, and an intracellular C terminus. Each of the EP receptors are coupled to a different G_α subunit (Figure 6) where activation initiates downstream signaling via cAMP or phospholipase C. Additionally, each receptor possesses distinct biochemical properties and

cellular/tissue localization. The localization patterns of the EP receptors in the epidermis of SKH-1 mice after UV exposure has been described (Lee, et al., 2005). Under normal conditions, EP1 expression is low and localized throughout the stratum corneum. After UV exposure, EP1 expression increases throughout the suprabasal layers. Basal EP2 expression is low and is localized throughout the suprabasal layers. UV exposure increases its expression throughout the epidermis. Both EP3 and EP4 expression were undetectable under the experimental conditions tested.

Strong evidence indicates that EP1, EP2, and EP4 activation is involved in tumor promotion. Numerous studies have demonstrated that the upregulation of the EP1 receptor promotes tumorigenesis while genetic or pharmacological inhibition of EP1 receptors suppresses tumor formation (Watanabe, et al., 1999; Thompson, et al., 2001; Tober, et al., 2006; Surh, et al., 2011). Similarly, EP2 and EP4 receptors are both reported to regulate epithelial tumor development. A study showed that genetic deletion of the EP2 receptor, but not EP3, blocked DMBA/TPA-induced carcinogenesis in mice (Sung, et al., 2005). In another study, EP4 receptor inhibition reduced aberrant crypt foci formation induced by azoxymethane or spontaneous polyp development in Min mice (Mutoh, et al., 2002). These findings illustrate the role that the EP receptors play in tumor formation and identify novel therapeutic targets for the prevention/treatment of skin cancer.

1.7 Cancer chemoprevention by natural compounds

The use of botanicals as therapeutic agents predates medical recorded history and has led to the identification of various natural compounds as chemopreventative and chemotherapeutic agents. Several anticancer drugs that are used clinically were originally derived from plants including vincristine and vinblastine (derived from *Catharanthus roseusI)*, paclitaxel and docetaxal (*Taxus baccata* or European yew), and etoposide (*Podophyllum peltatu*) (Nobili, et al., 2009). Natural agents such as resveratrol and epigallocatechin gallate (EGCG), which is derived from green tea, are known to possess antioxidant and anti-inflammatory properties and may serve as a potential chemopreventive agent (Ahmed, et al., 2002; Kundu, et al., 2006). Development of natural compounds for preventing and treating cancer is an area of intense study as these agents are generally low in toxicity and safe to consume (Gupta and Mukhtar, 2002). In addition, there is also growing evidence that the use of natural products as adjunctive therapy may produce synergistic effects when combined with current cancer treatments.

1.8 Apigenin

Apigenin (4', 5, 7, - trihydroxyflavone), whose chemical structure is shown in Figure 6, is a natural plant flavone that is present in a variety of fruits and vegetables with the highest concentrations found in parsley and chamomile. Apigenin has been used in alternative medicine for years to treat ailments ranging from asthma to neuralgia

and is now being evaluated for its chemopreventative potential. In the last few years, significant progress has been made in determining the pharmacological effects of apigenin at the cellular and molecular level. This non-mutagenic bioflavonoid displays several anticancer properties in tumor cells and also prevents skin tumorigenesis induced by UV light or DMBA/TPA (Figure 7) (Wei, et al., 1990; Birt, et al., 1997; Shukla and Gupta, 2010). Previous data from our laboratory show that apigenin suppresses both TPA- and UVB-induced COX-2 expression in cultured keratinocytes (Van Dross, et al., 2005; Van Dross, et al., 2007). Additional antitumor effects of apigenin have been attributed to features including its ability to activate tumor suppressor genes, induce cell cycle arrest, and initiate apoptosis (Lepley, et al., 1996; McVean, et al., 2000; Abu-Yousif, et al., 2008; Kaur, et al., 2008; Choi and Kim, 2009).

To begin to understand the role of COX-2 in the antitumor activity of apigenin we conducted a tumor study using the two-stage chemical carcinogenesis protocol and evaluated COX-2 expression, PGE_2 production, and EP receptor expression in tumors and in the surrounding epidermis. Apigenin suppressed DMBA/TPA-induced COX-2/PGE_2 signaling in the surrounding epidermis but did not alter COX-2/PGE_2 signaling in the tumors. Furthermore, apigenin increased chemically-induced terminal differentiation and decreased proliferation within the epidermis of the surrounding skin of tumor bearing animals. We also demonstrate in this study that apigenin effectively blocks COX-2 expression and signaling in mouse skin acutely exposed to TPA. Because suppression of COX-2 expression/activity prevents tumor development, the reduction in COX-2/PGE_2 signaling that we observed here implies that apigenin blocks tumor development by inhibiting COX-2 expression, PGE_2 production, and EP1 receptor

expression. As such, apigenin or apigenin derivatives could provide additional options for treatment of various types of cancer including NMSC.

Figure 1: Anantomy of the human skin. Figure obtained from http://en.wikipedia.org/wiki/File:HumanSkinDiagram.jpg.

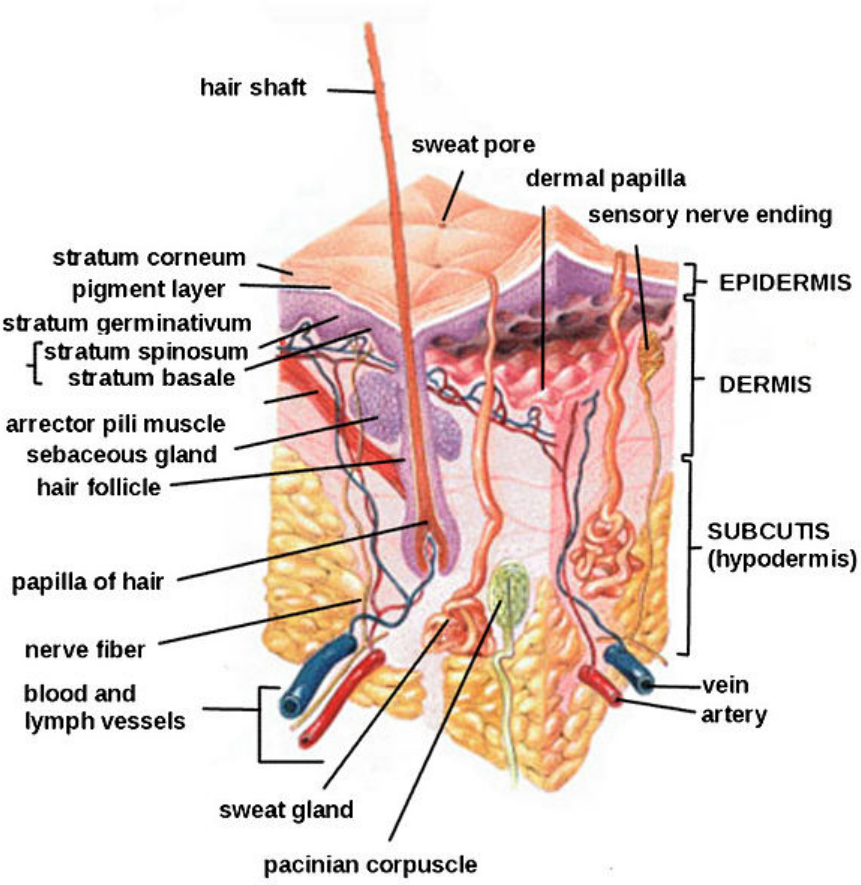

hair shaft
sweat pore
dermal papilla
sensory nerve ending
stratum corneum
pigment layer
stratum germinativum
stratum spinosum
stratum basale
arrector pili muscle
sebaceous gland
hair follicle
papilla of hair
nerve fiber
blood and
lymph vessels
sweat gland
pacinian corpuscle
EPIDERMIS
DERMIS
SUBCUTIS
(hypodermis)
vein
artery

Figure 2: Histological examination of the structural layers within the epidermis. Figure obtained from http://en.wikipedia.org/wiki/File:Epidermal_layers.png.

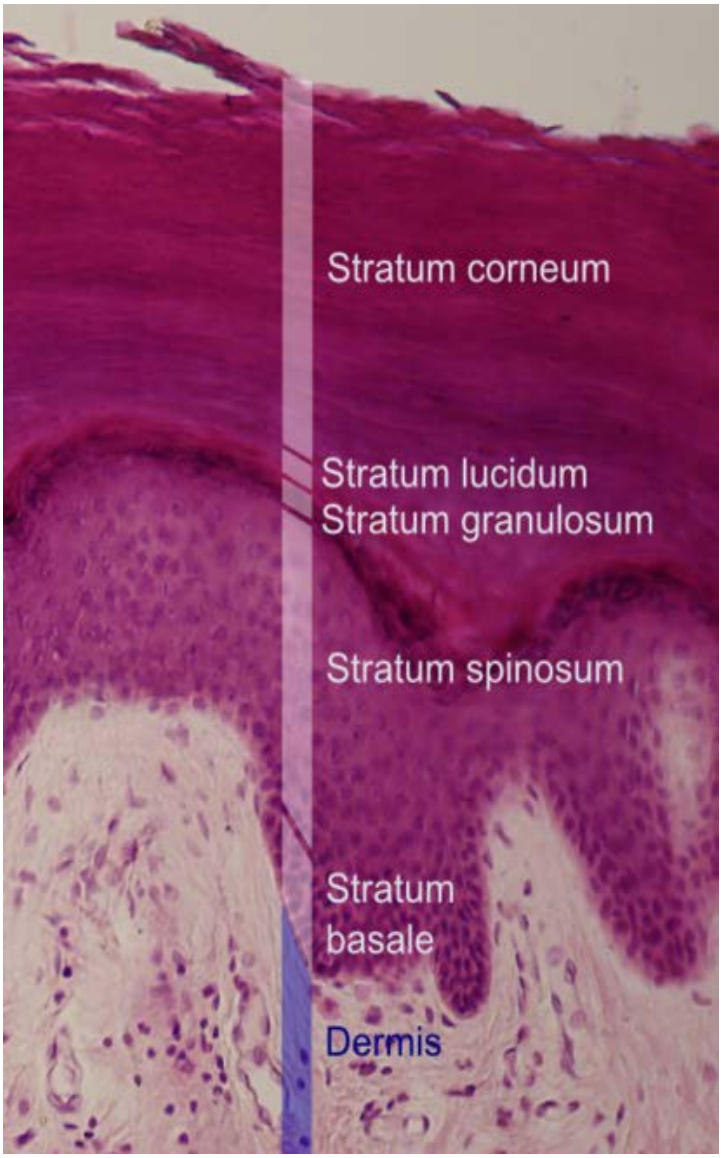

Stratum corneum
Stratum lucidum
Stratum granulosum
Stratum spinosum
Stratum basale
Dermis

Table 1: Examples of tumor initiating and tumor promoting agents used in mouse skin tumorigenesis studies (DiGiovanni, 1992).

.

Tumor Initiating Agents	Tumor Promoting Agents
Benzo[a]pyrene	Croton oil
7, 12-dimethylbenz[a]anthracene	12-*O*-tetradecanoylphorbol-13-acetate
Dibenzanthracene	Phenol
β-propiolactone	Antrahlin
3-methylcholanthrene	Lodoacetic acid
Ultraviolet radiation	

Figure 3: The two-stage chemical carcinogenesis animal model. Tumor initiation can be achieved with a single application of the chemical initiator, DMBA, to the dorsal skin. Next, tumor promotion occurs after repeated topical applications of the pro-inflammatory phorbol ester, TPA, which stimulates cell proliferation and clonal expansion of the initiated cell. The final stage is progression. Tumor progression occurs spontaneously and represents the point at which benign lesions transform to malignant tumors.

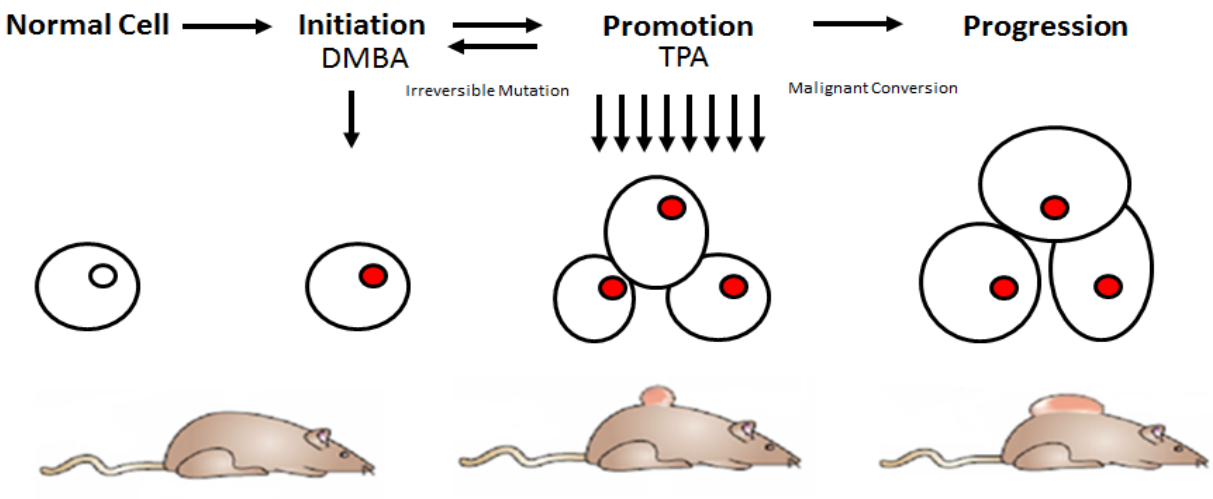

Normal Cell
Initiation
DMBA
Irreversible Mutation
Promotion
TPA
Malignant Conversion
Progression

Figure 4: Prostaglandin E2 synthesis pathway. Dermal exposure to pro-inflammatory agents such as TPA, leads to $cPLA_2$ activation which catalyzes the release of arachidonic acid from membrane phospholipids. Arachidonic acid is then metabolized to PGH_2 by COX-2 and further metabolized to PGE_2 by PGE Synthase 1 and 2. PGE_2 plays a critical role in cancer formation by promoting cell proliferation, cell survival, and angiogenesis.

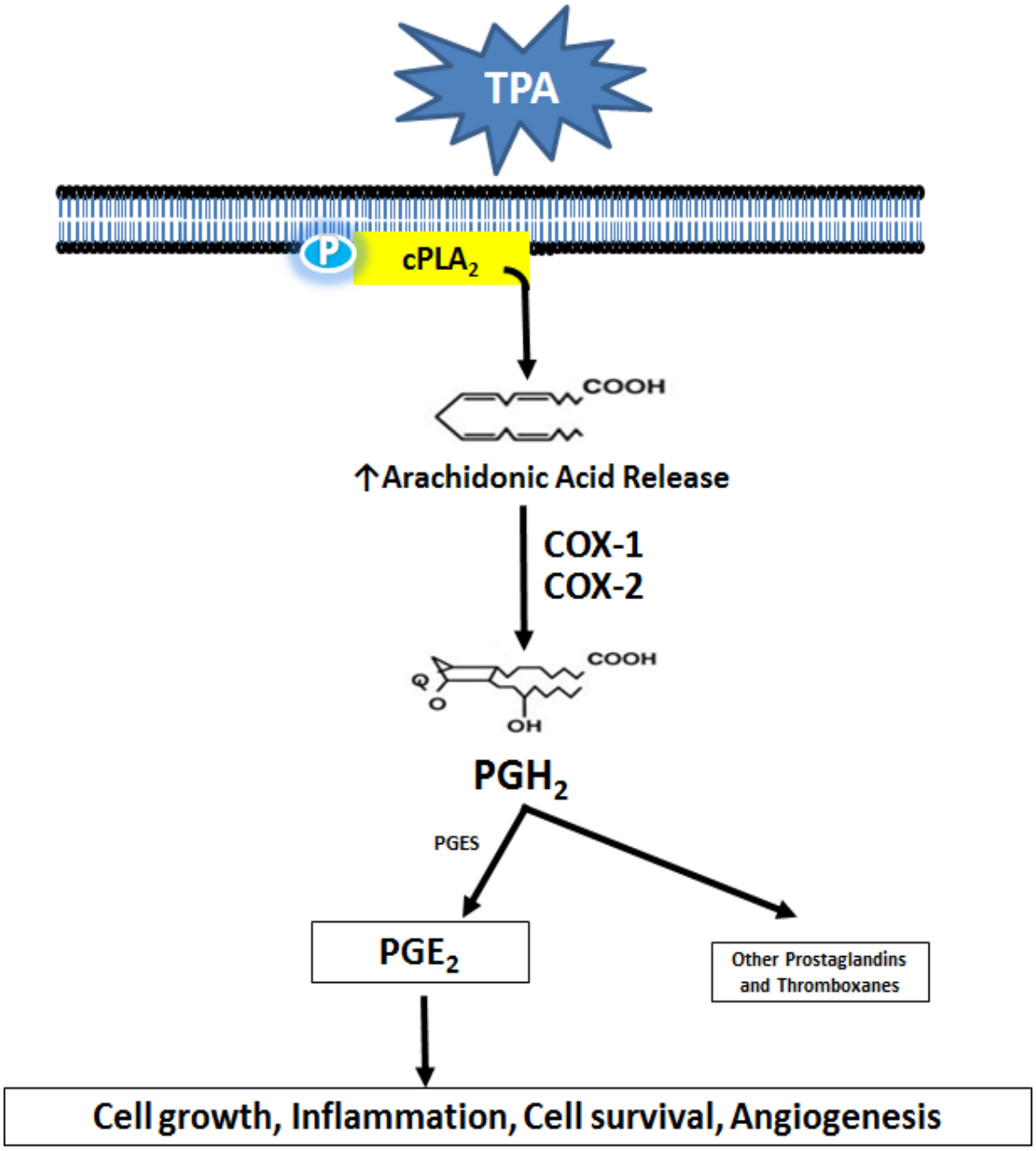

TPA
P
cPLA2
COOH
↑Arachidonic Acid Release
COX-1
COX-2
COOH
O
OH
PGH2
PGES
PGE2
Other Prostaglandins and Thromboxanes
Cell growth, Inflammation, Cell survival, Angiogenesis

Figure 5: Genetic deficiency of COX-1 or COX-2 reduces tumorigenesis in the two-stage chemical carcinogenesis protocol (adapted from (Tiano, et al., 2002). In this experiment, F_1 agouti wild-type and COX-deficient mice were subjected to DMBA-initiated TPA-promoted skin tumorigenesis. Tumor incidence decreased by 60% in COX-2 knockout mice and 30% in COX-1 knockout mice compared to wild-type, suggesting that COX-2 plays a role in skin tumor formation.

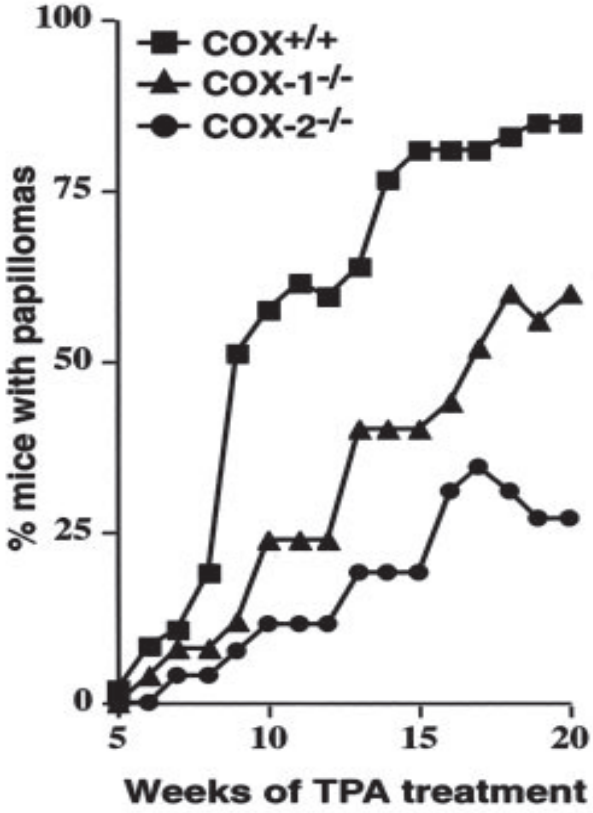

100
75
50
25
0
% mice with papillomas
COX+/+
COX-1-/-
COX-2-/-
5
10
15
20
Weeks of TPA treatment

Figure 6: EP receptor signaling (adapted from Rundhaug, et al., 2011). The four EP receptor subtypes are G-protein-coupled receptors that are activated by PGE_2. EP1 is a G_q-coupled receptor that activates phospholipase C (PLC), resulting in the production of 1, 2-diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3). DAG activates protein kinase C (PKC) and IP3 increases $[Ca^{2+}]$. Both EP2 and EP4 are G_s-coupled receptors that activate adenylyl cyclase (AC) and increase both cAMP synthesis and protein kinase A (PKA). EP3 is a G_i-coupled receptor that reduces cAMP levels upon activation. Activation of EP1, EP2, and EP4 receptors have all been shown to promote skin tumor formation by increasing cell proliferation, angiogenesis, and cell survival.

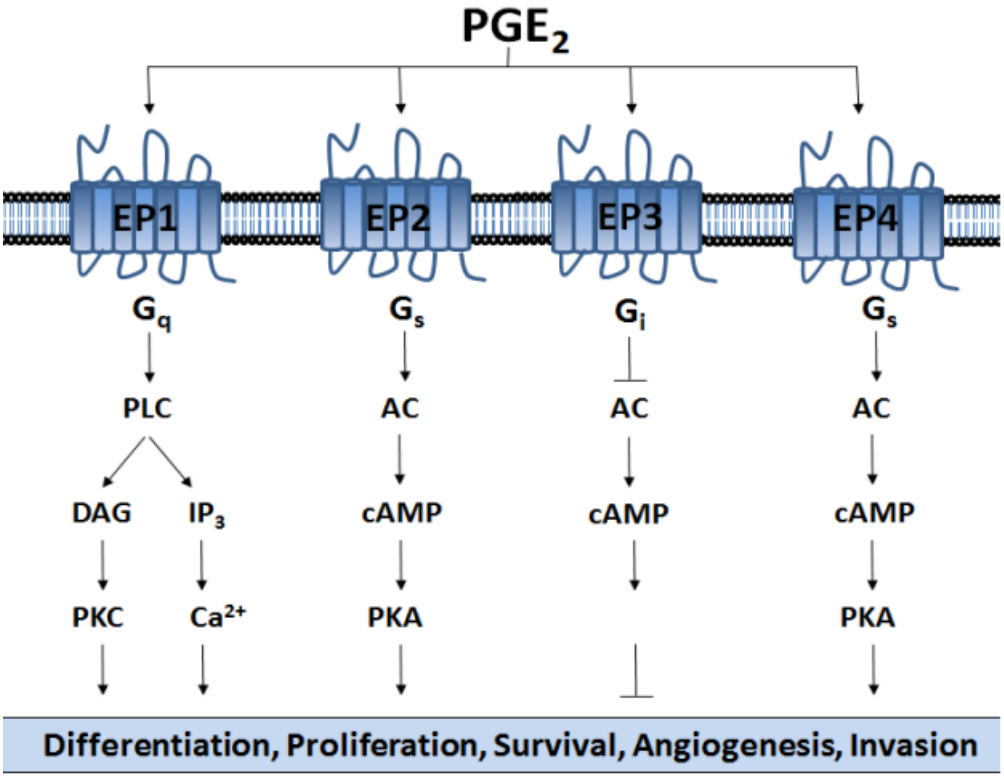

PGE2
EP1
EP2
EP3
EP4
Gq
Gs
Gi
Gs
PLC
AC
AC
AC
DAG
IP3
cAMP
cAMP
cAMP
PKC
Ca2+
PKA
PKA
Differentiation, Proliferation, Survival, Angiogenesis, Invasion

Figure 7: Apigenin inhibits DMBA/TPA-induced skin tumor formation (obtained from Birt, et al., 1997). Female SENCAR mice were exposed to a single dose of DMBA. Animals were pretreated with apigenin (5 or 20 µmol) or DMSO (vehicle control) before TPA exposure twice a week for 20 weeks and tumor development evaluated up to week 40. Apigenin inhibited skin tumor formation in a concentration dependent fashion.

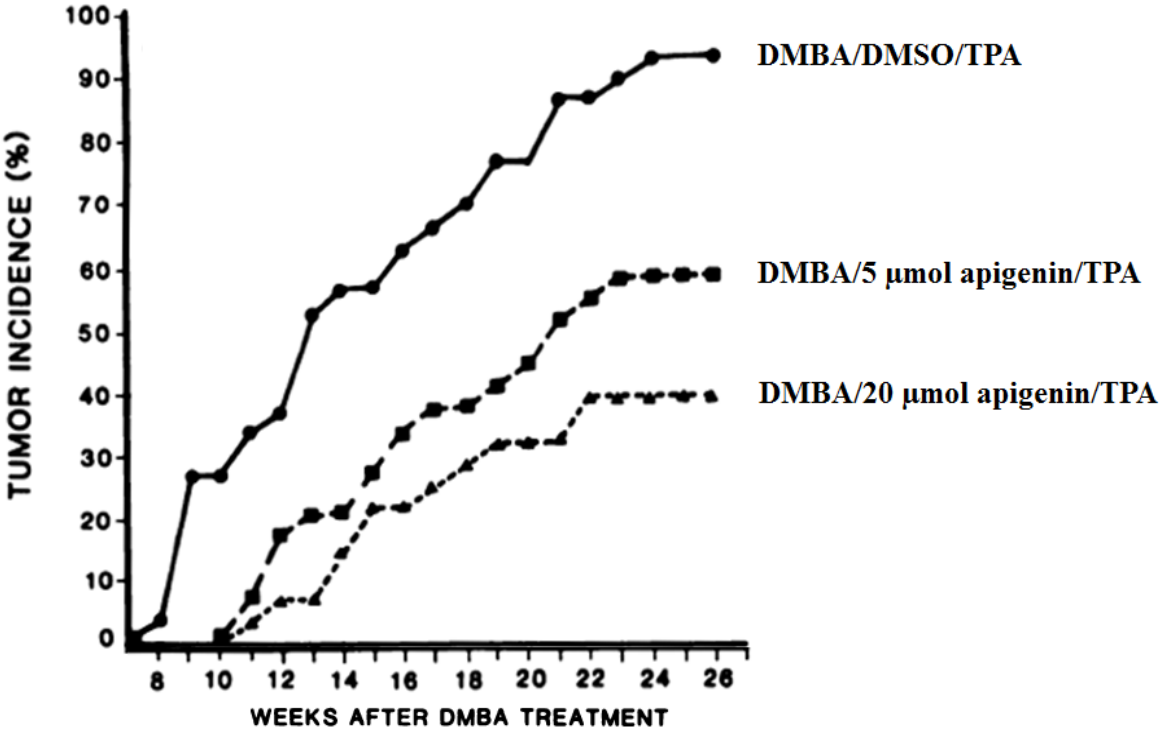
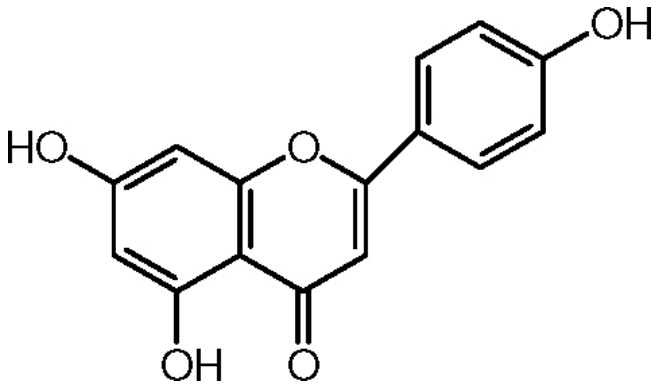

TUMOR INCIDENCE (%)
100
90
80
70
60
50
40
30
20
10
0
8
10
12
14
16
18
20
22
24
26
WEEKS AFTER DMBA TREATMENT
DMBA/DMSO/TPA
DMBA/5 µmol apigenin/TPA
DMBA/20 µmol apigenin/TPA

Figure 8: Structure of apigenin (4', 5, 7, - trihydroxyflavone).


OH
HO
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Chapter 2

Material & Methods

2.1 Materials and reagents

Apigenin was purchased from Indofine Chemical Company (Hillsborough, NJ). Acetone, dimethyl sulfoxide (DMSO), and TPA were purchased from Sigma-Aldrich (St. Louis, MO). PGE_2 EIA kits as well as COX-2, EP receptor, mPGES-1, and mPGES-2 antibodies were from Cayman Chemical (Ann Arbor, MI). Anti-active caspase-3 antibody was from BD Biosciences (Chicago, IL). Ki67 antibody was purchased from Abcam (Cambridge, MA). Keratin-1 (K1) and -10 (K10) antibodies were purchased from Covance (Princeton, NJ).

2.2 Animals

SKH-1 hairless mouse breeding pairs were generously provided by Dr. Susan M. Fischer (MD Anderson Cancer Center; Smithville, TX). Animals were bred and maintained within the Department of Comparative Medicine of East Carolina University. Room temperature of the housing unit was maintained at 70-75°F with 40-60% humidity and on a 12 hour light/dark cycle. Water and food were provided *ad libitum*. The mice were fed a bioflavonoid-free diet (AIN-76A; Dyets, Bethelham, PA) one week prior to experimentation. All experimental procedures used for this study were approved by the Institutional Animal Care and Use Committee of East Carolina University.

2.3 Breeding and genotyping SKH-1 mice

SKH-1 hairless mouse breeding pairs consisted of one pair COX-2 wild-type (COX-2 +/+) and one pair COX-2 heterozygous (COX-2 +/-). Animals were bred to produce only wild-type and heterozygous mice. COX-2 homozygous (COX-2-/-) knockout mice develop renal failure and survive only a few weeks after birth and therefore were not bred. SKH-1 pups were weaned from the mothers at approximately 21 days after birth. At this time, each mouse was ear tagged and tail snipped. Individual tail snips were added to 100 µl of tail digestion buffer (TDB) consisting of; 50 mM KCl, 10 mM Tris-Hcl (pH 9), 0.1% Triton-X, and 0.4 mg/ml proteinase K). Samples were then incubated overnight at 55°C. Upon digestion, samples were incubated at 94°C for 10 minutes to inactivate proteinase K. Samples were then centrifuged at top speed for 15 minutes. Next, 1.0 µl of genomic DNA was placed into the following PCR mixture: 5 µl 10x PCR buffer, 10 mM dNTP mixture, 50 mM $MgCl_2$, 10 µM primer, 0.5 µl Taq polymerase (Invitrogen), and sterile dH_2O. The following set of primers was used to validate SKH-1 COX-2 wild-type allele; forward-COX2 WT: ACACACTCTATCACTGGCACC, reverse-COX-2: ATCCCTTCACTAAATGCCCTC. The following set of primers was used to validate SKH-1 COX-2 heterozygous allele; forward-COX2 WT: ACGCGTCACCTTAATATGCG, reverse-COX-2: ATCCCTTCACTAAATGCCCTC. The following GAPDH primers were used to validate the PCR reaction; forward-GAPDH: TGCACCTCTGGTAACTCCGC, reverse-GAPDH: GCATCACCTGGCCTACAGGA. Samples were then placed into a thermal cycler and incubated at 95°C for 3 minutes to denature the template followed by 25-35 cycles of PCR amplification. Once complete,

amplified PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.4 Two-stage chemical carcinogenesis protocol

Female SKH-1 mice ranging from 5 to 10 weeks of age were used (n = 19 per treatment group). Female mice were used to prevent fighting/injury to one another as they tend to be docile compared in comparison to males. All animals received a single initiating dose of 100 nmol of DMBA topically applied in 200 µl of acetone using a non-abrasive swab (Texwipe, Mahwah, NJ). One week after initiation, all animals received twice weekly applications of 3.2 nmol of TPA in 200 µl of acetone for 25 weeks. Apigenin was applied topically at 5 µmol and 20 µmol in 200 µl of DMSO one hour prior to TPA exposure. Concentrations of apigenin were determined from the work of Diane Birt (Wei, et al., 1990). Animals in the control group were pretreated with DMSA (vehicle control) one-hour prior to TPA application. After 25 weeks of treatments, animals were euthanized via cervical dislocation and CO_2 asphyxiation. Subsequently, tumors and surrounding skin were measured in two dimensions (length x width) using digital calipers and then harvested and analyzed by a board certified pathologist, Dr. Allan Smith. Tumors and surrounding skin were then labeled and stored in skin fixative (Amersco, Solon, OH) or frozen in liquid nitrogen. Statistical analysis for tumor incidence and tumor multiplicity was conducted using repeated measure ANOVA with GraphPad Prism Software (La Jolla, CA) and Microsoft Excel 2010 (Mission Viejo, CA).

2.5 Immunohistochemistry

Fixed epidermal skin and tumors were incubated overnight in a 40% sucrose solution before being embedded in TissueTek OCT compound (Sakura Finetek, Torrence, CA), sectioned, and mounted on positively charged slides. The tissues were blocked and incubated in 3.0% H_2O_2 for 30 minutes to quench endogenous peroxidase activity. Sections were then incubated overnight with primary antibody and were incubated with secondary antibody for 30 minutes. Preliminary experiments were conducted to optimize conditions for primary antibody concentrations. Staining was visualized using the VectaStain ABC kit (Burlingame, CA) and diaminobenzidine (DAB) (Sigma, St. Louis, MO) and the tissues counterstained in Harris' hematoxylin. Quantification of optical densities was measured from non-counterstained sections of the epidermis at 40x magnification and at 4x for tumor samples. Color images from 10 random fields showcasing the epidermis were captured using ImagePro software under identical exposure conditions. Each image captured was then background corrected to a control slide and converted to an 8-bit gray scale picture before analyzing the densities using the software. Color images of random whole tumor samples were captured and processed as described above. Statistical analysis was completed using one-way ANOVA and Student's T-test using GraphPad Prism software (La Jolla, CA) and Microsoft Excel 2010 software (Mission Viejo, CA).

2.6 Western analysis

The dorsal skin from SKH-1 mice in each experimental group was scraped, pooled (n = 3) and homogenized in a buffer containing 60 mM Tris pH 8.6, 5 mM EDTA, 5 mM EGTA, 300 mM sucrose, 1% NP-40, 5 mM DTT, 2 mM PMSF, 10 mM sodium molybdate, 20 µg/ml aprotinin, 20 mM sodium fluoride, and 100 mM sodium orthovanadate. Epidermal extracts were then centrifuged at 3000 x g for 10 minutes and the supernatant collected for determination of protein concentration using the BCA method (Thermo Scientific, Rockford, IL). Proteins were resolved in SDS-PAGE gels, protein transferred to PVDF membranes, and blots probed with the appropriate primary and secondary antibody. Preliminary experiments were conducted to optimize conditions for antibody concentrations. Bands were visualized on X-ray film using Enhanced ChemiLuminesence reagents (Amersham, Piscataway, NJ) according to the manufacturer's protocol. The intensity of each band was determined using Image J image Processing and Analysis Software (http://rsbweb.nih.gov/ij/).

2.7 PGE_2 assays

For PGE_2 analysis of the surrounding skin and tumors, epidermis from tumor bearing mice was isolated and prostaglandins extracted as described by Fisher et al (Fischer, et al., 2007). Briefly, the epidermis was removed and homogenized in Tris buffer containing 5 µg/ml of indomethacin. For the PGE_2 assays of tumor samples, random tumors from each treatment group were selected. Tumors were minced and homogenized in Tris buffer containing 5 µg/ml of indomethacin. Samples were then

centrifuged at 3000 x g for 10 minutes, and an aliquot of the extract removed for protein concentration determination. Lysates were then acidified using HCl and applied to a preconditioned C18 SPE cartridge (Grace Davison Discovery Science, Deerfield, IL). Columns were then rinsed with 15% ethanol to remove any polar substances. Afterwards, columns were rinsed with petroleum ether (Sigma-Aldrich, St. Louis, MO) to remove all traces of water and to allow PGE_2 molecules to adhere to the silica in the column. Methyl formate (Sigma-Aldrich, St. Louis, MO) was then used to elute PGE_2 from the column and then PGE_2 was dried under a gentle stream of nitrogen gas. Samples were reconstituted in EIA immunoassay buffer provided by the manufacturer. PGE_2 concentrations were then determined by ELISA as directed by the manufacturer (Cayman, Ann Arbor, MI). Statistical analysis was computed using GraphPad Prism Software (La Jolla, CA) and Microsoft Excel 2010 (Mission Viejo, CA).

2.8 Statistical Analysis

Data is presented as mean ± SD. Repeated analysis of variance (ANOVA), Tukey post-hoc analysis, and Student's T-test was carried out using GraphPad Prism and Microsoft Excel 2010.

Chapter 3

Results

Aim 1: Acute Effects of Apigenin on TPA-mediated COX-2 Expression in Mouse Epidermis

3.1 Apigenin inhibits TPA-mediated COX-2 expression in an acute setting in the epidermis of SKH-1 mice

Previous reports from our laboratory show that apigenin reduces TPA- and UVB-induced COX-2 expression and PGE_2 production in cultured keratinocytes (Van Dross, et al., 2005; Tong, et al., 2007; Van Dross, et al., 2007). To determine the optimal conditions for the induction of COX-2 in the epidermis, different concentrations of TPA were applied to the dorsal surface of SKH-1 mice and the skin isolated after 8 hours. Western blot analysis showed that TPA caused a 1.9-, 4.4-, and a 4.7-fold increase in COX-2 protein expression when compared to vehicle-treated epidermis (Figure 9A). Because topical application of 3.4 nmol and 6.8 nmol TPA produced a similar fold increase in COX-2 expression, 3.4 nmol of TPA was used throughout this study. Next, a time course analysis of COX-2 induction was conducted by topically treating animals with 3.4 nmol TPA or with vehicle for 2, 4, or 8 hours (Figure 9B). TPA caused a 4.0-fold increase in epidermal COX-2 expression at 8 hours. A modest increase in COX-2 expression was observed in animals treated with TPA for 2 and 4 hours.

Next, our goal was to confirm that TPA-induced COX-2 expression is reduced in COX-2 heterozygous knockout mice (COX-2 +/-) compared with wild-type (COX-2 +/+) mice. In Figure 10A, different concentrations of TPA were applied to the dorsal surface of SKH-1 COX-2 +/+ and SKH-1 COX-2 +/- mice and the skin isolated after 8 hours. Western blot analysis revealed no difference in COX-2 expression between the genotypes. Also, in our time course study, differences in the expression of COX-2 in COX-2 +/+ and COX-2 +/- mice were not observed (Figure 10B). Based on these results, the COX-2 +/- animals were excluded from this study.

Next, we wanted to determine the effect of apigenin on TPA-induced COX-2 expression, PGE_2 production, and EP receptor expression *in vivo*. Apigenin was topically applied to mouse skin 1 hour prior to the application of TPA and epidermal COX-2 expression measured by Western analysis (Figure 11A). Our data show that apigenin suppressed TPA-induced COX-2 expression in a concentration-dependent manner. Further, the level of expression of COX-2 in animals treated with TPA plus 20 µmol apigenin (TPA + AP20) was comparable to the level of COX-2 present in the epidermis of animals treated with vehicle or apigenin alone. Next, we conducted immunohistochemical analysis to examine the distribution (Figure 11B) and to quantify the relative expression of COX-2 (Figure 11C) in the skin. Similar to other reports, TPA increased COX-2 expression in each epidermal layer with the most prominent staining located in the basal layer (Tripp, et al., 2003; Funk, et al., 1993). Apigenin at 5 and 20 µmol dramatically reduced TPA-induced COX-2 expression throughout the epidermis. Also, COX-2 expression was absent in vehicle treated skin and skin treated with

apigenin alone. These findings show that apigenin restores basal level COX-2 expression in TPA-exposed epidermis.

3.2 Apigenin inhibits TPA-mediated PGE_2 production in the mouse epidermis.

To determine if apigenin also blocks PGE_2 production *in vivo*, mice were topically treated with TPA, TPA plus apigenin, apigenin or vehicle and PGE_2 levels measured (Figure 12A). As anticipated, TPA increased PGE_2 production. Pretreatment of the epidermis with 5 or 20 µmol apigenin blocked TPA-induced PGE_2 synthesis with both concentrations of apigenin producing a statistically significant decrease in PGE_2 production. The synthesis of PGE_2 is dependent on the sequential enzymatic activity of COX and PGES. To determine if apigenin inhibits TPA-induced PGE_2 production by decreasing mPGES expression, epidermal mPGES levels were measured by Western analysis (Figure 12B). The steady-state levels of mPGES-1 or mPGES-2 were not changed by TPA and mPGES-1 and mPGES-2 were only slightly altered in animals treated with TPA plus 20 µmol apigenin. These findings that apigenin does not inhibit PGE_2 by blocking the expression of mPGES1 or mPGES2.

3.3 Apigenin decreases EP1 receptor expression in TPA-treated mouse skin

In Figure 13, we analyzed the expression of each EP receptor in the epidermis using Western analysis. A 3-fold increase in EP1 receptor expression was observed in mice topically treated with TPA. Apigenin suppressed the TPA-induced increase in EP1 receptor expression in a concentration-dependent manner. Next, we examined the

expression of the EP2 receptor which appears as two separate bands on the Western blot likely due to post translational modification of the receptor. In each treatment group, no significant changes were observed in EP2 receptor expression. In contrast, no consistent pattern of EP3 and EP4 receptor expression was observed in any of the treatment groups. We also examined the expression of EP receptors in cultured keratinocytes. Interestingly, EP1 receptor expression was elevated in the tumorigenic JWF-2 cell line while the expression of this receptor was barely detectable in non-tumorigenic HaCaT cells. Expression of only the high molecular weight form of the EP2 receptor was observed in JWF2 cells while the level of each EP2 receptor band in HaCaTs was similar to the levels found in epidermal extracts. On the other hand, JWF2 and HaCaT cells did not express the EP4 receptor. Thus, the EP1 receptor displays a similar expression pattern in TPA-treated epidermis and in cultured tumorigenic keratinocytes.

Next, we conducted immunohistochemical analysis to examine the expression and distribution of the EP receptors in the epidermis following exposure to TPA and apigenin (Figure 14). Topical application of TPA increased EP1 receptor expression in each epidermal layer with the highest intensity staining in the suprabasal epidermis. In agreement with our data from the epidermal lysates, apigenin suppressed TPA-induced EP1 receptor expression. EP2 receptor expression was intense in each treatment group with a slight increase in EP2 expression in the outermost layers of the epidermis in TPA-treated mice. In contrast, EP3 and EP4 expression were barely detectible in each treatment group. These results show that apigenin suppresses TPA-mediated

EP1 induction and this pattern of expression is consistent with an inhibitory effect on tumor formation. Overall, apigenin inhibits TPA-mediated COX-2/PGE_2 signaling pathway in an acute setting.

Aim 2: Apigenin Inhibits Chemically-induced Tumorigenesis; Role of COX-2/PGE_2 Signaling and the EP1 Receptor Expression in Non-melanoma Skin Cancer

3.4 Apigenin inhibits DMBA/TPA-induced carcinogenesis in SKH-1 mice

To investigate the chemopreventative activity of apigenin, the two-stage chemical carcinogenesis protocol was utilized. Female SKH-1 mice were exposed to a single topical application of 100 nmol DMBA followed by twice weekly applications of 3.2 nmol of TPA for 25 weeks. One hour prior to TPA exposure, animals were topically treated with 5 µmol apigenin (AP5 + DMBA/TPA), 20 µmol apigenin (AP20 + DMBA/TPA), or vehicle/DMSO (DMBA/TPA). In animals treated with DMBA/TPA alone, approximately 70% of the mice developed tumors 25 weeks post-initiation (Figure 15). Animals in the AP5 + DMBA/TPA group displayed an approximate 35% decrease in tumor incidence at the same time point. In the AP20 + DMBA/TPA treatment group, tumor incidence was significantly reduced ($p < 0.05$) and tumor development delayed by 3 weeks compared with DMBA/TPA-treated animals. In addition, the average number of tumors per mouse in AP20 + DMBA/TPA- compared with DMBA/TPA-exposed animals was significantly decreased (0.18 and 3.37 respectively). In Table 2, tumors were measured and categorized according to their size to determine the effect of apigenin on tumor size.

More than 50% of the tumors obtained from the animals in the DMBA/TPA treatment group were 5 mm^2 or greater with successively decreasing numbers of tumors in the 2.5 - 4.9 mm^2 and 0.1 – 2.4 mm^2 size categories. A similar tumor distribution is also observed in both apigenin 5 and 20 µmol groups. Overall, apigenin significantly decreased tumor incidence and multiplicity in DMBA/TPA treated animals but did not change the tumor size distribution. Furthermore, apigenin at both concentrations had no significant effect on body weight in comparison to DMBA/TPA treated animals (Figure 16).

3.5 Apigenin blocks COX-2 expression and PGE_2 production in the surrounding epidermis

Numerous studies show that COX-2 promotes tumorigenesis and that the inhibition of COX-2 decreases tumor formation. Birt et al determined that apigenin prevents tumor development and work from this laboratory has shown that apigenin blocks COX-2 expression and activity in cultured keratinocytes (Birt, et al., 1997; Van Dross, et al., 2005; Tong, et al., 2007; Van Dross, et al., 2007). Therefore, we examined COX-2 expression and activity in isolated tumors and in the tissue surrounding the tumors (referenced throughout as surrounding epidermis) to further investigate the chemopreventative properties of apigenin and whether they are related to the inhibition of COX-2 expression. In the surrounding epidermis, apigenin at both 5- and 20-µmol significantly suppressed DMBA/TPA-induced COX-2 expression throughout the epidermis Figure 17A). In addition, we observed strong COX-2

expression at the epidermal-dermal interface that was not decreased by apigenin at either concentration. In Figure 17B, we examined COX-2 expression in isolated tumors from each treatment group. Our data show that COX-2 expression is elevated in all of the tumors isolated from the DMBA/TPA, AP5 + DMBA/TPA, and AP20 + DMBA/TPA treatment groups demonstrating the importance of COX-2 expression in tumor development.

The COX-2 metabolic product PGE_2 is known to play a prominent role in tumor promotion. As such, we examined PGE_2 production in the surrounding epidermis (Figure 17C) and tumors (Figure 17D). In the surrounding epidermis, apigenin inhibited DMBA/TPA-induced PGE_2 production in a concentration-dependent fashion ($p < 0.05$ for AP20 + DMBA/TPA). In tumors, PGE_2 production was not decreased by 5- or 20-µmol apigenin. These results show that apigenin inhibits COX-2 expression and activity in the surrounding epidermis but not in developed tumors.

3.6 Apigenin decreases EP1 and EP2 receptor expression in the surrounding epidermis

PGE_2 produces its cellular effects by interacting with EP receptors that are expressed in various tissues including the epidermis. Both TPA and UV-light are known to upregulate EP receptor expression (Tober, et al., 2006; Black, et al., 2008). In Figure 18, we analyzed EP1 and EP2 receptor expression in the surrounding epidermis and tumors of animals in each of our treatment groups. In the surrounding epidermis, apigenin at 5- and 20-µmol significantly inhibited DMBA/TPA-induced EP1 expression throughout the epidermal layers (Figure 18A). In contrast, EP1 expression in isolated

tumors from the DMBA/TPA, AP5 + DMBA/TPA, and AP20 + DMBA/TPA treatment groups was strongly expressed throughout the epidermis (Figure 18B). Figure 18C shows that AP20 + DMBA/TPA significantly inhibited EP2 receptor expression in the surrounding epidermis; however, apigenin did not decrease EP2 expression in DMBA/TPA-induced tumors (Figure 18D). Overall, these results show that apigenin suppresses DMBA/TPA-induced EP1 and EP2 expression in the surrounding epidermis but not in existing tumors. These findings are consistent with other reports which demonstrate that EP receptor inhibition effectively prevents tumor formation (Kawamori, et al., 2001; Kawamori, et al., 2005; Tober, et al., 2006).

3.7 Apigenin's effect on epidermal differentiation and proliferation

In the epidermis, the basal layer represents the proliferative zone of keratinocytes. Once basal cells divide, a daughter cell moves upward to the squamous layer where cytokeratins that are needed for terminal differentiation are expressed. Keratin-1 (K1) and keratin-10 (K10) are two critical cytokeratins expressed in the early phases of terminal differentiation. K1 and K10 are normally expressed in the suprabasal epidermis and not in basal keratinocytes which have proliferative capacity. K1 and K10 form a heterodimer whose interaction in a 1:1 ratio is critical for its function to maintain the structural integrity of keratinocytes. Thus, expression of K1 and K10 in the basal keratinocytes indicates a loss in proliferative capacity and premature entry into the terminal differentiation program. In the surrounding epidermis obtained from DMBA/TPA treated animals, K1 staining is predominantly located in the suprabasal

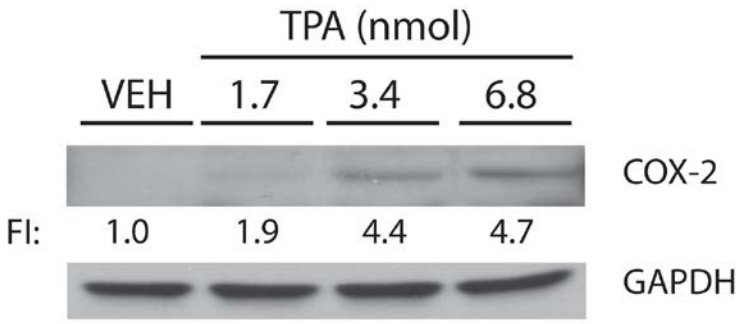
layers (Figure 19A). In contrast, the surrounding epidermis of AP5 + DMBA/TPA- and AP20 + DMBA/TPA-treated animals contained numerous K1 positive basal keratinocytes. Moreover, in AP20 + DMBA/TPA treated animals, the intensity of K1 expression was significantly increased. A similar staining pattern was observed for K10 with prominent suprabasal signal in the surrounding epidermis of DMBA/TPA animals and K10 positive basal and suprabasal keratinocytes in apigenin + DMBA/TPA treated animals (Figure 19C). Also, apigenin significantly increased K10 expression intensity within the epidermis. In tumors (Figure 19B), K1 staining was limited to the suprabasal region and was not observed in the basal epidermal cell layer in DMBA/TPA treated animals and apigenin increased K1 expression throughout the tumors. In Figure 19D, K10 expression was elevated in DMBA/TPA-treated animals but decreased in the presence of apigenin. These data show that apigenin increases K1 and K10 expression in the surrounding epidermis of mice treated with DMBA/TPA. These findings suggest that apigenin may prevent tumor development by increasing terminal differentiation.

Keratinocyte proliferation is known to be induced by TPA and PGE_2 (Furstenberger and Marks, 1980; Pentland and Needleman, 1986). Therefore we investigated the effect of apigenin on keratinocyte proliferation. Figure 20 shows the expression of Ki67, a proliferation marker, in the surrounding epidermis and tumors of the animals from each of our treatment groups. In the surrounding epidermis, DMBA/TPA-induced Ki67 expression was inhibited in animals treated with 5- or 20-µmol apigenin (Figure 20A). Similarly in isolated tumors, apigenin reduced the number of

Ki67 positive cells (Figure 20B). These data imply that the apigenin-mediated decrease in COX-2/PGE_2 signaling may alter induce cells to switch from a proliferating state to a differentiating state.

Figure 9: Concentration and time course analysis of TPA-induced COX-2 expression in mouse epidermis. (A) SKH-1 mice (n = 3) were topically treated with 1.7, 3.4, or 6.8 nmol of TPA dissolved in acetone or mice were treated with acetone alone (vehicle; VEH). Animals were euthanized after 8 hours and the epidermis was isolated. Epidermal extracts were then pooled and protein concentration was determined as described in the material and methods section. Western blot analysis was carried out with antibodies directed towards COX-2 and GAPDH (loading control). (B) Time-dependent induction of COX-2 protein expression by TPA. Mice were treated topically with 3.4 nmol TPA and euthanized after 2, 4, or 8 hours. Western analysis was carried out using antibodies directed towards COX-2 or GAPDH. Fold induction (FI) in TPA-treated samples compared to time-matched vehicle treated samples is indicated below the figure.

A.


TPA (nmol)
VEH
1.7
3.4
6.8
COX-2
FI:
1.0
1.9
4.4
4.7
GAPDH

B.

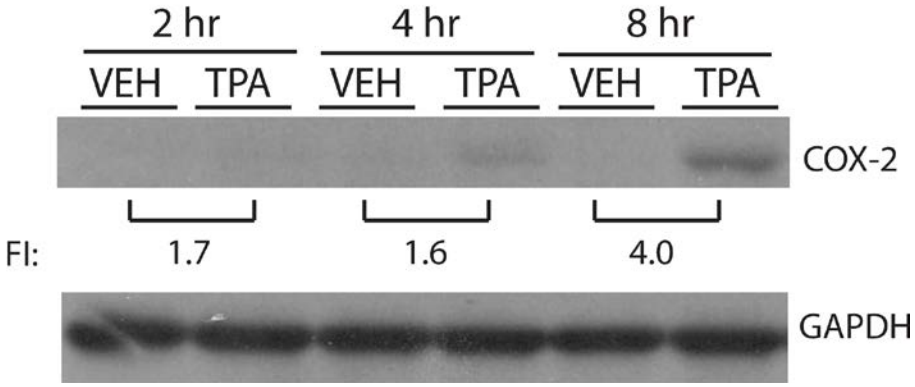
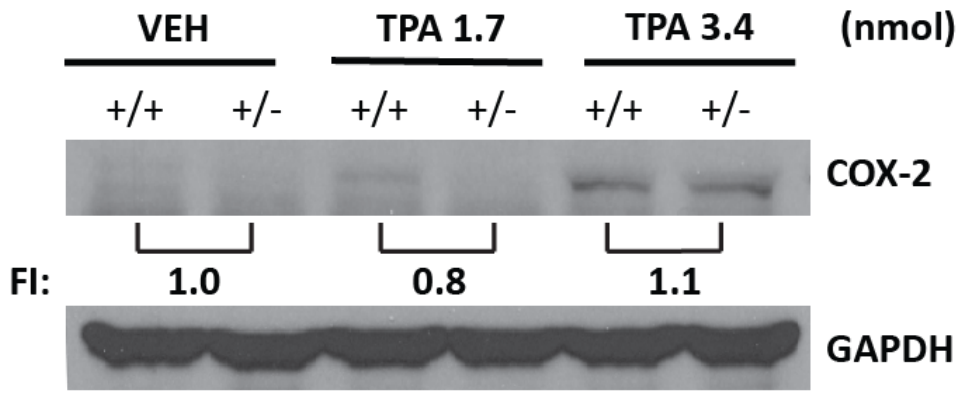

2 hr
4 hr
8 hr
VEH
TPA
VEH
TPA
VEH
TPA
COX-2
FI:
1.7
1.6
4.0
GAPDH

Figure 10: Concentration and time course analysis of TPA-induced COX-2 expression in COX-2 WT and COX-2 HET mouse epidermis. (A) Both COX-2 +/+ and COX-2 +/- mice were topically treated with 1.7 or 3.4 nmol of TPA dissolved in acetone or mice were treated with acetone alone (vehicle; VEH). Animals were euthanized after 8 hours, the epidermis isolated, pooled (n = 3) and Western analysis carried out with antibodies directed towards COX-2 and GAPDH (loading control). (B) Time-dependent induction of COX-2 protein expression by TPA. COX-2 +/+ and COX-2 +/- mice were treated topically with 3.4 nmol TPA and euthanized after 2, 4, or 8 hours. Western analysis was carried out using antibodies directed towards COX-2 or GAPDH. Fold induction (FI) in TPA-treated samples compared to time-matched vehicle treated samples is indicated below the figure.

A.


VEH
TPA 1.7
TPA 3.4
(nmol)
+/+
+/-
+/+
+/-
+/+
+/-
COX-2
FI:
1.0
0.8
1.1
GAPDH

B.

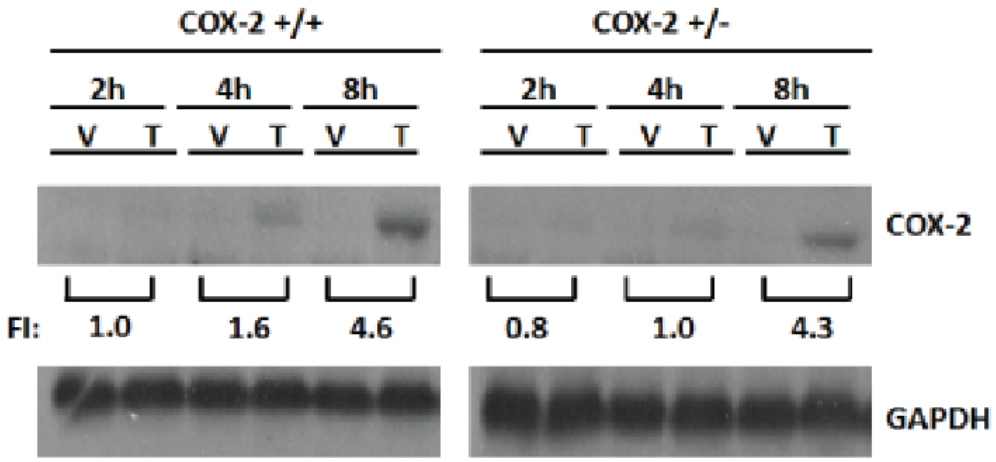
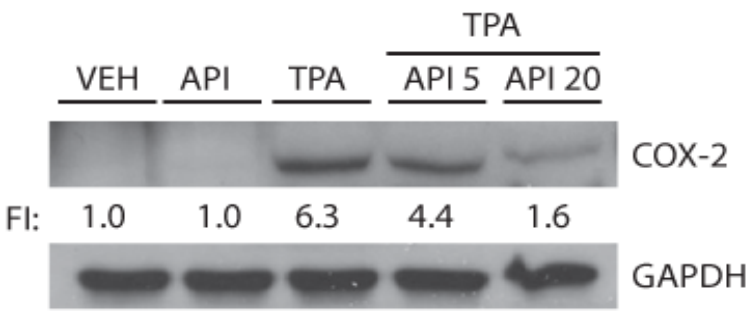
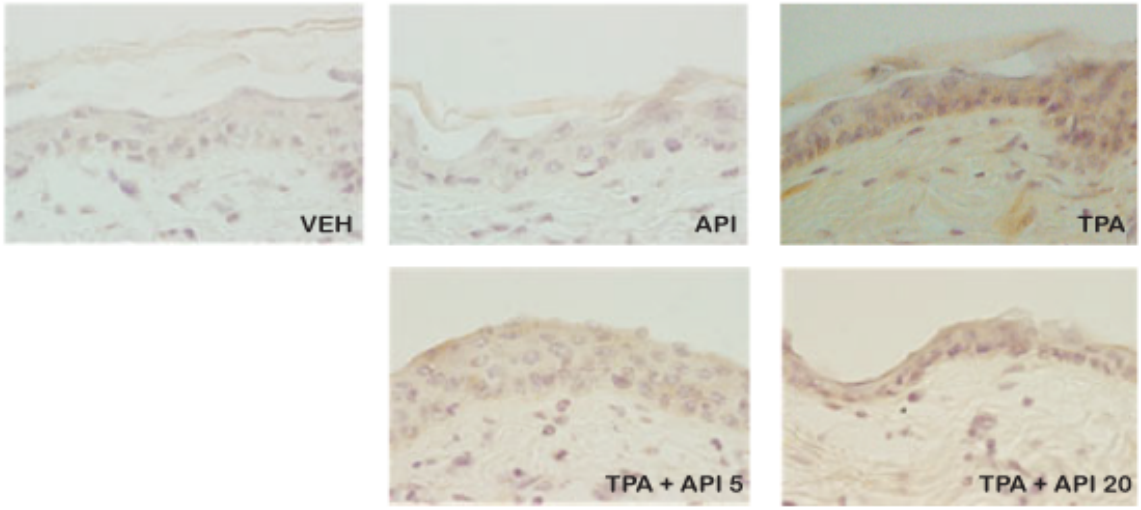

COX-2 +/+
COX-2 +/-
2h
4h
8h
2h
4h
8h
V
T
V
T
V
T
V
T
V
T
V
T
COX-2
FI:
1.0
1.6
4.6
0.8
1.0
4.3
GAPDH

Figure 11: Apigenin inhibits TPA-induced COX-2 expression in the epidermis of SKH-1 mice. SKH-1 mice (n = 3) were topically treated with 20 µmol apigenin (API), 3.4 nmol TPA (TPA), 3.4 nmol TPA + 5 µmol apigenin (TPA + AP5), 3.4 nmol TPA + 20 µmol apigenin (TPA + AP20), or vehicle (VEH). Animals were euthanized after 8 hours and the epidermis isolated. (A) Western analysis was carried out using antibodies directed towards COX-2 and GAPDH. Fold induction (FI) of TPA- or TPA + Apigenin-treated samples compared to the vehicle-treated sample is indicated below the figure. (B) Epidermal sections from mice treated as indicated above were analyzed for COX-2 protein content by immunohistochemical analysis. Tissue sections were observed at 40X magnification and (C) mean optical density of 10 images (n = 3) calculated using ImagePro software (ǂ $p < 0.05$ TPA compared to vehicle, * $p < 0.05$ apigenin compared to TPA, data presented as mean ± SD).

A.


TPA
VEH
API
TPA
API 5
API 20
COX-2
FI:
1.0
1.0
6.3
4.4
1.6
GAPDH

B.


VEH
API
TPA
TPA + API 5
TPA + API 20

C.

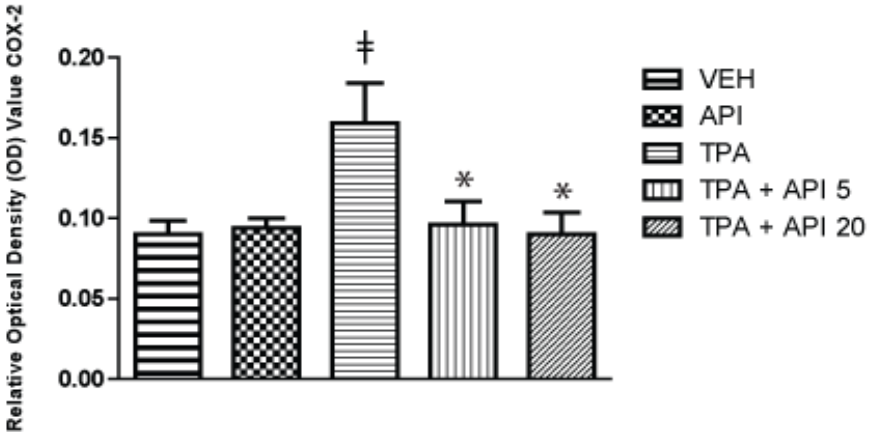
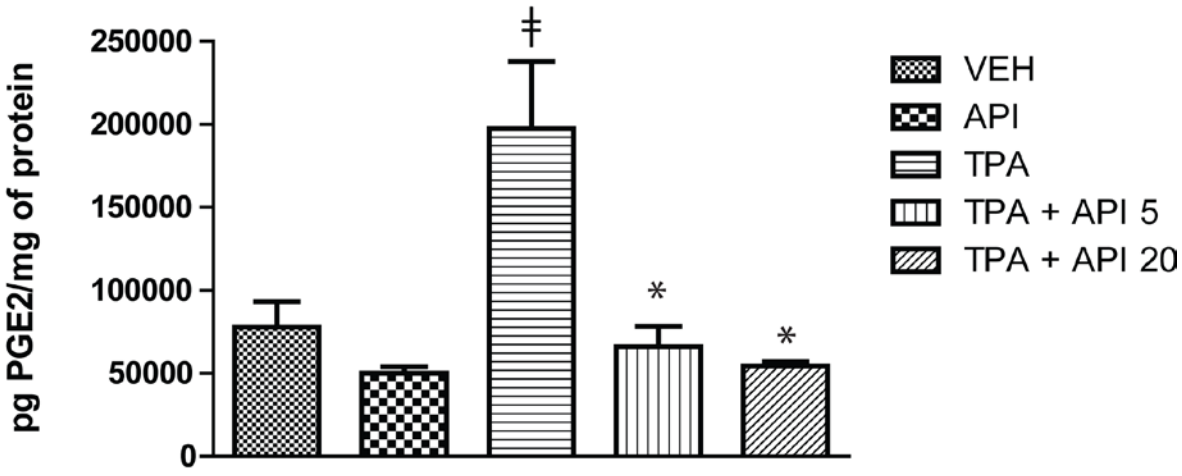

Relative Optical Density (OD) Value COX-2
0.20
0.15
0.10
0.05
0.00
‡
*
*
VEH
API
TPA
TPA + API 5
TPA + API 20

Figure 12: Apigenin inhibits TPA-mediated COX-2 activity in the mouse epidermis. (A) Mice were topically treated as described in Figure 16. Epidermal PGE_2 levels were assessed by conducting ELISA analysis as directed by the manufacturer. Statistical analysis was conducted using one-way ANOVA and Student's T-test (ǂ $p < 0.05$ TPA compared to vehicle, * $p < 0.05$ apigenin compared to TPA). (B) Effect of apigenin on mPGES expression. Animals were treated as indicated above. mPGES-1 and mPGES-2 expression was evaluated by Western blot analysis. Fold induction (FI) of TPA- or TPA + Apigenin-treated samples compared to the vehicle-treated sample is indicated below the figure.

A.


pg PGE2/mg of protein
250000
200000
150000
100000
50000
0
‡
*
*
VEH
API
TPA
TPA + API 5
TPA + API 20

B.

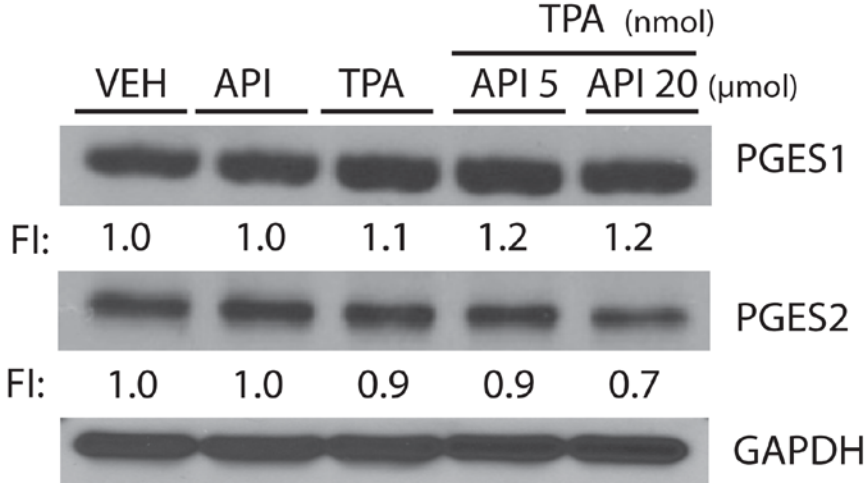

TPA (nmol)
VEH
API
TPA
API 5
API 20 (μmol)
PGES1
Fl: 1.0 1.0 1.1 1.2 1.2
PGES2
Fl: 1.0 1.0 0.9 0.9 0.7
GAPDH

Figure 13: Apigenin inhibits TPA-mediated expression of the EP1 receptor in the epidermis. SKH-1 mice were topically treated as described previously. Western blot analysis was carried out using antibody directed towards EP1, EP2, EP3, and EP4 receptors. Cell lysates from untreated JWF-2 and HaCaT cell lines were also loaded in the SDS-PAGE gel. GAPDH was measured to confirm equal loading of cell lysates. Fold induction (FI) of TPA- or TPA plus apigenin-treated samples compared to the vehicle-treated sample is indicated below the figure.

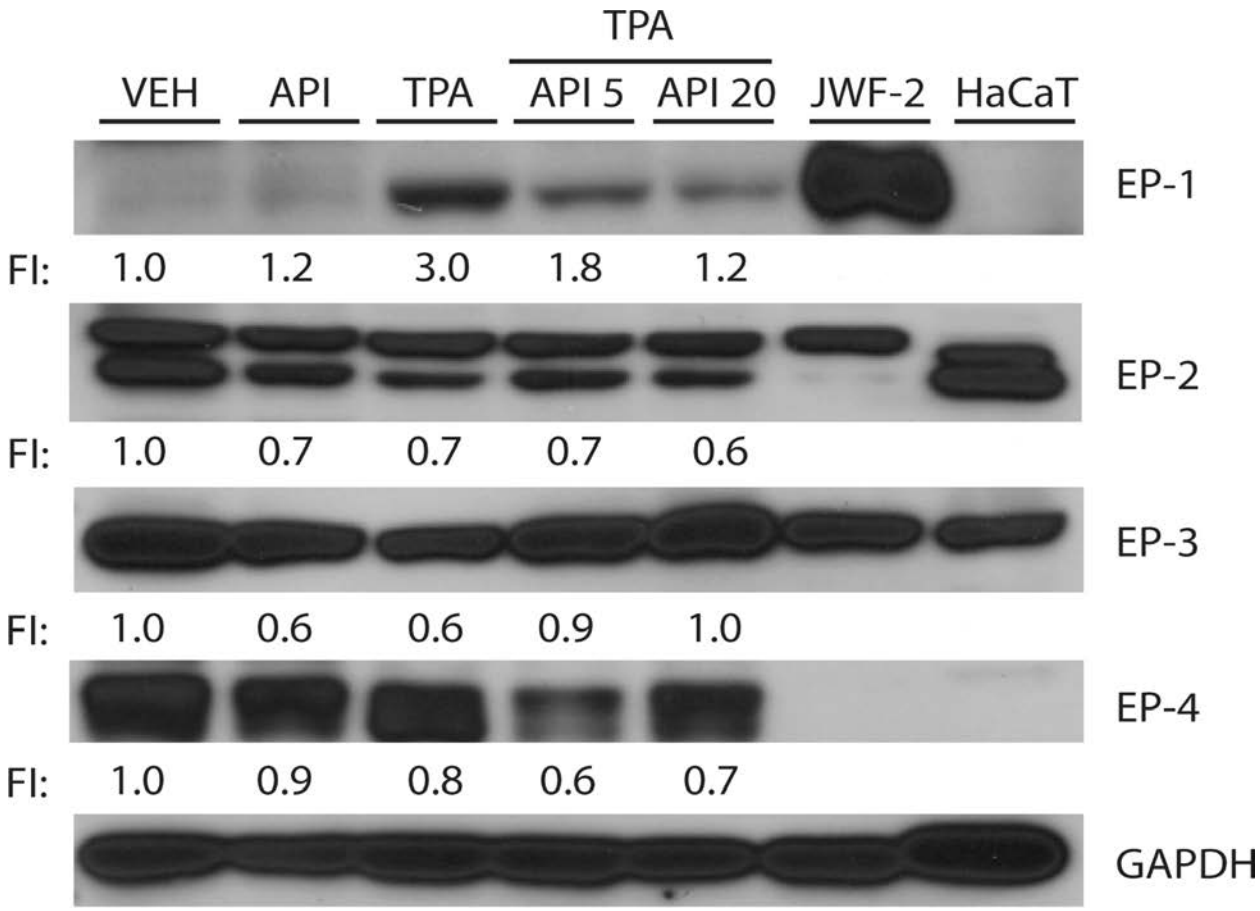

TPA
VEH
API
TPA
API 5
API 20
JWF-2
HaCaT
EP-1
FI: 1.0 1.2 3.0 1.8 1.2
EP-2
FI: 1.0 0.7 0.7 0.7 0.6
EP-3
FI: 1.0 0.6 0.6 0.9 1.0
EP-4
FI: 1.0 0.9 0.8 0.6 0.7
GAPDH

Figure 14: Examination of EP receptor expression and localization in the epidermis. SKH-1 mice were treated with 20 µmol apigenin (API), 3.4 nmol TPA (TPA), 3.4 nmol TPA + 20 µmol apigenin (TPA + AP20), or vehicle (VEH). Frozen epidermal sections (n = 3) were analyzed for (A) EP1, (B) EP2, (C) EP3, and (D) EP4 expression by immunohistochemistry as described in Materials and Methods (* $p < 0.05$ in comparison to TPA treated animals).

.

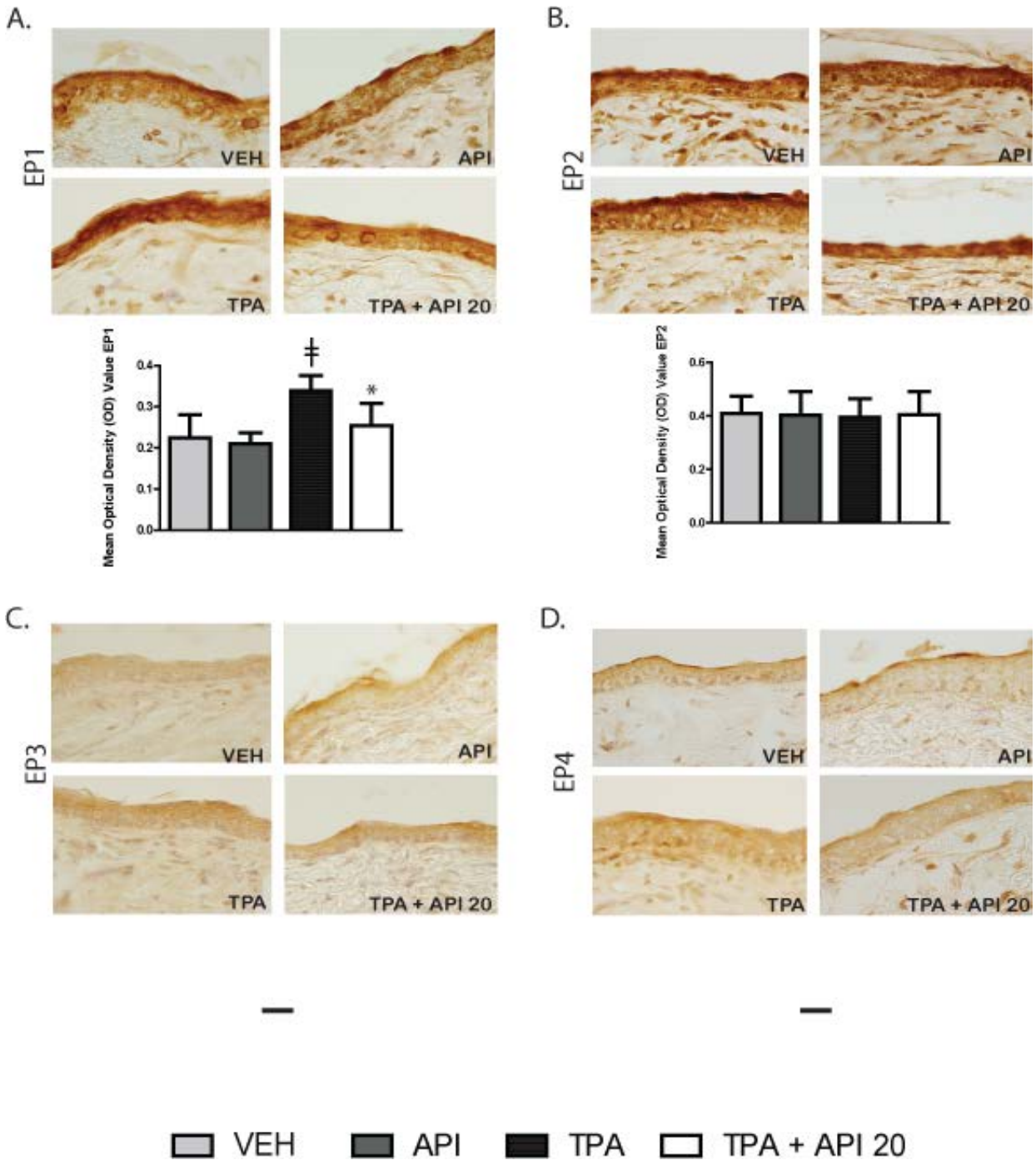
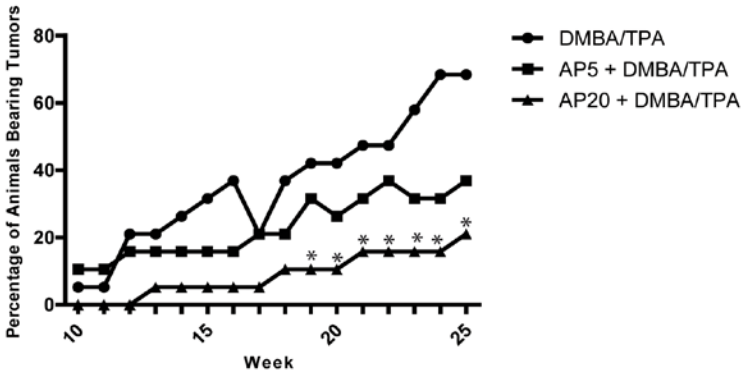
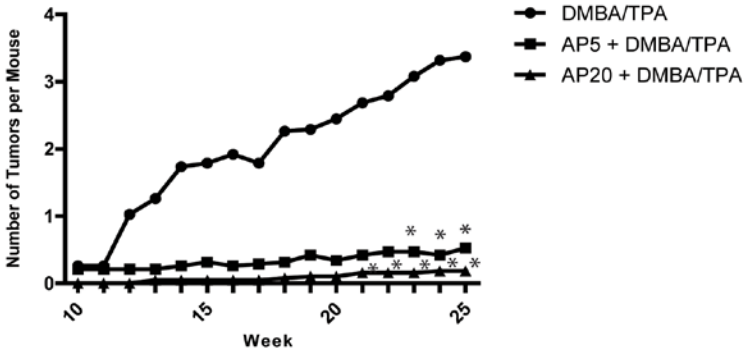

A.
EP1
VEH
API
TPA
TPA + API 20
Mean Optical Density (OD) Value EP1
0.4
0.3
0.2
0.1
0.0
‡
*
B.
EP2
VEH
API
TPA
TPA + API 20
Mean Optical Density (OD) Value EP2
0.6
0.4
0.2
0.0
C.
EP3
VEH
API
TPA
TPA + API 20
D.
EP4
VEH
API
TPA
TPA + API 20
VEH
API
TPA
TPA + API 20

Figure 15: Apigenin inhibits chemically-induced tumorigenesis in SKH-1 female mice. A single topical application of 100 nmol of DMBA was applied to the dorsum of SKH-1 mice to achieve tumor initiation. One-hour prior to TPA exposure animals were treated with apigenin at 5 µmol (AP5 + DMBA/TPA) or 20 µmol (AP20 + DMBA/TPA) twice weekly for 25 weeks. Tumors were counted on a weekly basis. AP20 + DMBA/TPA significantly reduced (A) the percentage of animals bearing tumors (tumor incidence) at 24 and 25 weeks post-initiation, and (B) the number of tumors per animal (tumor multiplicity) at 23-25 weeks post-initiation. (C) Representative images from each treatment group. Statistical analysis was conducted using repeated measure ANOVA with Tukey's post-hoc analysis (* $p < 0.05$).

A.


Percentage of Animals Bearing Tumors
80
60
40
20
0
10
15
20
25
Week
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

B.


Number of Tumors per Mouse
4
3
2
1
0
10
15
20
25
Week
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

C.


DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Figure 16: Weekly analysis of animal weight by treatment group throughout the two-stage chemical carcinogenesis protocol. Apigenin at both concentrations had no significant effect on body weight throughout the study.

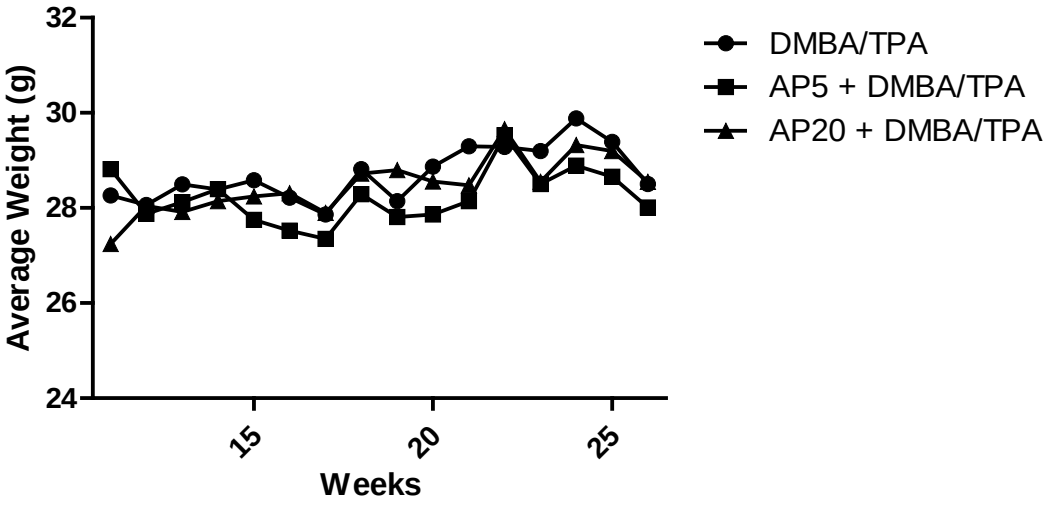

32
30
28
26
24
Average Weight (g)
15
20
25
Weeks
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Table 2: Comparison of tumor sizes for each treatment group. At the conclusion of the tumor study, the size of each tumor was measured in two dimensions (length x width) using a digital caliper. Tumors from each treatment group (n = 19) were then categorized according to size (listed as mean ± SD) and compared. Statistical analysis was conducted using one-way ANOVA and Student's T-test analysis (* $p < 0.05$ apigenin compared to DMBA/TPA, ǂ $p < 0.05$ AP20 + DMBA/TPA to AP5 + DMBA/TPA).

Table 2: Tumor Size

	0.1-2.4 mm^2	2.5-4.9 mm^2	>5.0 mm^2
DMBA/TPA	0.47 ± 0.61	1.11 ± 1.85	2.37 ± 3.71
DMBA/TPA + API 5	0.12 ± 0.33*	0.21 ± 0.42	0.32 ± 0.58*
DMBA/TPA + API 20	0.05 ± 0.23*	0.00 ± 0.00*‡	0.16 ± 0.50*

Figure 17: Examination of apigenin's effect on COX-2 expression and PGE_2 production in the surrounding epidermis and in tumors. Animals were subjected to the two-stage chemical carcinogenesis protocol as described in the methods section. (A) Samples of the surrounding epidermis and (B) tumors from DMBA/TPA, AP5 + DMBA/TPA, and AP20 + DMBA/TPA were analyzed for COX-2 expression by immunohistochemistry and optical densities were determined using ImagePro software. Epidermal PGE_2 levels were assessed in the (C) surrounding epidermis and (D) tumor samples from each treatment group by conducting ELISA analysis as directed by the. Tissue sections were observed at 40x for the surrounding epidermis and 20x for tumors (optical densitometry performed at 4x for tumors only). Asterisk indicates statistical significance (* $p < 0.05$ compared to the DMBA/TPA treatment group).

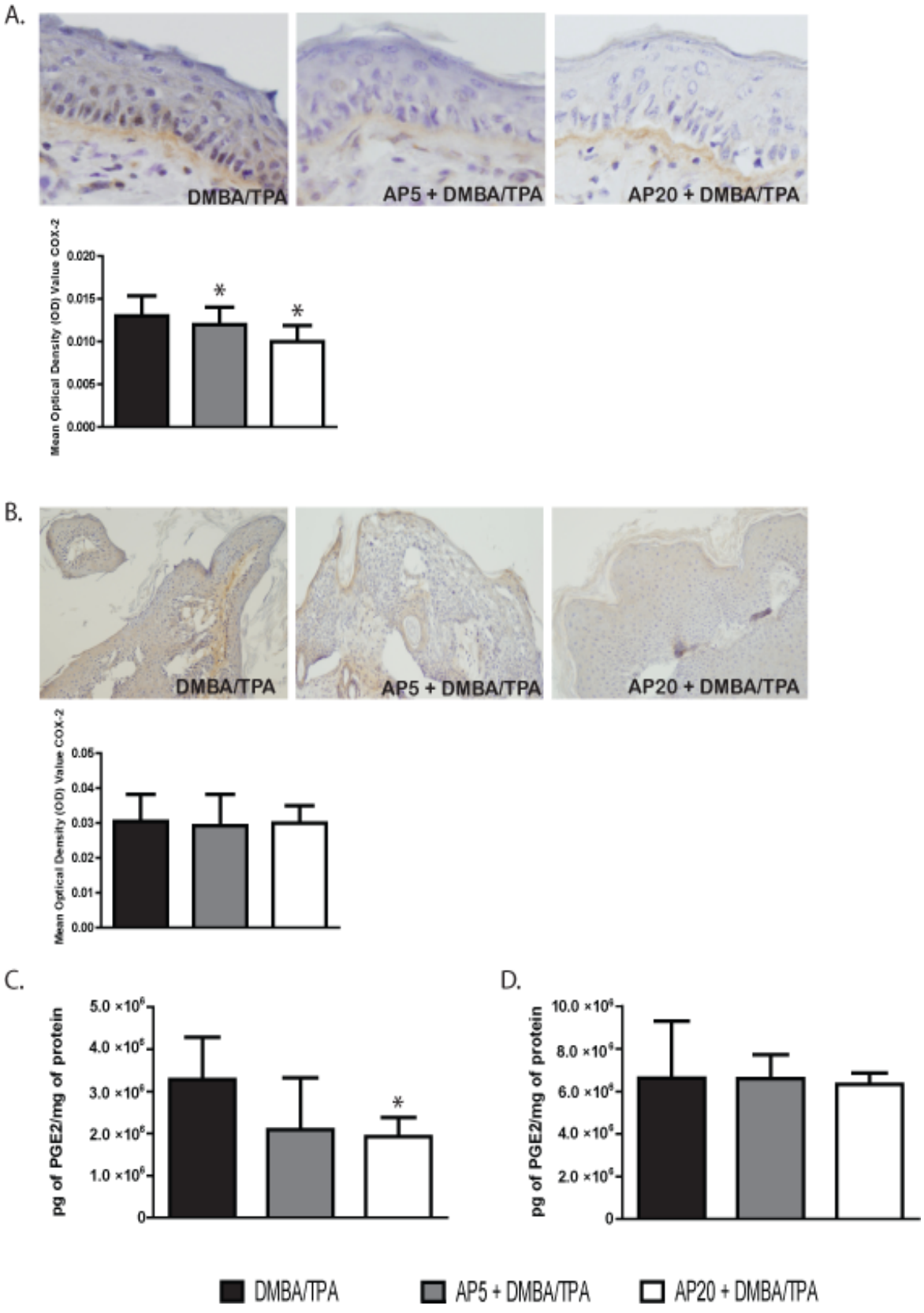

A.
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value COX-2
0.020
0.015
0.010
0.005
0.000
*
*
B.
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value COX-2
0.05
0.04
0.03
0.02
0.01
0.00
C.
pg of PGE2/mg of protein
5.0 ×10⁵
4.0 ×10⁵
3.0 ×10⁵
2.0 ×10⁵
1.0 ×10⁵
0
*
D.
pg of PGE2/mg of protein
10.0 ×10⁵
8.0 ×10⁵
6.0 ×10⁵
4.0 ×10⁵
2.0 ×10⁵
0
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Figure 18: Apigenin suppresses DMBA/TPA-mediated EP1 and EP2 receptor expression throughout the epidermis. Frozen epidermal sections (A & C) and tumors (B & D) were analyzed for EP1 and EP2 receptor expression and optical densities were determined using ImagePro software. Tissue sections were observed at 40x for normal skin and 20x for tumor (optical densitometry performed at 4x for tumors only). Asterisk indicates statistical significance (* $p < 0.05$ compared to DMBA/TPA treatment group).

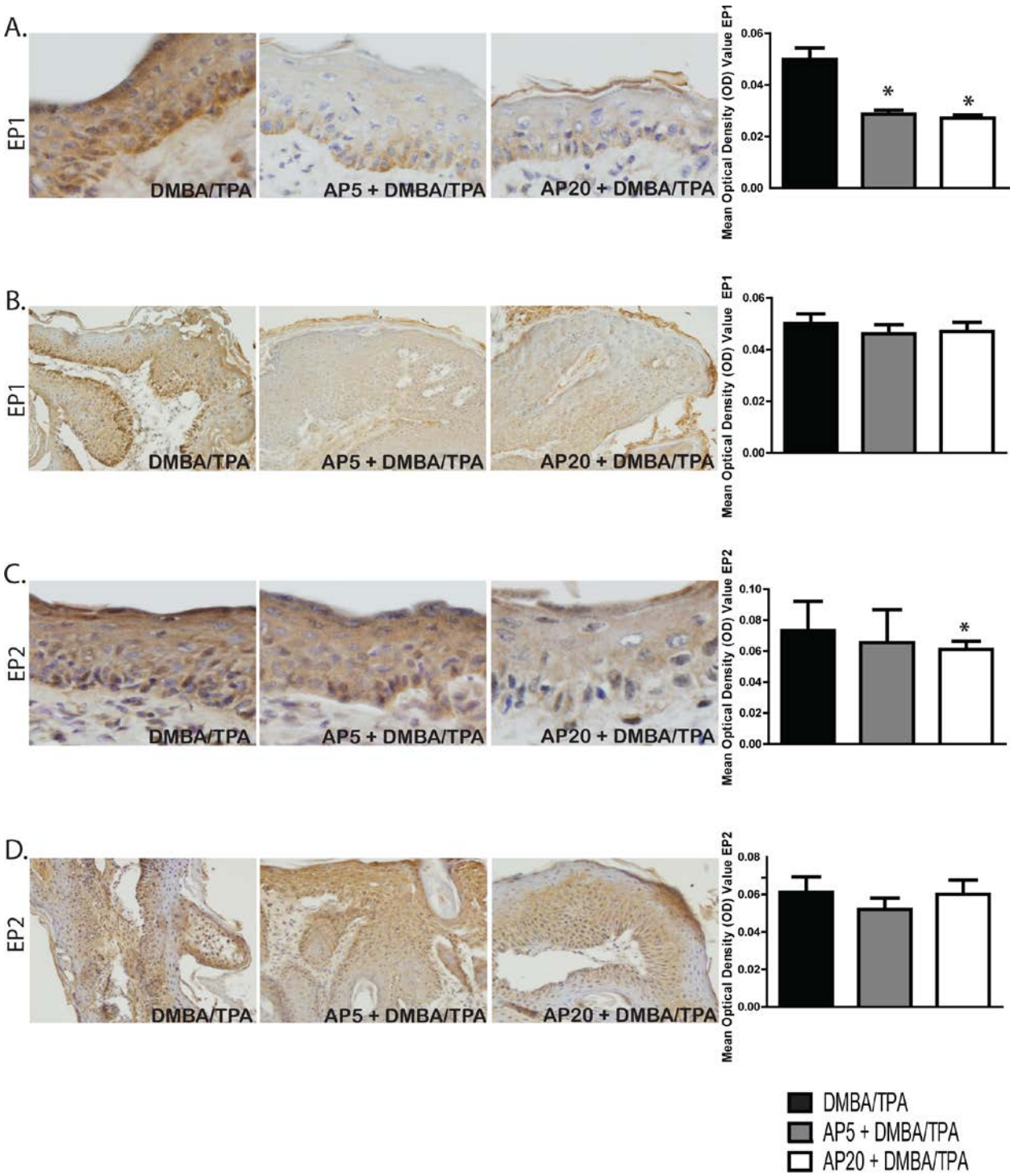

A.
EP1
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value EP1
0.06
0.04
0.02
0.00
*
*
B.
EP1
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value EP1
0.06
0.04
0.02
0.00
C.
EP2
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value EP2
0.10
0.08
0.06
0.04
0.02
0.00
*
D.
EP2
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value EP2
0.08
0.06
0.04
0.02
0.00
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Figure 19: Apigenin increases keratin-1 (K1) and keratin-10 (K10) expression throughout the epidermis. Frozen epidermal sections (A & C) and tumors (B & D) from each treatment group were subjected to immunohistochemical analysis using antibodies towards K1 and K10 as described previously. Tissue sections were observed at 40x for normal skin and 20x for tumor (optical densitometry performed at 4x for tumors only). Asterisk indicates statistical significance (* $p < 0.05$ compared to DMBA/TPA treatment group).

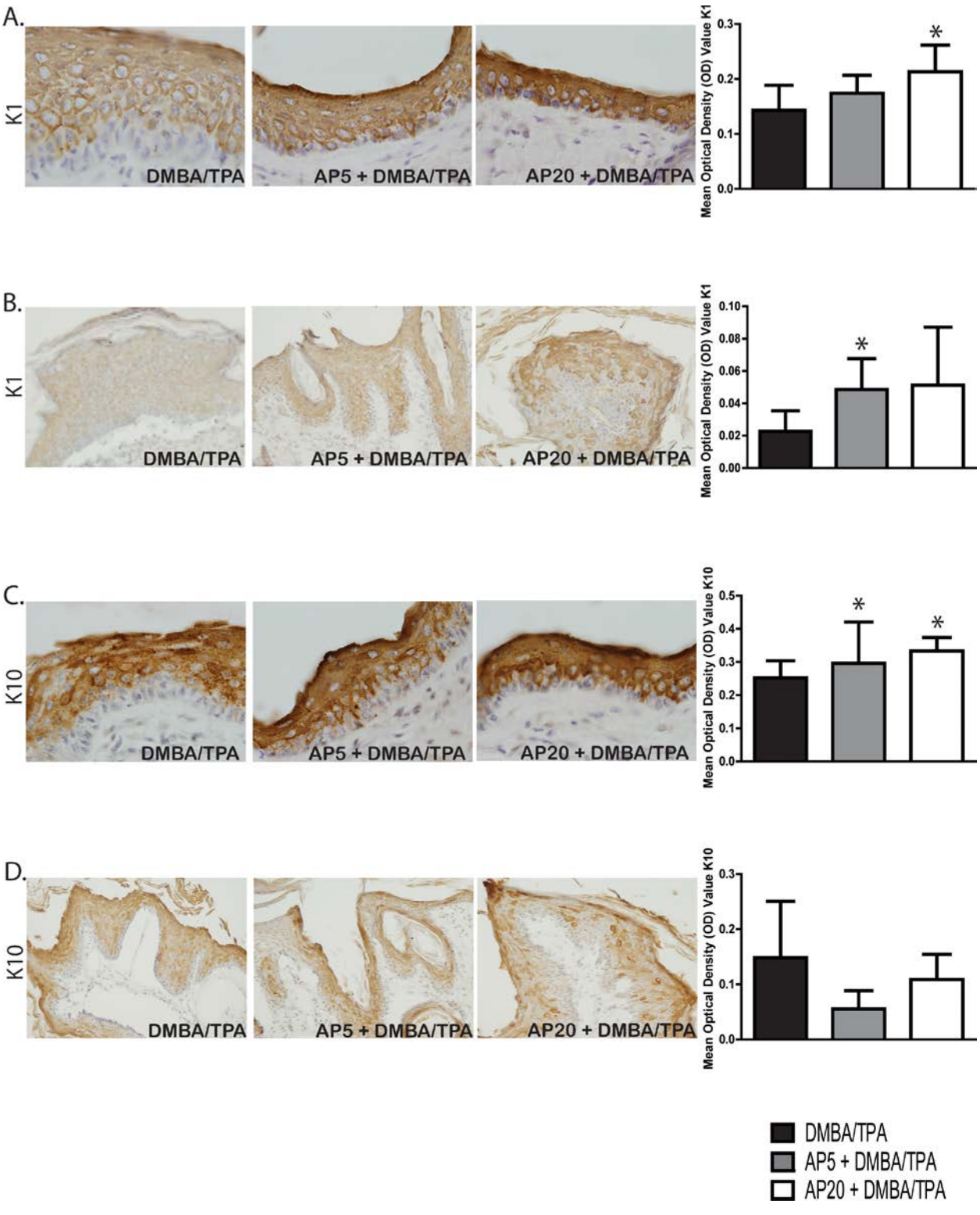
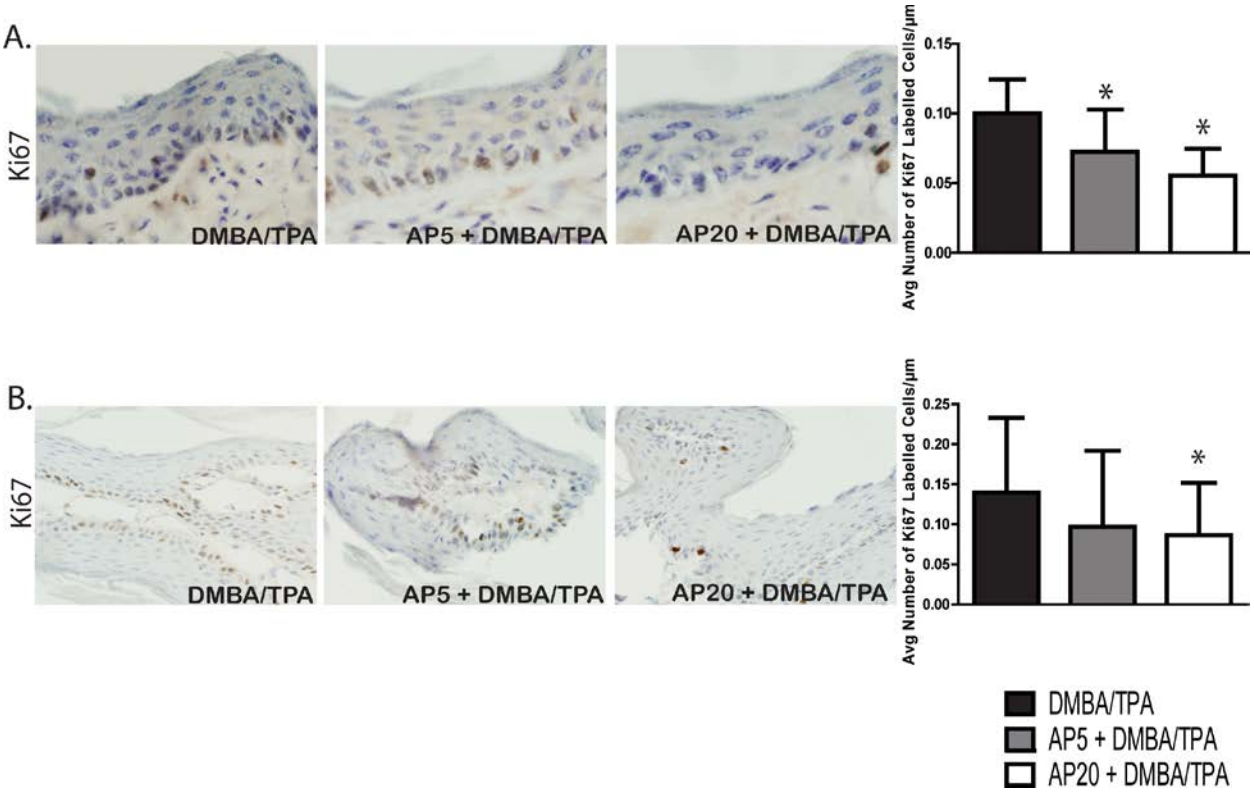

A.
K1
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value K1
0.3
0.2
0.1
0.0
*
B.
K1
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value K1
0.10
0.08
0.06
0.04
0.02
0.00
*
C.
K10
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value K10
0.5
0.4
0.3
0.2
0.1
0.0
*
*
D.
K10
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Mean Optical Density (OD) Value K10
0.3
0.2
0.1
0.0
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Figure 20: Apigenin inhibits keratinocyte proliferation in surrounding epidermal tissue and in tumors. Frozen sections from DMBA/TPA, AP5 + AMBA/TPA, or AP20 + DMBA/TPA treated animals were analyzed for Ki67. The number of Ki67 positive cells per micrometer (μm) of basal epidermis was counted in the (A) surrounding epidermis and (B) in tumors. Tissue sections were observed at 40x for normal skin and 20x for tumor. Asterisk indicates statistical significance (* $p < 0.05$ compared to DMBA/TPA treatment group).


A.
Ki67
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Avg Number of Ki67 Labelled Cells/µm
0.15
0.10
0.05
0.00
*
*
B.
Ki67
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA
Avg Number of Ki67 Labelled Cells/µm
0.25
0.20
0.15
0.10
0.05
0.00
*
DMBA/TPA
AP5 + DMBA/TPA
AP20 + DMBA/TPA

Chapter 4

Discussion

Apigenin is natural plant flavonoid that inhibits both DMBA/TPA- and UVB-induced skin tumor formation. In addition, numerous laboratories have reported that apigenin also blocks breast, colon, and prostate tumorigenesis (Wang, et al., 2000; Gupta, et al., 2002; Van Dross, et al., 2003; Way, et al., 2004; Au, et al., 2006; Shukla, et al., 2007; Choi and Kim, 2009). However, the exact mechanism by which this occurs is unclear. In this proposal we found that apigenin inhibited DMBA/TPA-induced mouse skin tumor promotion and chemically-induced COX-2 expression and activity in the epidermis of SKH-1 mice. We also showed that apigenin decreases DMBA/TPA-mediated induction of EP1 and EP2 receptors. Furthermore, we determined that the apigenin-regulated decrease in COX-2/PGE_2 signaling occurred coincident with an increase in terminal differentiation and decrease in keratinocyte proliferation. We also demonstrated in acute exposure studies that apigenin inhibits TPA-induced COX-2 expression, PGE_2 production, and EP receptor expression in the mouse epidermis. These findings suggest that apigenin blocks COX-2 expression and signaling in DMBA/TPA treated animals resulting in an induction of terminal differentiation which ultimately inhibits tumor development.

Our major goal in this study was to determine if apigenin inhibits chemically-induced tumor formation in SKH-1 mice by blocking COX-2/PGE_2 signaling. Our results clearly show that apigenin significantly decreases tumor multiplicity and tumor incidence in animals subjected to the DMBA/TPA chemical carcinogenesis protocol. We also

determined that apigenin decreases epidermal COX-2 expression, PGE_2 production, and EP receptor expression. Other groups have shown that the inhibition of COX-2 by genetic deletion or pharmacological inhibition leads to a decrease in skin tumor formation. For instance, Fischer et al. reported that heterozygous COX-2 knockout mice displayed a 50-65% reduction in tumor incidence compared to wild-type mice when subjected to the UV-light tumorigenesis protocol (Fischer, et al., 2007). In another study, a 75% decrease in tumor incidence was observed in homozygous COX-2 knockout mice (strain unrelated to SKH-1 hairless mice) exposed to the DMBA/TPA skin tumorigenesis protocol compared to wild-type animals (Tiano, et al., 2002). In contrast, transgenic mice that overexpress COX-2 developed significantly more tumors in comparison to their wild-type counterparts in UV-exposed animals (Fischer, et al., 2007). Topical application of the non-selective COX inhibitor indomethacin reduced chemically-induced skin tumor formation in mice (Muller-Decker and Furstenberger, 2007). In addition, topically or orally administered celecoxib or indomethacin also significantly reduced tumor formation and tumor size in animals exposed to the UV-light induced carcinogenesis protocol (Fischer, et al., 1999; Wilgus, et al., 2003). These experimental findings show that the inhibition of COX-2 expression or activity blocks epidermal tumor growth. Since we have shown that apigenin inhibits COX-2 expression its chemopreventative activity may be mediated by inhibition of COX-2.

To definitively determine whether apigenin inhibits tumor formation by blocking COX-2, additional studies utilizing COX-2 knockout animals would be needed. SKH-1 homozygous COX-2 knockout mice (COX-2 -/-) could not be included in our study because these animals die shortly after birth due to an inability to close the ductus

arteriosus and due to various renal pathologies (Morham, et al., 1995; Loftin, et al., 2001; Rundhaug, et al., 2007a). As such, we planned to use SKH-1 heterozygous COX-2 knockout mice (COX-2 +/-) in our tumorigenesis study. Our acute TPA exposure experiments showed that COX-2 +/- mice displayed levels of COX-2 expression that were similar to COX-2 +/+ mice (Figure 10). We believe that the recessive allele of the COX-2 gene was knocked down rather than the dominant allele and as a result, the COX-2 gene was expressed at wild-type levels. For this reason we omitted the heterozygous COX-2 animals from our tumorigenesis study.

As an alternative approach to understand the role of COX-2 in the antitumor activity of apigenin, the expression of COX-2 was measured in tumors and in the surrounding epidermis. Although the surrounding epidermis is non-tumor tissue it is abnormal because the animals in each experimental group were initiated with DMBA and chronically exposed to TPA. Thus, the surrounding epidermis actually represents initiated skin. We compared the expression of COX-2 in the surrounding epidermis of animals treated with DMBA/TPA or apigenin + DMBA/TPA and found that apigenin significantly decreased COX-2 expression. Interestingly, another report showed that in DMBA-initiated mouse skin, the overexpression of COX-2 was sufficient to induce tumor formation (Muller-Decker, et al., 2002). As such, apigenin's ability to suppress COX-2 expression in DMBA-initiated surrounding epidermis is likely a major event that is responsible for its chemopreventative activity.

Various studies have also shown that EP1 receptor expression is critical for skin tumor development. Results from our tumorigenesis study revealed that apigenin effectively blocked the DMBA/TPA-induced increase in EP1 receptor expression in the

surrounding epidermis. We also observed that EP1 receptors were overexpressed in the tumorigenic keratinocyte cell line JWF-2 and not expressed in the non-tumorigenic cell line, HaCaT (Figure 13). This expression pattern suggests that similar to COX-2, down regulation of EP1 is critical for antitumor activity of apigenin. Accumulating evidence from many studies support this idea. For instance, Watanabe et al. revealed that azoxymethane-induced aberrant crypt foci development in the colon was significantly inhibited in EP1 knockout versus wild-type mice or in wild-type mice treated with the EP1 antagonist, ONO-8711 (Watanabe, et al., 1999). Additionally, topical application of an alternative selective EP1 antagonist, ONO-8713, reduced UVB-induced skin tumor multiplicity (Tober, et al., 2006). In contrast, overexpression of the EP1 receptor in the DMBA/anthralin (non-PKC activating tumor promoter) skin tumor model enhanced tumor development compared to DMBA/TPA treated animals, indicating that the tumor promoting activity of the EP1 receptor may be stimulus dependent (Surh, et al., 2011).

The EP2 or EP4 receptors also promote tumor development. For example, both DMBA/TPA- and UVB-induced carcinogenesis was blocked in EP2 heterozygous knockout animals compared to wild-type mice (Sung, et al., 2005; Chun, et al., 2007). Fewer tumors developed in UVB-exposed EP2 homozygous knockout mice compared with wild-type animals however the tumors from the EP2 knockout animals displayed a more aggressive phenotype (Brouxhon, et al., 2007). Results from our tumorigenesis study show that apigenin inhibits DMBA/TPA-induced tumor development and the expression of EP2 in the surrounding skin again suggesting that apigenin-mediated down regulation of COX-2/PGE_2 signaling is important for its antineoplastic activity. In

another report, EP4 receptor inhibition by genetic ablation or chemical inhibition suppressed aberrant crypt formation induced by azoxymethane or spontaneous polyp development in Min mice (Mutoh, et al., 2002). In contrast, the EP3 receptor does not appear to regulate carcinogenesis (Sung, et al., 2005). In our investigation of the acute effect of apigenin on EP receptor expression, positive staining for EP3 and EP4 was not observed in our immunohistochemical data possibly due to the lack of epitope recognition by the antibody in intact tissue. Therefore, the expression of EP3 and EP4 was not examined in our tumor study. These combined results suggest that the suppression of EP1 receptor expression mediated by apigenin is a likely mechanism by which DMBA/TPA-induced carcinogenesis is prevented.

When analyzing COX-2/PGE_2 signaling in epithelial tumors; COX-2, PGE_2, EP1, and EP2 receptor expression were all elevated in DMBA/TPA treated animals. Apigenin at both concentrations did not reduce COX-2 signaling in the tumors. The expression of COX-2 is regulated by various signaling proteins including protein kinase B (PKB/Akt), upstream stimulatory factors (USF1/USF2), and the T-cell-restricted intracellular antigen 1-related protein (TIAR) (Van Dross, et al., 2005; Tong, et al., 2007; Van Dross, et al., 2007). Akt is a serine/threonine kinase that functions downstream of phosphatidylinositol 3-kinase (PI3K) and induces COX-2 expression by activating transcription factors such as nuclear transcription factor kappa B (NF-kB) (Van Dross, et al., 2005; St-Germain, et al., 2004). The transcription factors USF1 and USF2 increase UVB-light induced COX-2 expression in cultured keratinocytes (Van Dross, et al., 2007). Also, post-transcriptional regulation of COX-2 expression occurs through binding of TIAR to AU-rich elements (ARE) of the 3'-untranslated region of COX-2 mRNA

regulating its stability (Cok and Morrison, 2001; Tong, et al., 2007). Our previous work shows that apigenin inhibits the synthesis of COX-2 by blocking these signaling pathways. Since other signaling pathways also regulate COX-2 expression it is possible that COX-2/PGE_2 signaling in AP5- and AP20 + DMBA/TPA animals was not decreased in the tumors due to the upregulation of alternative signaling pathways. Upregulation of alternative signaling pathways is a common mechanism of resistance for a number of chemotherapeutic agents. For example, gefitinib and erlotinib are epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors used to treat non-small cell lung cancer. These tumors can become resistant through activation of c-MET, a receptor tyrosine kinase, which can bypass EGFR and activate signaling molecules down-stream of EGFR (Garofalo, et al., 2012).

Several reports show that upregulation of COX-2/PGE_2 signaling promotes tumor development due to its ability to suppress terminal differentiation, promote proliferation, and/or block apoptotic signaling (Fischer, et al., 1989; Yuspa, et al., 1990; Sarasin, 1999). Under normal physiological conditions, terminal differentiation occurs in keratinocytes that lose their ability to self-renew after transition to the squamous epidermal layer. Tumor cells are unable to terminally differentiate in order to promote cell survival. COX-1 or COX-2 deficiency inhibits mouse skin tumor formation by inducing pre-mature terminal differentiation of keratinocytes after DMBA/TPA exposure (Tiano, et al., 2002; Lao, et al., 2012). Another study showed that topical application of celecoxib induced premature terminal differentiation in mouse epidermis after acute TPA exposure (Akunda, et al., 2004). Apigenin increases the number of K1 and K10 positive keratinocytes in the basal layer of the surrounding epidermis. These findings

imply that apigenin-mediated inhibition of COX-2 expression, PGE_2 production, and EP receptor expression is likely responsible for the induction of premature terminal differentiation in our study.

Cancer is a disease characterized by uncontrolled cell proliferation and numerous signaling molecules regulate this process. COX-2 overexpression increases cell proliferation and epidermal hyperplasia in various *in vivo* and *in vitro* models through increased production of PGE_2. For example, addition of endogenous PGE_2 to subconfluent cultured breast cells and keratinocytes resulted in an increased rate of proliferation (Pentland and Needleman, 1986; Konger, et al., 1998). Tiano et al determined that papillomas from COX-2 knockout animals subjected to chemical carcinogenesis contained reduced levels of PGE_2 as well as decreased BrdU-labeled proliferating keratinocytes compared to wild-type mice (Tiano, et al., 2002). Furthermore, recent studies have shown that EP1 and EP2 receptor activation induces cellular proliferation. In EP1-/- mice a decrease in cell proliferation was observed in azoxymethane-induced colon tumors versus EP1+/+ animals (Kawamori, et al., 2005). Similar results were also observed in EP2 -/- mice exposed to TPA which displayed reduced keratinocyte proliferation in the epidermis compared to EP2 wild-type mice (Sung, et al., 2005). Therefore, it is not surprising that fewer proliferating keratinocytes were observed in the epidermis of animals from the AP5 + DMBA/TPA and AP20 + DMBA/TPA treatment groups compared to animals treated with DMBA/TPA alone. Since we also determined that apigenin caused premature terminal differentiation, the blockade in cell proliferation may have triggered unscheduled entry into the terminal differentiation program. Alternatively, premature activation of terminal differentiation

may have caused the block in cell proliferation. This differentiation-proliferation switch has been described previously and could explain the mechanism of apigenin-mediated prevention of tumor development (Liu, et al., 2008; Zhu, et al., 2009).

Several published reports and our previous data show that apigenin is a potent inducer of cellular apoptosis (Chen, et al., 1996; Van Dross, et al., 2005; Vargo, et al., 2006; Balasubramanian and Eckert, 2007; Abu-Yousif, et al., 2008; Choi and Kim, 2009). Apoptosis is also known to be a functional consequence of the suppression of COX-2/PGE_2 signaling (Arico, et al., 2002; Wu, et al., 2004; Akunda, et al., 2007). We attempted to evaluate the induction of apoptosis in the surrounding epidermis and in tumors of DMBA/TPA-, AP5 + DMBA/TPA- and AP20 + DMBA/TPA-treated mice and observed a strong signal for activated caspase-3 in all of the analyzed samples. Since differences in signal intensity among the treatment groups and within the individual epidermal layers were expected, these results suggest a lack of specificity in antibody target recognition. In our studies, the induction of apoptosis was measured using an antibody directed against activated caspase-3. The antibody recognizes a conformational epitope that is exposed in the activated protein or in the inactive protein that is denatured. Therefore, it is critical that proteins in the tissue sections maintain the appropriate three-dimensional structure. As such, only frozen tissue sections, but not paraffin embedded samples (which must be heated) are appropriate for this antibody. Because the staining in each of the samples was heavy and did not contain distinct patterns, we suspect that the proteins in our frozen sections may have become denatured at some stage during IHC tissue processing. Our attempts to measure apoptosis with TUNEL assays in intact skin were also unsuccessful due to an

unacceptable level of background signal using a fluorescence approach. The scarcity of studies that measure apoptosis in intact skin lead us to believe that the currently available techniques are not optimal for detection of apoptosis in the skin.

Overall, results from specific aim 1 demonstrate that apigenin inhibits the acute TPA-mediated increase in COX-2 expression and activity in mouse epidermis dermis. Also, apigenin inhibits TPA-mediated induction in PGE_2 production and EP1 receptor expression in SKH-1 mice. Specific aim 2 shows that apigenin prevents DMBA/TPA-mediated skin tumor formation and also prevents COX-2 expression, PGE_2 production, EP1, and EP2 receptor expression in the surrounding epidermis of DMBA/TPA treated SKH-1 mice. In addition, apigenin appears to prevent tumor development by promoting terminal differentiation and inhibiting cell proliferation in basal keratinocytes. Because numerous studies have shown that the suppression of COX-2 expression/activity blocks tumor development, the reduction in COX-2/PGE_2 signaling that we observed suggests that apigenin may prevent tumor development by inhibiting COX-2 expression, PGE_2 production, and EP receptor expression. Thus, apigenin could provide additional treatment options for NMSC.

In order to overcome the pitfalls of this study, future experiments will be required. In our tumorigenesis study, a vehicle control group was not included, because it is well known that SKH-1 mice do not spontaneously develop tumors. By not including a vehicle-only group, data and statistical analysis was limited to comparing only apigenin + DMBA/TPA treated animals to DMBA/TPA-treated animals. In this study we were unable to utilize COX-2 +/- SKH-1 mice to determine the role of COX-2 in apigenin-mediated inhibition of DMBA/TPA-induced carcinogenesis. Other groups have utilized

the COX-2 +/- and COX-2 -/F1 agouti mouse strain. However, hairless mice are preferred in our research because hair removal can produce scarring and inflammation which may confound our results. To overcome this problem, a different COX-2 knockout hairless strain will need to be identified. Lastly, to overcome the issues we experienced determining apigenin's effect on apoptosis, it will be necessary to identify an appropriate probe to detect apoptosis in intact skin in order to assess whether the inhibition of tumor development might also be regulated by apigenin's ability to induce apoptosis.

Our future goal is to develop a topical preparation of apigenin for prevention of skin cancer. Apigenin could be useful for preventing skin cancer in individuals at high risk for developing skin cancer including the elderly, kidney transplant recipients, and xeroderma pigmentosa (XP) patients. For example, aging individuals with a single tumor are at increased risk (44% increase for BCC and 18% increase for SCC) of developing a second tumor within 3 years (Marcil and Stern, 2000). Approximately 30% of kidney transplant patients develop skin cancer as a likely result of chronic immunosuppression (Carroll, et al., 2003; Euvrard, et al., 2003). Finally, XP is a rare disorder characterized by inherited mutations in the XP family of DNA repair enzyme genes. On average, XP patients develop skin cancer by age 8 and succumb to cancer by age 20. Apigenin could provide additional treatment options for these individuals who are at increased risk of developing skin cancer.

The use of apigenin in a topical preparation also provides the benefit of selectively inhibiting COX-2 expression in the epidermis. This route of administration avoids potential adverse cardiovascular effects observed with systemic administration of

COX-2 inhibitors. Collectively, the use of apigenin or apigenin derivatives in a topical cream may be an effective and safe strategy to prevent the formation of cancer.

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APPENDIX

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

November 21, 2008

Rukiyah Van Dross, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Van Dross:

Your Animal Use Protocol entitled, "Role of COX-2 in Apigenin-Mediated Inhibition of DMBA/TPA-Induced Skin Tumorigenesis," (AUP #W216) was reviewed by this institution's Animal Care and Use Committee on 11/21/08. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

October17, 2011

Rukiyah Van Dross, Ph.D.
Department of Pharmacology
Brody 6S-10
ECU Brody School of Medicine

Dear Dr. Van Dross:

Your Animal Use Protocol entitled, "Role of COX-2 in Apigenin-Mediated Inhibition of DMBA/TPA-Induced Skin Tumorigenesis" (AUP #W216a) was reviewed by this institution's Animal Care and Use Committee on 10/17/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure