Men and Women Are Not Just From Different Planets: The Role of Sex-Based Differences in the Prevention of Non-Melanoma Skin Cancer

DISSERTATION

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By

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Abstract

There are over 3.5 million new diagnoses of skin cancer each year in more than 2 million patients, making skin cancer more prevalent than all other cancers. While the risk of developing cutaneous squamous cell carcinoma, the more deadly type of non-melanoma skin cancer, is three times greater in men than women, the mechanisms that contribute to this disparity are unclear. Our previous studies showed that after equivalent, chronic ultraviolet light B (UVB) exposure, male mice had greater tumor multiplicity, burden, and grade compared to female mice. Additionally, our acute UVB studies revealed decreased antioxidant capacity and cutaneous inflammation in male mice, suggesting that topical antioxidant or anti-inflammatory agents may decrease tumor burden following chronic UVB exposure. In the current studies we examined the potential preventative effects of anti-inflammatory (diclofenac) and antioxidant (C E Ferulic and vitamin E) agents on UVB-induced carcinogenesis in male and female mice. Our results demonstrated that despite observed sex differences in the inflammatory response, prolonged topical diclofenac treatment of chronically UVB-damaged skin effectively reduced tumor multiplicity in both sexes. Unexpectedly, tumor burden was significantly decreased only in male mice. Further, our data demonstrated that topical C E Ferulic treatment effectively reduced tumor number and burden in both sexes. While topical vitamin E treatment provided moderate therapeutic benefits in male mice, it provided no preventative benefits for female mice, and in fact, resulted in accelerated tumor growth.
rate compared to vehicle-treated female mice. In addition, we sought to investigate the
efficacy of diclofenac, C E Ferulic, and vitamin E treatments in our model where UVB
exposure was continued throughout the study. Our results demonstrated that both male
and female mice that were exposed to 25 weeks of UVB developed more tumors, larger
tumors, and a higher percentage of malignant tumors compared to mice that were
exposed to 10 weeks of UVB. Further, mice treated with antioxidants exhibited no
beneficial effects in terms of tumor number and burden compared to vehicle-treated mice.
In contrast, diclofenac continued to be effective for decreasing both tumor number and
burden in male and female mice with extended UVB exposure. Unexpectedly, mice
treated with diclofenac developed a higher percentage of malignant tumors compared to
vehicle-treated mice. These data, in combination with our results demonstrating that the
use of previously reported visual criteria resulted in the false identification of 67% of
tumors as SCC, indicate that tumor size and appearance are not reliable predictors of
tumor grade and underscore the importance of histologically examining every tumor to
correctly evaluate the efficacy of treatments in decreasing cutaneous tumor development
and progression. Collectively, these studies demonstrate that topical anti-inflammatory
and antioxidant treatments exert differential outcomes in the sexes. Overall, these data
support the commonly assumed, but not demonstrated, fact that cumulative UVB
exposure is a risk factor for UVB-induced SCC and highlight the fact that changing sun
worshiping habits, even after early chronic sun exposure and skin damage, may be crucial
for experiencing therapeutic benefits and ultimately preventing tumor development in
patients.
Dedication

This document is dedicated to my family. I could not have done this without your support.
Acknowledgements

I would like to begin by thanking my family for always supporting me in everything I have done. You have encouraged me and never doubted that I would be successful even when I doubted myself. Thank you for asking about my research and being excited about what I said even if you were not sure what exactly I was talking about.

I am grateful to my committee members, Drs. Traci Wilgus, Mandy Toland, and Greg Lesinski, for their support and involvement in my research. Monday morning group meetings have been helpful for making me think and be able to discuss my project from its early stages. You have given me invaluable input and experiences that will help me be successful in the future, and I thank you so much.

I would like to thank the members of the Wilgus Lab, especially Dr. Brian Wulff and soon-to-be Dr. Kelly Johnson, for the use of lab equipment, troubleshooting suggestions, friendship, and the storage of lab furniture.

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To the members of the Oberyszyn Lab, I do not know where to begin. From the day I first interviewed for a rotation, I felt like I was at home, and I cannot say how amazing it has been to have a lab family throughout graduate school. I do not think that other people would have appreciated my crazy hair styles and colors and nail designs as much as you have. Thank you all for your support, humor, and friendship, for which I will always be grateful. I would like to thank Dr. Kathy Tober for always being there to answer my random questions about my research, to plan experiments with an excessive number of controls, or just to talk about life. I hope that I will be able to attend Pigatonia in the future, because who can say no to too much food, perfect margaritas (and occasionally getting “iced”), and great entertainment?!??! To Judy Riggenbach, the Western Queen, thank you for helping me find everything in the lab and keeping the lab in order. Thank you for always being so cheerful and “bothering” me when I had been working for too long. I hope you will keep singing “Soft Mousie” on sac days. I would also like to thank Dr. Nick Sullivan for his advice and suggestions in my first couple years in the lab. I do not think I would have made it through candidacy if you had not answered all my questions over and over again, so Thank You! I know you will have a successful career. To Dr. Priya Nagarajan, it has been so wonderful to have you in the lab! You gave me the courage to pursue a different field for my postdoc (hopefully it works out), and I cannot even begin to count the questions that you have answered and the ways you have helped
me over the past 8 months. I could not have finished my data collection without the help of my lab “slaves,” Kristen Duckro, Katie Samijlenko, and Evan Sommer. I am so, so grateful for all of your help, and thank you for being such fun lab members! I would like to thank Jodie Kinney for her administrative help as well as her humor. You definitely are an asset to the lab and it’s more fun when you’re around! Thank you also to previous lab members Jonathan Schick, Dr. Michelle Creamer, Keith Lamping, and Paul Cipriani for your help and input over the years.

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Area of Emphasis: Cancer Biology
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<th>Full Form</th>
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<tbody>
<tr>
<td>8-oxo-dG</td>
<td>8-oxo-deoxyguanosine</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AK</td>
<td>actinic keratosis</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
</tr>
<tr>
<td>c</td>
<td>centi-</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CPD</td>
<td>cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
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</table>
G guanine
GI gastrointestinal
GPx glutathione peroxidase
H&E hematoxylin and eosin
IL interleukin
J Joule
k kilo-
l liter
m milli-; meter
M molar
MDSC myeloid-derived suppressor cell
MED minimal erythemic dose
MPO myeloperoxidase
NMSC nonmelanoma skin cancer
n nano-
NSAID non-steroidal anti-inflammatory drug
OCT optimal cutting temperature
PBS phosphate-buffered saline
PBST PBS containing 0.1% Tween-20
PG prostaglandin
RNS reactive nitrogen species
ROS reactive oxygen species
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>µ</td>
<td>micro-</td>
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<tr>
<td>UV</td>
<td>ultraviolet light</td>
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Chapter 1: Introduction

1.1 Sex Differences

With the trend towards personalized medicine, sex differences have been found in several diseases including cardiovascular disease [1,2] and chronic kidney disease [1,2,3]. Men have been found to have higher morbidity and mortality rates following trauma and sepsis [4] while women exhibit increased immune responses following hemorrhagic shock resulting in better tolerance compared to a decreased responsiveness in males that correlates with increased susceptibility [5,6]. According to the most recent SEER statistics, men have increased incidence and mortality rates compared to women for all sites of cancer. With the statistics excluding nonmelanoma skin cancer (NMSC) [7], the incidence rate for men is 541.8 per 100,000 while for women, the incidence rate is 412.3 per 100,000. The mortality rate for men for all sites of cancer is 219.4 per 100,000 and 151.1 per 100,000 for women. These sex differences have also been found to hold true for both melanoma and nonmelanoma skin cancer. Compared to men, women with malignant melanoma exhibit a decreased risk of metastasis and therefore, a survival advantage [8]. Further, men are twice as likely to develop basal cell carcinoma (BCC) and three times as likely to develop squamous cell carcinoma (SCC) as women [9]. However, the mechanisms that contribute to this sex disparity are poorly understood.
1.2 Skin Cancer

There are over 3.5 million new diagnoses of NMSC each year in more than 2 million patients, making NMSC more prevalent than all other cancers [10]. In fact, more people have had a type of skin cancer (melanoma and nonmelanoma) than all other cancers combined in the last 30 years [11]. Risk factors for developing NMSC include amount of UV exposure, number of sunburns, race, age, immunosuppressive status, and sex [12]. Higher amounts of UV exposure and sunburns, especially blistering sunburns before age 18, increase a person’s risk of developing NMSC by over 50%. Fair-skinned people with red or blonde hair and blue or green eyes have a higher incidence of skin cancer compared to darker skinned people with dark hair and dark eyes. Older people (70+ years old) are at a high risk for NMSC due to the buildup of damage from sun exposure over their lifetimes; however, recently more and more young people (under age 40) are being diagnosed with NMSC [13]. Immunosuppressed patients such as transplant patients have between a 60-250-fold increased skin cancer incidence [14,15,16,17]. Men are twice as likely to develop BCC and three times as likely to develop SCC as compared to women [9].

Skin cancer is composed of both melanoma and nonmelanoma skin tumors. Melanoma contributes to about 5% of skin cancer diagnoses, with approximately 76,000 new cases diagnosed in 2012, and approximately 9,000 deaths annually, which accounts for the majority of skin cancer deaths [18].
NMSCs account for over 3,000 deaths annually. NMSC consists of BCC and SCC. Between 40-50% of Americans who live to age 65 will have at least one BCC or SCC diagnosis [19]. Over 90% of NMSC are associated with exposure to UV from the sun [20]. BCC contributes to 80% of all skin cancers, with over 2.8 million new diagnoses each year in the US [21]. While rarely fatal, 50% of patients will develop another lesion within 5 years. BCC usually develops on sun-exposed sites and has been linked with intense UVB exposure in early childhood and adolescence. Actinic keratoses (AK) are flat, scaly patches and the most common precancerous lesion, affecting more than 58 million Americans [22]. SCC, the focus of these studies, contributes to 16% of all skin cancers with 700,000 diagnoses annually and is the deadly type of nonmelanoma skin cancer that can metastasize more frequently. SCC occurs on sun-exposed sites and is correlated with cumulative UVB exposure. The number of women under age 40 diagnosed with BCC has increased more than two-fold over the last 30 years while the number of women under age 40 diagnosed with SCC has increased almost 700% [13].

As previously mentioned, while NMSC has a relatively low mortality rate, there is a large economic burden. Over $1.4 billion is spent on the treatment and management of NMSC yearly, making it the fifth most costly cancer in the Medicare population in 2004. An additional $1.1 billion is spent on the treatment and management of AK lesions [22]. Further costs accrue because NMSC may also contribute to considerable morbidity, especially on the visible, sun-exposed sites such as the face, arms, and legs, resulting in disfiguration and disability [23,24,25,26,27]. The morbidity caused by NMSC is perhaps
so striking due to the lack of successful non-invasive therapies. The most common treatments for SCC include excision, Mohs micrographic surgery, cryosurgery, curettage and electrodessication, and radiation therapy, which, when detected early and promptly removed, are effective and cause minimal damage. If left untreated, the lesions can grow exponentially or metastasize, spreading into underlying tissues and other parts of the body leading to more invasive procedures and the removal of larger apparently normal skin margins around the tumor. Surgery may not be an option with recurring lesions or a patch of lesions where the remaining skin margin around the tumors may not be large enough or the skin may be too damaged to proceed.

1.3 The skin

The skin—the largest organ in the body—is our first line of defense from the environment and is composed of three layers, the hypodermis, the dermis, and the epidermis [28]. The epidermis, which is the outer layer that serves as the physical and chemical barrier, is primarily comprised of keratinocytes, which is the cell type involved in NMSC. There are four distinct layers of the epidermis. The innermost basal layer, the stratum basale, consists of actively proliferating keratinocytes, characterized by a dispersed network of keratin filaments. Proliferation ceases and terminal differentiation begins as basal keratinocytes move toward the surface of the skin through the spinous and granular layers, stratum spinosum and stratum granulosum, respectively, ending in the outermost cornified layer, the stratum corneum. Beneath the epidermis lies the dermis,
which provides structural support to the skin. The dermis consists primarily of fibroblasts that produce collagen, elastin, and structural proteoglycans, in addition to mast cells and macrophages. 70% of the dermis consists of collagen fibers that provide strength and toughness. Flexibility and elasticity are maintained by elastin and proteoglycans provide hydration and viscosity. The dermal vasculature, lymphatics, nerve cells, sweat glands, hair roots, and a small amount of striated muscle are housed within this fibrous tissue. The hypodermis is made up of loose connective tissue and adipose, which is an important fat depot. Besides acting as a protective barrier, the skin prevents moisture loss, protects against UV, acts as a sensory organ, helps to regulate temperature control, and plays a role in immunological surveillance.

While the basic processes and structure and function of the skin are very similar in men and women, there are clear phenotypic differences driven by hormones. While testosterone is important for hair growth, sebum production, and overall masculine features, estrogen suppresses body hair growth, maintains the dermal matrix, influences the deposition of body fat, and is beneficial for decreasing the time it takes to heal wounds [29]. Besides hormone regulated differences, in male skin, the dermis is consistently thicker throughout life compared to the dermis in female skin [30,31]. Male skin has been found to thin at a steady rate with increasing age while female skin remains relatively stable until menopause, indicating that skin thickness may be hormonally regulated [32]. Women have more subcutaneous fat than men [33], which decreases during menopause in correlation with estrogen status. Male skin has larger pores,
generates four times more sebum [34], and releases twice the amount of sweat during exercise as compared to female skin. Additionally, the basal blood flow is higher in male skin compared to female skin [35,36] and vasodilation will occur at hotter temperatures in men [37]. In regards to skin pigmentation, women overall have lighter skin coloring compared to men, reflected in all skin types across all ethnicities [38,39,40,41].

Because obtaining detailed sun exposure histories is difficult with human subjects, we have used the hairless Skh-1 mouse model for our studies. Human skin and murine skin are similar, but there are some important differences to consider. The epidermis in human skin consists of approximately ten layers of differentiating squamous cells while murine skin consists of only a few layers of differentiating cells [42]. The panniculus carnosus, a thin layer of striated muscle, separates murine skin from other structures and is not found in human skin. Human skin contains eccrine sweat glands; however, these glands are only found in the mouse footpad, not in the skin.

The hairless mouse model is an appropriate and accepted model for experimental skin carcinogenesis [43]. Haired mice provide difficulties in that the hair causes the skin to be nearly impenetrable by UV, and shaving the hair could introduce unwanted confounding effects on tumor formation. Because the mice are hairless, tumors can easily be observed and their progression tracked over time with relatively no discomfort for the mice. Importantly, the induction of tumors via chronic exposure of low UVB doses can be directly correlated to regular occurrences in human life. Skh-1 hairless mice have fully
functioning immune systems, so they should not be confused with nude mice. UV radiation induces skin carcinomas and precursors originating from the epidermis almost exclusively in these mice, which is also seen in human skin cancer induced by UV exposure. In haired mice that have been shaved, fibrosarcomas originating from the dermis are also found [44], something rarely described in humans. Importantly, because Skh-1 mice are albino, no melanomas develop due to the absence of melanocytes.

1.4 UV light

Solar radiation is the most common carcinogen to which we are exposed with the skin being its primary target. Both the US Department of Health and Human Services and the World Health Organization have identified UV as a proven human carcinogen [45]. Sunlight is considered to be a complete carcinogen, meaning that it can initiate and promote the carcinogenesis process [46]. Sunlight consists of three ranges of UV light, including UVC (100-280 nm), UVA (315-400 nm), and UVB (280-315 nm) [47,48]. UVC is mostly absorbed by the ozone layer and does not reach the Earth’s surface. UVA rays account for over 95% of the UV radiation reaching the Earth’s surface. UVA rays are relatively equally present during daylight hours throughout the year and are able to penetrate both clouds and glass. Because UVA penetrates deep into the dermis and is about 10 times less effective at causing an erythematous response, UVA was not believed to play a role in skin carcinogenesis, though its role in skin wrinkling and aging is well documented [49,50,51]. In the last two decades, however, studies have demonstrated that
UVA is able to damage basal keratinocytes in the epidermis, which is where many skin cancers arise. While only 2% of sunlight reaching our skin is made up of the UVB wavelength, it is believed to contribute to the greatest amount of damage to our skin [52,53,54]. Between 10 AM and 4 PM from April to October, UVB rays are the most intense in the United States. Importantly, UVB rays can damage the skin at any time, especially in the colder months in the presence of snow, ice, or other reflective surfaces that reflect 80% of the rays onto exposed skin.

Contrary to popular belief, only about 23% of lifetime sun exposure is acquired by age 18. Based on a 78 year lifespan, 47% of lifetime exposure occurs by age 40, 74% by age 60, and the remainder by age 78 [55]. This misconception that the majority of lifetime sun exposure was acquired by age 18 may have contributed to the continuing increased skin cancer incidence because adults believed that there was no way to prevent tumor development. Because cumulative sun exposure is thought to contribute to SCC development, it is possible to make lifestyle changes to eliminate sun exposure and decrease the accumulating damage and the risk of developing SCC. Further, this information highlights the importance of sun protection throughout an individual’s lifetime.

1.5 Inflammation

It is known that the inflammatory response following both brief periods of high intensity
sun exposure and chronic sun exposure play roles in the formation of NMSC [56,57,58,59]. It has been well documented that exposure to UVB light is associated with an inflammatory response characterized by increased blood flow and vascular permeability, resulting in erythema and edema, the infiltration of neutrophils into the dermis, and the induction of pro-inflammatory cytokines [60,61,62]. The first cell type to migrate to the site of inflammation is the neutrophil. Chemoattractants such as IL-8 released by keratinocytes and dermal cells drive neutrophil migration. In response to inflammatory stimuli, neutrophils degranulate, therefore releasing many signaling molecules as well as producing an oxidative burst consisting of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Myeloperoxidase (MPO) is an enzyme that converts the ROS, hydrogen peroxide (H₂O₂), released by neutrophils into hypochlorous acid, which is a potent microbicidal agent. MPO levels in the skin can be used as a marker for neutrophil infiltration and activation [63].

Chronic UVB exposure induces the proinflammatory cytokines TNF-alpha and IL-1beta. These cytokines induce constitutive expression of cyclooxygenase 2 (COX-2), the primary source of elevated prostaglandin E2 (PGE₂) levels in the skin [63,64], and also results in a stable increase in oxidant synthesis and further inflammatory cell infiltration and activation. The prostaglandin synthesis increase contributes to the functional alteration of both resident skin cells and infiltrating inflammatory cells [63,65] and is believed to play a key role in the carcinogenesis process by contributing to the uncontrolled proliferation of damaged cells that ultimately form tumors [66,67,68,69,70].
PGE$_2$ can bind to any of four G-protein-linked receptors, each of which is coupled to different intracellular effector molecules. The binding of PGE$_2$ to its receptors mediates the effects on the body, including the induction of fever or edema, mediation of the pain response, proliferation, migration, or the activation of several different cell types.

Cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2) serve to catalyze the conversion of arachidonic acid (AA) to prostaglandins [71]. COX-1 is constitutively expressed and is important for maintaining homeostasis [72]. COX-1 plays a cytoprotective role in the skin and the gastrointestinal (GI) tract where epithelial cells proliferate and differentiate on a continual basis. COX-1 is also involved in kidney and renal function as well as platelet aggregation. COX-2 is inducible and is involved in immediate-early gene response to stimuli such as growth factors, hormones, and UVB light [66,73,74]. Both COX-1 and COX-2 are targets for the action of non-steroidal anti-inflammatory drugs (NSAIDs).

NSAIDs have analgesic, antipyretic, and anti-inflammatory actions and they function by inhibiting the COX enzymes. NSAIDs prevent the formation of prostaglandins from AA by binding at Arg 120 [75], which causes a physical block of the hydrophobic channel that surrounds the active site, preventing the entrance of AA into the channel and therefore, its conversion to prostaglandins. Problems with the chronic use of oral NSAIDs are associated with the inhibition of homeostatic functions of COX-1 including the common side effects of nausea and vomiting, as well as more serious toxic effects.
resulting in nephrotic syndrome, acute renal insufficiency, prolonged bleeding time, fluid retention, and the risk of drug interactions [76]. As a result, COX-2 inhibitors were developed, which have much milder GI side effects but can still cause cardiac problems. Some cardiac side effects can potentially be avoided by using topical forms of NSAIDs, resulting in lower serum concentrations, thus avoiding these harmful effects [77].

COX-2 also contributes to oxidative damage, both directly and indirectly. COX-2 directly generates ROS upon reaction with its substrate, AA. During the production of prostaglandins from AA, oxygen is incorporated to produce PGG$_2$, which is reduced to PGH$_2$ by the peroxidase activity of COX-2 [78]. During this reaction, oxygen-derived free radicals are produced that can cause oxidative DNA damage [79]. COX-2 contributes to oxidative DNA damage indirectly through the promotion of the inflammatory process including the infiltration, activation, and release of ROS by inflammatory cells.

1.6 DNA damage

In addition to inducing cutaneous inflammation, exposure to UVB results in the formation of both direct and indirect DNA damage. Direct DNA damage is the result of photons of UV light energy being absorbed by the DNA. This induces the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pirimidone photoproducts [80]. C to T and CC to TT transitions at neighboring pyrimidine bases are the signature
UV mutations [81]. These mutations are found in up to 90% of BCC and SCC and nearly 100% of UV-induced murine skin cancers in the p53 tumor suppressor gene [82]. (6-4) photoproducts form 3-40 times less frequently than CPDs after UV exposure and are repaired at a much faster rate than CPDs. Therefore, it is thought that CPDs contribute to mutation formation to a greater extent than (6-4) photoproducts. CPDs are repaired via the nucleotide excision repair pathways [83]. Transcription coupled repair (TCR) occurs at sites of damage within the transcribed portion of the genome at a rapid rate. Global genome repair occurs anywhere in the genome but at a much slower rate than TCR.

Exposure to UVB also indirectly induces oxidative DNA damage, partially through generation of ROS by infiltrating inflammatory cells including neutrophils and macrophages, and also by activated epidermal keratinocytes [84,85,86]. Without proper repair, indirect DNA damage causes G to T transitions, which leads to one of the most mutagenic DNA lesions, 8-oxo-deoxyguanosine (8-oxo-dG). While there are several photooxidative DNA products, 8-oxo-dG is considered a stable and sensitive marker for evaluating oxidative DNA damage [87]. Base excision repair is a DNA repair pathway capable of repairing lesions caused by ROS.

DNA damage following exposure to stimuli, such as UVB, induces the expression of wild-type p53, a tumor suppressor gene, by a post-translational mechanism [88]. The TP53 gene encodes a 53-kDa phosphoprotein that plays an important role in the control of cell proliferation [89]. Increased levels of p53 protein block cells in the G1/S stage of
the cell cycle, allowing DNA repair to occur before DNA replication [90]. If the damage is beyond repair, p53 will induce pro-apoptotic proteins. Therefore, p53 plays a key physiological role in limiting mutagenic damage. After repeated UV exposures, however, the TP53 gene may become mutated, leading to a dysfunctional protein product. The mutant protein becomes resistant to degradation, resulting in the loss of the ability to halt cell cycle progression and resulting in the accumulation of p53-positive proliferating cells. Thus, mutations in TP53 would allow for an increased rate of accumulation of genetic damage in the cell. The loss of function of the TP53 tumor suppressor gene by mutation or allele loss is a common finding in many human malignancies including SCC of the skin [82,91]. Failure to adequately repair DNA lesions, both direct and indirect, can result in the formation of skin cancers [92]. Clusters of cells expressing mutant p53 are causally related to chronic UVB and appear to be early carcinogenesis events in the skin.

### 1.7 Antioxidants

The skin is constantly exposed to both endogenous and exogenous agents that may stimulate reactive oxygen species production. For example, exposure to UV results in the formation of ROS including singlet oxygen, peroxy radicals, superoxide anion, and hydroxyl radicals. Low levels of ROS are beneficial for normal cellular processes such as cell differentiation and proliferation, growth arrest, apoptosis, and microorganism defense. High levels of ROS can overwhelm the antioxidant networks in the skin,
resulting in oxidative stress that can lead to cellular damage [93]. The induction of oxidative stress as well as an imbalance in antioxidant activity is associated with inflammation, rheumatoid arthritis, immunotoxicity, photoaging, and skin cancer.

There are multiple antioxidant networks in the skin designed to remove ROS. These cutaneous antioxidants include the enzymatic activities of catalase, glutathione reductase, glutathione-S-transferase, glutathione peroxidase, and superoxide dismutase as well as the non-enzymatic effects of glutathione, alpha tocopherol, and ascorbic acid. Catalase, which contains four porphyrin heme (iron) groups that allow the enzyme to react with $H_2O_2$, converts $H_2O_2$ to water and molecular oxygen. Catalase is usually located in peroxisomes and is found at particularly high concentrations in the liver. Catalase expression and activity decrease with UV exposure in human and murine skin [94,95,96,97,98,99]. Glutathione reductase reduces glutathione disulfide back to glutathione. UV exposure decreases glutathione reductase activity in the skin. Glutathione-S-transferase detoxifies foreign compounds, exhibits peroxidase activity, and binds reactive metabolites. Glutathione peroxidase converts $H_2O_2$ to water and molecular oxygen using glutathione as a co-factor. As a by-product of this conversion, glutathione is oxidized to glutathione disulfide. Glutathione peroxidase activity and glutathione levels in human epidermis are decreased after UV exposure [100]. Superoxide dismutase (SOD) catalyzes the dismutation of super oxide anion to molecular oxygen and $H_2O_2$. UV decreases SOD activity in the skin while the upregulation of SOD decreases sunburn cell formation, which are cells that have UV-induced DNA damage, resulting in chromatin
condensation in the nucleus, large amounts of cytoplasm, and apoptosis [101]. Because our bodies do not produce all of these antioxidants, we rely on our diet, lotions, and cosmetics to supply the exogenous antioxidants we need.

Alpha tocopherol is the most biologically active form of vitamin E. It is the second most common form found in the North American diet, with the most common form being gamma tocopherol. Alpha tocopherol is a fat-soluble antioxidant that stops the production of ROS formed when fat undergoes oxidation. It functions in the glutathione peroxidase pathway and protects the cell membrane from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. As vitamin E quenches free radicals, it becomes oxidized. Vitamin C is able to reduce oxidized vitamin E in order to regenerate its activity, and mixing these vitamins together stabilizes the topical formulation [102]. Vitamin E can act as a pro-oxidant in the absence of vitamin C, causing oxidative damage by attacking lipids in lipoproteins. Ferulic acid, an ingredient in some cosmeceutical antioxidant formulations, is a potent antioxidant found in the cell walls of fruits, vegetables, and grains that exerts its antioxidant effects by supplying hydrogens to free radicals with phenolic hydroxyl groups [103]. Ferulic acid also has an active oxygen erasing function that protects against the toxicity of active oxygen.
1.8 Chapter summaries

The focus of the following chapters is to investigate sex-based mechanistic differences in the response between male and female skin to UVB, specifically in the areas of inflammation, antioxidant status, and DNA damage. Further, we examine the efficacy of potential treatment strategies for UVB-induced cutaneous SCC development in the sexes in hopes of making available more appropriate prevention and treatment strategies for patients. Chapter 2 investigates the efficacy of diclofenac, a topical NSAID and COX-2 selective inhibitor currently used for patients with SCC precursor lesions, in decreasing tumor development in male and female mice. Our results indicate that diclofenac decreases tumor number in both male and female mice, but only significantly decreases tumor burden in male mice, with catalase playing an important role in this process. Chapter 3 examines the differential effects of topical antioxidants on SCC development in females and males. While vitamin E, a popular cutaneous antioxidant, seems to contribute to SCC development through increased DNA damage and angiogenesis in females, C E Ferulic, a stable combination cosmeceutical antioxidant formula, decreases tumor burden. Though the antioxidant treatments decreased tumor burden in males, the underlying mechanisms remain to be elucidated in future studies. Chapter 4 focuses on the relationship between the length in weeks of UVB exposures and tumor development. We demonstrate that mice exposed to UVB for 25 weeks develop more tumors, larger tumors, and more malignant tumors compared to mice exposed to UVB for 10 weeks. The foci of Chapter 5 are to compare the relationship between tumor size and grade, investigate the possibility of using visual criteria to identify SCC, as well as examine
potential markers for indicating malignancy. We found that there is no direct correlation between tumor size and grade; however, in general, fully invasive SCCs seem to be larger. Further, we demonstrate that visual criteria do not offer a high success rate for correctly identifying SCC. Finally, Chapter 6 summarizes the aforementioned studies and discusses their significance in regards to our goal of providing a better understanding of sex-based mechanistic differences and their influence on offering more appropriate prevention and treatment strategies for both current patients and people predisposed to developing cutaneous SCC.
Chapter 2: Topical Diclofenac Treatment Differentially Decreases Tumor Burden in Male and Female Skh-1 Mice in a Model of UVB-Induced Cutaneous Squamous Cell Carcinoma

2.1 Introduction

According to recent SEER Cancer Statistics, between 2005-2009 the overall cancer incidence and mortality rate was higher in men than in women [7]. This sex difference is also observed in non-melanoma skin cancer (NMSC), which includes both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Epidemiological studies demonstrate that men have twice the incidence of BCC and three times the incidence of SCC as women [9]. There are over 3.5 million NMSC diagnoses in approximately 2 million patients each year [10] with treatment costs reaching over $1.4 billion [22], making it the fifth most costly cancer in the Medicare population [104]. While SCC only makes up 16% of all NMSC, it can metastasize more frequently, killing approximately 2500 people each year, of which men are the majority [46].

A component of solar radiation, UVB is the major environmental carcinogen contributing to the development of both BCC and SCC [52,53,54]. Though the increased SCC incidence observed in men has been attributed to lifestyle differences with higher levels of sun exposure and less protection from the sun compared to women [105,106,107], we have previously shown using a murine model that when exposed to equivalent UVB
doses, this sex bias still holds true [108]. With the increasing incidence of NMSC and the knowledge that men and women respond differently to UVB, it is imperative to examine the efficacy of potential treatments in both sexes.

It is well documented that UVB exposure is associated with an inflammatory response [60,61,62], which following both acute and chronic exposure, is known to play a role in the formation of NMSC [57,58,109]. Chronic UVB exposure induces constitutive expression of cyclooxygenase 2 (COX-2), which is the primary source of elevated prostaglandin E2 (PGE2) in the skin [63,64]. This prostaglandin synthesis increase plays a key role in carcinogenesis by contributing to uncontrolled proliferation of damaged cells that ultimately form tumors [66,67,68,69,70]. The inflammatory response that coincides with elevated PGE2 levels is linked to increased DNA damage, which following exposure to stimuli such as UVB, induces the expression of wild type p53, a tumor suppressor gene [88]. The loss of function of p53 by mutation or allele loss is a common finding in many human malignancies, including cutaneous SCC [82,91].

UVB exposure induces the production of reactive oxygen species such as hydrogen peroxide, which if not maintained at a homeostatic level by antioxidant networks, can cause oxidative stress and cellular damage. The main cutaneous antioxidant, catalase, has the role of detoxifying hydrogen peroxide. Skin antioxidant activity decreases with UVB exposure, and specifically, catalase activity level decreases have been associated with both skin carcinogenesis and progression [94,96,97,98,99,110,111]. Previously, we
reported decreased antioxidant activity [108], and specifically, catalase activity [112], in the skin of male mice compared to female mice that may contribute to increased tumorigenesis in males.

We previously demonstrated that treating female mice topically with the COX-2 inhibitor, celecoxib, decreased PGE₂ levels as well as the percentage of p53-positive epidermal cells in the acute UVB-induced cutaneous response [113]. Chronic UVB exposure and subsequent topical celecoxib treatment decreased both tumor number and grade in female mice; however, its efficacy in males has not been described. Since male mice display lower levels of inflammation and antioxidant activity as compared to female mice after UVB exposure [108,112], we sought to investigate the extent to which a topical anti-inflammatory drug would affect tumor burden as well as PGE₂ or catalase levels in the skin of male versus female mice after chronic UVB exposure.

Because celecoxib is not available to patients in a topical formulation, we conducted studies using topical diclofenac, an NSAID and COX-2 inhibitor, which is currently prescribed to patients with actinic keratotic lesions—precursors of SCC. Topical formulations are better tolerated and cause fewer adverse effects compared to oral COX inhibitors as the systemic levels are greatly decreased with topical treatments [77]. Open label studies have demonstrated that diclofenac is indeed well tolerated and effective short-term for treating actinic keratotic lesions [114,115]. However, its efficacy as a preventative agent in patients without evidence of precursor lesions but with significant
UVB-induced cutaneous damage has not been examined. Additionally, potential differences between men and women in the efficacy of diclofenac treatment have not been reported.

A recent case-control study suggested a dose-dependent, cumulative, protective effect for the reduction of skin cancer risk, specifically SCC, with the use of a number of NSAIDs [116]. In the current study, we have modeled both male and female compliant patients who eliminated sun exposure and began using topical diclofenac regularly to reverse existing damage resulting from chronic UVB exposure and prevent tumor development. Our results demonstrate that despite the observed sex differences in the inflammatory response, prolonged topical diclofenac treatment of chronically UVB-damaged skin effectively reduced tumor multiplicity in male and female Skh-1 hairless mice. Interestingly, with topical diclofenac treatment, tumor burden was significantly decreased and tumors were of lower histologic grade only in male mice. While PGE2 levels were decreased in both sexes, levels of the antioxidant, catalase, were increased with diclofenac treatment in male mice only. These data support inflammation as a key factor contributing to UVB-induced tumor development in both male and female mice. Taken together, our study emphasizes differences in the response of male and female murine skin and demonstrates a potential new therapeutic use for this currently available topical treatment as a preventative intervention for patients predisposed to cutaneous SCC development.
2.2 Materials and Methods

2.2.1 Animal treatments and experimental design. Outbred, male and female Skh-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee before the initiation of any studies. Mice were dorsally exposed to 2240 J/m² UVB, previously determined to be 1 MED, 3× weekly on non-consecutive days for 10 weeks. UVB dose was calculated using UVX radiometer and UVB sensor (UVP, Upland, CA) and emitted by Phillips FS40 UV bulbs (American Ultraviolet Company, Lebanon, IN). After 10 weeks of UVB exposure, mice were treated topically with vehicle (Surgilube®; Savage Laboratories, Melville, NY; n=20 males and 20 females) or 500 µg diclofenac (Solaraze®, 3% diclofenac sodium; Doak Dermatologics, Fairfield, NJ; n=10 males and 10 females) in vehicle for 15 weeks with no additional UVB exposure. This dose of diclofenac was chosen based on previous studies in our laboratory. Surgilube is an inert vehicle used clinically as a surgical lubricant. Tumors larger than 1 mm in diameter were measured weekly with calipers. After sacrifice, 0.5cm² section of dorsal skin and all tumors were fixed as previously described [112] while remaining dorsal skin was snap frozen in liquid nitrogen.

2.2.2 Tumor Grading. Hematoxylin and Eosin (H&E)-stained tissue sections of tumors isolated from male and female mice were graded in a blinded manner by Dr. Donna
Kusewitt, a board-certified veterinary pathologist as previously described [108]. Briefly, papillomas were exophytic tumors (tumors that grow outward from the originating epithelium) that showed no invasion of the stroma. A grade 1 papilloma primarily was composed of epithelium without a pronounced papillary pattern. Grade 2 papillomas were well-differentiated papillary masses while grade 3 papillomas contained a few finger-like projections of atypical cells at the base of the mass. Microinvasive squamous cell carcinomas grades 1-3 were distinguished by the depth of penetration into the dermis. Squamous cell carcinomas displayed a more endophytic (growing inward) appearance, with the loss of basement membrane continuity, the development of a dermal inflammatory response, and stromal invasion. Fully invasive squamous cell carcinomas were tumors that invaded the panniculus carnosus. Papillomas were considered benign while microinvasive and fully invasive squamous cell carcinomas were considered malignant.

2.2.3 Catalase Activity Assay. Frozen dorsal skin was crushed in liquid nitrogen using a mortar and pestle and 15 mg was weighed out and used for analysis of catalase activity using the Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s instructions. Briefly, 25 µl extraction buffer per milligram sample weight was added to the crushed skin in a microcentrifuge tube on ice. Tissue was homogenized followed by 3 rounds of sonication, pulsating for 10 seconds each round. Samples were vortexed for 10 seconds every 5 minutes for a total of 50 minutes, followed by two rounds of centrifugation at 10,000 x g for 10 minutes at 4 °C, discarding the pellet after
each centrifugation. Samples were diluted 1:10 in Sample Buffer contained in the Catalase Assay Kit. Samples were loaded onto the plate in triplicate and the plate absorbance was read at 540 nm. This assay measures the peroxidatic function of catalase in the reaction between catalase and methanol in the presence of an optimal hydrogen peroxide concentration. This reaction produces formaldehyde, which is measured colorimetrically with the chromogen 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). Catalase activity was determined by comparing the samples to the standard curve of the formaldehyde standard.

2.2.4 Prostaglandin E2 Enzyme Immunoassay. Frozen dorsal skin was crushed in liquid nitrogen using a mortar and pestle and 15 mg was weighed out and used for analysis of PGE$_2$ levels using the Prostaglandin E$_2$ EIA Kit-Monoclonal (Cayman Chemical) according to manufacturer’s instructions. Briefly, 25 µl extraction buffer per milligram sample weight was added to the crushed skin in a microcentrifuge tube on ice. Tissue was homogenized followed by 3 rounds of sonication, pulsating for 10 seconds each round. Samples were vortexed for 10 seconds every 5 minutes for a total of 50 minutes, followed by two rounds of centrifugation at 10,000 x g for 10 minutes at 4 °C, discarding the pellet after each centrifugation. Samples were diluted 1:10 in extraction buffer for use in a BCA (bicinchoninic acid) assay where the samples were compared to a serial dilution of BSA (bovine serum albumin) of known concentrations in order to determine the protein concentration of each sample and then prepare stock dilutions of each sample at either 1 µg/µl (UV irradiated samples) or 2 µg/µl (unirradiated samples).
Because the plate is coated with goat anti-mouse antibody, we incubated the plate with the monoclonal PGE₂ capture antibody overnight at 4 °C in order to avoid non-specific binding of the capture antibody with mouse IgG in the tissue samples. The plate was washed with kit wash buffer, samples were added in triplicate, and the plate was incubated for 18 hours at 4 °C. Next, the plate was washed with kit wash buffer, Ellman’s Reagent was added to the wells, and the plate was incubated for 60-90 minutes or until the control wells had an absorbance greater than 0.3 at 415 nm. This is a competitive assay between PGE₂ and a PGE₂-acetylcholinesterase conjugate (PGE₂ Tracer) for a fixed amount of the monoclonal PGE₂ antibody. Since the amount of PGE₂ varies from sample to sample while the amount of PGE₂ Tracer is always the same, the amount of PGE₂ Tracer that is able to bind to the monoclonal PGE₂ antibody will be inversely proportional to the amount of PGE₂ in the well. Sample values were compared to a standard curve of the PGE₂ standards at known dilutions in order to determine the concentration of PGE₂ in each sample well.

2.2.5 Histological Techniques. After sacrifice, 0.5 cm² sections of dorsal skin and all tumors were fixed for 4 hours at 4 °C in 4% paraformaldehyde prepared in sodium phosphate buffer. Fixed skin was then incubated in sodium phosphate buffer containing 20% sucrose overnight at 4 °C. Next, the skin was incubated in sodium phosphate buffer containing 20% sucrose and 5% glycerol for 2 hours at 4 °C followed by embedding in OCT freezing compound (Sakura, Torrance, CA). Paraformaldehyde-fixed/OCT-embedded dorsal skin sections were cut (10 µm) onto Superfrost Plus® microscope slides.
(Fisher Scientific) and stored at -80°C for future analysis. Before use, slides were thawed overnight at room temperature, baked at 60°C for 30 min, and then rehydrated in Clear Rite 3 (Richard-Allen Scientific) and a graded series of ethanol.

**Immunohistochemical detection of p53**

After rehydration, sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 25 min in a vegetable steamer reaching 95°C. Slides were cooled for 20 minutes at room temperature and blocked in 1× Casein (Vector Laboratories) for 10 minutes followed by incubation with primary p53 antibody (clone CM5p, Novocastra (Leica Microsystems Inc.), Buffalo Grove, IL) at a 1:500 dilution in 1× Casein at room temperature for 1 hour. Slides were incubated in Rabbit Link and Label (Biogenex, Fremont, CA), each for 30 minutes at room temperature. Slides were washed in PBS containing 0.05% Tween (PBST) between the various incubation steps described above. Following a final PBST wash, slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at room temperature. Slides were washed in deionized water, counterstained with Hematoxylin 2, dehydrated in an increasing series of ethanol followed by Clear Rite 3, and then cover slipped with VectaMount mounting medium (Vector Laboratories). p53 foci were counted as 3 or more adjacent p53-positive cells and examined in 5 fields of view at 20× magnification.
**Immunohistochemical detection of Ki67**

After rehydration, sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 25 min in a vegetable steamer reaching 95°C. Slides were cooled for 20 minutes at room temperature and blocked in 1× Casein (Vector Laboratories) for 10 minutes followed by incubation with primary Ki67 antibody (Dako, Carpinteria, CA) at a 1:200 dilution in 1× Casein overnight at 4°C in a humid chamber. Slides were incubated in biotinylated IgG (Vector Laboratories) at a 1:200 dilution in 1× Casein for 30 minutes at room temperature. Slides were incubated in ABC Elite (Vector Laboratories) for 30 minutes at room temperature. Slides were washed in PBST between the various incubation steps described above. Slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at room temperature. Slides were washed in deionized water, counterstained with Hematoxylin 2, dehydrated in an increasing series of ethanol followed by Clear Rite 3, and then cover slipped with VectaMount mounting medium (Vector Laboratories). Ki67-positive cells were examined in 5 fields of view at 60× magnification.

**2.2.6 Statistical Analysis.** The results presented in this paper were part of a larger experiment involving four treatment groups (of which Diclofenac was one) and a single control group. Dunnett’s adjustment [117,118] for multiplicity was used for comparing the primary outcome of tumor burden at 24 weeks between the treatment groups and
control in order to restrict the probability of a type I error to 5%. The number of control mice was inflated compared to the treatment groups to increase the power of the comparison [117]. Residual plots verified the model assumptions of normality and homoscedasticity and a logarithmic transformation was utilized if necessary. Continuous outcome data were analyzed using an ANOVA approach with linear contrasts for testing the comparisons of interest. A mixed-effects regression model with a random slope and intercept by subject was used to model tumor growth from the time of tumor origination. For count data, Poisson regression was used. All analyses were conducted in SAS version 9.2 (SAS Institute, Cary, NC). \( p \)-values ≤0.05 were considered statistically significant.

2.3 Results

2.3.1 Diclofenac topical treatment decreased tumor number and burden. To examine the effects of topical diclofenac treatment as a preventative agent against tumor development, we exposed male and female Skh-1 hairless mice to 2240 J/m\(^2\) UVB (previously determined to be 1 MED in our laboratory) for ten weeks to model chronic sun exposure. Mice were then topically treated with either surgilube (vehicle) or diclofenac for 15 weeks without further UVB exposure to model a lifestyle change. Non-irradiated male and female mice treated with either vehicle or diclofenac did not develop tumors. Additionally, mice that received no vehicle treatment did not exhibit a significantly different tumor burden compared to mice treated with vehicle, indicating that the vehicle had no significant effect on tumorigenesis in this study (Figure 2.1).
Figure 2.1 Topical vehicle treatment has no significant effect on tumor burden in male or female mice. Mice were exposed to 2240 J/m² three times weekly for 10 weeks followed by 15 weeks of topical treatment (black bars) or no topical treatment (gray bars) with no further UVB exposures. Tumors were examined and measured with calipers weekly.

Following 10 weeks of only UV exposure and 15 weeks of preventative topical treatment with diclofenac, male mice exhibited decreased tumor multiplicity with 57.8% fewer tumors compared to the mice treated with vehicle (p<0.0001, Figure 2.2A). At week 24, male mice treated topically with diclofenac exhibited significantly decreased tumor burden by 82.5% (p<0.0001) compared to the mice treated with vehicle (Figure 2.2B). Female mice treated topically with diclofenac also demonstrated decreased tumor multiplicity with 63.1% times fewer tumors compared to mice treated with vehicle.
Figure 2.2 Diclofenac decreases tumor multiplicity and burden in male and female mice. Tumors with a diameter larger than 1 mm were measured weekly. (A) Male and female mice treated topically with diclofenac for 15 weeks after 10 weeks of UVB exposure exhibited decreased tumor multiplicity compared to the mice treated with vehicle (*p<0.0001). (B) At week 24, male mice treated topically with diclofenac exhibited significantly decreased tumor burden (*p<0.0001) compared to the mice treated with vehicle while the decrease in female mice was not statistically significant (p=0.1098).
Female mice treated topically with diclofenac displayed a 51.7% decrease in average tumor burden that was not statistically different ($p=0.1098$) compared to mice treated with vehicle (Figure 2.2B). However, female mice treated with vehicle exhibited approximately half the tumor burden as vehicle-treated males, which was significantly different ($p=0.0033$).

Examining the change in tumor burden over time, we found that male mice treated topically with diclofenac had a significantly decreased tumor growth rate of 6.9% while mice treated with vehicle exhibited a growth rate of 15.9% ($p=0.0057$, Figure 2.3A). Though female mice treated topically with diclofenac also exhibited a slight decrease in tumor growth rate compared to mice treated with vehicle, 8.6% versus 11.1%, respectively, this decrease was not significant ($p=0.3949$, Figure 2.3B).

### 2.3.2 Diclofenac topical treatment decreased tumor severity in male mice.

Tumors were isolated from mice after 10 weeks of UVB exposure followed by 15 weeks of topical treatment and scored by a board-certified veterinary pathologist (DFK). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. Male mice treated with vehicle developed tumors of each grade while male mice preventatively treated with diclofenac developed tumors graded as papilloma (grades 1-3) or microinvasive SCC (grade 1 only). The estimated per tumor malignancy rate for male mice treated with vehicle was 0.20, indicating that one out of five tumors could be expected to be malignant, as compared to
Figure 2.3 Diclofenac decreases tumor growth rate in male mice. Tumors with a diameter larger than 1 mm were measured weekly. (A) Male mice treated topically with diclofenac had a decreased tumor growth rate (*p=0.0057) compared to mice treated with vehicle. (B) Female mice treated topically with diclofenac exhibited a slight decrease in tumor growth rate compared to mice treated with vehicle that was not statistically significant (p=0.3949).
the malignancy rate of 0.06 for mice treated with diclofenac. There was a trend towards a lower rate, but it was not statistically significant \( (p=0.2413) \). Importantly, male mice treated with diclofenac developed no fully invasive SCC lesions while mice treated with vehicle developed 5.3% of graded tumors that were fully invasive SCC lesions.

Female mice treated with vehicle developed all grades of papilloma, microinvasive SCC grade 1 and fully invasive SCC. Mice preventatively treated with diclofenac developed all grades of papilloma, microinvasive SCC grades 1 and 2, and fully invasive SCC. Interestingly, while female mice treated topically with diclofenac exhibited decreased tumor burden compared to vehicle-treated mice, the estimated per tumor malignancy rate was 0.25 compared to 0.11 for mice treated with vehicle, which trended towards an increased malignancy rate but was not statistically significant \( (p=0.2574) \).

### 2.3.3 Diclofenac topical treatment increased catalase activity in male mice.

Protein was extracted from tumor-free, dorsal skin in order to examine catalase activity levels. Even after 15 weeks without UVB exposure, catalase activity levels were significantly decreased in the vehicle-treated male skin as compared to the non-irradiated controls \( (p=0.0004, \text{ Figure 2.4A}) \), but not in female skin \( (p=0.2121, \text{ Figure 2.4B}) \). Topical diclofenac treatment applied to male skin significantly increased catalase activity from levels observed in the vehicle-treated skin \( (p=0.0035, \text{ Figure 2.4A}) \), and in fact, catalase activity is almost restored to levels observed in non-irradiated skin. In contrast, catalase
Figure 2.4 Diclofenac increases catalase activity in male skin. Even after 15 weeks without UVB exposure, catalase activity levels were significantly decreased in the vehicle-treated male skin (A) as compared to the non-irradiated controls (*p=0.0004). With topical diclofenac treatment in male skin, catalase activity levels were significantly increased from levels observed in the vehicle-treated skin (*p=0.0035). (B) Catalase activity levels were not significantly altered in female vehicle-treated skin (p=0.2121) or diclofenac-treated skin (p=0.159).
activity levels in female skin treated topically with diclofenac were not significantly altered compared to those observed in vehicle-treated skin \((p=0.1590, \text{Figure 2.4B}).\)

2.3.4 **Diclofenac topical treatment decreased cutaneous Prostaglandin E2 (PGE}_2\) levels.** To confirm that topical diclofenac treatment preventatively applied effectively inhibited COX-2-mediated PGE\(_2\) production, cutaneous PGE\(_2\) levels were determined by enzyme immunoassay. In chronically irradiated male (Figure 2.5A) and female (Figure 2.5B) vehicle-treated skin, even after 15 weeks without UVB exposure, PGE\(_2\) levels were significantly increased from levels observed in non-irradiated skin \((p<0.0001).\) Skin of male (Figure 2.5A) and female (Figure 2.5B) mice exposed to UVB for 10 weeks and treated topically with diclofenac for 15 weeks exhibited significantly decreased PGE\(_2\) levels as compared with chronically irradiated, vehicle-treated mice \((p=0.0001).\)

2.3.5 **Male mice have increased numbers of p53-positive foci compared to female mice.** Tumor-free, dorsal skin sections were examined for p53-positive foci via immunohistochemistry with an antibody detecting both wild type and mutant p53 (Figure 2.6A and B). The number of p53-positive foci measured over five fields was not significantly altered with preventative topical diclofenac treatment in male skin \((2.5 \text{ for vehicle-treated vs 1.8 for diclofenac-treated, } p=0.2323)\) or female skin \((0.89 \text{ for vehicle-treated vs 0.63 for diclofenac-treated, } p=0.4918).\) Corresponding with the increased tumor burden, the mean number of foci was significantly higher in vehicle-treated male skin compared to vehicle-treated female skin \((2.5 \text{ vs 0.89, } p=0.0004, \text{Figure 2.6C}).\)
Figure 2.5 Diclofenac topical treatment decreases cutaneous PGE$_2$ levels in male and female mice. Cutaneous PGE$_2$ levels were determined by enzyme immunoassay after 10 weeks of UVB exposure and 15 weeks subsequent topical treatment. In both male (A) and female (B) mice, PGE$_2$ levels were significantly increased with UVB exposure compared to levels observed in unirradiated mice (*$p<0.0001$). Skin of male (A) and female (B) mice exposed to UVB for 10 weeks and treated topically with diclofenac for 15 weeks exhibited significantly decreased PGE$_2$ levels as compared with chronically irradiated vehicle-treated skin (*$p=0.0001$).
Male mice have increased numbers of p53-positive foci compared to female mice. Dorsal, tumor-free, skin sections were examined for p53-positive foci via immunohistochemistry with an antibody detecting both wild type and mutant p53. Representative (A) male and (B) female vehicle-treated skin after 10 weeks of UVB and 15 weeks of treatment. Scale bar = 10 μm. The mean number of foci per field of view was significantly higher (C) in vehicle-treated male skin compared to vehicle-treated female skin (*p=0.0004).
2.3.6 Diclofenac does not affect proliferation 15 weeks after cessation of UVB exposure. Tumor-free, dorsal skin sections were stained for Ki67. The percentage of Ki67-positive cells was not different ($p=0.4708$) between the vehicle-treated and diclofenac-treated male skin after 10 weeks of UVB exposure followed by 15 weeks of topical treatment with no additional UVB exposure. In female skin, the percentage of Ki67-positive cells exhibited a decreasing trend from 13.4% to 9.4% with preventative topical diclofenac treatment compared to vehicle-treated mice ($p=0.0596$). Interestingly, the percentage of Ki67-positive cells was significantly higher in male skin compared to female skin with both vehicle and diclofenac topical treatment ($p<0.006$ and $p=0.0004$, respectively, Figure 2.7).
Figure 2.7 Male skin displays higher levels of proliferation regardless of treatment. Dorsal, tumor-free, skin sections were stained for Ki67. Male skin exhibited higher percentages of Ki67-positive cells compared to female skin with both vehicle (*p<0.006) and diclofenac topical treatment (**p=0.0004).
2.4 Discussion

Our previous studies have demonstrated that when exposed to equivalent, chronic UVB light, male mice exhibit a higher tumor burden compared to female mice [108], which we confirmed in the current study. We also have previously shown that female mice preventatively treated topically with the anti-inflammatory drug, celecoxib exhibited decreased tumor number, grade, PGE\textsubscript{2} levels, and the number of p53-positive cells [63,113]. In the current study, we show that the readily available and currently used NSAID, diclofenac, elicits similar results in male mice treated topically with diclofenac after sustaining significant UVB damage. We demonstrate that prolonged, topical diclofenac treatment of chronically UVB-damaged skin effectively reduced tumor multiplicity in both sexes. Unexpectedly, tumor burden was only significantly decreased in male mice, where we also observed a slower rate of growth and a trend toward a lower malignancy rate, with no fully invasive squamous cell carcinomas developing in diclofenac-treated male mice. We also observed a sex difference in catalase activity in that male mice treated with diclofenac exhibited increased catalase activity levels compared to vehicle-treated male mice while catalase activity levels in female mice remained unchanged.

The observed increase in catalase activity levels in male skin may be an important factor because we have observed endogenously lower antioxidant activity levels [108] and specifically, lower catalase activity levels in male skin [112]. Additionally, both decreased antioxidant enzyme levels and the resulting oxidative protein damage in
chronically UV-exposed skin have been demonstrated [97]. Recently we reported a link between decreased skin catalase activity, increased Gr-1⁺CD11b⁺ myeloid cell infiltration, and increased tumorigenesis in male mice but not in female mice [112]. Elevated levels of PGE₂ have been shown to induce higher levels and more suppressive Gr-1⁺CD11b⁺ myeloid cells that produce high levels of reactive oxygen species (ROS) [119,120]. By decreasing PGE₂ levels in male skin with topical diclofenac treatment, Gr-1⁺CD11b⁺ myeloid cell infiltration is decreased (unpublished data), therefore decreasing myeloid-associated ROS levels. As a result, catalase activity levels may be increased or maintained due to the overall lower levels of cutaneous ROS production. The increased skin catalase activity in male mice treated with diclofenac compared to male mice treated with vehicle may indicate that restoration of this antioxidant is important for decreasing tumorigenesis in male but not in female mice.

In addition to increasing catalase activity, diclofenac topical treatment also reduced the number of p53-positive foci observed in male mice. Though not statistically significant, there is a clear trend towards fewer foci. As p53 is indicative of overall DNA damage, a decrease in the number of p53-positive cells observed demonstrates that diclofenac treatment may be promoting repair or preventing damage altogether. Previous studies demonstrate that ingenol mebutate, a well-tolerated topical antineoplastic drug used for treatment of both NMSC and actinic keratotic lesions [121,122,123], effectively reduced UVB-induced lesions and p53 patches in Skh-1 mice [124]. However, the side effects of this treatment included scar formation and skin tightening, which were not observed in
the current study. Because the mice in the current study were exposed to UVB for 10 weeks and then treated for 15 weeks without further UVB exposures prior to examining tissue, further studies examining various time points in addition to examining levels of mutated p53 would be necessary to determine the role diclofenac is playing in the formation of p53 foci.

Importantly, preventative diclofenac treatment led to decreased tumor burden in male mice and significantly lower PGE$_2$ levels in both sexes compared to vehicle-treated mice, underscoring the importance of COX-2-mediated inflammation in the UVB-induced carcinogenesis process. PGE$_2$ also contributes to angiogenesis [125] and invasion [126,127,128,129], in addition to inflammation. Notably, angiogenesis has been reportedly increased in actinic keratoses compared to adjacent skin [130]. Further, studies have indicated that an “angiogenic switch” is turned on during this stage, which contributes to the invasive nature and progression of these lesions toward becoming SCCs [131,132]. While not examined in the current study, blocking PGE$_2$ production with diclofenac treatment may be decreasing angiogenesis and invasive properties that contribute to the lack of development of fully invasive SCC lesions in male mice that are present in the vehicle-treated mice.

We have previously shown that males have lower levels of acute UVB-induced inflammation compared to female mice and suggested that the inflammatory response was not as important to tumorigenesis in male mice [108]. However, this study offers
support to the fact that the inflammatory response in male mice, regardless of magnitude, is indeed important for tumorigenesis, since decreasing levels of inflammatory mediators and increasing the antioxidant capacity within male skin correlated with decreased tumor number and burden in male mice topically treated with diclofenac.

Recent studies have refocused on the preventative effects of NSAIDs against skin cancer [116,133], highlighting the relevance of our study. While many studies focus on oral NSAIDs that may result in harmful side effects over time, the use of topical NSAIDs results in lower systemic levels and therefore, a lower risk of these side effects including gastrointestinal hemorrhage and peptic ulcer disease [76,77]. Our data demonstrate a potential NSAID alternative for patients with cardiac or other health issues that may be heightened with oral NSAID use.

In summary, we have shown in a model of UVB-induced SCC that topically treating male and female Skh-1 hairless mice prophylactically with the NSAID and COX-2 inhibitor, diclofenac, decreased tumor multiplicity and PGE2 production in both sexes. In addition, topically applied diclofenac also increased skin catalase levels and decreased tumor burden as well as the percentage of malignant tumors in male mice. These findings are especially relevant because in humans, men have a greater risk and incidence of cancer overall [7], with a three-fold greater incidence of SCC [9]. To the best of our knowledge, this is the first report of a sex-associated difference in the efficacy of topical diclofenac treatment, implying that it may be beneficial to retrospectively dissect out
potential sex-based differences in previously reported clinical outcomes in order to more effectively treat each sex. As we previously observed that male mice have lower antioxidant activity as compared with female mice, further studies are needed to determine if the increased catalase levels are contributing to the therapeutic effects of the diclofenac treatment, or if inhibiting inflammation is indeed the primary factor in both sexes. Overall, our data suggest that sustained diclofenac treatment applied topically to chronically UVB-damaged skin before the appearance of precursor lesions, in combination with the elimination of UVB exposure, may be beneficial to patients who are predisposed to developing SCC.
Chapter 3: Negative Effects of Topical Alpha Tocopherol Treatment on Ultraviolet Light B-Induced Cutaneous Tumor Development in Skh-1 Mice

3.1 Introduction

Over two million people are diagnosed with a form of non-melanoma skin cancer (NMSC) each year in the United States, making skin cancer more prevalent than all other cancers combined [10]. Squamous cell carcinoma (SCC), a malignant form of NMSC, makes up about 16% of all skin cancers. While the mortality rate from SCC is relatively low—about 3000 deaths per year—SCC can be quite disfiguring since most lesions are located on sun exposed body parts such as the face and arms. Additionally, current topical therapies used to treat precursor actinic keratotic lesions are not effective on invasive SCC, necessitating invasive surgeries. With the increasing NMSC incidence, there has been a renewed focus on other methods of preventing skin cancer, including antioxidant supplementation in food, sunscreens, and lotions.

The skin is constantly exposed to both endogenous and exogenous agents that may stimulate reactive oxygen species production. For example, following UVB exposure, both infiltrating inflammatory cells and activated epidermal keratinocytes generate reactive oxygen species (ROS). There are multiple antioxidant networks in the skin designed to remove ROS. These cutaneous antioxidants include the enzymatic activities
of catalase, glutathione reductase, glutathione-S-transferase, glutathione peroxidase, and superoxide dismutase as well as the non-enzymatic effects of glutathione, alpha tocopherol, and ascorbic acid. When ROS levels overwhelm the cutaneous antioxidant networks, the cells will be subjected to oxidative stress [93]. Major mechanisms by which ROS foster skin tumor development include induction of DNA damage [134,135], inflammation [136], and angiogenesis [137,138,139].

It has been demonstrated that UVB-induced ROS production, and specifically hydrogen peroxide production, is required for the activation of the epidermal growth factor receptor (EGFR) [140,141]. In mice, epidermal growth factor (EGF), a ligand for EGFR, is produced in the submaxillary gland [142] and then stored in granules found in tubular duct cells. Upon binding to its receptor, EGFR, cell growth, proliferation, and differentiation are stimulated [143]. Members of the EGFR family have been implicated in several human cancers including head and neck, prostate, breast, lung, SCC, and BCC, among others, with EGFR levels being quite variable [144]. In tumors, high levels of EGFR protein have been linked with aggressive disease and poor response to therapy [145]. Previous studies have demonstrated that Trolox, a vitamin E analog, modulates UVB-induced EGFR-dependent signaling pathways in normal human epidermal keratinocytes [146].

Endogenous antioxidants in the skin include the enzyme catalase as well as ascorbic acid and alpha tocopherol (vitamins C and E, respectively) [95,97,98,147,148]. Catalase, the
main cutaneous antioxidant, detoxifies hydrogen peroxide. Decreased catalase activity has been linked with both skin carcinogenesis and progression [97,98]. A decrease in vitamin C levels following UVB exposure [149] results in increased DNA damage and apoptosis [150]. Both human and animal studies have demonstrated decreased SCC formation with diets containing supplemental vitamin C [151,152]. Previous studies of the effects of exogenous vitamin E treatment on skin carcinogenesis have resulted in a variety of observations, including a 50% decrease in skin cancer incidence with topical application of vitamin E [153] and an increase in photocarcinogenesis following treatment with more stable vitamin E esters [154]. Other studies have reported no significant association between vitamin E treatment and SCC development [155,156,157,158,159]. As vitamin E quenches free radicals, it becomes oxidized. Vitamin C is able to reduce oxidized vitamin E thus regenerating its activity; therefore, mixing vitamins E and C stabilizes topical formulations of vitamin E [102].

Additionally, ferulic acid has been shown to further stabilize vitamins C and E. Ferulic acid, a potent antioxidant found in the cell walls of fruits, vegetables, and grains, exerts its antioxidant effects by supplying protons or hydrogen ions to free radicals with phenolic hydroxyl groups [103]. Ferulic acid also protects against the toxicity of active oxygen, or superoxide, similarly to superoxide dismutase. Ferulic acid and several of its derivatives have been shown to decrease tumor formation in chemically-induced skin carcinogenesis models [160,161,162,163]. Previously, the combination of vitamin C, vitamin E, and ferulic acid was demonstrated to have photoprotective effects when
applied for four days prior to one UVB exposure [164]. Topical application of this antioxidant combination for four days prior to UVB exposure also significantly reduced UVB-induced thymine dimer formation in the epidermis 24 hours post-irradiation [165]. C E Ferulic is currently being marketed as an anti-aging treatment and sunscreen additive. However, the potential of C E Ferulic for preventing skin cancer in chronically UVB-damaged skin has not been examined.

Cosmeceuticals are topical cosmetics such as lotions or creams that contain biologically active ingredients that claim to have therapeutic benefits. The sales of these products are targeted primarily towards women; especially those who have a history of considerable prior UVB exposure and resultant skin damage. However, any beneficial effect of topical antioxidant application to previously sun damaged skin on skin tumor development has remained controversial. Because our previous studies have demonstrated that male mice have lower endogenous antioxidant activity compared to female mice and this activity is differentially altered with UVB exposure in the sexes [108,112,166], we sought to examine the efficacy of two topical antioxidant formulations in preventing UVB-induced cutaneous SCC in both males and females. Our model mimicked women and men who were exposed to UVB regularly in childhood and early adulthood and then markedly reduced their sun exposure and began applying topical antioxidants prior to the formation of any lesions. The current study demonstrated that topical C E Ferulic treatment effectively reduced tumor number and burden in both female and male Skh-1 mice. Topical vitamin E treatment provided no preventative benefits for female mice, and in
fact, resulted in accelerated tumor growth rate compared to vehicle-treated female mice. This difference may be explained by the resultant increase in catalase activity levels and DNA damage present in the mice treated with vitamin E compared to those treated with C E Ferulic. Interestingly, vitamin E topical treatment demonstrated more beneficial effects in male mice, which may be a result of the lower endogenous antioxidant levels in male mice that were significantly elevated compared to vehicle-treated mice after topical antioxidant treatment. Our study demonstrates both the potential detrimental effects of treating chronically UVB-damaged female skin with topical vitamin E alone and the potential benefits of topically treating male and female skin with a stable combination antioxidant compound for the prevention of UVB-induced SCC.

3.2 Materials and Methods

3.2.1 Animal Treatments and Experimental Design. Outbred, male and female Skh-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee before the initiation of any studies. Mice were dorsally exposed to 2240 J/m² UVB, previously determined to be 1 MED, 3x weekly on non-consecutive days for 10 weeks. UVB dose was calculated using UVX radiometer and UVB sensor (UVP, Upland, CA) and emitted by Phillips FS40 UV bulbs (American Ultraviolet Company, Lebanon, IN). After 10 weeks of UVB exposure, mice were treated topically with vehicle (Surgilube®; Savage Laboratories,
Melville, NY; n=20 males and 20 females), 5mg vitamin E (d-alpha tocopherol; Sigma-Aldrich, St. Louis, MO; n=10 males and 10 females) in vehicle, or 0.1mL C E Ferulic (SkinCeuticals; n=10 males and 10 females) for 15 weeks with no additional UVB exposure. Surgilube is an inert vehicle used clinically as a surgical lubricant. Tumors larger than 1 mm in diameter were measured weekly with calipers. After sacrifice, 0.5cm$^2$ section of dorsal skin and all tumors were fixed as previously described [112] while remaining dorsal skin was snap frozen in liquid nitrogen.

3.2.2 Tumor Grading. Hematoxylin and Eosin (H&E)-stained tissue sections of tumors isolated from mice were graded in a blinded manner by a board-certified veterinary pathologist (DFK) as previously described [108] and in Chapter 2.

3.2.3 Catalase Activity Assay. Frozen dorsal skin was crushed in liquid nitrogen using a mortar and pestle and 15 mg was weighed out and used for analysis of catalase activity using the Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer’s instructions. This assay was described in detail in Chapter 2.

3.2.4 Total Antioxidant Power (Activity) Assay. Frozen dorsal skin was crushed in liquid nitrogen using a mortar and pestle and 10-15 mg was weighed out and used for analysis of total antioxidant activity using the Total Antioxidant Power Kit (Oxford Biomedical Research, Oxford, MI) according to manufacturer’s instructions. This assay measures the enzymatic activities of superoxide dismutase, catalase, and glutathione
peroxidase, the large molecules albumin, ferritin, and ceruloplasmin, as well as the small molecules ascorbic acid, alpha tocopherol, beta carotene, and uric acid. Briefly, 15 µl cold PBS, pH 7.0, per milligram sample weight was added to the crushed skin in a microcentrifuge tube on ice. Tissue was subjected to 3 rounds of sonication, pulsating for 10 seconds each round. Samples were centrifuged at 3,000 x g for 12 minutes at 4 °C and the supernatant was transferred to a new tube and stored at -80 °C. The uric acid standard curve was prepared, ranging from a concentration of 2 mM to 0 mM. Samples and standards were diluted 1:40 in the provided dilution buffer and loaded onto the plate in triplicate. The plate was read at 450 nm for a reference measurement and then incubated for 3 minutes at room temperature with copper solution. After adding stop solution to end the reaction, the plate was read again at 450 nm. Cu$^{+2}$ is effectively converted to Cu$^{+1}$ by the reduction potential of the samples and standards, which changes the absorption characteristics of the Cu ion. Cu$^{+1}$, the reduced form of copper, will selectively form a stable complex with the chromogenic reagent contained in the copper solution. This complex has a maximum absorption around 450 nm. The net absorbance was calculated by subtracting the reference absorbance measurement from the final absorbance measurement. Sample values were calculated in terms of mM uric acid equivalents by plotting the net absorbance values versus the uric acid concentrations. These values were then multiplied by 2189 µM to express the data in µM copper reducing equivalents.

3.2.5 Histological Techniques. Samples were fixed, embedded, and mounted on slides as described previously in Chapter 2.
Immunohistochemical detection of p53
Samples were examined for p53-positive foci as a measure of overall DNA damage as previously described in [166] and Chapter 2.

Immunohistochemical detection of Ki67
Samples were examined for Ki67-positive cells as a marker of cellular proliferation as previously described in [166] and Chapter 2.

Immunohistochemical detection of CD31
After rehydration, sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 15 min in a microwave. After slides were cooled, they were blocked with avidin D and biotin (Vector Laboratories), each for 15 minutes, 1× Casein for 30 minutes, and incubated with primary CD31 antibody (Abcam) at a 1:50 dilution in 1× Casein for 1 hour at room temperature. Slides were then incubated with biotinylated IgG (Vector Laboratories) at a 1:200 dilution in 1× Casein, followed by ABC Elite. Slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at RT. Slides were washed in deionized water, counterstained, and dehydrated. CD31-positive vessels were examined in 7 fields of view at 60× magnification.
3.2.6 Mouse Angiogenesis Array. Frozen dorsal skin was crushed in liquid nitrogen using a mortar and pestle and 20-40 mg was weighed out and used for analysis of angiogenesis-related proteins. Briefly, 800 µl NP-40 buffer (20mM Tris-HCl pH 8.0, 137mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA, 1X Protease Inhibitor, 1X Phosphatase Inhibitor, in dH₂O) was added to each sample in a microcentrifuge tube on ice and quickly homogenized. Samples were vortexed for 10 seconds every 5 minutes for a total of 50 minutes, followed by two rounds of centrifugation at 10,000 x g for 10 minutes at 4 °C, discarding the pellet after each centrifugation. Samples were diluted 1:10 in NP-40 buffer for use in a BCA (bicinchoninic acid) assay where the samples were compared to a serial dilution of BSA (bovine serum albumin) of known concentrations in order to determine the protein concentration of each sample. Each nitrocellulose membrane, spotted with duplicate angiogenesis-related and control antibodies, was incubated with Array Buffer 6 (included in the kit) for 1 hour on a rocking platform shaker, which served as a blocking buffer. Meanwhile, 300 µg of each protein sample in Array Buffer 6 was incubated with 0.5 ml Array Buffer 4 and 10 µl Detection Antibody Cocktail for 1 hour at room temperature. After the incubations, buffer was aspirated from the membranes and the appropriate sample/antibody mixtures were added and incubated overnight at 4 °C on a rocking platform shaker. Membranes were washed 3 times with Wash Buffer (included in the kit) for 10 minutes each to remove unbound material, after which each membrane was incubated with diluted Streptavidin-HRP for 30 minutes at room temperature on a rocking platform shaker. After repeating the wash steps, each membrane was incubated with West Femto Reagent Mix and chemiluminescence
imaging was carried out using the Bio-Rad ChemiDoc XRS to detect signals produced at each spot, which corresponds to the amount of protein bound. After imaging, membranes were stripped through incubating with Western Stripping Solution (stock solution-0.06M Trizma Base, 2% SDS; 70 µl Beta-Mercaptoethanol per 10 ml stock solution) for 30 minutes at 50 °C. Membranes were washed with Wash Buffer and imaged to ensure that the membranes were sufficiently stripped. This procedure was then repeated with a second set of samples. The average signal (pixel density) was determined for each pair of duplicate spots using ImageJ software (NIH). This value was divided by the average signal of the reference spots to normalize the values among the membranes. The adjusted values were then compared among the sample groups to determine potential differences in angiogenesis-related proteins as a result of the different topical treatments.

3.2.7 Statistical Analysis. The results presented in this paper were part of a larger experiment involving four treatment groups (of which vitamin E and C E Ferulic were two) and a single control group. The analyses were carried out by Gregory Young from the Center for Biostatistics at The Ohio State University, as described previously in Chapter 2. $p$-values $\leq 0.05$ were considered statistically significant.

3.3 Results

Effects of Topical Antioxidant Treatments on Tumor Development in Female Mice

3.3.1 C E Ferulic topical treatment decreased tumor number and burden in female mice. To examine the effects of topical vitamin E or C E Ferulic treatment as
preventative agents against tumor development, we exposed female Skh-1 hairless mice to 2240 J/m² UVB (previously determined to be 1 MED in our laboratory) three times weekly for ten weeks to model chronic sun exposure. Female mice were then treated topically with vehicle, vitamin E, or C E Ferulic for 15 weeks without further UVB exposure to model a lifestyle change. Non-irradiated female mice treated with either vehicle, vitamin E, or C E Ferulic did not develop tumors. Additionally, mice that were exposed to UVB but received no vehicle treatment did not exhibit a significantly different tumor burden compared to female mice treated with vehicle, indicating that the vehicle had no significant effect on tumorigenesis in this study (Figure 2.1).

Following 10 weeks of UV exposure and 15 weeks of preventative topical treatment with C E Ferulic, female mice developed 30.7% fewer tumors compared to the mice treated with vehicle ($p=0.0340$, Figure 3.1A). At the end of the study, female mice treated topically with C E Ferulic exhibited a 34% decrease in tumor burden compared to mice treated with vehicle (Figure 3.1B); however, probably as a result of variability due to the outbred nature of this strain of mice, the difference was not statistically significant ($p=0.6047$). Female mice treated topically with vitamin E demonstrated a trend toward increased tumor multiplicity with 14.9% more tumors compared to mice treated with vehicle ($p=0.3193$, Figure 3.1A). Female mice treated topically with vitamin E displayed a 20.7% increase in average tumor burden that was not statistically different from that in mice treated with vehicle ($p=0.9566$, Figure 3.1B).
Examining the change in tumor burden over time, we found that tumor growth rates did not significantly differ between female mice treated topically with C E Ferulic and those treated with vehicle (Figure 3.1C). Mice treated topically with vitamin E exhibited an

Figure 3.1 C E Ferulic decreases tumor number in female mice. Tumors with a diameter larger than 1mm were measured weekly with calipers. Mice were exposed to 2240 J/m² UVB three times weekly for 10 weeks followed by 15 weeks of topical C E Ferulic or vitamin E treatment. (A) Mice treated with C E Ferulic developed fewer tumors compared to mice treated with vehicle or vitamin E (*p=0.0283). (B) At the end of the study, mice treated with C E Ferulic exhibited a 34% decrease in tumor burden that was not statistically significant. (C) Mice treated with vitamin E exhibited an increased growth rate compared to mice treated with vehicle or C E Ferulic that approached significance (p=0.0649).
increase in tumor growth rate compared to mice treated with vehicle, 16.6% increase in tumor burden per week versus 11.1%, respectively, which approached significance \((p=0.0649, \text{Figure } 3.1C)\).

3.3.2 Female mice treated topically with C E Ferulic developed no malignant tumors. Tumors were isolated from mice after 10 weeks of UVB exposure followed by 15 weeks of topical treatment and scored as previously described in ([108] and Ch. 2) by a board-certified veterinary pathologist (DFK). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. As seen in Table 1, female mice treated with vehicle developed both benign and malignant lesions. Female mice preventatively treated with C E Ferulic developed only benign papillomas. Mice preventatively treated with vitamin E developed both papillomas and malignant lesions (Table 3.1).

<table>
<thead>
<tr>
<th></th>
<th>% Benign Tumors</th>
<th>% Malignant Tumors</th>
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</thead>
<tbody>
<tr>
<td>UVB/Vehicle</td>
<td>89.7</td>
<td>10.3</td>
</tr>
<tr>
<td>UVB/C E Ferulic</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>UVB/Vitamin E</td>
<td>97.1</td>
<td>2.9</td>
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Table 3.1 Distribution of benign and malignant tumors in female mice.

3.3.3 Topical vitamin E treatment increased catalase activity levels in female mice.

Protein was extracted from tumor-free, dorsal skin in order to examine catalase activity
levels. Catalase activity levels in female skin treated topically with C E Ferulic were not significantly altered compared to those observed in vehicle-treated skin \( (p=0.7631, \text{Figure 3.2A}) \). In contrast, catalase activity levels were significantly increased in female skin treated with vitamin E compared to vehicle-treated skin \( (p<0.0001, \text{Figure 3.2A}) \). Interestingly, total antioxidant activity levels were increased with both C E Ferulic and vitamin E treatment \( (p=0.0522 \text{ and } p=0.0074, \text{respectively; Figure 3.2B}) \). Recently, decreased catalase activity was linked with increased tumor burden, while increased catalase activity corresponded with a decrease in tumor burden in male Skh-1 mice [112]. This link was not observed in female mice [166].

3.3.4 Vitamin E topical treatment increased cutaneous proliferation in female mice.

To examine changes in proliferation rates, tumor-free, dorsal skin sections were stained for Ki67. The percentage of Ki67-positive cells was not significantly altered in C E Ferulic-treated female skin compared to vehicle-treated skin after 10 weeks of UVB exposure followed by 15 weeks of topical treatment with no additional UVB exposure. In contrast, the percentage of Ki67-positive cells exhibited a significant increase with topical vitamin E treatment compared to vehicle-treated mice \( (p=0.0004, \text{Figure 3.3}) \).

3.3.5 Topical vitamin E treatment increased the number of p53-positive foci in female skin. As a measure of total DNA damage, tumor-free, dorsal skin sections were examined for p53-positive foci via immunohistochemical analysis. Because the antibody used detected both wild type and mutant p53, some p53-positive foci represented
Figure 3.2 Vitamin E increases cutaneous catalase activity levels and total antioxidant activity. (A) Compared to mice treated with vehicle or C E Ferulic, topical vitamin E significantly increased catalase activity levels in female skin (*p<0.0001). (B) Mice treated with either C E Ferulic or vitamin E exhibited elevated total antioxidant activity (p=0.0522 and *p=0.0074, respectively).
Figure 3.3 Female skin treated with vitamin E displays increased levels of proliferation. Dorsal, tumor-free skin sections were stained for Ki67. Mice treated with vitamin E exhibited higher percentages of Ki67-positive cells compared to mice treated with vehicle or mice treated with C E Ferulic (*p=0.0004).
expanding clones of keratinocytes with mutated p53. The density of p53-positive foci in hairless mice has been shown to correlate well with skin tumor risk [167]. The mean number of p53 foci was not significantly altered with topical C E Ferulic treatment (Figure 3.4A) compared to vehicle-treated female mice (Figure 3.4B). The average number of p53-positive foci was significantly increased with preventative topical vitamin E treatment in female skin \( (p=0.0216, \) Figure 3.4C, quantified in Figure 3.4D), suggesting a greater risk of skin tumors in mice treated with vitamin E alone.

3.3.6 Topical vitamin E treatment increased the number of CD31-positive blood vessels in female skin. To examine changes in vasculature, tumor-free dorsal skin sections were examined for CD31-positive blood vessels via immunohistochemical analysis. Several studies have demonstrated a link between microvessel density, tumor growth, and metastasis [168,169,170]. The mean number of CD31-positive blood vessels was not significantly altered with topical C E Ferulic treatment (Figure 3.6A) compared to vehicle-treated female mice (Figure 3.6B). However, the average number of blood vessels was significantly increased with topical vitamin E treatment \( (p<0.0001, \) Figure 3.6C, quantified in Figure 3.6D).
Figure 3.4 Mice treated with topical vitamin E have increased numbers of p53-positive foci. Dorsal, tumor-free skin sections were examined via immunohistochemistry with an antibody detecting both wild type and mutant p53. Representative images of skin from mice treated with (A) vehicle, (B) C E Ferulic, and (C) vitamin E for 15 weeks after 10 weeks of UVB exposure. (D) The average number of p53-positive foci per field of view was significantly higher in mice treated with vitamin E compared to vehicle-treated mice (*p=0.0216).
Figure 3.5 Mice treated with topical vitamin E have increased numbers of CD31-positive vessels. Dorsal, tumor-free skin sections were examined for CD31-positive vessels via immunohistochemistry. Representative images of skin from mice treated with (A) vehicle, (B) C E Ferulic, and (C) vitamin E for 15 weeks after 10 weeks of UVB exposure. (D) The average number of CD31-positive vessels per field of view was significantly higher in mice treated with vitamin E compared to vehicle-treated mice (*p<0.0001).
3.3.7 C E Ferulic topical treatment decreased tumor burden in male mice. To examine the effects of topical vitamin E or C E Ferulic treatment as preventative agents against tumor development, we exposed male Skh-1 hairless mice to 2240 J/m² UVB (previously determined to be 1 MED in our laboratory) three times weekly for ten weeks to model chronic sun exposure. Mice were then treated topically with vehicle, vitamin E, or C E Ferulic for 15 weeks without further UVB exposure to model a lifestyle change. Non-irradiated male mice treated with either vehicle, vitamin E, or C E Ferulic did not develop tumors. Additionally, mice that were exposed to UVB but received no vehicle treatment did not exhibit a significantly different tumor burden compared to male mice treated with vehicle, indicating that the vehicle had no significant effect on tumorigenesis in this study (Figure 2.1).

Following 10 weeks of UV exposure and 15 weeks of preventative topical treatment with C E Ferulic, male mice developed 25% fewer tumors (Figure 3.6A) and exhibited a 54.8% decrease in tumor burden compared to mice treated with vehicle (Figure 3.6B); however, probably as a result of variability due to the outbred nature of this strain of mice, the difference was not statistically significant ($p=0.1945$ and $p=0.0836$, respectively). Male mice treated topically with vitamin E demonstrated a trend toward increased tumor multiplicity with 15.2% more tumors compared to mice treated with
Figure 3.6 C E Ferulic topical treatment decreases tumor burden in male mice. Tumors with a diameter larger than 1mm were measured weekly with calipers. Mice were exposed to 2240 J/m$^2$ UVB three times weekly for 10 weeks followed by 15 weeks of topical C E Ferulic or vitamin E treatment. (A) Mice treated with C E Ferulic or vitamin E did not develop fewer tumors compared to mice treated with vehicle. (B) At the end of the study, mice treated with C E Ferulic exhibited a 54.8% decrease in tumor burden that approached significance due to the outbred nature of this strain of mice (*$p=0.0836$). Mice treated with vitamin E exhibited a 25.5% decrease in tumor burden that was not significant ($p=0.8288$).
vehicle \((p=0.3060, \text{ Figure 3.6A})\). Interestingly, male mice treated topically with vitamin E displayed a 25.5\% decrease in average tumor burden that was not statistically different from that in mice treated with vehicle \((p=0.8288, \text{ Figure 3.6B})\).

### 3.3.8 Male mice treated topically with vitamin E developed the lowest percentage of malignant tumors.

Tumors were isolated from mice after 10 weeks of UVB exposure followed by 15 weeks of topical treatment and scored by a board-certified veterinary pathologist (DFK) as previously described in ([108] and Ch. 2). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. As seen in Table 2, male mice treated with vehicle developed both benign and malignant tumors including papillomas, microinvasive SCC, and fully invasive SCC. Male mice preventatively treated with C E Ferulic developed a lower percentage of microinvasive SCC, and therefore, a lower percentage of malignant tumors. Mice preventatively treated with vitamin E developed the lowest percentage of both fully invasive SCC and malignant tumors (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>% Benign Tumors</th>
<th>% Malignant Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB/Vehicle</td>
<td>80.7</td>
<td>19.3</td>
</tr>
<tr>
<td>UVB/C E Ferulic</td>
<td>86.8</td>
<td>13.2</td>
</tr>
<tr>
<td>UVB/Vitamin E</td>
<td>91.8</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Table 3.2 Distribution of benign and malignant tumors in male mice.
3.3.9 Topical vitamin E and C E Ferulic treatment increased total antioxidant activity levels in male mice. Protein was extracted from tumor-free, dorsal skin in order to examine total antioxidant activity levels. Interestingly, catalase activity levels were not significantly altered with either C E Ferulic or vitamin E treatment (Figure 3.7A). Total antioxidant activity levels were significantly increased in male skin treated topically with C E Ferulic and vitamin E ($p=0.0008$ and $p=0.0109$, Figure 3.7B) compared to those observed in vehicle-treated skin.

3.3.10 Topical C E Ferulic treatment decreased cutaneous PGE2 levels in male mice. To examine whether topical C E Ferulic or vitamin E treatment preventatively applied effectively inhibited COX-2-mediated PGE2 production, cutaneous PGE2 levels were determined by enzyme immunoassay. Skin of male exposed to UVB for 10 weeks and treated topically with C E Ferulic for 15 weeks exhibited significantly decreased PGE2 levels as compared with chronically irradiated, vehicle-treated mice ($p<0.0001$, Figure 3.8). Mice topically treated with vitamin E exhibited no significant change in PGE2 levels compared to mice treated with vehicle ($p=0.8314$, Figure 3.8).

3.3.11 Topical C E Ferulic and vitamin E treatment decreased cutaneous proliferation in male mice. To examine changes in proliferation rates, tumor-free, dorsal skin sections were stained for Ki67. The percentage of Ki67-positive cells was significantly decreased in C E Ferulic-treated skin and vitamin E-treated skin ($p<0.0001$ and $p=0.0109$, respectively; Figure 3.9) compared to vehicle-treated skin after 10 weeks.
Figure 3.7 Topical C E Ferulic and Vitamin E increase cutaneous total antioxidant activity in male mice. (A) Compared to mice treated with vehicle, catalase activity levels in male skin topically treated with C E Ferulic or vitamin E were not significantly altered. (B) Mice treated with either C E Ferulic or vitamin E exhibited elevated total antioxidant activity (*p=0.0008 and *p=0.0109, respectively).
Figure 3.8 Male skin treated with C E Ferulic displays decreased cutaneous PGE₉ levels. Cutaneous PGE₂ levels were determined by enzyme immunoassay after 10 weeks of UVB exposure and 15 weeks subsequent topical treatment. Skin of male mice exposed to UVB for 10 weeks and treated topically with C E Ferulic for 15 weeks exhibited significantly decreased PGE₂ levels as compared with chronically irradiated vehicle-treated skin (*p<0.0001).
Figure 3.9 Male skin treated with C E Ferulic and vitamin E display decreased levels of proliferation. Dorsal, tumor-free skin sections were stained for Ki67. Mice treated with both C E Ferulic and vitamin E exhibited decreased percentages of Ki67-positive cells compared to mice treated with vehicle (*p<0.0001 and *p=0.0109, respectively).
Figure 3.10 Male skin treated with C E Ferulic and vitamin E exhibit increased EGF. After 10 weeks of UVB exposure and 15 weeks of topical antioxidant treatment, protein was extracted from dorsal, tumor-free skin sections and subjected to the angiogenesis array. Representative arrays are shown. Compared to mice treated with vehicle (A), mice treated with both C E Ferulic (B) and vitamin E (C) exhibited increased percentages of EGF.
of UVB exposure followed by 15 weeks of topical treatment with no additional UVB exposure.

3.3.12 Topical C E Ferulic and vitamin E treatment increased angiogenesis-related proteins in male mice. To examine changes in angiogenesis-related proteins, relative cutaneous levels were determined using the Mouse Angiogenesis Array. Compared to mice treated with vehicle (Figure 3.12A), male mice topically treated with C E Ferulic (Figure 3.12B) and vitamin E (Figure 3.12C) exhibited increased amounts of epidermal growth factor (EGF).

3.4 Discussion

Previously, we demonstrated that treating female Skh-1 mice with Celebrex®, a topical anti-inflammatory drug, decreased DNA damage, tumor number and tumor grade [113]. We also recently showed decreased tumor number and burden in male and female mice treated with diclofenac, a different anti-inflammatory compound [166]. In the current study we examined the potential efficacy of topically applied antioxidant compounds in preventing tumor development in previously UVB-exposed skin of male and female mice. We showed that topically applied C E Ferulic, a stable antioxidant compound, protects chronically UVB-damaged male and female skin against skin tumor development. Tumor number and burden were decreased in C E Ferulic-treated mice compared to vehicle-treated mice. Confirming the results of several previous studies [155,156,157,158,159], topical vitamin E provided no beneficial effects with regard to
tumor burden in female mice; in fact, topical vitamin E alone resulted in increased tumor number and burden as well as increased DNA damage as indicated by p53 stabilization. Additionally, topical vitamin E treatment contributed to increased proliferation of epidermal cells, as well as an increase in angiogenesis in female mice. In contrast, in male mice, vitamin E topical treatment offered a trend toward an increase in tumor number while still contributing to moderately decreased cutaneous proliferation and tumor burden. Interestingly, while total antioxidant activity was increased with both vitamin E and C E Ferulic topical treatment in male and female skin, catalase activity was increased only with vitamin E treatment in female mice.

Because the skin is constantly exposed to external stimuli, it has developed a system of endogenous antioxidants to deal with environmental challenges. Antioxidants have been shown to effectively inhibit the initiation phase of carcinogenesis by detoxifying carcinogens, especially in chemically induced models [171]. Antioxidants also have an inhibitory role in the promotion phase where they scavenge ROS and work to prevent antioxidant network depletion [171].

Of these antioxidants, catalase has been the best studied in terms of its role in cutaneous homeostasis. Recently, we demonstrated an important link between restoring catalase activity after chronic UVB exposure and decreasing tumor burden in male but not female mice [112,166]. After chronic UVB exposure, catalase activity in male skin is decreased, which corresponds with an increase in tumor burden; however, topical treatments that
resulted in a restoration of catalase activity correlated with decreased tumor burden compared to vehicle-treated male mice. In female mice, however, catalase activity was not significantly altered with topical treatments that resulted in a decreased tumor burden [166]. In contrast, the current experiments have demonstrated that catalase activity levels in male mice are not changed with either topical antioxidant treatment compared to vehicle-treated mice though there were observed decreases in tumor burden. In the current study, the observed increase in catalase activity in female mice treated with vitamin E suggests that this antioxidant may actually be acting as a pro-oxidant. Antioxidant activity and efficacy depend heavily on the preexisting redox status of the environment [172], with several studies demonstrating that vitamin E may exert a pro-oxidant effect when it is administered under low or even normal levels of oxidative stress both in vitro [173,174] and in vivo [175,176]. Although we did not specifically measure oxidative stress in the current study, it is possible that low levels of oxidative stress in the skin following the cessation of UVB exposure resulted in the pro-oxidant effect with the application of the vitamin E treatment. The fact that female mice treated with C E Ferulic did not exhibit increased catalase activity levels but did demonstrate decreased tumor number and burden, while mice treated with vitamin E exhibited increased catalase activity levels and no beneficial effects on tumor burden, suggests that the observed increased catalase activity levels were not beneficial for decreasing tumor burden in female mice.
Interestingly, both male and female mice treated with either vitamin E or C E Ferulic demonstrated increased total antioxidant activity compared to vehicle-treated mice. This suggests that the observed increase in total antioxidant activity in the C E Ferulic-treated groups resulted from increases in additional endogenous antioxidants, such as SOD or glutathione. This increase in total antioxidant activity in the C E Ferulic-treated groups may have restored an appropriate balance of total antioxidant capacity. Manganese superoxide dismutase activity has been found to be decreased in hyperproliferative keratinocytes in squamous cell carcinoma [177]. Other cutaneous antioxidants, including vitamin C, vitamin E, and reduced glutathione, have also been shown to be decreased by up to 93% after UVB exposure [95]. Because the cutaneous antioxidant networks are exceedingly complex, further studies are needed to fully understand the interactions among the different endogenous antioxidants in the skin, as well as with exogenously applied antioxidants.

In addition to increasing catalase activity and total antioxidant activity, topical vitamin E increased the number of p53-positive foci observed in the epidermis of female mice, reflecting an increase in DNA damage. If vitamin E is exerting a pro-oxidant effect, increased alpha tocopheroxyl radicals may be contributing to increased levels of DNA damage. However, previous studies in male pigs as well as previous in vitro studies indicate that both vitamins E and C decrease the amount of UVB-induced oxidative DNA damage, specifically 8-hydroxy-2-deoxyguanosine adducts [150,178]. The fact that we saw no change in the level of overall DNA damage in C E Ferulic-treated mice but
elevated levels of DNA damage in vitamin E-treated mice, in addition to the fact that we observed no alterations in DNA damage in male skin, suggests that the concentrations of the vitamins utilized, as well as the delivery and treatment schedule, may play important roles in determining the efficacy of these antioxidants.

ROS, including alpha tocopheroxyl radicals, contribute to oxidative stress after UVB exposure. Previous studies suggested that alpha tocopheroxyl radicals generated from alpha tocopherol play a pivotal role in antioxidant-induced angiogenesis [179]. In the current study, the increased vessel density observed in vitamin E-treated mice further supports the mounting evidence that oxidative stress can act as a trigger for angiogenesis. Interestingly, increased levels of vitamin C have been demonstrated to prevent the increased angiogenesis observed with high vitamin E treatment concentrations; the suggested mechanism is scavenging of alpha tocopheroxyl radicals [172]. While we only observed a change in angiogenesis with the vitamin E treatment in female mice, it is possible that the C E Ferulic is scavenging tocopheroxyl radicals at a rate that prevents an increase in angiogenesis but is not sufficient to cause a decrease. Interestingly, we observed increased levels of epidermal growth factor in male skin treated with both C E Ferulic and vitamin E compared to vehicle-treated mice. The highest levels of EGF were observed in male mice topically treated with vitamin E, which correlates to the increased tumor number we observed. EGF is a ligand for the EGFR, whose activation induces a signaling pathway important for the tumorigenesis process, affecting proliferation, cell survival, and angiogenesis. It has previously been demonstrated that UVB-induced
hydrogen peroxide production is an important mediator required for the phosphorylation and thus, activation of EGFR [140,141], implicating a potential therapeutic role for antioxidants in combating the activation of EGFR and the resulting enhanced tumorigenesis. Trolox, a vitamin E analog, enhanced UVB-induced EGFR phosphorylation despite its hydrogen peroxide-suppressing activity, suggesting that other radicals, including Trolox radicals, as well as the interaction of Trolox with antioxidants generated by normal cellular processes may affect the status of Trolox as an EGFR inhibitor. This study provides evidence for Trolox differentially modulating signaling pathways in response to UVB due to the complex nature of antioxidant effective concentrations [146]. This previous report may help to explain the differential tumor effects of vitamin E and C E Ferulic treatment in the current study due to the differing concentrations and stability of the antioxidants.

With the ever-increasing skin cancer incidence, antioxidant supplementation in food, sunscreens, and lotions has become widespread. While some animal models suggest beneficial effects from antioxidants, it is important to note that antioxidants are delivered prior to any UV exposure in many of these studies [149,153], which is not the usual way humans are exposed to antioxidants and UV. Clinical trials examining potential effects of antioxidant supplementation have yielded contradictory results. The Supplementation in Vitamins and Mineral Antioxidants (SU.VI.MAX) study revealed that daily supplementation with nutritional doses of antioxidants decreased the overall incidence of cancer in men, but had no effect in women [180]. Further, the impact of antioxidant
supplementation on skin cancer incidence, specifically, was examined within the framework of the SU.VI.MAX study [181], revealing an increased incidence of skin cancer in women and a trend towards decreased skin cancer in men receiving antioxidant supplements. The current study supports these findings in that there is a trend towards increased tumor burden in female mice treated with vitamin E, a single antioxidant. Also in support of these findings, male mice treated topically with C E Ferulic, a combination antioxidant, exhibited decreased tumor burden. In contrast to these findings, female mice treated with C E Ferulic exhibited decreased tumor number. Interestingly, male mice treated with vitamin E exhibited a trend toward a decrease in tumor burden and, in contrast, an increase in tumor number.

These data may partially be explained by the fact that cutaneous proliferation is decreased in male mice treated topically with vitamin E. Hypoxia inducible factor (HIF-1alpha), a transcription factor that plays a key role in cutaneous homeostasis, induces apoptosis independently of p53, which was not found to be significantly altered in male mice. HIF-1alpha has been implicated in the blockage of keratinocyte proliferation under hypoxic conditions [182,183]. ROS have been shown to be key players in the regulation of HIF-1alpha accumulation in UVB-damaged keratinocytes, but it is not clear which type of ROS or the exact redox-regulated signaling mechanisms are involved [184]. Multistage murine skin carcinogenesis was greatly inhibited in transgenic mice with a HIF-1alpha gain of function that was targeted in the basal keratinocytes. These mice demonstrated papilloma outgrowth that was attributed to the pro-angiogenic nature of the
function of HIF-1alpha; however, these premalignant lesions were more differentiated than control mice, had decreased proliferation rates, and a lower percentage of tumors became malignant [182].

The delivery method of antioxidant supplements also seems to play a role in the study outcomes. A systematic review of randomized controlled trials reported that there was no beneficial effect of oral vitamin or antioxidant supplementation on skin cancer prevention but that topical antioxidant application did offer some degree of protection in high risk individuals [185]. Our current study supported these findings, in that male and female mice with chronically UVB-damaged skin that were treated topically with C E Ferulic had decreased tumor burden compared to vehicle-treated mice. The current study also demonstrated that vitamin E topical treatment decreased tumor burden in male mice, but increased tumor burden in female mice, highlighting a sex-based difference in response to antioxidant topical treatments. Further, the antioxidant concentration and activity in the various products varied greatly; thus, standardized testing and labeling will be required to allow consumers to more easily compare these products [186]. Further studies are needed to understand antioxidant activity in vivo and to measure topical antioxidant efficacy with the consideration of differences between males and females.

In summary, we have shown in a model of UVB-induced SCC that topically treating male and female Skh-1 hairless mice with C E Ferulic decreased tumor number and burden and suppressed the formation of malignant tumors in female mice. In contrast,
treating with topical vitamin E had no therapeutic benefits for female mice, and, in fact, resulted in increased overall DNA damage and vessel density, and tended to increase tumor number, burden and growth rate, possibly due to the pro-oxidant effects of vitamin E. In male mice, however, we observed decreased tumor burden and a decrease in the percentage of fully invasive SCCs.

Because of the focus on antioxidant supplementation in many products targeted towards women, these findings are especially relevant. Overall, our data suggest that topically treating chronically UVB-damaged skin with 5% vitamin E alone may actually promote SCC development in females but may offer moderate beneficial effects in males. Since many previous studies do not describe which sex was used, our findings may help explain previous contradictory evidence regarding antioxidant supplementation and cancer incidence.
Chapter 4: Mice Develop More Tumors When Exposed to UVB for 25 Weeks Compared to 10 Weeks

4.1 Introduction

The development of cutaneous squamous cell carcinoma has been linked with cumulative, lifetime sunlight exposure. These data have come from epidemiological studies where patients self-report the amount of sun they have been exposed to over their lifetime. While informative, with patients historically developing these lesions in their 70s, these self-reports may not reflect the actual exposure history over the course of their lifetime. Interestingly, we could not find any studies reporting the effects of UVB exposure length on the extent of tumor development.

Skin carcinogenesis experiments utilizing animal models, especially hairless mice, have contributed greatly to understanding how skin tumorigenesis depends on the wavelength of UV radiation, dose, and time [43]. The Skh-1 hairless mouse model has proven to be an appropriate and accepted model for experimental skin carcinogenesis. The drawback to using haired mice is that the hair-covered skin is nearly impenetrable by UV, and shaving or using depilatories to remove the hair can introduce unwanted confounding effects on tumor formation. Because the Skh-1 mice are hairless, tumors can easily be observed and their progression tracked over time with relatively no discomfort for the
mice. Importantly, unlike in haired mice, the induction of tumors via chronic exposure of non-burn-inducing low UVB levels can be directly correlated to regular occurrences in human life. Furthermore, in this mouse strain repeated UV exposure induces precursors and skin carcinomas originating from the epidermis, which is also seen in UV-induced human skin cancer.

Skin tumors induced by chronic exposure to UV radiation progress from focal epithelial hyperplasia to papillomas and finally squamous cell and spindle cell carcinomas [187]. Previous studies in our laboratory using female mice have demonstrated that papilloma growth begins around 10-12 weeks of three times weekly UVB exposures. Approximately 90% of both male and female mice have developed at least one tumor with a diameter greater than 1 mm after 16 weeks of three times weekly UVB exposures, with males having approximately 50% more tumors than females [108]. Squamous cell carcinoma development has been observed around 25-30 weeks of three times weekly UVB exposures [113], with males having more malignant tumors compared to females [108]. However, the effects of the length of UVB exposure on tumor burden in males and females, while presumed, have not been formally investigated.

Previous studies have demonstrated that decreasing the minimal erythemic dose (MED) results in the delay of tumor onset, indicating that patients with SCC may benefit from decreasing the amount of UVB to which they are exposed [188,189]. Further, increasing the dose of UV resulted in a shorter latency period, increased DNA damage and p53
mutations, as well as increased tumor number [190]. The focus of these previous studies was mainly the time to tumor onset by altering the MED. What has not been investigated is whether the overall tumor number or burden, examined at the end of 25 weeks, will be different between mice exposed to 1 MED of UVB for either 10 weeks or the entire 25 weeks. That is, we were interested in investigating whether men and women who had large amounts of UVB exposure during childhood and early adolescence but made efforts to stay out of the sun in adulthood would lessen the number of tumors they develop compared to people who continue sun worshiping habits throughout adult life. Further, we wanted to examine the extent to which these outcomes would be the same between men and women.

We set out to examine possible differences in tumorigenesis at 25 weeks, comparing 10 or 25 weeks of exposure to 1 MED of UVB three times weekly on nonconsecutive days. In the current study, we have modeled both male and female compliant patients who have chronically UVB-damaged skin but have made lifestyle changes to eliminate further UVB exposure. We have also modeled both male and female non-compliant patients who continued to be exposed to UVB even after the appearance of lesions. In addition, we sought to investigate the efficacy of the anti-inflammatory (diclofenac) and antioxidant (C E Ferulic and vitamin E) treatments (as described in Chapters 2 and 3, respectively) in the non-compliant model where UVB exposure is continued throughout the study. Our results demonstrate that both male and female mice in the non-compliant patient model that were exposed to 25 weeks of UVB developed more tumors, larger tumors, and a
higher percentage of malignant tumors compared to mice in the compliant patient model that were exposed to 10 weeks of UVB. The percentage of both total p53- and mutant p53-positive cells was elevated in mice exposed to 25 weeks of UVB compared to mice exposed to 10 weeks of UVB. Further, diclofenac continued to be effective for decreasing both tumor number and burden in male and female mice with concurrent topical treatment and UVB exposure. In contrast, male and female mice treated with antioxidants exhibited no beneficial effects in terms of tumor number and burden compared to vehicle-treated mice. Taken together, these data support the commonly assumed, but not demonstrated, fact that cumulative UVB exposure is a risk factor for UVB-induced SCC and highlight the fact that changing sun worshiping habits, even after early chronic sun exposure and skin damage, may ultimately decrease tumor development in patients.

4.2 Materials and Methods

4.2.1 Animal Treatments and Experimental Design. Outbred, male and female Skh-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee before the initiation of any studies. Mice were dorsally exposed to 2240 J/m² UVB, previously determined to be 1 MED, 3× weekly on non-consecutive days for 10 (compliant patient model) or 25 (non-compliant patient model) weeks. UVB dose was calculated using UVX radiometer and UVB sensor (UVP, Upland, CA) and emitted by Phillips FS40 UV bulbs (American
Ultraviolet Company, Lebanon, IN). After 10 weeks of UVB exposure, mice in the compliant patient model were treated topically with vehicle (Surgilube®; Savage Laboratories, Melville, NY), 500 μg diclofenac (Solaraze®) in vehicle, 5mg vitamin E (d-alpha tocopherol; Sigma-Aldrich, St. Louis, MO) in vehicle, or 0.1mL C E Ferulic (SkinCeuticals) for 15 weeks with no additional UVB exposure. After 10 weeks of UVB exposure mice in the non-compliant patient model were treated topically with the aforementioned agents immediately following each UVB exposure for the remaining 15 weeks of the study. Surgilube is an inert vehicle used clinically as a surgical lubricant. Tumors larger than 2 mm in diameter were measured weekly with calipers. After sacrifice, 0.5cm\(^2\) section of dorsal skin and all tumors were fixed as previously described [112] while remaining dorsal skin was snap frozen in liquid nitrogen.

4.2.2 Tumor Grading. Hematoxylin and Eosin (H&E)-stained tissue sections of tumors isolated from mice were graded in a blinded manner by a board-certified veterinary pathologist (DFK) as previously described [108] and in Chapter 2.

4.2.3 Histological Techniques. Samples were fixed, embedded, and mounted on slides as described previously in Chapter 2.

_Immunohistochemical detection of p53_

Samples were examined for p53-positive foci as a measure of overall DNA damage as previously described in [166] and Chapter 2.
Immunohistochemical detection of Ki67

Samples were examined for Ki67-positive cells as a marker of cellular proliferation as previously described in [166] and Chapter 2.

Immunohistochemical detection of mutant p53

After rehydration, sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 15 min in a microwave. After slides were cooled, they were blocked with avidin D and biotin (Vector Laboratories), each for 15 minutes, 1× Casein for 30 minutes, and incubated with primary CD31 antibody (Abcam) at a 1:50 dilution in 1× Casein for 1 hour at room temperature. Slides were then incubated with biotinylated IgG (Vector Laboratories) at a 1:200 dilution in 1× Casein, followed by ABC Elite. Slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at RT. Slides were washed in deionized water, counterstained, and dehydrated.

4.2.4 Statistical Analysis. The results presented in this paper were part of two separate experiments involving four treatment groups and a single control group. The major limitation of these comparisons is that the groups were not randomized to the two exposure protocols and the experiments were run at different times. The analyses were carried out by Gregory Young from the Center for Biostatistics at The Ohio State University. No adjustments were made for multiple comparisons. A Poisson regression
was used to model the number of tumors present at the end of the study. *p*-values ≤0.05 were considered statistically significant.

### 4.3 Results

4.3.1 Effects of topical antioxidant and anti-inflammatory treatments on tumor number and burden in male mice after 25 weeks of UVB exposure. To examine the efficacy of topical diclofenac, C E Ferulic, or vitamin E treatment as preventative agents in a non-compliant patient model, we exposed male Skh-1 hairless mice to 2240 J/m² UVB (previously determined to be 1 MED in our laboratory) three times weekly for a total of 25 weeks to model chronic sun exposure. Mice were exposed only to UVB for 10 weeks and then treated topically with vehicle, diclofenac, C E Ferulic, or vitamin E immediately following each UVB exposure for 15 weeks to model a man who is frequently exposed to UVB but is attempting to prevent the development of tumors. Non-irradiated male mice treated with vehicle, diclofenac, vitamin E, or C E Ferulic did not develop tumors.

Following 10 weeks of UV exposure alone and 15 weeks of preventative topical treatment with concurrent UVB exposures, male mice treated topically with diclofenac developed 77% fewer tumors compared to mice treated with vehicle (*p*<0.0001, Figure 4.1A). At the end of the study, male mice treated topically with diclofenac exhibited an 80% reduction in tumor burden compared to vehicle-treated mice (*p*=0.0002, Figure 4.1C). Male mice treated topically with C E Ferulic did not exhibit a significant alteration
in tumor number (Figure 4.1A) but displayed a 60% increase in tumor burden compared to vehicle-treated mice, which due to the variability in this outbred mouse strain, did not reach statistical significance ($p=0.5590$, Figure 4.1C). Male mice treated with vitamin E exhibited a 20% increase in tumor number compared to mice treated with vehicle that trended toward significance ($p=0.0813$, Figure 4.1A). Male mice treated topically with vitamin E displayed a 30% increase in average tumor burden; however, this decrease was not statistically different from that in mice treated with vehicle ($p=0.9100$, Figure 4.1C).

4.3.2 **Effects of topical antioxidant and anti-inflammatory treatments on tumor number and burden in female mice after 25 weeks of UVB exposure.** To examine the efficacy of topical diclofenac, C E Ferulic, or vitamin E treatment as preventative agents in a non-compliant patient model, we exposed female Skh-1 hairless mice to 2240 J/m$^2$ UVB (previously determined to be 1 MED in our laboratory) three times weekly for 25 weeks to model chronic sun exposure. Mice were treated topically with vehicle, diclofenac, C E Ferulic, or vitamin E immediately following each UVB exposure, beginning after week 10, for 15 weeks to model a woman who is frequently exposed to UVB but is attempting to prevent the development of tumors. Non-irradiated female mice treated with vehicle, diclofenac, vitamin E, or C E Ferulic did not develop tumors.

Following 10 weeks of UV exposure alone and 15 weeks of preventative topical treatment with concurrent UVB exposures, female mice treated topically with diclofenac developed 55% fewer tumors compared to mice treated with vehicle ($p<0.0001$, Figure
Figure 4.1 Diclofenac topical treatment decreased tumor number and burden in male and female mice after 25 weeks of UVB exposure. Mice were exposed to UVB for 25 weeks and beginning after week 10, received topical treatment immediately following each UVB exposure for the remaining 15 weeks. Tumors were examined weekly. (A) Male mice treated topically with diclofenac exhibited decreased tumor multiplicity compared to vehicle-treated mice (*p<0.0001). (B) Female mice treated with diclofenac exhibited decreased tumor multiplicity compared to vehicle-treated mice (p<0.0001). (C) Male mice topically treated with diclofenac exhibited decreased tumor burden compared to vehicle-treated mice (p=0.0002). (D) Female mice treated with topical diclofenac exhibited decreased tumor burden compared to mice treated with vehicle (p=0.0186).
4.1B). At the end of the study, female mice treated topically with diclofenac exhibited a 68% reduction in tumor burden compared to vehicle-treated mice ($p=0.0186$, Figure 4.1D). Female mice treated topically with C E Ferulic did not exhibit a significant alteration in tumor number (Figure 4.1B) or average tumor burden (Figure 4.1D). Female mice treated with vitamin E did not display a significant alteration in tumor number (Figure 4.1B). Female mice treated topically with vitamin E displayed a 43% increase in average tumor burden compared to mice treated with vehicle; however, probably due to the outbred nature of the Skh-1 mouse strain, was not statistically different ($p=0.7961$, Figure 4.1D).

4.3.3 Diclofenac topical treatment increased the malignancy rate in female mice. After 25 weeks of UVB exposure, tumors were isolated from mice and scored as previously described in ([108] and Ch. 2) by a board-certified veterinary pathologist (DFK). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. Female mice treated topically with diclofenac displayed a 90% higher malignancy rate compared to vehicle-treated mice ($p=0.0227$, Table 4.1). Male mice treated topically with diclofenac also exhibited a larger percentage of malignant tumors, with a 50% increase in malignancy rate compared to vehicle-treated male mice, which due to the variability observed in this outbred mouse strain, was not statistically significant ($p=0.1016$). Female mice treated topically with vitamin E exhibited a 28% increase in malignancy rate compared to vehicle-treated mice and male mice treated topically with C E Ferulic displayed a 20%
increase in malignancy rate compared to vehicle treated mice, neither of which reached statistical significance ($p=0.2816$ and $p=0.3391$, respectively).

<table>
<thead>
<tr>
<th></th>
<th>% Benign Tumors</th>
<th>% Malignant Tumors</th>
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Table 4.1 Distribution of benign and malignant tumors in male and female mice after 25 weeks of UVB exposure.

4.3.4 Male mice treated topically with an anti-inflammatory drug or with antioxidants in the non-compliant model developed more tumors compared to mice in the compliant model. To examine the effects of increased UVB protocol length on tumor development, we exposed male Skh-1 hairless mice to 2240 J/m² UVB (previously determined to be 1 MED in our laboratory) three times weekly for 10 or 25 weeks to model chronic sun exposure. Mice were then treated topically with vehicle, diclofenac, vitamin E, or C E Ferulic for 15 weeks without further UVB exposure to model a lifestyle change in the compliant model or immediately after each UVB exposure for 15 weeks in
the non-compliant model. Non-irradiated male mice treated with vehicle, diclofenac, vitamin E, or C E Ferulic did not develop tumors.

Male mice treated with vehicle in the non-compliant model developed 1.53-fold more tumors compared to vehicle-treated mice in the compliant model ($p=0.0002$, Figure 4.2A). Mice treated with topical diclofenac in the non-compliant model developed a 1.18-fold higher tumor number compared to mice treated with diclofenac in the compliant model that was not significant, possibly due to the effectiveness of the treatment ($p=0.4800$, Figure 4.2B). Mice treated topically with C E Ferulic displayed a 1.69-fold increase in average tumor number in the non-compliant model compared to mice in the compliant model ($p=0.0009$, Figure 4.2C). Mice treated topically with vitamin E in the non-compliant model demonstrated 1.58-fold increased tumor number compared to mice in the compliant model ($p=0.0007$, Figure 4.2D).

4.3.5 Female mice treated topically with C E Ferulic in the non-compliant model developed more tumors compared to mice in the compliant model. To examine the effects of increased UVB protocol length on tumor development, we exposed female Skh-1 hairless mice to 2240 J/m$^2$ UVB (previously determined to be 1 MED in our laboratory) three times weekly for 10 or 25 weeks to model chronic sun exposure. Mice were then treated topically with vehicle, diclofenac, vitamin E, or C E Ferulic for 15 weeks without further UVB exposure to model a lifestyle change in the compliant model or immediately after each UVB exposure for 15 weeks in the non-compliant model. Non-
irradiated female mice treated with vehicle, diclofenac, vitamin E, or C E Ferulic did not develop tumors.

Female mice treated with vehicle in the non-compliant model developed 1.15-fold more tumors compared to vehicle-treated mice in the compliant model ($p=0.2280$, Figure 4.2A). Mice treated with topical diclofenac in the non-compliant model developed a 1.43-fold higher tumor number compared to mice treated with diclofenac in the compliant model that was not significant, possibly due to the effectiveness of the treatment ($p=0.1838$, Figure 4.2B). Female mice treated topically with C E Ferulic displayed a 1.92-fold increase in average tumor number in the non-compliant model compared to mice in the compliant model ($p=0.0003$, Figure 4.2C). Mice treated topically with vitamin E in the non-compliant model demonstrated 1.02-fold increased tumor number compared to mice in the compliant model ($p=0.9258$, Figure 4.2D).

### 4.3.6 Male mice in all treatment groups in the non-compliant model exhibited a larger tumor burden compared to mice in the compliant model.

Male mice treated with vehicle in the non-compliant model developed 2.80-fold higher tumor burden compared to vehicle-treated mice in the compliant model ($p=0.0011$, Figure 4.3A). Mice treated with topical diclofenac in the non-compliant model developed a 3.12-fold higher tumor burden compared to mice treated with diclofenac in the compliant model ($p=0.0061$, Figure 4.3B). Mice treated topically with C E Ferulic displayed a 9.87-fold increase in average tumor burden in the non-compliant model compared to mice in the
compliant model ($p<0.0001$, Figure 4.3C). Mice treated topically with vitamin E in the non-compliant model demonstrated a 4.85-fold increased tumor burden compared to mice in the compliant model ($p=0.0002$, Figure 4.3D).

4.3.7 Female mice in all treatment groups in the non-compliant model exhibited a larger tumor burden compared to mice in the compliant model. Female mice treated with vehicle in the non-compliant model developed 3.82-fold higher tumor burden compared to vehicle-treated mice in the compliant model ($p<0.0001$, Figure 4.3A). Mice treated with topical diclofenac in the non-compliant model developed a 2.56-fold higher tumor burden compared to mice treated with diclofenac in the compliant model ($p=0.0258$, Figure 4.3B). Mice treated topically with C E Ferulic displayed a 5.38-fold increase in average tumor burden in the non-compliant model compared to mice in the compliant model ($p<0.0001$, Figure 4.3C). Mice treated topically with vitamin E in the non-compliant model demonstrated a 4.53-fold increased tumor burden compared to mice in the compliant model ($p=0.0004$, Figure 4.3D).

4.3.8 Male and female mice in the non-compliant model developed more malignant tumors compared to mice in the compliant model. Tumors were isolated from mice at the end of 25 weeks from both the compliant and non-compliant models and scored as previously described in ([108] and Ch. 2) by a board-certified veterinary pathologist (DFK). Tumors classified as papilloma were considered benign and those classified as microinvasive or fully invasive SCC were considered malignant. Male and female mice
Mice were exposed to 2240 J/m² three times weekly for 10 weeks followed by 15 weeks of topical treatment with no further UVB exposures (black bars) or mice were exposed to UVB for 25 weeks and beginning after week 10, received topical treatment immediately following each UVB exposure for the remaining 15 weeks (gray bars). Tumors were examined weekly. (A) Male mice treated topically with vehicle exhibited increased tumor multiplicity after 25 weeks of UVB exposure compared to mice exposed to 10 weeks of UVB (*p=0.0002). (B) Mice treated with diclofenac did not develop significantly different numbers of tumors between the UVB protocols. (C) Male and female mice topically treated with C E Ferulic developed more tumors after 25 weeks of UVB exposure compared to 10 weeks of UVB exposure (p=0.0009). (D) Male mice treated with topical vitamin E developed more tumors after 25 weeks of UVB exposure compared to 10 weeks of UVB exposure (p=0.0007).
Figure 4.3 Male and female mice exposed to 25 weeks of UVB exhibit increased tumor burden. Mice were exposed to 2240 J/m² three times weekly for 10 weeks followed by 15 weeks of topical treatment with no further UVB exposures (black bars) or mice were exposed to UVB for 25 weeks and beginning after week 10, received topical treatment immediately following each UVB exposure for the remaining 15 weeks (gray bars). Tumors were measured weekly with calipers. (A) Male (*p=0.0011) and female (*p<0.0001) mice treated topically with vehicle exhibited increased tumor multiplicity after 25 weeks of UVB exposure compared to mice exposed to 10 weeks of UVB. (B) Male (*p=0.0061) and female (*p=0.0258) mice treated with diclofenac developed significantly larger tumor burden after 25 weeks of UVB exposure. (C) Male and female mice topically treated with C E Ferulic developed more tumors after 25 weeks of UVB exposure compared to 10 weeks of UVB exposure (*p<0.0001). (D) Male (*p=0.0002) and female (*p=0.0004) mice treated with topical vitamin E developed more tumors after 25 weeks of UVB exposure compared to 10 weeks of UVB exposure.
treated topically with vehicle in the non-compliant model developed more malignant
tumors per mouse compared to vehicle-treated mice in the compliant model, with males
developing 1.9-fold more malignant tumors and females developing 3.05-fold more
tumors ($p=0.0467$ and $p=0.0319$, respectively, Table 4.2). Male mice topically treated
with diclofenac developed 9.79-fold more malignant tumors ($p=0.0262$), mice treated
with C E Ferulic developed 3.46-fold more tumors ($p=0.0087$), and mice treated topically
with vitamin E developed 4.08-fold more tumors ($p=0.0028$) compared to vehicle-treated
male mice. Female mice treated topically with diclofenac developed 2.43-fold more
malignant tumors compared to vehicle-treated mice; however, due to both the smaller
amount of tumors developed and the inherent variable nature of this outbred strain, this
difference was not statistically significant ($p=0.1565$). Female mice treated with C E
Ferulic developed 10.71-fold more malignant tumors ($p=0.0196$) and mice treated
topically with vitamin E developed 14.38-fold more malignant tumors ($p=0.0087$)
compared to vehicle-treated female mice.
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Table 4.2 Comparison of malignant tumor grade distribution after 10 and 25 weeks of UVB exposure in male and female mice.

4.3.9 Male and female mice in the non-compliant model exhibited elevated levels of p53. As a measure of overall DNA damage, tumor-free, dorsal skin sections were examined for p53-positive cells via immunohistochemical analysis. Male mice treated with vehicle exhibited a 3.74-fold increase in p53-positive area after 25 weeks of UVB exposure compared to vehicle-treated mice after 10 weeks of UVB exposure ($p=0.0002$, Figure 4.4A), while females exhibited a 2.52-fold increase after 25 weeks of UVB exposure compared to female mice after 10 weeks of UVB exposure ($p=0.011$, Figure 4.4A). Neither female mice treated with topical antioxidants nor male mice treated topically with diclofenac exhibited significant alterations in p53-positive area between the two models. However, female mice treated topically with diclofenac exhibited a 2-fold increase in p53-positive area, which due to the variability in this mouse strain, was not statistically significant ($p=0.1683$, Figure 4.4B). Interestingly, male mice treated with
topical C E Ferulic \((p=0.062, \text{ Figure 4.4C})\) or vitamin E \((p=0.0023, \text{ Figure 4.4D})\) exhibited increased p53-positive cells after 25 weeks of UVB exposure compared to the mice in the compliant model.

4.3.10 Male and female mice in the non-compliant model displayed increased mutant p53-positive cells. To further investigate DNA damage, tumor-free, dorsal skin sections were examined for mutant p53-positive cells via immunohistochemistry. Both male and female mice in the non-compliant model exhibited increased levels of cutaneous mutant p53 after 25 weeks of UVB exposure; however, due to the variability resulting from this outbred mouse strain, many of the comparisons were not statistically significant. Male and female mice treated with vehicle displayed increased levels of mutant p53-positive cells after 25 weeks of UVB exposure compared to mice after 10 weeks of UVB exposure \((p<0.0001, \text{ Figure 4.5A})\). Male and female mice treated topically with diclofenac (Figure 4.5B) or vitamin E (Figure 4.5D) did not exhibit significantly altered mutant p53 staining. Female mice treated with C E Ferulic exhibited increased mutant p53-positive cells after 25 weeks of UVB exposure compared to female mice exposed to UVB for 10 weeks \((p=0.0183)\).
Figure 4.4 Male and female mice in the non-compliant model exhibit elevated levels of p53. Dorsal, tumor-free skin sections were examined via immunohistochemistry with an antibody detecting p53. ImageJ software was used to calculate the % positive area. Black bars represent mice in the compliant model and gray bars represent mice in the non-compliant model. (A) Male (*p=0.002) and female (*p=0.011) mice treated with vehicle in the non-compliant model exhibited elevated p53 staining. (B) Male and female mice treated with diclofenac did not exhibit significantly altered p53-positive area. (C) Male mice treated topically with C E Ferulic exhibited increased levels of p53-positive area in the non-compliant model (*p=0.0062) (D) Male mice treated with vitamin E exhibited increased p53-positive area in the non-compliant model compared to mice in the compliant model (*p=0.0023)
Figure 4.5 Male and female mice in the non-compliant model display increased levels of mutant p53-positive cells. Dorsal, tumor-free skin sections were examined via immunohistochemistry with an antibody detecting mutant p53. ImageJ software was used to calculate the % positive area. Black bars represent mice in the compliant model and gray bars represent mice in the non-compliant model. (A) Male and female mice treated with vehicle in the non-compliant model exhibited elevated mutant p53 staining (*p<0.0001). (B) Male and female mice treated with diclofenac did not exhibit significantly altered mutant p53-positive area. (C) Female mice treated topically with C E Ferulic exhibited increased levels of mutant p53-positive area in the non-compliant model (*p=0.0183). (D) Male and female mice treated with vitamin E did not exhibit significantly altered mutant p53-positive area in the non-compliant model compared to mice in the compliant model.
4.4 Discussion

Previously, we demonstrated that the topical anti-inflammatory drug, diclofenac, applied preventatively to male and female mice with chronically UVB-damaged skin, but without the appearance of lesions, and with no further UVB exposure, significantly decreased tumor number and burden compared to vehicle-treated, UVB-exposed mice [166]. In the current study, we showed in a non-compliant model where mice were exposed to UVB alone for 10 weeks, followed by 15 weeks of topical diclofenac treatment immediately following each further UVB exposure, that topical diclofenac treatment continued to effectively decrease tumor number and burden in both male and female mice compared to mice treated with vehicle. In contrast, male and female mice treated with topical antioxidants did not exhibit any beneficial effects in terms of tumor number or burden, and in fact, trended toward increases in both parameters. Interestingly, the previously reported trend toward an increased malignancy rate in female mice treated topically with diclofenac [166] was exacerbated in the current study, where mice exposed to UVB for 25 weeks and treated topically with diclofenac exhibited a 90% increase in malignancy rate compared to vehicle-treated female mice. Male mice exposed to UVB and treated with topical diclofenac were previously demonstrated to have a decreased malignancy rate compared to vehicle-treated mice [166]; however, with 25 weeks of UVB exposure, male mice exhibited a 50% increase in malignancy rate compared to vehicle-treated mice. Many previous studies that examine possible therapeutics or preventative treatments for cancers examine the number and size of tumors that appear but do not discuss the possibility of small tumors being of a malignant grade. These data highlight the
importance of histologically examining tumors that form in groups where treatment was effective for decreasing tumor number or burden.

In Chapter 3, we demonstrated that the combination antioxidant, C E Ferulic, exerted potential benefits in terms of decreased tumor number and burden in both male and female mice. Further, we revealed that while moderate benefits were observed with vitamin E topical treatment in male mice, female mice treated topically with vitamin E exhibited increased overall DNA damage, cutaneous proliferation and angiogenesis, as well as trends toward increased tumor growth rate, number, and burden. With 25 weeks of UVB exposure in the current study, however, any potential benefits of antioxidant treatment in terms of tumor number, burden, and grade were lost in both male and female mice. These data highlight the importance of eliminating UVB exposure in order to experience therapeutic benefits of antioxidant treatment.

A previous report demonstrated that increasing the dose of UV resulted in a shorter latency period, increased DNA damage and p53 mutations, as well as increased tumor number [190]. The current study demonstrated an increased tumor number, burden, and grade in male and female Skh-1 mice exposed to 2240 J/m² UVB three times weekly for 25 weeks compared to 10 weeks. The current study validates data extracted from patient questionnaires in experiments where the extent of UVB exposure was directly monitored and recorded. The current study also supports previous reports demonstrating that overall
DNA damage and p53 mutations increase with 25 weeks of UVB exposure compared to 10 weeks of UVB exposure.

We set out to examine possible differences in tumorigenesis at 25 weeks, comparing 10 or 25 weeks of exposure to 1 MED of UVB three times weekly on nonconsecutive days. In the current study, we have modeled both male and female compliant patients who have chronically UVB-damaged skin but have made lifestyle changes to eliminate further UVB exposure. We have also modeled both male and female non-compliant patients who continued to be exposed to UVB even after the appearance of lesions. Taken together, these data support the commonly assumed, but not demonstrated, fact that cumulative UVB exposure is a risk factor for UVB-induced SCC and highlight the fact that changing sun worshiping habits, even after early chronic sun exposure and skin damage, may ultimately decrease tumor development in patients.
Chapter 5: Size is Not a Reliable Predictor of Tumor Grade in a Murine UVB-Induced Carcinogenesis Model

5.1 Introduction

It has been assumed that larger tumors are malignant while smaller tumors have a more benign phenotype; however, it is unclear whether tumor size can be a reliable predictor for tumor grade. A recently reported study attempted to define visual criteria that could be used to predict the grade of cutaneous tumors. They defined fully invasive SCCs as tumors having a diameter greater than 3mm, with bleeding and ulceration [191]. In order to track the development of malignant tumors during the course of preventive or therapeutic treatments, it would be useful to be able to visually identify malignant tumors without biopsy. We set out to use these criteria in our model and to validate our visual observations with histological grading as well as other markers of tumor progression including COX-2, p53, and blood vessel formation.

The risk of developing cutaneous squamous cell carcinoma (SCC) is 3 times greater in males than females [9]; however, the mechanisms that contribute to this disparity are unclear. Our previous studies have demonstrated that after equivalent, chronic UVB exposure, male mice had larger tumors and more malignant tumors compared to female
mice [108]. We also showed that treatment with the anti-inflammatory agent, diclofenac, effectively decreased tumor growth [166].

Diclofenac functions by selectively inhibiting the cyclooxygenase-2 (COX-2) enzyme. COX-2 is induced by chronic UVB exposure and is the major source of elevated cutaneous prostaglandin E2 (PGE₂) [63, 64]. Increased PGE₂ synthesis is known to play a key role in carcinogenesis by contributing to the uncontrolled proliferation of damaged cells that ultimately form tumors in several organ systems including the skin [66, 67, 68, 69, 70]. Previous studies revealed that COX-2 staining in cutaneous SCC tumors is not always apparent, illustrating that the magnitude of COX-2 expression may not be proportional to the malignancy of a tumor [192, 193]. Interestingly, COX-2 has been detected via immunohistochemical staining in actinic keratoses, which are precursor lesions for SCC, implying that COX-2 may be used as a marker for detecting malignant change at an early time point but then may be lost as the tumor progresses and the cells become less differentiated [183].

UVB exposure also induces the expression of wild type p53, a phosphoprotein encoded by the TP53 tumor suppressor gene [88]. The loss of function of TP53, whether by mutation or allele loss, is commonly found in many human malignancies including cutaneous SCC [82, 91]. The percentage of mutated TP53 expressed in SCC has been reported to be between 15 and 90% in several studies [193, 194]. Interestingly, COX-2 expression has been found to be decreased in tumors containing elevated levels of wild
type p53, while elevated COX-2 expression has been detected in tumors with mutated TP53 genes, suggesting a link between the two [195]. However, this relationship has been contradicted by several studies in which no significant relationship was elucidated [193,196]. However, these studies did not take tumor size into account as a possible explanation for the observed variability in COX-2 or p53 expression and their potential interaction in SCC tumors. Thus, though both may be playing important roles in tumorigenesis, the validity of an interaction between p53 and COX-2 may depend on tumor size and remains unclear.

Previous studies have demonstrated that increased COX-2 expression can alter apoptosis, cell differentiation, and angiogenesis [197]. Further, COX-2 inhibitors have been demonstrated to effectively inhibit inflammatory cells that activate angiogenesis and to down-regulate the expression of pro-angiogenic factors [198,199,200,201,202]. Previous studies with small sample sizes were not able to detect a significant correlation between angiogenesis and SCC progression [203,204,205,206], suggesting that the angiogenic switch may be occurring late in the process of SCC carcinogenesis instead of in premalignant or early invasive lesions as had been suggested [207,208,209,210]. In contrast, a recent study described a significant correlation between COX-2 expression and angiogenesis in both SCC and precursor lesions, indicating that these are indeed early events in SCC carcinogenesis [131]. While angiogenesis is known to be important for the development and progression of tumors, the relationship between tumor size and grade in the context of blood vessel content has not been fully investigated.
While COX-2, p53, and angiogenic expression have been studied in SCC, the tumor sizes and specific tumor grades are often ignored. In the current study we exposed male and female Skh-1 mice to UVB for 25 weeks and tracked tumors over time to directly match tumor size, appearance, and grade. We found that while the above criteria successfully identified many SCCs, 16.7% of male and 16.7% of female tumors proved to be non-ulcerated SCCs smaller than 3mm and 33.3% of male tumors and 26.1% of female tumors were non-ulcerated papillomas larger than 3mm. Further, we examined p53, mutant p53, COX-2, and CD31 via immunohistochemical staining in order to investigate potential relationships with size, grade, or interaction with another marker. Our study demonstrates that while visual observation may provide preliminary data, it is critical to histologically examine every tumor regardless of size or appearance to correctly evaluate the efficacy of treatments in decreasing cutaneous tumor progression.

5.2 Materials and Methods

5.2.1 Animal Treatments and Experimental Design. Outbred, male and female Skh-1 mice (6-8 weeks old, Charles River Laboratories, Wilmington, MA) were housed in the vivarium at The Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by the Institutional Animal Care and Use Committee before the initiation of any studies. Mice were dorsally exposed to 2240 J/m² UVB, previously determined to be 1 MED, 3× weekly on non-consecutive days for 25 weeks. UVB dose was calculated using UVX radiometer and UVB sensor (UVP, Upland, CA) and emitted by Phillips
FS40 UV bulbs (American Ultraviolet Company, Lebanon, IN). After 10 weeks of UVB exposure mice in the non-compliant patient model were treated topically with vehicle (Surgilube®; Savage Laboratories, Melville, NY) immediately following each UVB exposure for the remaining 15 weeks of the study. Surgilube is an inert vehicle used clinically as a surgical lubricant. Tumors larger than 2 mm in diameter were measured weekly with calipers and photographs were taken of each mouse in order to map tumors. After sacrifice, tumors were harvested and fixed in individual cassettes in formalin for 4 hours followed by PBS until paraffin embedding.

5.2.2 Tumor Grading. Hematoxylin and Eosin (H&E)-stained tissue sections of tumors isolated from mice were graded in a blinded manner by a board-certified veterinary pathologist (DFK) as previously described [108] and in Chapter 2.

5.2.3 Histological Techniques. Samples were fixed, embedded, and mounted on slides as described previously in Chapter 2.

_Immunohistochemical detection of p53_

Samples were examined for p53-positive foci as a measure of overall DNA damage as previously described in [166] and Chapter 2.
*Immunohistochemical detection of mutant p53*

Samples were examined for mutant p53-positive cells as a measure of advanced progression as previously described in Chapter 4.

*Immunohistochemical detection of CD31*

Samples were examined for CD31-positive blood vessels as a measure of angiogenesis as previously described in Chapter 3.

*Immunohistochemical detection of COX-2*

After rehydration, sections were circled with Immedge pen (Vector Laboratories), and endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes at room temperature. Slides were incubated in Antigen Unmasking Solution (Vector Laboratories) for 15 min in a microwave. After slides were cooled, they were blocked with 1× Casein for 10 minutes followed by an overnight incubation at 4 °C with primary COX-2 antibody (Cayman) at a 1:500 dilution. Slides were then incubated with Rabbit Link and Label (Vector Laboratories) for 30 minutes each at room temperature. Slides were incubated in DAB solution (Vector Laboratories) for 10 minutes at RT. Slides were washed in deionized water, counterstained, and dehydrated.

**5.2.4 Statistical Analysis.** The analyses were carried out by Gregory Young from the Center for Biostatistics at The Ohio State University. No adjustments were made for multiple comparisons. *p*-values ≤0.05 were considered statistically significant.
5.3 Results

5.3.1 Previously reported criteria for visually identifying fully invasive squamous cell carcinomas were not accurate in our model. To investigate the efficacy of using previously reported criteria to visually identify fully invasive squamous cell carcinomas, we exposed male and female Skh-1 mice to 2240 J/m² UVB (previously determined in our laboratory to be 1 MED) three times weekly on non-consecutive days for 25 weeks to induce SCC development. Upon examination of tumors from male mice that fulfilled the criteria of a diameter over 3 mm and bleeding and ulceration of the tumor, 21/31 or 67.7% were not fully invasive SCC while 10/31 32.3% were correctly identified as fully invasive SCC (Figure 5.1). Importantly, 10 tumors graded as fully invasive SCC were missed using the visual criteria. Of these 10 tumors, 9 had a diameter less than 3 mm with 5 having an ulcerated appearance, and 1 was not ulcerated but had a diameter larger than 3 mm.

Upon examination of tumors from female mice that fulfilled the visual criteria, 19/29 or 65.5% were not fully invasive SCC while 10/29 or 34.5% were correctly identified as fully invasive SCC (Figure 5.2). There were 9 tumors graded as fully invasive SCC that were missed using the visual criteria. Of these 9 tumors, 7 had a diameter less than 3 mm, with 1 having an ulcerated appearance, and 2 tumors were not ulcerated with a diameter larger than 3 mm.
Previously reported criteria for visually identifying fully invasive SCC were not accurate in male mice. After 25 weeks of UVB exposure, tumors were photographed, measured, and isolated from male mice. Of the tumors that met the criteria of a tumor diameter greater than 3 mm, bleeding from the tumor, and ulceration/erosion/cratering, only 32.3% were correctly identified as fully invasive SCC. The circled tumor on the mouse on the top left is a fully invasive SCC while the circled tumor on the bottom right is a papilloma.
Figure 5.2 Previously reported criteria for visually identifying fully invasive SCC were not accurate in female mice. After 25 weeks of UVB exposure, tumors were photographed, measured, and isolated from female mice. Of the tumors that met the criteria of a tumor diameter greater than 3 mm, bleeding from the tumor, and ulceration/erosion/cratering, only 34.5% were correctly identified as fully invasive SCC. The circled tumor on the mouse on the top left is a fully invasive SCC while the circled tumor on the bottom right is a papilloma.
5.3.2 Tumor size and grade are not directly correlated. In order to further investigate the assumption that larger tumors have a more malignant phenotype, we tracked tumors over time to correlate tumor size and grade. Upon examination of tumor size across each grade (papilloma 1-3, microinvasive SCC grades 1-3, and fully invasive SCC), we did not observe a direct correlation between increasing tumor size and tumor progression in male or female mice (Figure 5.3A). When we grouped tumors as either benign (papilloma grades 1-3) or malignant (microinvasive SCC grades 1-3 or fully invasive SCC), we observed a 3.6-fold increase in average tumor area between benign and malignant tumors in female mice ($p=0.0382$, Figure 5.3B). We observed a 4-fold increase in average tumor area between benign and malignant tumors in male mice (Figure 5.3B), which was not statistically significant due to the variability of this outbred mouse strain ($p=0.1030$).

5.3.3 Large tumors isolated from female mice display decreased COX-2-positive staining. In order to investigate the correlation of COX-2-positivity with the malignancy of a tumor, we examined COX-2 staining via immunohistochemical analysis. Upon examination of the average % COX-2-positive area of the tumors across each grade, we did not observe a loss of positivity with tumor progression (Figure 5.4A). When tumors were grouped as either benign or malignant, we observed a slight increase in COX-2 positivity in the malignant tumors in both male and female mice that was not statistically significant (Figure 5.4B). When we examined the correlation between COX-2 staining positivity and tumor size, we found that tumors from male mice exhibited no alteration in COX-2 positivity, regardless of tumor size (Figure 5.4C). Interestingly, tumors isolated
Figure 5.3 Tumor size and grade are not directly correlated across all grades. After 25 weeks of UVB exposure, tumors were measured and isolated from male and female mice. Tumors were graded by a veterinary pathologist and grades were matched with tumor sizes. (A) P = papilloma, M = micro-invasive SCC, SCC = squamous cell carcinoma, SCT = spindle cell tumor. (B) Benign = papilloma, malignant = M, SCC, SCT. (*p=0.0382).
Figure 5.4 Large tumors isolated from female mice display decreased COX-2-positive staining. After 25 weeks of UVB exposure, tumors were measured and isolated from male and female mice. Tumors were graded by a veterinary pathologist and grades were matched with tumor sizes. Tumor sections were examined for COX-2 via immunohistochemistry and analyzed using ImageJ software. (A) P = papilloma, M = micro-invasive SCC, SCC = squamous cell carcinoma, SCT = spindle cell tumor. (B) Benign = papilloma, malignant = M, SCC, SCT. (*p = 0.0382). (C) <25mm = tumor area less than 25mm$^2$, >25mm = tumor area greater than 25mm$^2$. 

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from female mice displayed a 2.4-fold decrease in COX-2-positive area in tumors that had an area larger than 25 mm$^2$ compared to tumors of an area less than 25 mm$^2$ though this decrease was not statistically significant (Figure 5.4).

**5.3.4 Large tumors isolated from female mice display increased p53-positive staining.** To examine the relationship between p53-positivity and tumor progression, we examined tumors isolated from male and female mice for p53-positive cells via immunohistochemistry. Tumors isolated from male and female mice exhibit increased p53-positivity through microinvasive SCC grade 2, followed by a step-wise decrease toward fully invasive SCC (Figure 5.5A). Both male and female tumors exhibited larger amounts of p53-positive area in malignant tumors compared to benign tumors (Figure 5.5B) that approached statistical significance ($p=0.0589$ and $p=0.0844$, respectively). Tumors isolated from male mice did not exhibit any significant alteration in p53-positivity, regardless of tumor size (Figure 5.5C), while female tumors exhibited increased p53-positive area in tumors with an area larger than 25 mm$^2$ compared to tumors with an area less than 25 mm$^2$ ($p=0.0254$, Figure 5.5C).

**5.3.5 Malignant tumors exhibit higher levels of mutant p53-positive staining.** To further investigate the relationship between COX-2 and p53 with tumor malignancy, we examined tumors isolated from male and female mice for mutant p53 via immunohistochemical analysis. Tumors isolated from male or female mice did not exhibit any significant trends in mutant p53-positive staining across all grades (Figure
Figure 5.5 Large tumors isolated from female mice display increased p53-positive staining. After 25 weeks of UVB exposure, tumors were measured and isolated from male and female mice. Tumors were graded by a veterinary pathologist and grades were matched with tumor sizes. Tumor sections were examined for p53 via immunohistochemistry and analyzed using ImageJ software. (A) P=papilloma, M=micro-invasive SCC, SCC=squamous cell carcinoma, SCT=spindle cell tumor. (B) Benign=papilloma, malignant=M, SCC, SCT. (C) <25mm=tumor area less than 25mm$^2$, >25mm=tumor area greater than 25mm$^2$. (*$p=0.0254$).
Malignant tumors isolated from male mice exhibited increased mutant p53-positive staining which due to the variable nature of this outbred mouse strain, was not statistically significant ($p=0.5036$, Figure 5.6B). However, malignant tumors isolated from female mice displayed a 1.5-fold increase in mutant p53-positive staining compared to benign tumors ($p=0.0395$, Figure 5.6B). Further, tumors isolated from male mice with an area larger than 25 mm$^2$ displayed increased mutant p53-positive staining compared to smaller tumors that approached statistical significance ($p=0.0734$, Figure 5.6C). Tumors isolated from female mice did not exhibit significant alterations in mutant p53-positive staining based on tumor size (Figure 5.6C). Interestingly, male tumors exhibited elevated levels of mutant p53-positive staining compared to female tumors, regardless of tumor grade or size ($p=0.0021$).

**5.3.6 Large tumors isolated from male mice exhibit decreased numbers of CD31-positive blood vessels.** To examine the relationship between COX-2 and angiogenesis with tumor progression, we examined tumors isolated from male and female mice for CD31-positively staining blood vessels via immunohistochemistry. Tumors isolated from male or female mice did not exhibit any significant trends in the number of CD31-positive blood vessels across all tumor grades (Figure 5.7A). Analyzing benign and malignant tumors isolated from male and female mice did not provide any significant correlation between tumor progression and the number of CD31-positive blood vessels present (Figure 5.7B). Interestingly, tumors isolated from male mice with an average area larger than 25 mm$^2$ exhibited a decreased number of CD31-positive blood vessels.
compared to smaller male tumors ($p=0.0324$, Figure 5.7C). In contrast, tumors isolated from female mice exhibited a slight increase in the observed number of CD31-positive blood vessels in tumors having an area larger than 25 mm$^2$ compared to smaller tumors, which due to the variability of this outbred mouse strain, was not statistically significant ($p=0.4283$, Figure 5.7C). Additionally, large tumors isolated from female mice displayed an approximately 2-fold increase in the number of CD31-positive blood vessels compared to those observed in large tumors isolated from male mice ($p=0.0029$).
Figure 5.6 Malignant tumors exhibit higher levels of mutant p53-positive staining. After 25 weeks of UVB exposure, tumors were measured and isolated from male and female mice. Tumors were graded by a veterinary pathologist and grades were matched with tumor sizes. Tumor sections were examined for mutant p53 via immunohistochemistry and analyzed using ImageJ software. (A) P=papilloma, M=micro-invasive SCC, SCC=squamous cell carcinoma, SCT=spindle cell tumor. (B) Benign=papilloma, malignant=M, SCC, SCT. (*p=0.0395). (C) <25mm=tumor area less than 25mm$^2$, >25mm=tumor area greater than 25mm$^2$. 
Figure 5.7 Large tumors isolated from male mice exhibit decreased numbers of CD31-positive blood vessels. After 25 weeks of UVB exposure, tumors were measured and isolated from male and female mice. Tumors were graded by a veterinary pathologist and grades were matched with tumor sizes. Tumor sections were examined for CD31-positive blood vessels via immunohistochemistry and counted at 20× magnification. (A) P=papilloma, M=micro-invasive SCC, SCC=squamous cell carcinoma, SCT=spindle cell tumor. (B) Benign=papilloma, malignant=M, SCC, SCT. (C) <25mm=tumor area less than 25mm², >25mm=tumor area greater than 25mm². Large tumors isolated from male mice exhibited significantly decreased blood vessel numbers compared to smaller tumors (*p=0.0324). Large tumors isolated from female mice exhibited approximately twice the blood vessels observed in large tumors isolated from male mice (**p=0.0029).
5.4 Discussion

Herein, we have demonstrated that utilizing the previously reported clinical criteria for visually identifying cutaneous squamous cell carcinomas [191] is not effective in our murine UVB-induced carcinogenesis model. We also demonstrated that tumor size and individual grade are not directly correlated; however, examining overall malignant versus benign tumor groups in females revealed an increased average tumor size in malignant tumors. We found that there was a slight increase in COX-2 staining in malignant tumors from males and females while tumors having an average area larger than 25 mm$^2$ displayed decreased COX-2 positivity. Malignant tumors isolated from both male and female mice exhibited elevated p53 levels compared to benign tumors. Elevated p53 staining was also observed in large, female tumors. Further examination of tumors revealed increased mutant p53 positivity in male tumors overall compared to female tumors, malignant tumors compared to benign tumors isolated from female mice, and large tumors compared to smaller tumors isolated from male mice. We found no significant alterations in the number of blood vessels present in benign versus malignant tumors in male or female mice. Interestingly, while we observed a decrease in blood vessel density in large male tumors, in contrast, in large female tumors we observed an increased blood vessel density compared to smaller tumors.

The previously reported clinical criteria for visual identification of SCC, which required a tumor diameter greater than 3 mm, bleeding from a tumor, and tumor ulceration/crating/erosion [191], was ineffective in our model. However, it is important
to note that different strains of mice were used in the two studies. In the current study, we used male and female Skh-1 hairless mice while the previous report used the haired FVB strain. It is possible that shaving the FVB mice weekly confounded the effects of the UVB exposures, resulting in an altered carcinogenesis process. Additionally, the tumors in the two studies were graded by different pathologists, allowing for slight differences in the identification of tumor grades, which may account for some of the observed differences.

A recent study reported that COX-2-positive staining was observed in both AK and SCC [211], suggesting that COX-2 may not be functioning as an early marker of malignancy, but it supports COX-2 as an important therapeutic target for SCC. These findings correlate with the current study in that COX-2-positive staining was observed in malignant tumors, and did not dissipate with tumor progression as had been previously suggested [193]. Further studies support the current data demonstrating slightly higher or similar staining in both precursor lesions such as AK, keratoacanthoma, or Bowen’s Disease, and SCC [131,192,193,212,213,214]. In contrast to a previous report demonstrating that COX-2 staining was positively correlated with SCC tumor size [215], in the current study, we observed decreased COX-2 positivity in large tumors. Interestingly, there was no correlation between COX-2 staining and the size of AK precursor lesions.
There have been several controversial reports regarding potential relationships between COX-2 and p53. In the current study, COX-2, p53, and mutant p53 staining increased in malignant tumors compared to benign tumors. These data correlate with a previous report of increased COX-2 expression along with p53 in both BCC and SCC [215], but they partially contradict a previous report demonstrating that COX-2 staining is decreased in tumors expressing wild type p53 but elevated in tumors with p53 mutations [167]. Interestingly, in the current study, large female tumors exhibited increased p53 staining while large male tumors exhibited increased mutant p53 staining. Because many studies do not report data based on sex, these findings may help to explain the variable reports discussing the relationship between COX-2 and p53 staining.

Several previous studies with small sample sizes were not able to detect a significant correlation between angiogenesis and SCC progression [202,203,204,205], and these studies are supported by our current data that found no alteration in the number of blood vessels present in benign versus malignant tumors from male or female mice. Interestingly, large male tumors exhibited decreased vessel density while large female tumors exhibited an increase. We thought that the decreased vessel density in large tumors isolated from male mice may be due to necrosis, which has been described as part of the angiogenesis process as a tumor grows [216]. Therefore, we examined Hematoxylin and Eosin-stained sections but found only two tumors with geographic necrosis, which did not account for the vessel differences observed in the large tumors.
Further studies investigating the balance between peritumoral and intratumoral vessel density may further explain this finding.

Overall, we have demonstrated that tumor size, even in combination with appearance, is ineffective for visually identifying SCC without biopsy. Further, we found no direct correlation between tumor size and grade, though we did observe increased size of malignant tumors isolated from female mice. Our data examining COX-2, p53, and blood vessel formation could be used to further characterize malignant tumors in terms of sex-based differences. This information could be expanded by investigating these parameters in tumors isolated from mice after topical treatment with diclofenac, a COX-2 inhibitor. Further studies are needed to examine the relationships between COX-2, p53, and angiogenesis.
Chapter 6: Discussion

With 3.5 million new diagnoses of non-melanoma skin cancer each year in approximately 2 million patients [10], in the past three decades more people have been diagnosed with skin cancer than all other cancer types combined [11]. Epidemiological evidence has revealed that men have three times the risk for developing SCC [9], the more deadly type of NMSC that can metastasize more frequently. This sex difference was previously attributed to the fact that men spent more time working in the sun and used less sun protection compared to women. However, we previously demonstrated in a murine model of UVB-induced skin carcinogenesis that when chronically exposed to equal amounts of UVB, male mice developed more tumors, larger tumors, and more malignant tumors compared to female mice [108].

The treatment and management of all NMSCs costs over $1.4 billion each year, with an additional $1.1 billion annually for the treatment of AK precursor lesions [22]. Further costs accrue because NMSC contributes to considerable morbidity which can result in disfiguration and disability [10,11,18,19,20]. The morbidity caused by NMSC is perhaps so striking due to the lack of successful non-invasive therapies, especially in the case of recurring lesions or a large lesion or patch of lesions that may not leave enough skin to easily close the wound. Though the incidence of many cancers is decreasing, skin cancer
incidence continues to increase especially in young women as evidenced by the nearly 700% increased incidence of SCC in women under 40 [13], resulting in a renewed focus on prevention methods. Based on our previous data demonstrating lower levels of inflammation and antioxidant capacity and higher levels of DNA damage in male mice compared to female mice after exposure to UVB, we sought to examine the efficacy of topical anti-inflammatory and antioxidant compounds as preventative agents for UVB-induced SCC in our murine model. We hypothesized that an anti-inflammatory agent would be more efficacious in female mice due to increased levels of inflammation compared to male mice after UVB exposure, and topical antioxidants would be more beneficial for prevention in male mice due to the decreased level of antioxidants and increased tumor incidence after UVB exposure.

To investigate these hypotheses in the current studies, we first modeled men and women who spent significant time in the sun in childhood and adolescence and then made lifestyle changes to eliminate UV exposure and to begin using topical antioxidant or anti-inflammatory agents in hopes of reversing existing UVB-induced skin damage and preventing tumor development in adulthood. We exposed male and female Skh-1 hairless mice to 2240 J/m² UVB three times weekly on non-consecutive days, resulting in chronically UVB-damaged skin, followed by 15 weeks of topical anti-inflammatory or antioxidant treatment to skin begun before the appearance of lesions, without further UVB exposures. We demonstrated that despite the observed sex differences in the inflammatory response, preventative application of the topical COX-2 inhibitor and
NSAID, diclofenac, to chronically UVB-damaged skin effectively decreased tumor multiplicity in both male and female mice. Interestingly, tumor burden was significantly decreased and tumors were of lower histologic grade in male mice only. Further, the observed increase in skin catalase activity in male mice treated with diclofenac compared to male mice treated with vehicle, combined with the lack of significant alterations in female mice, may indicate that restoration of this antioxidant is important for decreasing tumorigenesis in male but not in female mice. Taken together, our study emphasizes differences in the response of male and female murine skin and demonstrates a potential new therapeutic use for diclofenac, a currently available topical treatment as a preventative intervention for patients predisposed to cutaneous SCC development.

We also demonstrated that topical C E Ferulic, a commercially available combination antioxidant treatment, effectively reduced tumor number and burden in both female and male Skh-1 mice. In accordance with several previous studies, topical vitamin E treatment provided no preventative benefits for female mice [155,156,157,158,159], and in fact, resulted in accelerated tumor growth rate compared to vehicle-treated female mice. This difference may be explained by the resultant increase in catalase activity levels and DNA damage present in the female mice treated with vitamin E compared to those treated with C E Ferulic. Interestingly, vitamin E topical treatment demonstrated more beneficial effects in male mice, which may be a result of the lower endogenous antioxidant levels in male mice that were significantly elevated compared to vehicle-treated mice after topical antioxidant treatment. Our study demonstrates both the potential
detrimental effects of treating chronically UVB-damaged female skin with topical vitamin E alone and the potential benefits of topically treating male and female skin with a stable combination antioxidant compound for the prevention of UVB-induced SCC. Because of the focus on antioxidant supplementation in many products targeted towards women, these findings are especially relevant. Since many previous studies do not describe which sex was used, our findings may help explain previous contradictory evidence regarding antioxidant supplementation and cancer incidence.

Despite the known link between UVB exposure and skin cancer development, skin cancer incidence continues to increase due to the societal belief that people look better and healthier with a tan [217]. Several previous studies have investigated the time to tumor onset by altering the dose of UVB [188,189,190]; however, the effects of the length of UVB exposure on tumor burden in males and females, while presumed, have not been formally investigated. Thus, we were interested in investigating whether men and women who had large amounts of UVB exposure during childhood and early adolescence but made efforts to stay out of the sun in adulthood would lessen the number of tumors they develop compared to people who continue sun worshiping habits throughout adult life. Further, we wanted to examine the extent to which these outcomes would be the same between men and women. In addition, we sought to investigate the efficacy of the anti-inflammatory (diclofenac) and antioxidant (C E Ferulic and vitamin E) treatments in the non-compliant model where UVB exposure is continued throughout the study. Our results demonstrate that both male and female mice in the non-compliant
patient model that were exposed to 25 weeks of UVB developed more tumors, larger tumors, and a higher percentage of malignant tumors compared to mice in the compliant patient model that were exposed to 10 weeks of UVB. While diclofenac continued to be effective for decreasing both tumor number and burden in male and female mice with concurrent topical treatment and UVB exposure, the tumors that formed were of a more malignant grade compared to vehicle-treated mice. In contrast, male and female mice treated with antioxidants exhibited no beneficial effects in terms of tumor number and burden compared to vehicle-treated mice. Taken together, these data support the commonly assumed, but not demonstrated, fact that cumulative UVB exposure is a risk factor for UVB-induced SCC and highlight the fact that changing sun worshiping habits, even after early chronic sun exposure and skin damage, may ultimately decrease tumor development in patients. Further, these data indicate the importance of eliminating UVB exposure in order to experience possible preventative or therapeutic benefits of antioxidant treatment. These data also bring up the idea that tumors escaping treatment may in fact be aggressive lesions that require further investigation.

It has been assumed that larger tumors are malignant while smaller tumors have a more benign phenotype; however, it is unclear whether tumor size can be a reliable predictor for tumor grade. A recently reported study attempted to define visual criteria that could be used to predict the grade of cutaneous tumors. They defined fully invasive SCCs as tumors having a diameter greater than 3mm, with bleeding and ulceration [191]. In order to track the development of malignant tumors during the course of preventive or
therapeutic treatments, it would be useful to be able to visually identify malignant tumors without biopsy. We set out to use these criteria in our model and to validate our visual observations with histological grading as well as other markers of tumor progression including COX-2, p53, and blood vessel formation. Our main findings demonstrate that while visual observation may provide preliminary data, it is critical to histologically examine every tumor regardless of size or appearance to correctly evaluate the efficacy of treatments in decreasing cutaneous tumor progression.

Overall, we have provided evidence to support the link between cumulative UVB exposure and SCC development and highlighted the importance of eliminating UVB exposure to experience therapeutic benefits of topical antioxidant agents. Though diclofenac successfully decreased tumor number and burden in male and female mice with both 10 and 25 weeks of UVB exposure, more malignant tumors were observed in female mice from both groups and male mice after 25 weeks of UVB. These data, in combination with the examination of the previously described visual criteria for identifying SCC without biopsy, underscore the importance of histological examination of all tumors, especially the decreased number of tumors that escape therapeutic treatment.

In order to further examine the effects of both endogenous and topically applied antioxidants in our model, further studies are needed to investigate the interactions of the complex cutaneous antioxidant networks. It may also be beneficial to examine different
concentrations and forms of antioxidants like vitamin E to elucidate the most efficacious therapeutic combination. Because we saw decreased tumor number in female mice with both diclofenac and C E Ferulic treatment, and we observed an increased malignancy rate with diclofenac treatment while no malignant tumors formed in mice treated topically with C E Ferulic, combining the two treatments may result in decreased tumor number, burden, and grade. This combination may allow for further investigation of the relationship between cutaneous antioxidants and inflammation, resulting in an enhanced understanding of how sex-based response differences may affect treatment outcomes. Further, examining tumors that escape treatment in comparison to tumors isolated from vehicle-treated mice may help to isolate potential therapeutic targets that can supplement current treatment modalities.
References

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