Direct Effects of VEGF on Keratinocyte Function During Skin Carcinogenesis and Wound Healing

DISSERTATION

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Abstract

Epidermal keratinocytes, the predominant cell type in the epidermis, play a crucial role in two processes in the skin: skin carcinogenesis and cutaneous wound healing. Non-melanoma skin cancer (NMSC) is the most prevalent type of cancer, with 3.5 million cases diagnosed each year in the US. These cancers, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are primarily caused by exposure to ultraviolet (UV) light from the sun. Wound healing is a key process in many aspects of medicine. In addition to injury and trauma, millions of surgeries are performed each year. Chronic wounds, which do not heal properly, can lead to hospitalizations, amputations, death and affect 6.5 million patients every year at an estimated cost of $12 billion dollars. Therefore, it is critical to understand how keratinocytes function in both of these processes.

Angiogenesis, the growth and expansion of new blood vessels, occurs during both NMSC and wound healing. Vascular endothelial growth factor (VEGF) promotes angiogenesis by causing the proliferation, migration and survival of vascular endothelial cells. VEGF is produced by the skin in response to UV and promotes NMSC indirectly through the induction of angiogenesis. Additionally, wounds contain high levels of VEGF. VEGF receptor 1 (VEGFR-1) has now been identified on epidermal keratinocytes, suggesting that VEGF can affect keratinocytes directly. Therefore, we hypothesize that
VEGF may influence wound healing and skin carcinogenesis by directly affecting keratinocytes via VEGFR-1. To test this, a unique conditional knockout mouse with VEGFR-1-deficient keratinocytes (cKO) was developed and was utilized in acute and chronic UV-induced skin carcinogenesis studies as well as wound healing studies.

Immunohistochemical analysis of human and murine NMSC samples revealed that VEGFR-1 is highly expressed in skin tumors. Furthermore, in vitro studies indicated that keratinocyte VEGF and VEGFR-1 expression is regulated by UV light. To examine the direct effects of VEGF on keratinocytes in skin carcinogenesis, cKO and control mice were exposed to acute and long term UV radiation. Keratinocytes in the epidermis of cKO mice showed a significant increase in apoptosis 24 hours following a single UV exposure compared to controls, suggesting that VEGF may function as a survival factor in UV-irradiated keratinocytes. Additionally, macrophage recruitment to UV damaged skin was reduced in cKO mice. Long term UV-induced skin carcinogenesis studies are ongoing and suggest that cKO mice are resistant to UV-induced skin carcinogenesis compared to controls.

To examine the direct role of VEGF on keratinocytes in wound repair, excisional wounds were inflicted on cKO and control mice. A significant delay in reepithelialization was observed in cKO mice compared to controls 5 days after wounding. These results suggest that VEGF can stimulate epidermal keratinocytes directly to promote reepithelialization. Similar to acute UV studies, a significant decrease in the number of macrophages was observed in wounds from cKO mice compared to controls, indicating that VEGF can affect keratinocyte-mediated recruitment of macrophages. Overall, these
studies have uncovered novel roles of VEGF in two important processes in the skin: wound healing and skin carcinogenesis.
Dedication

This document is dedicated to David, for making all things seem possible.
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Chapter 1: Introduction

1.1 Skin

The skin is the largest organ in the body and serves several important functions. The skin forms a protective barrier against the outside environment, provides sensation, prevents moisture loss, secretes waste, senses and regulates temperature, and is involved in vitamin D synthesis [1]. It defends against invading pathogens through the release of antimicrobial peptides and provides surveillance through innate and adaptive immune cells within the skin. Structurally, the skin is comprised of three layers; the epidermis, dermis and subcutis [2]. The epidermis, the outermost layer, is composed primarily of epithelial cells called keratinocytes. These cells comprise 95% of the epidermis and form a stratified, squamous epithelium [2]. The epidermis is organized into layers, with proliferating keratinocytes found along the innermost basal layer. The basal layer is localized along the basement membrane that separates the epidermis from the dermis. Keratinocytes migrate upward away from the basement membrane and differentiate. The outermost, cornified layer contains terminally differentiated, anucleated keratinocytes [1]. In humans, the epidermis also contains melanocytes, the cells that give the skin its pigmentation. The epidermis is avascular and requires the diffusion and transport of nutrients from the underlying dermis.
The dermis contains connective tissue proteins such as collagen and elastin that give the skin its strength and flexibility. Collagen is produced primarily by dermal fibroblasts and makes up about 70% of the dermis [2]. Immune cells such as macrophages and mast cells also reside in the dermis. Structures such as blood vessels, lymphatic vessels, nerves, hair follicles and glands are also found within the dermal layer.

Located below the dermis, the innermost layer of the skin is the subcutis (or hypodermis). This layer is composed primarily of adipose and connective tissues [2]. Additionally in rodents, a thin layer of striated muscle called the panniculus carnosus is found below the subcutis and is not present in humans. Because the skin forms a barrier between our bodies and the outside environment, it is constantly under attack from a host of damaging agents. Therefore, the skin is particularly vulnerable to environmental carcinogens and to injury.

1.2 Skin Cancer

Non-melanoma skin cancer (NMSC) is the most commonly diagnosed type of cancer. More than 3 million new cases of NMSC are diagnosed in 2 million patients each year in the US alone [3-5]. Unlike many other types of cancer, the rates of NMSC continue to rise, indicating the need to increase research and identify new, more effective therapies [4, 6]. The risk of developing skin cancer is very high in the general population, as one in five people will develop skin cancer in their lifetimes [7]; however, certain populations such as those with light skin, fair or red hair and blue eyes as well as immunosuppressed patients are at an even greater risk [8-12].
NMSCs are primarily caused by chronic exposure to ultraviolet (UV) light from the sun, although chemical exposure, chronic wounds and viral infection can be risk factors as well [13-14]. There are two main types of NMSC: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCCs account for about 8 out of 10 skin cancers of all types [4] and, although these tumors are rarely metastatic, patients have a high rate of developing additional tumors within 5 years of diagnosis [15]. SCCs make up roughly 16% of all skin cancers [4]. SCCs are typically more aggressive than BCCs, posing a higher risk for metastasis [13] and lead to approximately 2,500 deaths annually [5]. Although NMSCs have a relatively low rate of mortality compared to many other types of cancer, they present a substantial burden on patients and on the health care system. Treatment of NMSC results in nearly $1.5 billion total direct costs annually [16]. Additionally, most currently available treatments for these tumors are surgical, resulting in permanent scars that can be disfiguring and decrease the mobility and strength of the skin [17-18].

1.3 Multi-Stage Skin Carcinogenesis

Skin carcinogenesis occurs as a step-wise process comprised of three well defined stages: initiation, promotion and progression [19-21]. Initiation takes place when a skin cell acquires a genetic mutation as the result of a DNA-damaging event. Ultraviolet (UV) light from the sun is the predominant mutagenic agent encountered by the skin [22]. The p53 gene, a central regulator of cell cycle arrest following DNA damage, is particularly vulnerable to damage from solar radiation; mutations in p53 are found in human non-melanoma skin tumors at a high rate [23-24]. During the promotion stage, initiated skin cells harboring mutations develop into a benign tumor. This is often the result of ongoing
exposure to proliferative stimuli such as growth factors or other soluble mediators. Following promotion, the benign tumor cells undergo additional alterations, often in the form of additional mutations that allow for a conversion from a benign to a malignant phenotype in a process called progression. During progression, the tumor acquires the ability to invade and metastasize. In humans, progression results in the conversion of a premalignant tumor called an actinic keratosis, to a malignant SCC. In murine models, chronic UV exposure leads to premalignant tumors called papillomas that progress to malignant SCCs.

1.4 UV and Skin Cancer

The primary causative agent for NMSC is UV radiation from the sun [22]. The sun emits three ranges of UV light. At 100-280 nm wavelengths, UVC has the highest energy and is the most damaging; however it is absorbed by ozone in the atmosphere and does not reach the earth’s surface. UVA (315-400 nm) and UVB (280-315 nm) are able to penetrate the atmosphere and reach the surface of the earth where they can damage our skin [25-26]. UVB can only penetrate into the epidermis where it causes DNA damage in the epidermal keratinocytes. UVB is the type of solar radiation that is most strongly implicated in skin carcinogenesis [22, 27]. UVA, which can penetrate down into the dermal layer and break down connective tissue proteins, is implicated in the photo-aging effects of sunlight and, more recently, has been shown to amplify the effects of UVB-induced damage [28-29].

UV light is classified as a complete carcinogen because of its ability to both initiate and promote cells [21]. UV causes direct and indirect DNA damage as well as a strong
inflammatory response in the skin. DNA damage is caused directly when photons of energy from UV are absorbed by DNA molecules. This reaction induces pyrimidine-pyrimidone (6-4) photoproducts and cyclobutane pyrimidine dimers (CPD) which lead to C to T and CC to TT transitions in DNA molecules if the lesions are not repaired [23]. These changes are considered to be hallmarks of UV-induced DNA damage and are found in 90% of skin tumors [30]. UV also damages DNA indirectly, primarily through the production of reactive oxygen species which are released from infiltrating neutrophils, macrophages and activated keratinocytes [31-33]. Oxidative damage from ROS causes 8-oxo-deoxyguanosine DNA lesions which [34], if not repaired properly, can lead to G to T transitions [35-36].

The strong inflammatory response induced by UV exposure is also characterized by the release of many soluble factors in addition to reactive oxygen species (ROS) [37]. The pro-inflammatory cytokines interleukin-1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) are produced in the skin in response to UV [38-39], causing the infiltration and activation of additional immune cells. Additional soluble mediators, including prostaglandins and the pro-angiogenic vascular endothelial growth factor (VEGF), are also produced in response to UV radiation and are involved in the promotion and progression of skin carcinogenesis [40-44].

1.5 Wound Healing

In addition to being vulnerable to UV radiation, as the body’s barrier to the outside world, the skin is also prone to injury from the environment. Therefore, the skin must be able to
repair wounds rapidly and effectively. Wound repair in healthy adults occurs in three overlapping phases: inflammation, proliferation and remodeling/scar formation [45].

The inflammatory phase begins when an injury is inflicted on the skin. Platelets enter the site of injury through damaged blood vessels, where they aggregate to form a platelet plug and release soluble factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and VEGF that mediate subsequent steps in the healing process [46]. A fibrin clot is formed to help restore homeostasis to the vasculature and to provide a matrix to facilitate the infiltration of immune cells. Resident macrophages and mast cells are activated and promote the recruitment of additional immune cells, particularly neutrophils. Neutrophils enter the wound site quickly where they release soluble mediators to kill bacteria and sterilize the wound [47]. Circulating monocytes are also recruited to wounds where they mature into macrophages and join resident macrophages in the healing process. Macrophages phagocytose debris, bacteria and dead cells, and release soluble factors such as PDGF, VEGF, TNF-\(\alpha\), IL-1, transforming growth factor alpha (TGF-\(\alpha\)) and transforming growth factor-beta (TGF-\(\beta\)) that promote the formation of granulation tissue and the initiation of the proliferative phase [48]. Proper coordination and function of the cells in the inflammatory phase is critical for proper healing and studies have demonstrated that alterations in the number of infiltrating immune cells can greatly impact healing outcomes [49-52].

During the proliferative phase of wound healing damaged tissues are repaired and replaced. Reepithelialization is achieved by proliferation of keratinocytes at the wound margin and their subsequent migration across the wound bed, forming a new epidermis
In the dermis, granulation tissue is produced, creating an immature matrix made primarily of type III collagen to facilitate cell migration [54]. Gradually, granulation tissue is replaced by a mature, type I collagen matrix produced by fibroblasts. Angiogenesis, the growth and expansion of the vasculature, also occurs during this phase to replace damaged vessels and to bring nutrients and oxygen into the site of injury. In wounds, angiogenesis is initially mediated by bFGF and later by VEGF [55].

Finally, a permanent scar is formed. Scars are formed through the extensive remodeling of the collagen matrix in granulation tissue by fibroblasts. This collagen tends to be excessive and poorly organized, leading to a decrease in tensile strength [56].

Wound healing is a crucial component of many aspects of medicine. In addition to injury and trauma, 40 million inpatient and 31 million outpatient surgeries are performed each year, all requiring the repair of a wound [57]. Even when wounds heal properly, patients are left with a permanent scar which can lead to psychological distress as well as loss of tissue function and decreased joint mobility. Inefficient or abnormal healing of wounds can also lead to significant health problems. Some wounds do not heal properly, leading to chronic wounds. Chronic wounds commonly arise in diabetics, people with poor nutrition and the elderly. Chronic wounds can remain open for months or even years, have a high rate of developing infection and can lead to hospitalizations, amputations and death [58-59]. Abnormally healing wounds affect 6.5 million patients every year at an estimated cost of $12 billion dollars [57]. In order to improve patient outcomes, it is critical to understand the biological mechanisms underlying the repair process.
1.6 Angiogenesis

Blood vessel growth is an important process in both carcinogenesis and wound healing. There are three distinct ways in which the vasculature is expanded: vasculogenesis, arteriogenesis and angiogenesis. Vasculogenesis is the formation of de novo blood vessels from endothelial precursor cells and is important in embryogenesis [60]. Arteriogenesis involves the organization and expansion of existing arterioles into mature arteries and occurs primarily in response to an obstructed vessel [61]. Angiogenesis, the formation of new blood vessels from the expansion of existing vasculature, plays a critical role in processes including development, the female reproductive cycle and wound healing [62-64]. Abnormalities in the angiogenic process have been implicated in many pathological conditions such as cancer, psoriasis, retinopathies, diabetes, hypertension and arthritis.

Mechanistically, there are two types of angiogenesis: intussusceptive and sprouting. Intussusceptive angiogenesis occurs when excess shear stress within a microvessel leads to luminal splitting, causing the formation of two vessels [65]. Sprouting angiogenesis occurs in response to pro-angiogenic stimuli, often produced in response to tissue hypoxia or ischemia [66-67]. Sprouting angiogenesis begins with the activation of specialized endothelial cells called “tip cells” by a pro-angiogenic factor such as VEGF [68-69]. Following angiogenic stimulation, tip cells orient toward the VEGF gradient. Endothelial cells release proteases to degrade the basement membrane and surrounding matrix to facilitate migration. Next, endothelial cells proliferate and migrate, forming sprouts that extend toward the angiogenic stimulus [70-71]. Tube formation then
occurs in which the sprouts develop a lumen. Finally, these newly formed tubes fuse together and mature into stable vessels via the inhibition of endothelial cell proliferation and migration, acquisition of pericyte coverage and the development of a mature basement membrane [72].

Tumor angiogenesis promotes the growth and spread of many cancers, including NMSC. Typically, angiogenesis is required for tumors to grow beyond 1-2 mm in size and offers a route for tumor cells to disseminate to secondary sites [73]. Because of this, tumor angiogenesis has been an attractive and promising therapeutic target [74]. To induce angiogenesis, tumor cells and cells within the tumor microenvironment must alter the balance of pro- and anti-angiogenic factors, favoring an ‘angiogenic switch’ [75]. When pro-angiogenic signals outweigh anti-angiogenic signals, it allows for capillary sprouting through the proliferation and migration of endothelial cells, eventually leading to the formation of new vessels which supply the growing tumor with oxygen and nutrients. Many pro-angiogenic factors have been identified that can play role in tumor angiogenesis, including bFGF, interleukin-8 (IL-8), PDGF, placental growth factor (PIGF), TGF-β and VEGF [76-81].

In wound healing, angiogenesis is required to replace damaged vessels and to supply oxygen and nutrients to the wound site. Angiogenesis is a hallmark of the proliferative phase of wound healing, but changes to the vasculature occur throughout the entire repair process. Vascular hyperpermeability occurs almost immediately following wounding and persists for days [82]. The pro-angiogenic factors PDGF, bFGF and VEGF are elevated in wounds and an increase in vascular density and the presence of
endothelial progenitor cells have been observed in healing wounds [55, 82-84]. Evidence shows that chronic wounds of diabetic patients display impaired angiogenesis, highlighting the importance of angiogenesis for proper healing [85].

1.7 Vascular Endothelial Growth Factor

Vascular endothelial growth factor A (VEGF-A, referred to as VEGF throughout this document) is a 45 kDa heterodimeric heparin-binding protein belonging to the family of vascular endothelial growth factors that also includes VEGF-B, VEGF-C, VEGF-D and PIGF. At least 5 splice variants of VEGF have been identified in humans including VEGF_{121}, VEGF_{145}, VEGF_{165}, VEGF_{189}, and VEGF_{206} [86-87]. VEGF is well characterized as a strong inducer of angiogenesis by functioning as a potent survival factor and mitogen for endothelial cells [88-89]. In the skin, keratinocytes are the predominant source of VEGF [82, 90-91], although macrophages and fibroblasts can produce VEGF as well [55, 92]. VEGF is constitutively expressed at low levels in the skin by epidermal keratinocytes both in vitro and in vivo and is upregulated during many processes such as wound healing, psoriasis and skin carcinogenesis [41-42, 82, 93]. Induction of keratinocyte VEGF can be caused by many stimuli including hypoxia, TGF-α, keratinocyte growth factor (KGF), UV radiation and skin injury [90, 92, 94-98].

VEGF binds to three known receptors: VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and neuropilin-1 (Nrp-1) [99-101]. VEGFR-1 and VEGFR-2 are tyrosine kinase receptors characterized by a seven immunoglobulin-like extracellular domain, a single transmembrane region and an intracellular tyrosine kinase domain [102-103]. Signaling through the VEGF receptors occurs through ligand binding leading to receptor
dimerization, autophosphorylation and activation of downstream pathways [104]. VEGFR-1 can bind to three members of the VEGF family: VEGF, VEGF-B and placental growth factor (PIGF) [105] while VEGFR-2 binds VEGF, VEGF-C, VEGF-D and VEGF-E. Additionally, VEGFR-1 splice variants create a full length, membrane-bound form of VEGFR-1 that can propagate a signal upon ligand binding and a truncated, soluble form consisting of the extracellular portion only that is secreted and can act as a “sink” for extracellular ligand to inhibit VEGF signaling [103]. VEGFR-1 has a 10 fold higher affinity for VEGF than VEGFR-2; however the tyrosine kinase activity is relatively weak compared to VEGFR-2, suggesting that VEGFR-2 is the dominant signaling receptor in endothelial cells which express both VEGFR-1 and -2 [106].

Several studies have established a critical role for VEGF in skin cancer [107-109]. VEGF is highly expressed in human SCCs and VEGF expression increases stepwise during tumorigenesis in murine skin [110-113]. Furthermore, VEGF transgenic mice develop more papillomas, squamous cell carcinomas and metastases [107, 114]. Conversely, mice lacking VEGF in keratinocytes develop fewer skin tumors [115]. To date, the cancer promoting effects of VEGF have been attributed to the ability of VEGF to induce angiogenesis, which nourishes the tumor and provides a route for metastasis.

Overwhelming evidence also supports an important role for VEGF in wound healing. VEGF is found at high levels in both murine and human wounds and is predominantly produced by keratinocytes [55, 82]. VEGF produced in wounds is a potent chemoattractant for endothelial progenitor cells [116]. Inhibition of VEGF or VEGF receptors has been shown to decrease vessel density in the wound bed, delay wound
closure and decrease tensile strength of healed tissue [115, 117-119]. VEGF also has a role in pathological wound healing; The elderly have impaired wound healing and VEGF levels at the wound site have been shown to decrease with age [84] Additionally, keloids, a type of pathological scar, have elevated levels of VEGF [120-122] Chronic wounds, like those seen in diabetic patients, display deficiencies in angiogenesis and wound closure and [85]. In diabetic mice, VEGF treatment significantly increases vessel density, granulation tissue and reepithelialization [116, 123-125].

The angiogenesis-promoting effects of VEGF on endothelial cells have been well characterized. However, VEGFR-1 has now been discovered on a wide variety of cell types, including monocytes, neutrophils, pericytes, neurons, bone marrow derived cells and keratinocytes [105, 126-132], indicating that VEGFR-1 is not endothelial cell-specific. Studies have shown that VEGFR-1 is functional in these cells; for instance, VEGF can influence neuronal cone growth and mediate monocyte migration via VEGFR-1 [126, 129]. Additionally there is mounting evidence that VEGF can directly influence keratinocyte proliferation, migration and survival in vitro.[132-135]. However, it remains unknown how VEGF may mediate wound healing or UV-induced skin carcinogenesis by directly affecting keratinocytes via VEGFR-1.

1.8 Chapter Summaries

Skin carcinogenesis and wound healing are known to be influenced by VEGF, but to date the effects have been primarily attributed to the pro-angiogenic effects of VEGF. VEGFR-1 has been identified on keratinocytes, suggesting that VEGF can influence keratinocyte function directly. In vitro studies have shown that VEGF can alter
keratinocyte proliferation and migration, two processes that play a critical role in both cancer and wound healing. Additionally, VEGFR-1 is overexpressed in the tumor cells of several types of cancer including lung, breast, and melanoma. Furthermore, studies have shown a direct role for VEGF in two mouse models of skin cancer: the K5-SOS model and the two step chemical carcinogenesis model. However, it remains unknown if VEGF/VEGFR-1 signaling in keratinocytes plays a role in UV-induced skin carcinogenesis or wound healing.

The studies described here aim to identify the functional role of VEGF in the response of keratinocytes to UV and wound healing, two processes in the skin in which VEGF is highly expressed. To achieve this, we have developed a unique, conditional knockout mouse in which VEGFR-1 has been deleted in epidermal keratinocytes. In Chapter 3, the effect of VEGF on keratinocyte and SCC tumor cell survival following UV exposure is examined in vitro. Chapter 4 explores the role of keratinocyte VEGFR-1 in the response to acute UV exposure. These studies identify a role for VEGF in keratinocyte survival and keratinocyte-mediated recruitment of macrophages in UV-irradiated skin in vivo. Additionally, in Chapter 4 we examine VEGFR-1 expression in human and murine non-melanoma skin tumors and use mice with VEGFR-1 deficient keratinocytes to explore a direct role of VEGF in skin carcinogenesis. Finally, Chapter 5 examines the direct effects of VEGF on keratinocytes in wound healing and describes evidence for keratinocyte VEGF/VEGFR-1 mediated wound closure and macrophage recruitment. Overall, these studies demonstrate previously unidentified roles for VEGF in the cutaneous response to UV and injury.
Chapter 2: Methods

2.1 Cell Culture

2.1.1 Primary human keratinocyte and SCC tumor cell line cultures. Normal human epidermal keratinocytes (NHEK) from pooled neonatal donors were purchased from Lonza and grown in KGM-2 complete medium (Lonza, Walkersville, MD). Cultures were maintained at 37°C and 5% CO₂ and subcultured according to manufacturer's guidelines. NHEK used in experiments were 70-80% confluent and passaged no more than 3 times. Human squamous cell carcinoma cell lines SCC-13 and SCC-12F were kindly provided by Dr. Laurie G. Hudson (University of New Mexico) and were grown in DMEM:F12 medium (Gibco, Carlsbad, CA) supplemented with 5% FBS, 2mM L-glutamine and 1% penicillin-streptomycin (Gibco) [136].

2.1.2 Primary murine keratinocyte isolation and culture. Primary murine keratinocytes were cultured from neonatal mice as described [137]. Briefly, 1 to 2 day old mice were euthanized by decapitation and whole skins were removed and floated dermis side down on 0.25% trypsin overnight at 4°C. The following day, skins were laid epidermis side down into clean, sterile petri dish lids. The dermis was then lifted away, leaving only epidermis behind. Sheets of epidermal cells were collected into high calcium (HiCa) medium MEM supplemented with 8% chelated fetal bovine serum and 1% penicillin-streptomycin (all from GIBCO) plus 2.6 ml of 0.25 M calcium stock solution.
per 500 ml bottle), minced, washed through a 100 μm cell stainer and resuspended in HiCa medium. Cells were plated into 6 well dishes at a density of 0.5 mouse equivalents per well. The following day, medium was removed, wells were washed with phosphate-buffered saline (PBS) and wells were replaced with low calcium (LoCa) medium (S-MEM supplement with 8% chelated fetal bovine serum and 1% penicillin–streptomycin). Fresh LoCa medium was replaced every 2 days thereafter. Primary murine keratinocyte cultures were used when 70% confluence was reached.

2.1.3 In vitro UV experiments. For UV studies, NHEK or SCC-13 cells were grown to 70-80% confluence in 100 mm tissue culture dishes and serum-starved in basal media overnight prior to UV irradiation. Cells were rinsed twice with PBS and irradiated in PBS at a dose of 150 or 300 J/m² UVB. Unirradiated cells were treated the same way except they were not exposed to UV. UVB dose was determined by a UVX meter (UVP Inc., Upland CA) and emitted by Philips FS25 UV lamps (American Ultraviolet Company, Lebanon, IN). Following UV irradiation, PBS was replaced with complete media or complete media plus goat IgG or neutralizing antibodies to VEGF or VEGFR-1 (5g/ml) or 100 ng/ml recombinant human VEGF 165 (all from R&D Systems, Minneapolis, MN.) For coated plate studies, NHEK were cultured and irradiated as described above in 6 well plates pre-coated with laminin, fibronectin or collagen IV (BD Biosystems, San Diego, CA).

2.2 Generation of Conditional VEGFR-1 Knockout Mice

2.2.1 Conditional targeting of the Vegfr1 gene using the Cre-lox system. To examine the direct effects of VEGF on keratinocytes in wound healing and skin
carcinogenesis, a unique conditional knockout mouse strain in which VEGFR-1 is deleted in epidermal keratinocytes using the Cre-lox system was generated [138]. InGenious Targeting Laboratories (Ronkonkoma, NY), was contracted to create mice with a “floxed” Vegfr1 gene. In the Vegfr1 gene of these mice, a loxP site was inserted upstream of exon 1 and a second loxP site was inserted downstream of exon 1 using homologous recombination. Thus, exon 1 and part of the Vegfr1 promoter were flanked by loxP sites (Figure 2.1A). Mice carrying a floxed Vegfr1 allele were bred to K14-Cre mice, a strain that expresses cre recombinase on the keratinocyte-specific keratin 14 promoter [139] (Figures 2.1B and 2.1C). K14 mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). Crossing floxed Vegfr1 mice and K14-Cre mice generated conditional knockout (cKO) mice in which exon 1 and a portion of the Vegfr1 promoter are deleted in the keratinocytes (Figure 2.1D). The resulting cKO mice were backcrossed onto the FVB background using marker-assisted accelerated backcrossing (Charles River, Wilmington, MA).

2.2.2 Genotyping strategy for K14-Cre/Vegfr1 floxed mice.

Mice were routinely genotyped for the presence of floxed Vegfr1 and K14-Cre alleles (see sections on DNA isolation and PCR). Briefly, tail snips were obtained from pups and DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to manufacturer’s guidelines. The presence of the floxed Vegfr1 allele was assessed by PCR using primers (Table 2.1) that flank the second loxP site (Figure 2.2A). These primers generate a 219 bp product in wild type mice, a 396 bp band in floxed mice and both bands in heterozygous mice (Figure 2.2C). The presence or absence of the K14-Cre transgene was confirmed by PCR using a primer set in which
the sense primer lies within the K14 promoter and the anti-sense primer lies within the cre recombinase gene (Figure 2.2B). These primers generate a 495 bp PCR product in mice that carry the transgene (Figure 2.2C) while no product is produced in wild type mice.

![Diagram](image)

**Figure 2.1 Conditional targeting of the Vegfr1 gene using the Cre-lox system.** The Cre-lox system was used to generate a strain of mice in which a portion of the Vegfr1 gene is deleted in K14-expressing keratinocytes. The Vegfr1 gene was “floxed” by the addition of two loxP sites that flank exon 1 and a portion of the promoter of the Vegfr1 gene (A). K14-Cre mice were obtained that carry a transgene in which the K14 promoter is fused to the cre recombinase gene (B). Floxed Vegfr1 mice were crossed with K14-Cre to generate offspring that carry both the floxed and cre alleles (C). When cre is expressed, the DNA in between the loxP sites is excised, removing exon 1 and a portion of the Vegfr1 promoter from the Vegfr1 gene, leaving a single loxP site (D).
Figure 2.2 Genotyping strategies for K14-Cre/Vegfr1 floxed mice. The presence or absence of a floxed Vegfr1 allele and the K14-Cre transgene were confirmed by PCR. A single PCR reaction was used to determine whether mice carry floxed Vegfr1 alleles using the sense primer Neo F and the anti-sense primer Neo R1 (A). The Neo F and Neo R1 primers bind to the Vegfr1 gene on either side of the second LoxP site such that the PCR products differ in size (219 bp for wild type and 396 bp for floxed alleles) depending on whether a wild type or floxed allele, with the loxP site, is present (A). The K14-Cre transgene is detected using primers in which the sense primer binds within the K14 portion and the anti-sense primer binds within the Cre portion of the transgene, generating a 496 bp product in K14-Cre mice but no PCR product in wild type mice (B). A representative agarose gel showing typical genotyping results is shown (C). Genotyping results for the floxed Vegfr1 allele are shown in lanes 2, 4 and 6; a heterozygote is shown in lane 2, a homozygous wild type mouse in lane 4 and homozygous floxed mouse in lane 6 (C). Detection of the K14Cre transgene is shown in lanes 3, 5 and 7; all three mice shown are positive for the K14Cre transgene (C).
2.2.3 Confirmation of Vegfr1 deletion in keratinocytes. Successful deletion of exon 1 of the Vegfr1 gene and subsequent loss of VEGFR-1 mRNA and protein were confirmed in cKO mice. Deletion of the floxed portion of the Vegfr1 gene was confirmed by PCR on DNA from tails snips. Primers (Table 2.1) were used the flank the floxed region of the Vegfr1 gene (Figure 2.3A). In wild type mice, these primers generate a 1834 bp product (Figure 3A). A 367 bp product is produced by these primers when the floxed region has been excised by cre recombinase (Figure 2.3A). The presence of the 367 bp products can be detected in DNA from cKO mice (Figure 2.3B). To confirm a reduction in Vegfr1 mRNA in cKO mice, RT-PCR was performed on RNA isolated from epidermal cells that had been separated from the dermis (see section on RNA isolation and RT-PCR). A decrease in Vegfr1 mRNA was observed in epidermal peels from cKO compared to control mice (Figure 2.3C). Additionally, VEGFR-1 protein was examined by Western Blot in lysates from cultured primary murine keratinocytes. VEGFR-1 protein was detected in keratinocytes from control but not cKO mice (Figure 2.3D).

After confirming that conditional deletion of Vegfr1 was successful, cKO mice were crossed to hairless FVB mice. Hairless SKH-1 mice are susceptible to UV-induced skin carcinogenesis [140-141]. Hairless FVB mice that carry the hairless (Hr) mutation were obtained from Dr. Donna Kuzewitt at MD Anderson. cKO mice, on an FVB background, were crossed to hairless FVB mice. Hairless cKO mice were used in UV and wound healing studies described in Chapters 4 and 5 of this document.
Figure 2.3 Confirmation of Vegfr1 deletion in keratinocytes. Deletion of the floxed portion of the Vegfr1 gene was confirmed by PCR. Primers in which the sense primer (Flt1) binds upstream of the first loxP site and the anti-sense primer (Neo R1) binds downstream of the second loxP site. In mice that lack Cre recombinase, exon 1 will still be present, yielding an 1834 bp product. In cells that express Cre recombinase, exon 1 will be deleted, yielding a 367 bp product. A representative gel showing PCR results from a control and a cKO mouse is shown (B). A control mouse is shown in lanes 2-4 to be homozygous for the wild type Vegfr1 gene (lane 3) and carry the K14 transgene (lane 3). In lane 4, an 1834 bp band is presence, indicating that the Vegfr1 gene is intact (B) However, the 1834 bp band is absent and a 367 bp band is present (lane 7) in cKO mice (which are homozygous for the floxed allele (lane 5) and carry the K14-Cre transgene (lane 6)), indicating that the floxed portion of the Vegfr1 gene has been deleted (B). Additionally, RNA was isolated from epidermal peels of cKO and control mice and a reduction in Vegfr1 mRNA was observed in cKO compared to controls by RT-PCR (C). At the protein level, VEGFR-1 was detected in cultured primary murine keratinocytes from control but not cKO mice by Western blot (D).
<table>
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<th>Product Size</th>
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<td>367 bp</td>
</tr>
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<td>AGATTTAACACAGGTGCTAG</td>
<td>Hairless gene (Hr)</td>
<td>400 bp</td>
</tr>
</tbody>
</table>

Table 2.1 List of primers used to genotype cKO and control mice.

2.3 Animal Experiments

2.3.1 Acute UV studies. All animal experiments were approved by The Ohio State University Institutional Animal Care and Use Committee. For assessment of VEGF levels in the skin following UV, female SKH-1 hairless mice (Charles River) were dorsally exposed to one minimal erythemal dose (MED) of UVB light (2,240 J/m²). UVB dose was determined by a UVX meter (UVP Inc., Upland, CA) and emitted by Philips FS40 UV lamps (American Ultraviolet Company, Lebanon, IN). Mice were euthanized and dorsal skin was collected 2, 6, 12, 24 and 48 hours following UV irradiation. Four mice were examined at each time point. Dorsal skin samples were embedded and frozen in TBS tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) for immunohistochemical analysis. Additionally, dorsal epidermis was separated from the dermis by submersion in 60°C water followed immediately by submersion in ice water [142]. Epidermal and dermal samples were flash frozen in liquid nitrogen and stored at -80°C until use.

For acute UV experiments using cKO and control mice, animals were exposed dorsally to one MED of UVB light and euthanized at 24 or 48 hours following a single exposure or were exposed to one MED three times on non-consecutive days and euthanized 1 week after the initial exposure. Dorsal skin samples were collected at time of sacrifice. Dorsal...
skin thickness was measured at time of sacrifice using calipers. Dorsal skin samples were collected and fixed in formalin for paraffin embedding, frozen in TBS and flash frozen in liquid nitrogen for further analysis. A minimum of 8 mice per group were examined.

2.3.2 Long Term UV-Induced Carcinogenesis Studies. For long term UV-induced skin carcinogenesis studies, cKO or control mice were exposed to 1 MED of UVB light three times per week on non-consecutive days for 15 weeks. Tumors were measured weekly starting after 10 weeks of UV exposure using digital calipers. Tumors measuring 1.5 mm in diameter or greater were used for analysis. At total of 15 control and 12 cKO mice were used in these studies.

2.3.3 Wound Healing Studies, Six week old male cKO or control mice were used in wound healing experiments. Following anesthetization with Isoflurane (Piramal Healthcare, Mumbai India) and cleaning of the dorsal skin surface with 70% isopropyl alcohol, six circular, full-thickness excisional wounds were generated on the dorsal skin of mice using a 3mm biopsy punch. Wounds were allowed to heal for 1, 3, 5, 7, or 14 day without intervention, at which point the mice were sacrificed and wounds were collected for analysis. A minimum of 7 mice per group were wounded. Normal skin was collected from n=6 unwounded control and cKO mice for use as control tissue.

2.4 DNA Isolation and Genotyping

2.4.1 DNA isolation. The genotype of all cKO and control mice used in experiments was determined. Tail snips (0.5 mm) were obtained from pups under 21 days of age. DNA
was isolated from tail snips using the DNeasy Blood and Tissue Kit (Quagen) according to the manufacturer's recommended protocol. Briefly, tail snips were digested overnight at 55°C in 200 µl of a 1:10 mixture of proteinase K solution in ATL buffer. Following digestion, tail lysate was centrifuged to remove debris and the supernatant was transferred to a spin column and centrifuged. The column filter (containing the DNA) was transferred to a new collection tube and washed with 500 µl of AW1, centrifuged, transferred to a new collection tube and washed with 500 µl AW2. DNA was eluated from the column by adding 100 µl AE buffer and centrifuging. The elution step was repeated, resulting in DNA in a final volume of 200 µl of AE buffer.

2.4.2 DNA precipitation and concentration. To precipitate the DNA, 20 µl of 3M sodium acetate (1/10 volume) and 400 µl (2x volume) of cold 100% ethanol were added to 200 µl of DNA in AE buffer. DNA was precipitated at 20°C for a minimum of 1 hour. To concentrate the DNA, samples were centrifuged at 14,000 x g at 4°C for 15 minutes and the supernatant was removed. The DNA pellet was washed in 200 µl of cold 75% ethanol and centrifuged. The supernatant was removed and the pellets were dried at room temperature. Dried pellets were resuspended in 30 µl AE buffer and heated at 55°C for 10 minutes. The final concentration of DNA was determined by spectrometry. Two µl of DNA was added to 98 µl of nuclease-free water (Ambion, Grand Island, NY) and transferred to the wells of a quartz microplate in duplicate. The absorbance were determined at 260 nm and 280 nm using a SpectraMax 190 plate reader and DNA concentrations were calculated using SoftMax Pro software (Molecular Devices, Sunnyvale CA).
2.4.3 Genotyping. Routine genotyping of mice was performed by PCR to confirm the presence or absence of the floxed VEGFR-1 gene and the K14-Cre transgene (See Table 1 for list of primers). PCR was performed in 20 μl reactions containing 1 μl of DNA, 12.5 μl of ReadyMix containing taq polymerase (Sigma, St. Louis, MO), 7 μl of nuclease-free water (Ambion), and sense and anti-sense primers at a final concentration of 1 μM. After a 6-minute incubation at 94°C to activate the Taq polymerase, 35 cycles of PCR were performed with a 30-second denaturation step at 94°C, a 30-second annealing step at 62°C, and a 1-minute extension at 72°C, with a final 7-minute extension at 72°C. PCR products were run on 2.5% agarose gels. Gels were stained in ethidium bromide (Amresco, Solon, Ohio) and bands were detected using the ChemiDoc XRS Imaging System (Bio Rad, Hercules, CA).

2.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

2.5.1 RNA Isolation. Total RNA was isolated from NHEK, primary murine keratinocytes and murine epidermis using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Briefly, cultured cells were lysed in 1 ml of TRIzol reagent on ice for 5 minutes and collected by pipetting. Next, 0.2 ml of chloroform was added per ml of TRIzol. After vigorous shaking, samples were incubated for 2 minutes at room temperature, and then centrifuged for 15 minutes at 12,000 x g at 4°C. The upper, clear aqueous layer was transferred to a clean microtube and 0.5ml 100% isopropanol was added and incubated at room temperature for 10 minutes. Murine epidermis was added to 1 ml of TRIzol containing 20 μl of proteinase K (Qiagen) and incubated on ice for 10 minutes, homogenized, then centrifuged for 10 minutes at 12,000 x g at 4°C to remove debris. The aqueous layer was
transferred to a new microtube, combined with 0.2 ml chloroform, rocked at 4°C for 30 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. The clear, upper aqueous layer was transferred to a new tube, combined with 0.5 ml of isopropanol and incubated at -20°C for 2 hours. Samples in isopropanol from either murine epidermis or cultured cells were centrifuged at 12,000 x g at 4°C for 10 minutes. The supernatant was removed and the pellets were washed twice in 1 ml of 75% ethanol by centrifuging at 7500 x g at 4°C for 5 minutes. Pellets were dried at room temperature and resuspended in 25 μl DNase/RNase free water and heated for 10 minutes in a 60°C water bath. RNA quality was determined by RNA 6000 Nano Assay (Agilent, Santa Clara, CA) and the final concentration was determined using a Nanodrop (Nanodrop, Wilminton DE) by the Ohio State University Nucleic Acid Shared Resource core facility.

2.5.2 Reverse transcription polymerase chain reaction. To examine the expression of VEGF and VEGFR-1, reverse transcription polymerase chain reaction (RT-PCR) was performed on RNA isolated from cultured cells or murine epidermis using the following primers: human VEGFR-1 sense 5' CTA GGA TCC GTG ACT TAT TTT TTC TCA ACA AGG 3', human VEGFR-1 anti-sense 5' CTC GAA TTC AGA TCT TCC ATA GTG ATG GGC TC 3' (240-bp product); human VEGF sense 5' TGG GTG CAT TGG AGC CTT GCC TTG CTG CTC 3', human VEGF anti-sense 5' TCT GGT TCC CGA AAC CCT GAG GGA GCC TTC 3'; mouse VEGF sense 5' GAA GTC CCA TGA AGT GAT CAA G 3', mouse VEGF anti-sense 5' AAA CCC TGA GGA GCC TTC TT 3'; β-actin sense 5' CCC TGG AGA AGA GCT ATG AG 3', β-actin anti-sense 5' GGC ATA AGA GTC TTT ACG GA 3'. Briefly, 2 μg of total RNA was reverse transcribed at 42°C for 15 minutes in 20 μl containing 12.5 mM Tris HCl, 18.75 mM KCl, 0.75 mM MgCl, 5 mM DTT, 0.5 mM...
dNTP mix, 0.5 μg oligo(dT) primers and 2 U M-MLV reverse transcriptase (all from Invitrogen Corp., Carlsbad, CA). Following reverse transcription, PCR was performed in 25 μl reactions containing 2 μl cDNA from the RT reaction, 12.5 μl ReadyMix Taq polymerase (Sigma) and 0.5 μM sense primer and 0.5 μM anti-sense primer (Invitrogen). After a 6-minute incubation at 95°C to activate the Taq polymerase, 30 (for VEGF primers) or 36 (for VEGFR-1 primers) cycles of PCR were performed with a 30-second denaturation at 94°C, 30-second annealing at 62°C, and a 1-minute extension step at 72°C, with a final 7-minute extension step at 72°C. PCR products were run on 2% agarose gels and bands were detected using the ChemiDoc XRS System (Bio Rad). Bands were quantified using Quantity One 1-D analysis software (Bio Rad).

2.6 Apoptosis Detection by Flow Cytometry
Apoptotic cells were detected by flow cytometry. Briefly, NHEK or SCC-13 adherent cells were removed from dishes by trypsinization and combined with detached cells from the supernatant 24 hours following UV irradiation or mock irradiation and washed 2 times in PBS. Cells were then resuspended at 1x10^6 cells per ml in 1x Binding Buffer (BD Biosystems) and 1x10^5 cells were stained with 5 μl FITC-conjugated annexin V and 5 μl propidium iodide (BD Biosystems, San Jose, CA) for 10 minutes at room temperature in the dark. Staining was detected within 1 hour using an LSR II flow cytometer and analyzed using FACS DIVA software (BD Biosystems).

2.7 Histological Techniques

2.7.1 Sample preparation for histological analysis. Doral skin, excisional wounds and skin tumors were collected from mice after sacrifice. For frozen sections, tissues were
embedded in Tissue Freezing Medium (Triangle Biomedical Sciences INC, Durham NC), frozen on dry ice and stored at -80°C until sectioned. Ten μm sections were cut from frozen samples onto microscope slides (Fisher Scientific, Pittsburgh, PA) using a CM 1950 cryostat (Leica, Buffalo Grove, IL) and stored at -80°C until use. For paraffin sections, tissues were fixed in 10% formalin (Fisher Scientific) overnight and then placed in cold PBS prior to embedding into paraffin. Paraffin blocks were cut onto microscope slides (4 μm sections) using a microtome, baked at 60°C for one hour and stored at room temperature until use.

2.7.2 Immunohistochemical detection of VEGFR-1, F4/80 and PECAM-1.

Immunohistochemistry was performed on frozen sections collected from various murine and human tissues for the detection of VEGFR-1, F4/80 or PECAM-1 (Table 2.2). Slides containing frozen sections were fixed in acetone for 15 minutes. After washing in PBS three times for 3 minutes each, sections were treated with 0.3% H2O2 in methanol for 30 minutes to quench endogenous peroxidase activity. The slides were washed again in PBS and blocked with normal serum of the appropriate species (Vector Laboratories, Burlingame, CA) diluted 1:10 in PBS for 30 minutes (Table 2.2). Sections were incubated overnight at 4°C with primary antibody (Table 2.2). Incubation with the same concentration of isotype-matched nonspecific antibody (R&D Systems) or with no primary antibody was used as a negative control. After washes in PBS, appropriate biotinylated secondary antibody (Vector Labs) was added at a 1:200 dilution in 10% serum and sections were incubated at room temperature for 30 minutes. Following washes in PBS, slides were incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 30 minutes, washed, then incubated with 3,3-
diaminobenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 8 minutes in the dark. Sections were counterstained with hematoxylin and coverslips were mounted with Permount (Fisher Scientific). F4/80 staining was quantified by manually counting the number of positively-stained cells within the dermis in five unique fields at 40x magnification per slide for acute UV studies. For wound studies, F4/80 positive cells were quantified at the left and right wound margin at 40x magnification. PECAM-1 staining was quantified using Image J software to determine the percent area occupied by positive staining, considered to be blood vessels, in a defined area of the dermis. For PECAM-1, five individual 20x fields were analyzed per slide for acute UV studies and 20x fields taken at the wound bed for wound studies.

2.7.3 Immunohistochemical detection of LyG6, active caspase-3, and Ki67.

Immunohistochemistry was performed on paraffin sections collected from various murine tissues for the detection of Ly6G, active caspase-3 or Ki67 (Table 2.2). Slides containing paraffin-embedded sections were deparaffinized in Clear-Rite 2 (Thermo Scientific) and rehydrated in a series of graded ethanols. Antigen retrieval was performed, if required, by steaming slides for 20 minutes in a 1:10 dilution of target retrieval solution (Dako, Carpinteria CA) or in antigen unmasking solution (Vector) and cooled at room temperature for 10 minutes. After washing in PBS, sections were treated with 3% H$_2$O$_2$ in water for 10 minutes to quench endogenous peroxidase activity, if required. The slides were washed in PBS and blocked with normal serum of the appropriate species (Vector) dilutes 1:10 in PBS for 30 minutes (See Table). Sections were incubated overnight at 4°C with primary antibody (See table). Incubation with the
same concentration of biotinylated isotype-matched nonspecific antibody (R&D Systems) was used as a negative control. After washes in PBS, appropriate biotinylated secondary antibody (Vector Labs, Burlingame CA) was added at a 1:200 dilution in 10% serum and sections were incubated at room temperature for 30 minutes. Following washes in PBS, slides were incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 30 minutes, washed, then incubated with 3,3-diaminobenzidine (Kirkegaard and Perry Laboratories) for 8 minutes. Sections were counterstained with hematoxylin 2 and coverslips were mounted with Permount (Fisher Scientific).

Ly6G staining was quantified by manually counting the number of positively-stained cells within the dermis in five unique fields at 40x magnification per slide for acute UV studies. For wound studies, Ly6G-positive cells were quantified at the left and right wound margin at 20x magnification using particle count feature of Image J software to determine number of positively stained cells. Active caspase-3 staining was quantified by manually counting the number of positively-stained cells in the epidermis of 10 unique fields at 40x magnification. Active caspase-3 staining was quantified by manually counting the number of positively-stained cells in the epidermis of 5 unique fields at 40x magnification.

2.7.4 Histological measurement of epidermal and dermal thickness. To determine the thickness of the epidermis and dermis following UV exposure, hematoxylin and eosin (H&E) staining was performed on paraffin embedded sections of murine dorsal skin.
Table 2.2 Table summarizing the immunohistochemical methods used with each primary antibody utilized in these studies

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<td>rabbit anti-mouse</td>
<td>Abcam</td>
<td>1:100</td>
<td>goat</td>
<td>biotinylated goat anti-rabbit</td>
<td>frozen</td>
<td>Dako</td>
<td>MeOH/0.3% H2O2</td>
</tr>
<tr>
<td>Ki67</td>
<td>rabbit anti-mouse</td>
<td>Abcam</td>
<td>1:250</td>
<td>goat</td>
<td>biotinylated goat anti-rabbit</td>
<td>frozen</td>
<td>Vector</td>
<td>water/3.0% H2O2</td>
</tr>
</tbody>
</table>

Briefly, sections were deparaffinized in Clear-Rite 2 (Thermo Scientific) a rehydrated in a series of graded ethanols and stained in Hemotoxylin 2 (Fisher Scientific) for 8 minutes. Slides were then cleared in running tap water, dipped 3 times in acid alcohol (1 ml concentrated hydrochloric acid in 200 ml of 80% ethanol) and cleared in water. Slides were dipped 10 times in ammonium hydroxide (6 drops of 29.8% ammonium hydroxide in 200 ml water) and cleared in water and dipped in 95% ethanol before being dipped 10 times into alcoholic eosin (Fisher Scientific) Samples were then dehydrated in alcohols, cleared in Clear-Rite and cover slipped with Permount (Fisher Scientific). AxioVision software (Zeiss, Oberkochen, Germany) was used to measure the distance from the epidermal/dermal border to the surface of the epidermis for epidermal measurements or from the epidermal/dermal border, down through the dermis, stopping at the muscle layer for dermal measurements. Five measurements were taken for the dermis and epidermis in each of three 10x fields per section.

2.7.5 Analysis of wound reepithelialization. Reepithelialization was measured in excisional wounds collected from cKO or control mice. H&E staining was performed on
frozen sections taken from the middle of the wounds. The width of the entire wound bed and the distance of the wound bed covered by new epidermis were measured at 5x magnification. The percent reepithelialization was calculated by dividing the reepithelialized distance by the width of the entire wound bed and multiplying by 100.

2.8 Enzyme-linked immunosorbent assay (ELISA)
Concentrations of VEGF in cell culture supernatants, cultured cell lysates, murine epidermal protein, and total protein from whole murine skin or whole wounds were determined using commercial ELISA kits (R&D Systems). Cell culture supernatants were diluted 1:2 in reagent diluent (1 x PBS containing 1% bovine serum albumin (Miltenyi Biotec, Auburn, CA)). For lysates, 100 μg of protein was loaded per well in a final volume of 100 μl in reagent diluent. All samples were run in duplicate. Assays were performed according to the manufacturer’s protocol. Plates were read on a SpectraMax 190 plate reader and results were analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

2.9 Western Blot Analysis
Cultured primary murine keratinocytes, primary human keratinocytes or SCC tumor cells were washed twice in PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Pierce, Rockford, IL) for 20 minutes on ice. Cell debris was removed from the lysates by centrifugation. Murine epidermis was homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors, sonicated and centrifugation to remove debris. Total protein content of lysates was measured by BCA protein assay (Pierce). Forty μg of protein was separated on 10% acrylamide gels (Bio
Rad) and transferred to PVDF membrane (Bio Rad). Following transfer, membranes were blocked in Tris-Buffered Saline with 1% Tween (TBST) containing 5% dry milk. Blots were incubated overnight at 4°C with anti-human VEGFR-1 antibody (R&D) at 0.2 μg/ml or anti-mouse VEGFR-1 antibody (Abcam) at 1:1000 in TBST with 5% milk. Membranes were washed in TBST and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody (R&D) diluted 1:1000 in TBST with milk. Membranes were developed using the Western C Chemiluminescence Detection Kit (Bio Rad) and bands were detected using the ChemiDoc XRS Imaging System (Bio Rad). Blots were stripped with Restore PLUS western blot stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with a β-actin antibody that reacts with both mouse and human β-actin (Cell Signaling) at 1:1000 in TBST with 5% BSA. Results were quantified by densitometry using Quantity One 1-D analysis software (Bio Rad).

2.10 Collection of human basal cell and squamous cell carcinomas

To examine VEGFR-1 expression in human non-melanoma skin tumors, tumor samples were obtained from patients. All studies were conducted according to protocols approved by The Ohio State University IRB and informed consent was obtained from all patients prior to sample collection. Frozen tumor samples were collected from patients undergoing Mohs micrographic surgery to remove basal cell carcinomas (BCC) or squamous cell carcinomas (SCC). Samples were embedded in TBS tissue freezing medium (Triangle Biomedical Sciences) and stored at -80°C until use. A total of 25 BCC samples and 25 SCC samples were collected.
2.11: Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) or Microsoft Excel. Statistical differences were determined by two-way analysis of variance with Bonferroni’s post-hoc tests or unpaired t-test, with \( p \)-values of < 0.05 considered statistically significant.
Chapter 3: Regulation of keratinocyte VEGF and VEGFR-1 by UV light and the role of VEGF on apoptosis in UV-irradiated keratinocytes in vitro

3.1. Introduction

Non-melanoma skin cancer is by far the most common type of cancer [4, 13]. In fact, an estimated 3.5 million new cases of non-melanoma skin cancer will be diagnosed in 2 million patients this year in the US alone, and rates continue to rise annually [4-6]. Ultraviolet light from the sun is the primary causative agent of these cancers [13-14, 22]. UV radiation is considered to be a complete carcinogen due to its ability to both initiate and promote skin tumors [21]. The effects of UV exposure on the skin include direct and indirect DNA damage, inflammation, and the production of growth factors, cytokines and other soluble mediators [30-31, 38-39].

One growth factor known to be produced by the skin in response to UV radiation is vascular endothelial growth factor A (VEGF) [41-43]. VEGF is a potent pro-angiogenic factor that belongs to the vascular endothelial growth factor family of small heparin binding glycoproteins of which VEGF-B, VEGF-C, VEGF-D and placental growth factor are also members. VEGF levels in the skin are normally low and have been shown to increase in response to UV exposure in both human and murine skin [41-42, 82, 93]. Epithelial keratinocytes in the epidermis of the skin have been identified as the primary source of VEGF [82], although macrophages and stromal cells can contribute some VEGF as well [55]. Several studies have demonstrated a link between VEGF and skin
carcinogenesis and have attributed these results to the pro-angiogenic effects of VEGF [107-109].

VEGF induces angiogenesis by interacting with VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2) on vascular endothelial cells. VEGF induces the proliferation, migration and survival of endothelial cells, leading to the growth and expansion of the vasculature [88-89]. VEGFR-1 has now been identified on a wide variety of cell types, including monocytes, pericytes, neurons, bone marrow-derived cells and keratinocytes [105, 126-127, 129-132], suggesting that VEGF may have functions outside of angiogenesis. Indeed, recent studies have shown that VEGF can directly affect keratinocyte proliferation and migration [132-135]. Because VEGF is a potent survival factor for endothelial cells [88] and is produced in response to UV light [41-42], the potential role of VEGF/VEGFR-1 signaling in keratinocyte and the SCC-13 tumor cell line survival following UV exposure was examined.

3.2: Results

3.2.1 UV-induced apoptosis is not affected by VEGF/VEGFR-1 neutralization in primary human keratinocytes. UV is known to induce apoptosis in keratinocytes both in vitro and in vivo [143-146]. To assess the role of VEGF on keratinocyte survival following UV radiation, primary normal human epidermal keratinocytes (NHEK) were exposed to 300 J/m^2 of UV light and treated with neutralizing antibodies to VEGF or VEGFR-1. Cells were collected 24 hours following UV exposure and stained with annexin-V and propidium iodide to detect apoptotic cells by flow cytometry. A representative histogram shows that UV irradiation caused more than a 2-fold increase
in the total number of NHEK undergoing apoptosis (annexin-V⁺ and annexin-V⁺/FITC⁺
cells) 24 hours following exposure (9.6% for unirradiated control (no UV); 24.2% for
irradiated (UV)) (Figure 3.1A). Treatment with neutralizing antibodies to VEGF or
VEGFR-1 did not significantly alter the percentage of apoptotic cells after UV exposure
compared to irradiated cells treated with IgG (Figure 3.1B).

3.2.2 UV inhibits VEGF production in keratinocytes in vitro. After discovering that
blocking VEGF did not impact UV-induced apoptosis, VEGF production in response to
UV was examined to confirm that VEGF levels were increased following UV as
described in the literature [41-43, 96]. NHEK were grown to 70% confluence and
supernatants, cell lysates and RNA were collected at 6 or 24 hours following 300 J/m²
UV. While unirradiated NHEK produced increasing levels of VEGF over time,
surprisingly, UV irradiation significantly inhibited secretion of VEGF into the supernatant
(Figure 3.2A). Examination of mRNA also showed a decrease in VEGF (isoform 165)
expression 24 hours following UV compared to unirradiated cells (Figure 3.2B). VEGF
protein was also significantly decreased in cell lysates collected from irradiated NHEK
compared to unirradiated controls (Figure 3.2C). The reduction in VEGF from cell lysates
suggests that the observed decrease in VEGF secretion (and was not simply due to an
increase in the number of apoptotic cells (Figure 3.1). These results are in contrast to
previously published studies demonstrating an increase in VEGF production in
epidermal keratinocytes of UV-irradiated human and murine skin [41-42]. Therefore, we
hypothesized that VEGF production may be controlled by association with components
of the extracellular matrix that would be present in the whole skin but absent in culture.
To explore this, NHEK were grown in 6-well culture dishes coated with laminin,
Figure 3.1 Neutralization of VEGF or VEGFR-1 does not affect UV-induced apoptosis in cultured primary keratinocytes. Primary human keratinocytes (NHEK) were collected 24 hours following exposure to 300 J/m² of UV and stained with annexin-V and propidium iodide (PI) to assess the percentage of cells undergoing apoptosis by flow cytometry. Representative histograms of unirradiated cells (0 J/m²) and cells irradiated with 300 J/m² UV are shown (A). NHEK cells were exposed to 300 J/m² of UV and treated with 5 µg/ml of anti-VEGF or anti-VEGFR-1 neutralizing antibodies or control IgG and assayed for apoptosis by flow cytometry 24 hours after UV exposure (B). Apoptotic cells were quantified as the percentage of cells in early apoptosis (annexin-V⁻/PI⁻) plus the percentage in late apoptosis (annexin-V⁺/PI⁺). 10,000 gated events were analyzed per sample. The mean percentages of apoptotic cells +/- standard error of the mean (S.E.M.) are denoted in the graphs (cells from n=3 separate cultures per group; *p<0.05 compared to unirradiated (No UV) cells). No significant differences (n.s.) were observed between treatment groups in cells exposed to the same dose of UV.
fibronectin or collagen IV and exposed to 300 J/m$^2$ of UV and VEGF levels were examined in the supernatant 24 hours after UV irradiation (Figure 3.2D). A significant decrease in VEGF production was observed following UV-irradiation in all cultures regardless of substrate, suggesting that the decrease in VEGF observed in Figures 3.2A-C was not due to lack of adherence to extracellular matrix substrates.

### 3.2.3 VEGF treatment does not alter UV-induced apoptosis in keratinocytes.

Because VEGF production is reduced in response to UV in this system, the effect of exogenous VEGF on UV-induced apoptosis was examined. Cultured NHEK cells were exposed to 300 J/m$^2$ of UV light, treated with or without 100 ng/ml of VEGF 165; and assayed for apoptosis by flow cytometry. Representative histograms from NHEK cells exposed to 300 J/m$^2$ and treated with or without VEGF are shown (Figure 3.3A). Although UV exposure caused a significant increase in apoptosis as expected, the addition of VEGF did not alter the percentage of apoptotic cells after UV irradiation compared to untreated, UV-irradiated NHEK (Figure 3.3B). Taken together with Figure 2.1, these results suggest that VEGF does not mediate the survival of primary human keratinocytes after UV exposure in vitro.

### 3.2.4 VEGFR-1 is expressed in NHEK.

Previous studies have demonstrated that primary keratinocytes express VEGFR-1 [131-132, 135]. Because VEGF had no effect on keratinocyte survival following UV irradiation, we sought to confirm the expression of VEGFR-1 on cultured keratinocytes. NHEK cells were grown to 70% confluence on chamber slides and positive staining for VEGFR-1 was observed by immunohistochemistry (Figure 3.4A). To determine VEGFR-1 expression following UV
Figure 3.2 UV inhibits VEGF production in cultured primary keratinocytes. NHEK cells were exposed to 300 J/m² of UV. Supernatants were collected 6 or 24 hours following irradiation and VEGF protein content was examined by ELISA (A; n=6 per group). RT-PCR was performed on RNA to determine relative VEGF expression in NHEK cells 24 hours following irradiation compared to unirradiated cells (n=3 per group) (B). The data are presented as the ratio of VEGF (isoform 165) mRNA to β-actin. VEGF protein content in NHEK lysates was determined by ELISA and is reported as pg of VEGF per mg of total protein in the lysate (C; n=3-4 per group). NHEK were grown on 6 well plates coated with laminin, fibronectin or collagen IV. After exposure to 300 J/m² of UV, VEGF secretion into the supernatant was examined by ELISA at 24 hours (D; n=12 per group). For all graphs, mean values +/- S.E.M. are denoted; *p < 0.05, **p < 0.01, ***p < 0.001 compared to unirradiated cells from the same time point.
Figure 3.3 VEGF treatment does not alter UV-induced apoptosis in cultured primary keratinocytes. To examine the effect of exogenous VEGF on survival following UV irradiation, NHEK cells were exposed to 300 J/m² of UV and treated with or without 100 ng/ml recombinant VEGF 165. The percentage of apoptotic cells was determined by flow cytometry 24 hours following irradiation. Apoptotic cells were quantified as the percentage of cells in early apoptosis (annexin-V⁻/PI⁻) plus the percentage in late apoptosis (annexin-V⁻/PI⁺). 10,000 gated events were analyzed per sample. Representative histograms of untreated control or VEGF-treated cells 24 hours after 300 J/m² UV are shown (A). The mean percentages of apoptotic cells +/- S.E.M. are denoted in the graph (cells from n=3 separate cultures per group; *p < 0.05 compared to unirradiated control cells receiving the same treatment). No significant differences (n.s.) were observed between treatment groups in cells exposed to the same dose of UV.
exposure, western blotting was performed on whole cell lysate obtained from irradiated (300 J/m²) and unirradiated NHEK cells (Figure 3.4B). VEGFR-1 was lower in irradiated cells but the difference did not reach statistical significance. Overall, these results indicate that keratinocytes express VEGFR-1 and that VEGFR-1 expression may be reduced 24 hours after UV exposure.

**Figure 3.4 VEGFR-1 is expressed by cultured primary keratinocytes.** To confirm VEGFR-1 expression, NHEK cells were grown to 70-80% confluence on chamber slides and VEGFR-1 protein expression was examined by immunohistochemistry (A). Positive staining for VEGFR-1 was observed compared to NHEK cells stained with a non-specific isotype control antibody (control). To examine the effect of UV on VEGFR-1 expression, NHEK cell lysates were collected 24 hours following irradiation and VEGFR-1 protein levels were determined by Western blot (B). The bar graph shown in B represents relative VEGFR-1 expression compared to β-actin as determined by densitometry. The mean values +/- S.E.M. are denoted in the graphs (n=3 separate cultures per group; No significant difference (n.s.) was observed between irradiated and unirradiated cells).
3.2.5 UV-induced apoptosis is not affected by VEGF/VEGFR-1 neutralization in SCC tumor cells in vitro. The results in NHEK cells suggest that VEGF does not affect primary keratinocyte survival following UV exposure. However, squamous cell carcinoma tumors cells, which are of keratinocyte origin, could respond differently. Therefore, the role of VEGF in SCC tumor cell survival following UV exposure was investigated. Human SCC-13 tumor cells were exposed to 150 or 300 J/m² of UV light and treated with neutralizing antibodies to VEGF or VEGFR-1. Cells were collected 24 hours following UV exposure and stained with annexin-V and propidium iodide to detect apoptotic cells by flow cytometry. Representative histograms show that 150 or 300 J/m² of UV irradiation caused an increase in the total number of SCC-13 cells undergoing apoptosis (annexin-V⁻ and annexin-V⁺/FITC⁺ cells) 24 hours following exposure (37.4% for unirradiated control, 0 J/m² UV; 79.3% for 150 J/m² UV; 84.2% for 300 J/m² UV) (Figure 3.5A). UV increased the percentage of SCC-13 cells undergoing apoptosis by approximately 2.5-fold, similar to NHEK cells (Figure 3.1); however, overall higher levels of apoptosis were seen in both unirradiated and irradiated SCC-13 cells compared to NHEK. Treatment with neutralizing antibodies to VEGF or VEGFR-1 did not significantly alter the percentage of apoptotic cells after UV exposure compared to irradiated cells treated with IgG (Figure 3.5B). These results show that UV induces apoptosis in SCC-13 cells and that they may be more sensitive to UV light than primary keratinocytes. Additionally, VEGF or VEGFR-1 neutralization did not significantly affect UV-induced apoptosis in SCC-13 tumor cells in vitro.

3.2.6 UV inhibits VEGF production in SCC cells. VEGF is produced at elevated levels in squamous cell carcinomas; however it is not known if VEGF production is altered in
Figure 3.5 Neutralization of VEGF or VEGFR-1 does not affect UV-induced apoptosis in cultured SCC tumor cells. SCC-13 tumor cells were collected 24 hours following exposure to 150 or 300 J/m² of UV and stained with annexin-V and propidium iodide (PI) to assess the percentage of cells undergoing apoptosis by flow cytometry. Representative histograms of unirradiated cells (0 J/m²) and cells irradiated with 150 or 300 J/m² UV are shown (A). SCC-13 cells were exposed to 150 or 300 J/m² of UV and treated with 5 µg/ml of anti-VEGF or anti-VEGFR-1 neutralizing antibodies or control IgG and assayed for apoptosis by flow cytometry 24 hours after UV exposure (B). Apoptotic cells were quantified as the percentage of cells in early apoptosis (annexin-V⁺/PI⁻) plus the percentage in late apoptosis (annexin-V⁺/PI⁺). 10,000 gated events were analyzed per sample. The mean percentages of apoptotic cells +/- S.E.M. are denoted in the graph (cells from n=3 separate cultures per group; *p< 0.05 compared to unirradiated control cells receiving the same treatment). No significant differences (n.s.) were observed between treatment groups in cells exposed to the same dose of UV.
Figure 3.6 UV inhibits VEGF production in SCC-13 cells. SCC-13 cells and SCC-12F cells were exposed to 150 or 300 J/m² of UV. Supernatants from SCC-13 cells (A) or SCC-12F cells (B) were collected 6 - 72 hours following irradiation and VEGF protein content was examined by ELISA. VEGF levels were also examined in SCC-13 RNA 6 - 24 hours following irradiation by RT-PCR and in SCC-13 cell lysates by ELISA 6 - 72 hours following irradiation (C). For all graphs, mean values +/- S.E.M. are denoted (samples from n=3-5 separate cultures per group; *p < 0.05, **p < 0.01, ***p < 0.001 compared to unirradiated cells from the same time point).
squamous cell carcinoma tumor cells in response to UV. To examine VEGF production following UV exposure in SCC tumor cell lines, SCC-13 cells were grown to 70% confluence and supernatants and cell lysates were collected at 6, 12, 24, 48 or 72 hours following exposure to 150 or 300 J/m² UV. While unirradiated SCC-13 cells produced increasing levels of VEGF over time, UV irradiation significantly inhibited secretion of VEGF into the supernatant at both UV doses (Figure 3.6A). VEGF inhibition following UV was confirmed in an additional squamous cell carcinoma cell line, SCC-12F (Figure 3.6B). Furthermore, VEGF protein levels were significantly decreased in cell lysates collected from irradiated SCC-13 cells compared to unirradiated controls (Figure 3.6C). The reduction in VEGF in lysates from irradiated cells suggests that the observed decrease in VEGF secretion was not simply due to an increase in the number of apoptotic cells (Figure 3.5). These results suggest that, similar to NHEK cells, VEGF production is inhibited by UV light in SCC tumor cell lines at the doses examined in this study.

3.2.7 VEGF treatment does not alter UV-induced apoptosis in SCC cells. Because VEGF production in SCC-13 and NHEK cells is reduced in response to UV, the effect of exogenous VEGF on UV-induced apoptosis was examined. Cultured SCC-13 cells were exposed to 150 or 300 J/m² of UV light, treated with PBS or 100 ng/ml recombinant VEGF 165, and assayed for apoptosis by flow cytometry. Representative histograms from SCC-13 cells exposed to 300 J/m² and treated with or without VEGF are shown (Figure 3.7A). Exposure to 150 or 300 J/m² caused an increase in the percentage of apoptotic cells; however, the addition of VEGF did not alter the percentage of apoptotic cells after UV irradiation compared to untreated, UV-irradiated control cells (Figure
3.7B). These results suggest that VEGF does not mediate the survival of SCC tumor cells after UV exposure *in vitro*.

### 3.2.8 UV down-regulates VEGFR-1 in SCC cells.

It is known that VEGFR-1 is expressed in keratinocytes and is up-regulated in human SCC tumors [132-133, 147]. To assess whether VEGFR-1 is regulated by UV exposure *in vitro*, SCC-13 cells were grown to 70% confluence and exposed to 300 J/m² of UV. Cell lysates were collected 6 or 24 hours following irradiation to examine VEGFR-1 expression. SCC-13 cells expressed VEGFR-1 and UV exposure caused a decrease in VEGFR-1 protein expression at 24 hours (Figure 3.8A). VEGF receptor expression is known to be regulated by VEGF in endothelial cells [148-149]; therefore, the reduction in VEGFR-1 levels following UV irradiation could potentially be due to a direct down-regulation by UV or an indirect result of the loss of VEGF. To explore the second possibility, SCC-13 cells were exposed to 300 J/m² UV and treated with 100 ng/ml of VEGF or PBS as a control. The addition of VEGF did not rescue the UV-induced decrease in VEGFR-1 expression (Figure 3.8B), suggesting that VEGFR-1 is not down-regulated after UV exposure solely due to low VEGF levels. Furthermore, the addition of a VEGF neutralizing antibody to SCC-13 cultures in the absence of UV, when VEGF levels are high, did not alter VEGFR-1 expression compared to cells treated with control IgG (Figure 3.8C). Together, the data suggest that VEGFR-1 expression is regulated independently of VEGF in SCC-13 tumor cells.
Figure 3.7 VEGF treatment does not alter UV-induced apoptosis in SCC-13 tumor cells. To examine the effect of exogenous VEGF on survival following UV irradiation, SCC-13 cells were exposed to 150 or 300 J/m² of UV and treated with or without 100 ng/ml recombinant VEGF 165. The percentage of apoptotic cells was determined by flow cytometry 24 hours following irradiation. Apoptotic cells were quantified as the percentage of cells in early apoptosis (annexin-V⁺/PI⁻) plus the percentage in late apoptosis (annexin-V⁺/PI⁺). 10,000 gated events were analyzed per sample (B). Representative histograms of untreated control or VEGF-treated cells 24 hours after 300 J/m² UV are shown (A). The mean percentages of apoptotic cells +/- S.E.M. are denoted in the graph (cells from n=3 separate cultures per group; *p < 0.05 compared to unirradiated control cells receiving the same treatment). No significant differences (n.s.) were observed between treatment groups in cells exposed to the same dose of UV.
Figure 3.8 UV decreases VEGFR-1 expression in SCC-13 cells. To examine the effect of UV on VEGFR-1 levels, SCC-13 cells were exposed to 300 J/m² of UV and treated with or without 100 ng/ml of VEGF 165. Cell lysates were collected 6 or 24 hours following irradiation and VEGFR-1 protein levels were determined by Western blot (A, B). A representative image of a Western blot is shown in A and quantification of the bands by densitometry is shown in B. Additionally, SCC-13 cell lysates were collected from unirradiated cells 6 or 24 hours following treatment with 5 μg/ml of anti-VEGF antibodies or control IgG (C). The bar graphs represent relative VEGFR-1 expression compared to β-actin as determined by Western blot. The mean values +/- S.E.M. are denoted in the graphs (n=3-6 separate cultures per group; **p< 0.01 compared to unirradiated cells). No significant differences (n.s.) were observed between VEGF treatment groups and control.
3.2.9 Keratinocytes upregulate VEGF and maintain VEGFR-1 expression following UV exposure in vivo. In contrast to the reduction in VEGF observed in cultured cells in Figures 3.2 and 3.6, previous reports have described an increase in VEGF in the epidermis of human and murine skin following exposure to UV [42-43]. Therefore, VEGF expression levels were examined in UV-irradiated keratinocytes in vivo. SKH-1 hairless female mice were exposed to 1 MED of UV light (2240 J/m²) and epidermal protein was collected to examine VEGF levels. The levels of VEGF in total epidermal protein increased over time following UV irradiation (Figure 3.9A). To confirm that the VEGF protein was originating from epidermal cells, RT-PCR was performed on RNA isolated from the epidermis. Two isoforms of VEGF were detected at high levels in the epidermis, VEGF 120 and VEGF 164. Expression of both isoforms began to increase as early as 2 hours after UV irradiation and peaked at 48 hours (Figures 3.9B and 3.9C). These results are in line with previously published studies showing an increase in VEGF post-irradiation in the skin, but differ from the results seen in cultured cells (Figures 3.2 and 3.6). Overall, these results suggest differential regulation of keratinocyte VEGF production in response to UV in vivo and in vitro.

In addition to VEGF, VEGFR-1 expression was also examined in murine skin following acute UV exposure. To date, the regulation of VEGFR-1 in response to UV has not been examined in UV-irradiated skin. VEGFR-1 was examined by immunohistochemistry in frozen sections of dorsal skin taken from UV-irradiated SKH-1 mice. Keratinocytes in the epidermis of unirradiated skin showed positive staining for VEGFR-1 (Figure 3.9D). Positive staining was also observed in dermal blood vessels, as expected. VEGFR-1 remained highly expressed in keratinocytes 24 and 48 hours following UV exposure.
indicating that VEGFR-1 expression is highly expressed in keratinocytes following UV exposure. Taken together, these results show that, following UV exposure, VEGF is upregulated by keratinocytes and VEGFR-1 is highly expressed, suggesting the potential for an autocrine signaling loop that could affect keratinocyte function in vivo.

Figure 3.9 VEGF production increases in keratinocytes following UV irradiation in vivo. Female SKH-1 hairless mice were exposed to 2240 J/m² of UV and epidermal protein was isolated from dorsal skin collected at 2, 6, 12, 24 or 48 hours following irradiation and from unirradiated skin. VEGF content was examined in epidermal protein by ELISA (A). Results are reported as pg of VEGF per mg of total protein. Relative expression of VEGF isoforms VEGF 120 and VEGF 164 were examined in RNA isolated from the epidermis by RT-PCR (B and C). Irradiated and unirradiated dorsal skin was stained for VEGFR-1 by immunohistochemistry (D). The data are presented as the ratio of VEGF to β-actin. For all graphs, mean values +/- S.E.M. are denoted; n=4 mice per group; *p < 0.05 compared to unirradiated skin.
3.3: Discussion

VEGF is an important pro-angiogenic factor that is known to be up-regulated in the skin in response to UV light [41-43]. VEGF receptors have been identified on non-endothelial cells, indicating a role for VEGF outside of angiogenesis [126-127, 129, 150-151]. Keratinocytes express VEGFR-1 and it has been shown that keratinocytes can respond to VEGF directly. For example, cultured NHEK have been shown to proliferate when treated with VEGF and cultured keratinocyte migration is increased by VEGF treatment in scratch assays [131-134].

In the present study, the effect of VEGF on keratinocyte survival in response to UV irradiation was examined. In culture, UV exposure induced apoptosis in both NHEK and SCC-13 cells as expected. However, neutralizing antibodies to VEGF or VEGFR-1 did not alter the percentage of apoptotic cells 24 hours following exposure to UV, suggesting that VEGF and VEGFR-1 do not affect the survival of these cells in vitro. This finding is in contrast to endothelial cells, for which VEGF is a potent survival factor [88]. One potential explanation for the lack of effect with neutralizing antibodies is that the expression and production of VEGF and VEGFR-1 were reduced after UV irradiation in vitro. To overcome the reduction in VEGF resulting from UV exposure, the effects of exogenous VEGF on UV-induced apoptosis were also examined. As with the neutralizing approaches, treatment with recombinant VEGF did not alter the percentage of apoptotic cells after UV exposure. Together, the data suggest that VEGF/VEGFR-1 signaling does not mediate UV-induced apoptosis in vitro. It is possible that VEGF could
promote the survival of keratinocytes *in vivo* but not in cultured cells. This possibility will be explored in Chapter 4.

Keratinocytes in the epidermis have been implicated as the primary source of VEGF in the skin [82, 90, 152] and several experiments have demonstrated that UV irradiation increases the production of VEGF in murine and human skin [41-43]. Contrary to these studies, the results presented here indicate a reduction in VEGF in both NHEK and SCC-13 cells after UV exposure *in vitro*. These conflicting data may be explained by the fact that VEGF appears to be regulated differently in response to UV under *in vitro* and *in vivo* conditions. In contrast to the reduction in VEGF expression after irradiation in cultured keratinocytes, epidermal samples from UV-irradiated mice showed increased VEGF expression compared to unirradiated controls. While studies in the skin have clearly shown that UV induces VEGF expression in keratinocytes, results in cultured cells have varied. Several published studies have shown that transformed keratinocyte cell lines, such as HaCaT cells and A431 cells, increase VEGF production in culture following UV irradiation [96-98]; however, two studies have suggested that the type of UV light can affect VEGF production in cultured keratinocytes and cell lines. Mildner, *et al* showed that UVB causes no change in VEGF production in primary keratinocytes, while UVA decreases VEGF [98]. In contrast, Longuet-Perret, *et al* demonstrated an increase in VEGF production in NHEK in response to UVB but no change following UVA [97]. Interestingly, both groups reported a sharp decrease in VEGF production when UVA and UVB are combined. In the experiments outlined in this report, the UV source used emitted predominantly in the UVB spectrum, but some UVA is emitted as well. While the dose of UVA in our studies was equivalent to less than 1% of that given by
Longuet-Perret, et al or Mildner, et al, we cannot rule out the possibility that the reduction in VEGF production observed in NHEK and SCC-13 cells was due to a combination of UVA and UVB in our studies.

It is possible that keratinocyte VEGF expression in response to UV differs between intact skin and cultured cells due to interactions with extracellular matrix molecules in the basement membrane that are present in the skin but absent in vitro. To explore this possibility, VEGF production was examined in NHEK grown on plates coated with extracellular matrix molecules including laminin, fibronectin or collagen IV. Even when NHEK were grown on surfaces coated with extracellular matrix components, the increase in VEGF production by keratinocytes after UV seen in whole skin could not be replicated. It could be that even on a coated surface two-dimensional tissue culture does not accurately reflect the behavior of keratinocytes in vivo. It is possible that different results would be obtained in a three-dimensional system, which better represents the in vivo environment. Differences in VEGF production were also observed between unirradiated keratinocytes in culture compared to whole skin. Epidermal VEGF production is low in normal, unirradiated skin; however, cultured keratinocytes, HaCaT cells and SCC tumor cell lines all produce high levels of VEGF under standard cell culture conditions [97-98, 110]. It is possible that keratinocytes experience a signal that inhibits VEGF production in normal skin that is absent in the in vitro cell culture environment.

Cutaneous SCC tumors have been shown to express high levels of VEGFR-1 [133, 153]. Recently Zhu, et al showed that VEGFR-1 expression is altered in response to
acute UV exposure *in vitro*, reporting that VEGFR-1 increases at lower doses of UV but not at higher doses (above 500 J/m²) [135]. We observed a decrease in VEGFR-1 expression 24 hours following UV-irradiation in SCC-13 tumor cells and NHEK cells. It is possible that subtle differences in the UV lights and dosing could lead to the differences between our results and those of Zhu, *et al.* Although VEGFR-1 expression is enhanced by VEGF in endothelial cells, VEGFR-1 expression appears to be regulated independently of VEGF levels in the present studies. The addition of recombinant VEGF was unable to rescue the loss of VEGFR-1 following UV exposure and VEGF neutralization in unirradiated cells had no effect on VEGFR-1 expression. These results are supported by a recent paper demonstrating that VEGF does not regulate VEGFR-1 expression in primary keratinocytes [135].

Overall, the results suggest that VEGF expression can be inhibited by UV exposure in primary human keratinocytes and SCC tumor cell lines and that the VEGF/VEGFR-1 pathway does not alter UV-induced apoptosis *in vitro*. Future studies will be required to elucidate the precise mechanisms by which VEGF is down-regulated by UV in cultured cells and to determine whether VEGF regulates keratinocyte apoptosis *in vivo*. A better understanding of the functional significance of VEGF and VEGFR-1 expression and signaling during UV-mediated skin carcinogenesis could shed light on novel mechanisms of human skin cancer development.
Chapter 4: The Role of VEGF in Keratinocyte Function Following Acute and Long Term UV Exposure In Vivo

4.1 Introduction

Vascular endothelial growth factor (VEGF) is an important pro-angiogenic factor in the skin [154]. The production of VEGF and the subsequent induction of angiogenesis in the skin occurs in response to various stimuli, including wounding, chemical exposure and ultraviolet (UV) radiation [41-42, 82, 155]. Epidermal keratinocytes are the primary source of VEGF in the skin, but macrophages, fibroblasts and other cell types can also produce VEGF [55, 82, 90-91]. Chronic exposure to UV light is the primary cause of skin cancers, although chemical exposure is a risk factor as well.

Several studies have linked elevated VEGF production to the promotion of skin carcinogenesis [107-108, 110-115]. VEGF is highly expressed in human squamous cell carcinomas (SCC) and VEGF expression increases stepwise during tumorigenesis in murine skin [111-112]. Furthermore, mice that overexpress VEGF in keratinocytes develop more papillomas, squamous cell carcinomas and metastases; conversely, mice lacking VEGF in keratinocytes develop fewer skin tumors [107, 114-115].

To date, the findings linking VEGF to increased skin carcinogenesis have been attributed primarily to the pro-angiogenic effects of VEGF. VEGF induces angiogenesis by signaling through a family of tyrosine kinase receptors on endothelial cells, causing
their activation, proliferation, migration and survival [88-89, 102, 156-157]. However, functional VEGF receptors have also been identified on a variety of non-endothelial cell types, including monocytes, smooth muscle cells, and neurons [127, 150-151]. VEGF has been shown to have a direct role in inducing the migration of monocytes and in the guidance of neuronal cones, indicating functions for VEGF outside of angiogenesis [126, 129, 158]. Recently, several studies have identified one or more VEGF receptors on epidermal keratinocytes in the skin and have shown that VEGF can affect keratinocyte function directly [131-132, 134, 147, 159]. We previously reported that cultured human keratinocytes proliferate in response to VEGF through VEGFR-1 [132] and others confirmed this in mouse keratinocytes and SCC tumor cell lines [133]. These results indicate that keratinocytes can respond directly to VEGF and led us to hypothesize that VEGF may be directly involved in skin carcinogenesis by signaling through VEGFR-1 on keratinocytes.

A link between VEGFR-1 and several types of cancers has been established. VEGFR-1 is overexpressed on several types of tumor cells including breast, lung and melanoma [160-162]. VEGFR-1 is also highly expressed on squamous cell and basal cell carcinoma tumor cells [133, 153, 163]. Additionally, VEGFR-1 has been shown to promote skin carcinogenesis in K5-SOS (Keratin 5-son of sevenless) mice, a model in which skin tumors spontaneously develop [133]. Taken together, these studies suggest that normal keratinocytes and skin cancer cells can respond directly to VEGF via VEGFR-1 and that VEGFR-1 is likely important in skin carcinogenesis. However, the role of VEGFR-1 in the response of keratinocytes to UV, the primary causative agent in skin carcinogenesis, has not been investigated.
The studies in this chapter test the hypothesis that VEGF promotes skin carcinogenesis by directing affecting keratinocyte function via VEGFR-1 in addition to promoting angiogenesis. To test this, a unique, conditional knockout mouse strain that lacks VEGFR-1 only in keratinocytes was generated and utilized in acute and long term UV studies.

4.2 Results

4.2.1 Experimental design for acute UV studies. To explore the direct effects of VEGF on keratinocytes while leaving other potential actions of VEGF intact, a unique conditional knockout mouse in which VEGFR-1 is deleted in keratinocytes was generated. The keratinocyte-specific keratin 14 (K14) promoter was used to drive cre recombinase expression in mice with floxed VEGFR-1 alleles, effectively deleting VEGFR-1 solely in keratinocytes. Keratinocytes of K14-Cre/VEGFR-1^{fl/fl} mice (cKO) cannot respond to VEGF; however, other cell types that express VEGF receptors such as endothelial cells and macrophages are still able to respond to VEGF. These mice were used in acute UV studies to determine the direct effects of VEGF on keratinocytes via VEGFR-1. To investigate the role of keratinocyte VEGFR-1 during UV exposure, 6 week old female cKO or control mice were exposed to an acute dose of UV. Specifically, mice were irradiated with 2,240 J/m² UVB (one minimal erythemal dose or MED) and sacrificed at 24 or 48 hours (Figure 4.1A) or exposed 3 times over the course of a week and sacrificed at day 7 (Figure 4.1B).
4.2.2 Skin thickness increases following UV exposure in both cKO and control mice. One of the hallmarks of UV exposure is an increase in the overall thickness of the skin. cKO mice and controls were acutely exposed to UV light as described (Figure 4.1). Immediately following sacrifice, calipers were used to measure the overall thickness of a fold of dorsal skin (Figure 4.2A). Skin thickness increased compared to unirradiated skin in both cKO and controls 24 hours after a single exposure and remained elevated at 48 hours (Figure 4.2A). A more substantial increase in skin thickness was observed following 3 exposures over the course of one week (Figure 4.2B). No difference in skin thickness was detected between cKO and control mice by this methodology at any of the conditions examined.

4.2.3 Epidermal and dermal thickness increase following UV exposure in both cKO and control mice. Additionally, the thickness of the epidermis and the dermis were measured in H&E stained sections of dorsal skin collected from cKO and control mice following acute UV exposure (Figure 4.3A). Control mice showed a significant increase in epidermal thickness at 48 hours following UV exposure and cKO mice displayed a significant increase by 24 hours that was maintained at 48 hours (Figure 4.3B). No significant differences were detected between cKO and control mice (Figure 4.3B). Epidermal thickness was substantially elevated following 3 exposures, although no differences were found between cKOs and controls (Figure 4.3C). Dermal thickness also increased significantly following a single exposure or three exposures to UV; however, no differences were observed between cKO and control mice (Figure 4.3D and Figure 4.3E). Overall, these results suggest that UV increases the thickness of the whole
Figure 4.1 Experimental design for acute UV studies. Acute UV experiments were performed on cKO and control mice. For acute, single exposure experiments, cKO and control mice were irradiated with 2,240 J/m² (1 MED) of UV light and sacrificed 24 or 48 hours following UV exposure (A). For week-long experiments, cKO and control mice were irradiated with 1 MED of UV on non-consecutive days for a total of three exposures and sacrificed at one week, 48 hours following the final exposure (B).

Figure 4.2 Skin thickness increases following UV exposure in both cKO and control mice. Skin thickness was determined by measuring a fold of dorsal skin with calipers immediately following sacrifice in cKO and control mice at 24 or 48 hours following a single UV exposure (A) or three exposures over the course of a week (B). Skin from unirradiated mice (No UV) was used as a control. Bars represent mean skin thickness +/- S.E.M (**p<0.001 compared to unirradiated skin of the same strain; n = 7-11 irradiated mice per group, n = 5-6 unirradiated mice per group).
Figure 4.3 Epidermal and dermal thickness increase following UV exposure in both cKO and control mice. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures and epidermal and dermal skin thickness was measured in H&E stained sections of dorsal skin. Representative H&E stained sections are shown for control (top) and cKO (bottom), along with unirradiated skin (A). Epidermal thickness was quantified in unirradiated skin and in irradiated skin following a single exposure (B) or three exposures (C). Dermal thickness was also quantified in unirradiated skin and in irradiated skin following a single exposure (D) or three exposures (E). Bars represent mean thickness in mm² +/- S.E.M (*p<0.05, **p<0.01, ***p<0.001 compared to unirradiated skin (No UV) of the same strain; n = 7-11 irradiated mice per group, n = 5-6 unirradiated mice per group).
skin and increases the epidermal and dermal thickness, but that keratinocyte VEGFR-1 does not contribute significantly to these changes.

4.2.4 Keratinocyte proliferation is not altered in VEGFR-1 knockout mice following UV exposure. Previous studies in our lab and others have shown that keratinocytes proliferate in response to VEGF in vitro and that this is mediated through VEGFR-1 [132-133]. Therefore, we examined the effect of VEGFR-1 loss on keratinocyte proliferation following UV exposure in vivo. Dorsal skin sections from acutely irradiated or unirradiated cKO and control mice were stained for the proliferation marker Ki67 (Figure 4.4A). Positive cells were quantified in the epidermis of 5 high power fields per mouse (Figure 4.4B). The number of proliferating cells was lower at 24 hours, although this decrease was only statistically significant in control mice. At 48 hours following a single UV exposure, proliferation returned to the rates seen in unirradiated skin. No significant differences in the number of proliferating epidermal cells were observed between cKO and control mice. After three exposures over the course of a week, there was a significant increase in proliferating epidermal cells in both control and cKO skin with no differences observed between the strains (Figure 4.4C). Overall, these results show that epidermal proliferation following acute UV exposure is similar between control and cKO mice, with both strains exhibiting a strong increase in epidermal cell proliferation following a week of UV exposure.

4.2.5 Loss of VEGFR-1 increases keratinocyte apoptosis following UV. Another hallmark of the cutaneous response to UV is the induction of keratinocyte apoptosis [143-146]. VEGF has been shown to be a survival factor for keratinocytes in vitro [135],
Keratinocyte proliferation is not altered in VEGFR-1 knockout mice following UV exposure. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures. Epidermal cell proliferation was determined by immunohistochemical staining for the proliferation marker Ki67. Representative Ki67 stained sections from the dorsal skin of irradiated control (top) or cKO (bottom) mice are shown at 40 x magnification (A). Proliferating cells were quantified by counting the number of Ki67 positive epidermal cells per field at 40 x magnification in 5 fields per section following a single UV exposure (B) or three exposures (C). Skin from unirradiated mice (No UV) was used as a control. Bars represent mean vessel density +/- S.E.M. (*p<0.05 compared to unirradiated skin (No UV) of the same strain; n = 6-9 irradiated mice per group, n = 4-5 unirradiated mice per group).

but it remains unknown if VEGF can affect the survival of keratinocytes exposed to UV in vivo. To explore this, the number of apoptotic cells in the epidermis of cKO and control mice was determined following acute UV exposure by immunohistochemical staining for active caspase-3 (Figure 4.5A). Positive cells were not detected in unirradiated, 48 hour or 1 week samples; therefore, only 24 hour samples were quantified (Figure 4.5B). Epidermal cells positive for active caspase-3 were quantified in 10 high power fields per
section. cKO mice displayed significantly more apoptotic cells than controls, with an average of 2.1 positive cells per field for cKOs compared to only 1.3 for controls \((p<0.027)\). These results indicate that loss of keratinocyte VEGFR-1 leads to increased apoptosis, suggesting that VEGF could be functioning as a survival factor for keratinocytes following UV exposure.

Figure 4.5 Loss of VEGFR-1 increases keratinocyte apoptosis following UV. cKO and control mice were exposed to 1 MED of UV and sacrificed at 24 hours. Apoptotic cells in the epidermis were detected by immunohistochemical staining for the apoptosis marker active caspase-3. Representative active caspase-3 stained sections from control (left) and cKO (right) mice are shown (A). Apoptotic cells were quantified by counting the number of active caspase-3 positive epidermal cells per field at 40 x magnification in 10 fields per section (B). Bars represent mean active caspase-3 positive cells per field +/- S.E.M \((*p<0.05\) compared to controls; \(n=6\) mice per group).
4.2.6 Loss of keratinocyte VEGFR-1 does not alter the angiogenic response following UV exposure. VEGF is an important pro-angiogenic factor known to be produced in the skin following UV exposure [82, 90]. The cKO mice used here lack VEGFR-1 on keratinocytes, but VEGF receptors remain intact on vascular endothelial cells. Therefore, the angiogenic response should remain unaltered. To confirm this, we stained sections of dorsal skin from unirradiated and acutely irradiated cKO and control mice for PECAM-1, a marker for endothelial cells (Figure 4.6A). Vessel density was determined in five fields per mouse at 20 x magnification using image analysis software. Vessel density was not significantly altered 24 or 48 hours following exposure to a single MED of UV in either control or cKO mice (Figure 4.6B). Three exposures over the course of a week lead to a significant increase in vessel density in both control and cKO mice (Figure 4.6C). No differences in vessel density were observed between cKO and control mice in any of the treatment conditions, suggesting that loss of keratinocyte VEGFR-1 does not alter the angiogenic response to UV light.

4.2.7 Loss of keratinocyte VEGFR-1 does not alter neutrophil infiltration following UV exposure. UV light induces a potent inflammatory response in the skin. Keratinocytes contribute to the inflammatory response following UV by producing soluble factors and cytokines that help recruit inflammatory cells. Therefore, it was possible that VEGF was altering keratinocyte function via VEGFR-1 in ways that could mediate the inflammatory response to UV. One of the first inflammatory cells that are recruited following UV exposure are neutrophils [164], so the infiltration of neutrophils following UV exposure in cKO and control mice was examined. Sections of dorsal skin were stained for the neutrophil marker Ly6G (Figure 4.7A) and neutrophils were quantified.
Overall, neutrophil numbers increase 24 hours after UV exposure and peak at 48 hours in both cKO and control mice (Figure 4.7B). Interestingly, neutrophil density was significantly increased from 24 to 48 hours in control mice but not in cKO mice. After a week of UV exposure, few neutrophils were detected in either group (Figure 4.7C). No significant differences in neutrophil counts were found between cKO and controls at any time point examined.

4.2.8 Loss of keratinocyte VEGFR-1 alters macrophage recruitment following UV exposure. Macrophages are another type of immune cell recruited to the skin following UV. Macrophages remove dead cells, secrete growth factors and cytokines and contribute to the angiogenic response. To investigate the effect of keratinocyte VEGFR-1 loss on macrophage recruitment, dorsal skin sections from acutely irradiated cKO and control mice were stained for the macrophage specific marker F4/80 (Figure 4.8A). No significant alterations in macrophage density were observed at 24 or 48 hours in either group compared to unirradiated skin (Figure 4.8B). However, a sharp increase in macrophage density was observed following three exposure of UV (Figure 4.8C). Interestingly, macrophage density was significantly lower in cKO mice compared to controls after a week of exposure. Overall, these results suggest keratinocyte stimulation by VEGF via VEGFR-1 contributes to macrophage recruitment following UV exposure.

4.2.9 VEGF levels following UV exposure are not altered by loss of keratinocyte VEGFR-1. Keratinocytes have been identified as the primary source of VEGF in the skin following UV exposure, although macrophages and stromal cells contribute some VEGF as well [55, 82, 90-91]. It is not known if VEGF production in keratinocytes is controlled
Figure 4.6 Loss of keratinocyte VEGFR-1 does not alter the angiogenic response following UV exposure. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures. Vessel density was determined by immunohistochemical staining for the endothelial cell marker PECAM-1. Representative PECAM-1 stained sections from the dorsal skin of irradiated control (top) or cKO (bottom) mice are shown at 20 x magnification, along with unirradiated skin (A). The percent area of PECAM-1-positive staining was quantified using image analysis and reported as vessel density for mice following a single UV exposure (B) or three exposures (C). Bars represent mean vessel density +/- S.E.M. (*p<0.05 compared to unirradiated skin (No UV) of the same strain; n = 6-9 irradiated mice per group, n = 4-5 unirradiated mice per group).
Figure 4.7 Loss of keratinocyte VEGFR-1 does not alter neutrophil infiltration following UV exposure. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures. Neutrophil density was determined by immunohistochemical staining for the neutrophil marker Ly6G. Representative Ly6G stained sections from the dorsal skin of irradiated control (top) or cKO (bottom) mice are shown at 40 x magnification, along with unirradiated skin (A). The density of neutrophils was calculated following a single UV exposure (B) or three exposures (C). Bars represent mean neutrophil density (the number of Ly6G positive -cells per mm²) +/- S.E.M. (*p<0.05, **p<0.01, ***p<0.001 compared to unirradiated skin (No UV) of the same strain; n = 7-8 irradiated mice per group, n = 3 unirradiated mice per group)
Figure 4.8 Loss of keratinocyte VEGFR-1 alters macrophage recruitment following UV exposure. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures. Macrophage density was determined by immunohistochemical staining for the macrophage marker F4/80. Representative F4/80 stained sections from the dorsal skin of irradiated control (top) or cKO (bottom) mice are shown at 40 x magnification, along with unirradiated skin (A). The density of macrophages was calculated following a single UV exposure (B) or three exposures (C). Bars represent mean macrophage density (the number of F4/80 positive -cells per mm²) +/- S.E.M. (*p<0.05, **p<0.001 compared to unirradiated skin (No UV) of the same strain, †p<0.01 for cKO mice compared to controls; n = 4-6 irradiated mice per group, n = 4-5 unirradiated mice per group).
by VEGF in an autocrine loop following UV exposure. If VEGFR-1-deficient keratinocytes produce less VEGF, it could potentially explain the differences in macrophage recruitment observed in cKO mice since VEGF may be a chemoattractant for macrophages [126-127]. Therefore, the amount of VEGF present in the dorsal skin of acutely irradiated and unirradiated cKO and control mice was determined by ELISA. Overall, VEGF increased compared to unirradiated skin at 24 and 48 hours (Figure 4.9A). VEGF was also elevated one week following three UV exposures in both cKO and controls (Figure 4.9B). However, there were no differences in the amount of VEGF produced by cKO compared to control mice at any time examined. Therefore, loss of keratinocyte VEGFR-1 does not appear to alter VEGF production following UV exposure.

Figure 4.9 VEGF levels following UV exposure are not altered by loss of keratinocyte VEGFR-1. cKO and control mice were sacrificed 24 or 48 hours following a single UV exposure or one week after three exposures. VEGF protein content was examined by ELISA in homogenates from 5 mm dorsal skin punches taken from mice following a single UV exposure (A) or three exposures (B). Bars represent mean VEGF pg per mg of total protein +/- S.E.M. (*p<0.05, ***p<0.001 compared to unirradiated skin; n = 5 per group).
**4.2.10 VEGFR-1 is highly expressed in murine hyperplastic skin and skin tumors.**
Results from acute UV studies suggest that VEGF plays a role in the cutaneous response to UV, in part, by affecting keratinocyte function directly. Because UV light is the primary causative agent of non-melanoma skin cancers, we hypothesized that direct effects of VEGF on keratinocytes may play a role in skin carcinogenesis. To test this hypothesis, VEGFR-1 expression was examined in chronically UV-irradiated murine skin and murine skin tumors from SKH-1 hairless mice by immunohistochemistry. VEGFR-1 is expressed in the epidermis of unirradiated skin (4.10A). In chronically irradiated, hyperplastic skin, preferential staining for VEGFR-1 was observed in the basal keratinocytes and in the adjacent stratum spinosum (4.10B). As foci of hyperplasia begin to develop into papillomas (Figures 4.10C-D), staining becomes more disuse throughout the layers, but some preferential staining of the granular and basal layers is observed. In murine papillomas, VEGFR-1 staining is diffuse and heterogeneous, with the most intense staining observed in basal cells and in cells that are keratinizing (Figures 4.10E-H). Overall, the results suggest that VEGFR-1 is expressed in the keratinocytes of normal skin, hyperplastic skin and skin tumors.

**4.2.11 VEGFR-1 is highly expressed in human basal cell carcinomas.** Because VEGFR-1 is expressed in keratinocytes and in murine skin tumors, the expression of VEGFR-1 in human non-melanoma skin tumors was examined. Basal cell carcinomas were obtained from patients undergoing Moh’s surgery and were stained for VEGFR-1 by immunohistochemistry. VEGFR-1 is expressed in differentiated keratinocytes of normal skin adjacent to tumors (Figure 4.11A). In superficial basal cell carcinomas, VEGFR-1 is highly expressed in the tumor cells throughout the tumor (Figures 4.11B-C).
Figure 4.10 VEGFR-1 is highly expressed in murine hyperplastic skin and skin tumors. Normal, unirradiated dorsal skin, hyperplastic skin and papillomas from SKH-1 mice were collected and stained for VEGFR-1 by immunohistochemistry. Representative images of VEGFR-1-stained unirradiated skin (A) and chronically irradiated hyperplastic skin (B) are shown. Hyperplastic skin in which papillomas are beginning to develop are shown in C and D. Two representative murine papillomas were stained for VEGFR-1 and are shown at lower (E and G) and at higher magnification (F and H).
Figure 4.11 VEGFR-1 is highly expressed in human basal cell carcinomas. Samples of human basal cell carcinomas were collected from patients undergoing Moh’s surgery and stained for VEGFR-1 by immunohistochemistry. VEGFR-1 staining in tumor-adjacent skin is shown (A). Representative images of superficial BCCs, nodular BCCs and infiltrative BCCs stained for VEGFR-1 are shown in the left panels (B, D and F, respectively). Higher magnification images of each BCC type are also shown in the right panels (C, E and G).
Nodular BCCs display VEGFR-1 staining localized primarily to cell at the tumor periphery (Figures 4.11D-E). Peripheral staining was also observed in infiltrative BCCs (F and G). These results indicate that VEGFR-1 is expressed in tumor cells, suggesting that VEGFR-1 could play a role in these cancers.

**4.2.12 VEGFR-1 is expressed in human squamous cell carcinomas.** In additional to BCCs, human squamous cell carcinomas (SCC) were also obtained from patients undergoing Moh’s surgery. SCC tumor samples were stained for VEGFR-1 by immunohistochemistry. Similar to what was observed in human BCCs, VEGFR-1 was detected in the epidermis of skin adjacent to tumors (Figures 4.12A-B). Furthermore, VEGFR-1 staining was observed in SCC tumor cells (Figures 4.12C-D), indicating that VEGFR-1 may be able to stimulate SCC cells directly.

**4.2.13 Conditional VEGFR-1 knockout mice may be less susceptible to UV-induced skin carcinogenesis.** Because differences in the response to acute UV exposure were observed in cKO mice and VEGFR-1 is expressed in human and murine skin tumors, we hypothesized that cKO mice may be less susceptible to UV-induced skin carcinogenesis. To test this, cKO and control mice were subjected to a long term UV-induced skin carcinogenesis protocol in which mice are exposed to 1 MED of UV light three times per week for 15 weeks and tumors are collected at 25 weeks (Figure 4.13A). These experiments are ongoing; however, preliminary data from mice at 21 weeks after the start of chronic UV-irradiation suggest that cKO mice may be more resistant to UV-induced skin carcinogenesis than controls. cKO mice developed significantly fewer tumors per mouse at 20 weeks compared to controls (Figure 4.13B). Furthermore, cKO
Figure 4.12 VEGFR-1 is expressed in human squamous cell carcinomas. Samples of human squamous cell carcinomas were collected from patients undergoing Moh’s surgery and stained for VEGFR-1 by immunohistochemistry. VEGFR-1 staining in tumor-adjacent skin is shown at low (A) and high (B) magnification. Representative images from SCCs stained for VEGFR-1 are shown at low (C) and high (D) magnification.

mice displayed a significant reduction in tumor burden (Figure 4.13C) and tumor size (Figure 4.13D) at 20 and 21 weeks. A difference in tumor onset between cKO and controls was also observed; control mice developed detectable tumors after 14 weeks, but cKO mice did not develop tumors until 16 weeks (Figures 4.13B-D). These preliminary results suggest that cKO may be less susceptible to UV-induced skin carcinogenesis.
Figure 4.13 Conditional VEGFR-1 knockout mice may be less susceptible to UV-induced skin carcinogenesis. Long term UV-induced skin carcinogenesis studies are being conducted using cKO and control mice. cKO and control mice were irradiated with 1 MED three times per week for 15 weeks and tumors were measured weekly beginning after 10 weeks of UV exposure (A). The endpoint of long term studies will be 25 weeks (A). Tumors with a diameter of 1.5 mm or greater were recorded. The average number of tumors was determined each week after 10 week of UV exposure for cKO and control mice (B). Additionally, the average area covered by tumors was determined and is reported as average tumor burden (mm²) for each strain (C). The average size of tumors was also determined for cKO and control mice (D). All graphs represent mean +/- S.E.M. (*p<0.05, **p<0.01, ***p<0.001 for cKO mice compared to controls; n = 12 cKO and 15 control mice).
4.3 Discussion

Overwhelming evidence has shown that VEGF promotes skin carcinogenesis through the induction of angiogenesis [107-108, 111, 115]. However, emerging evidence supports a broader range of targets and functions for VEGF outside of angiogenesis. Monocytes, bone-marrow-derived cells, neurons and keratinocytes all express VEGFR-1 and VEGF has been shown to directly promote the proliferation, migration and survival of multiple cell types [126, 129, 132, 165-166], in addition to endothelial cells. *In vitro* studies have shown that keratinocyte functions are directly affected by VEGF [133-135, 167]. UV light, the main causative agent for non-melanoma skin cancer, induces VEGF production in the skin [43]; however, it remains unknown whether VEGF directly affects the response of keratinocytes to UV light. To investigate the direct effects of VEGF on keratinocytes following UV exposure, a keratinocyte-specific VEGFR-1 knockout mouse strain was developed and used in acute and chronic UV studies. In acute studies, differences were observed in the response to UV exposure between cKO mice and controls. Additionally preliminary results from long term studies suggest that cKO mice, which lack VEGFR-1 in the keratinocytes, are more resistant to UV-induced skin carcinogenesis than controls.

One interesting difference that was observed between strains in UV studies is that loss of keratinocyte VEGFR-1 caused an increase in the number of apoptotic keratinocytes 24 hours following UV compared to control mice. These results suggest that VEGF may function as a survival factor for keratinocytes. The removal of damaged keratinocytes by apoptosis following UV exposure is critical to keeping the skin free of cells harboring
tumor-initiating mutations. If VEGF, which is produced in response to UV, is a survival factor for keratinocytes, it could be promoting skin carcinogenesis by increasing the survival and accumulation of UV-damaged cells. These results are supported by findings by Zhu, et al showing that VEGF protects cultured primary keratinocytes from apoptosis following moderate UV exposure [135]. Additionally, a recent paper by Riese, et al showed that keratinocyte apoptosis was elevated following acute UV exposure in mice lacking neuropilin-1, a co-receptor of VEGFR-1 [168].

In addition to an increase in UV-induced apoptosis, we observed alterations in the inflammatory response following acute UV exposure in cKO mice. Inflammatory cells play a critical role in the UV response; they are recruited to the skin where they dispose of dead and damaged cells, produce pro-inflammatory factors and cytokines, and release ROS that can cause oxidative damage [37]. Two types of inflammatory cells were examined in acute UV studies: neutrophils and macrophages. Neutrophil infiltration peaked at 48 hours and resolved by one week in both cKO and control mice, and no significant differences were found between the strains. Macrophage recruitment was also examined. Interestingly, there were significantly fewer macrophages in the cKO mice compared to controls at one week, with only about half the number of macrophages found in cKOs. This suggests that VEGF stimulates keratinocytes to recruit macrophages. One potential mechanism could be that VEGF stimulates keratinocytes to release soluble factors that, directly or indirectly, attract macrophages. VEGF is one mediator that has been shown to induce macrophage migration [126-127]; however, VEGF production was not altered by the loss of keratinocyte VEGFR-1, suggesting that other soluble mediators are involved. For instance, keratinocytes have
been shown to produce macrophage chemoattractants including chemokine (C-C motif) ligand 8 (CCL8), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein 1 alpha (MIP-1α) [169-171]. Future studies will explore the effect of VEGF on cytokine and chemokine production to identify differentially expressed chemoattractants in the skin of irradiated cKO and control mice. Importantly, alterations in macrophage recruitment or macrophage phenotype could influence skin carcinogenesis and this will be examined in long term studies.

Results from acute UV studies indicate that VEGF may play a previously unknown role in the response to UV light by affecting keratinocytes directly. Because the majority of non-melanoma skin cancers are caused by UV exposure, these results suggest that VEGF may influence skin carcinogenesis through direct effects on keratinocytes and tumor cells. One way in which this was investigated was by examining VEGFR-1 expression in murine and human skin tumors. Our results show that VEGFR-1 is expressed by keratinocytes in murine hyperplastic skin and papillomas, as well as in human BCC and SCC tumor cells, indicating that VEGF could potentially affect non-melanoma tumor cells directly.

To examine how the direct effects of VEGF on keratinocytes may impact skin tumor development, several cKO and control mice were utilized in long term UV-induced skin carcinogenesis experiments. Unfortunately, several difficulties were encountered during the course of these studies. First, the mice did not develop a large number of tumors during the 25 week protocol, making it difficult to determine differences in tumor numbers between cKO mice and controls with statistical significance. Second, both cKO
and control mice began developing skin infections late into the experimental protocol. These infections interfered with tumor identification and measurement and lead to the loss of several experimental animals prior to the end of the 25 week study. Preliminary studies from a new set of cKO and control mice that seem to be developing fewer infections than previous cohorts suggest that loss of VEGFR-1 may lead to some level of resistance against UV-induced skin carcinogenesis. However, more studies will have to be performed with altered experimental design to minimize confounding infections and increase the number of tumors that develop.

Overall, these studies add the growing body of research showing that VEGF can directly affect keratinocyte function and are the first to look at the direct role of VEGF on keratinocytes via VEGFR-1 following UV exposure \textit{in vivo}. Our results show that VEGF promotes keratinocyte survival and keratinocyte-mediated recruitment of macrophages following acute UV exposure. These are two previously unknown functions of VEGF that could influence the growth and development of skin carcinogenesis. Indeed, preliminary results indicate that mice with VEGFR-1-deficient keratinocytes may be less susceptible to UV-induced tumor development. Additional long term \textit{in vivo} studies need to be performed to confirm that VEGF directly promotes UV-induced skin carcinogenesis.
5.1 Introduction

Wound healing is a critical health care issue that affects millions of patients each year. In addition to injuries and traumas, 40 million inpatient and 31 million outpatient surgeries are performed each year, all requiring the repair of a wound [57]. Even when wounds heal properly, patients can be left with a permanent scar that has decreased tensile strength and can lead to impaired joint mobility and psychological distress [172]. Chronic wounds, which can remain unhealed for months or even years, are commonly found in patients with diabetes, poor nutrition and in the elderly. These wounds can lead to extensive hospitalization, amputations and even death [59]. Furthermore, abnormally healing wounds impose a substantial burden on the health care system, affecting 6.5 million patients at the cost of $12.5 billion annually [57]. Therefore, it is critical to understand the mechanisms of wound repair and develop new therapies to improve healing.

In healthy adults, wound healing occurs in three overlapping phases: inflammation, proliferation and scar formation [45]. The inflammatory phase is characterized by the infiltration of innate immune cells including neutrophils and macrophages. Neutrophils arrive rapidly following injury and serve to sterilize the wound by releasing soluble mediators and reactive oxygen species to kill bacteria [47]. Macrophages are also...
recruited to the wound site where they clear bacteria, dead cells and debris, in addition to releasing soluble factors that promote the subsequent proliferative phase [48, 173]. During the proliferation phase, keratinocytes proliferate and migrate across the wound bed, forming a new epithelium in a process called reepithelialization [53]. Angiogenesis, the growth and expansion of new blood vessels, also occurs during the proliferation phase. In the dermis, fibroblasts also proliferate and produce collagen to replace the damaged extracellular matrix. Finally, collagen in remodeled by fibroblasts, forming a permanent scar.

Wounds contain high levels of vascular endothelial growth factor (VEGF) [55, 82], a factor that promotes angiogenesis by causing the proliferation, migration and survival of vascular endothelial cells [88-89]. Keratinocytes are the primary source of VEGF in wounded skin [82]; however, some VEGF is produced in wounds by macrophages and fibroblasts [55, 92]. VEGF is known to play a critical role in wound repair, as studies have shown that altering VEGF levels in wounds can impact vessel density, reepithelialization, granulation tissue and even scar formation [115-116, 123-125, 167]. Interestingly, one of the VEGF receptors, VEGFR-1, has now been identified on keratinocytes [131-132]. Previous studies by our laboratory and others have shown that VEGF can directly affect keratinocyte proliferation, migration and survival [132-135]. However, it is not known how direct VEGF signaling in keratinocytes impacts wound healing.

Because VEGF can directly alter keratinocyte function via VEGFR-1 [132-135] and because VEGF is present at high levels in wounds [55, 82], we hypothesized that VEGF
influences wound healing through direct effects on keratinocytes. To test this hypothesis, a unique conditional knockout mouse strain was generated that lacks keratinocyte VEGFR-1 and utilized these mice in wound healing studies. The results described in the Chapter show that keratinocyte-specific deletion of VEGFR-1 caused impairments in reepithelialization and macrophage recruitment. These studies suggest that, in addition to promoting angiogenesis, VEGF plays a role in wound healing by directly affecting keratinocytes.

**5.2: Results**

**5.2.1 Experimental design for wound healing studies.** To explore the direct effects of VEGF on keratinocytes during wound healing, a conditional knockout (cKO) mouse strain was developed in which VEGFR-1 is deleted in keratinocytes using the cre-lox system. Additionally, mice that express cre recombinase but do not have floxed VEGFR-1 alleles were generated as controls. cKO and control mice were used in wound healing studies. Six excisional wounds were inflicted on the dorsal skin of anesthetized mice with a 3 mm biopsy punch and allowed to heal without intervention. Wounds were collected after 1, 3, 5, 7, or 14 days and assessed for various aspects of healing including wound closure, angiogenesis, inflammation and VEGF production (Figure 5.1).

**5.2.2 Reepithelialization is impaired in cKO mice.** During the healing process, a new epithelium is formed by the proliferation and migration of keratinocytes across the wound bed. VEGF has been shown to promote keratinocyte proliferation and migration *in vitro*; therefore, we hypothesized that reepithelialization may be altered in mice with VEGFR-1
Figure 5.1 Experimental design for wound healing studies. Wound healing experiments were performed on two groups of mice; experimental cKO and control mice. Six excisional wounds were inflicted on the dorsal skin of anesthetized mice. Wounded were left untreated and allowed to heal for 1, 3, 5, 7, or 10 days. At sacrifice, wounds were collected and assessed for reepithelialization, immune cell infiltration, angiogenesis and VEGF content.

deficient keratinocytes that cannot respond to VEGF. Reepithelialization was assessed in H&E stained sections from wounds of cKO and control mice by calculating the percentage of the wound bed covered by a new epithelium. Wounds collected on days 1 and 3 displayed minimal reepithelialization (5.2A). Additionally, wounds examined after seven or more days of healing showed complete reepithelialization (5.2A). After 5 days of healing, wounds showed varying degrees of closure; therefore, reepithelialization was quantified at this time point (5.2.2B). A significant delay in wound closure was observed in cKO compared to controls. Wounds from cKO mice were only 68% reepithelialized compared to 95% for control wounds (5.2B). Strikingly, only 20% of the cKO wounds showed complete healing compared to 85.7% of the control wounds (5.2.2C). These results suggest that VEGF may stimulate keratinocytes directly to promote reepithelialization.
Figure 5.2 Reepithelialization is impaired in cKO mice. cKO and control mice received 3 mm excisional wounds. Wounds were collected 1, 3, 5, 7, and 14 days following injury and reepithelialization was assessed in H&E stained sections taken from the center of the wound. Representative H&E stained wounds from control (left) or cKO (right) wounds are shown at 5 x magnification, along with unwounded skin (A). For 5 day wounds, percent reepithelialization (B) and incidence of complete healing (C) are shown. Bars represent mean values +/- S.E.M. (n = 7-10 per group; *p<0.05).
5.2.3 Loss of keratinocyte VEGFR-1 does not alter neutrophil infiltration during wound healing. Because a significant impairment in reepithelialization was observed in cKO mice, we hypothesized that other aspects of the healing process may be altered in cKO mice. To examine the effect of keratinocyte VEGFR-1 ablation on the inflammatory response, neutrophils, a type of inflammatory cell known to infiltrate rapidly following an injury, were examined in wounds from cKO and control mice. Immunohistochemical staining for the neutrophil marker Ly6G was performed on wound sections from cKO and control mice (5.3A). Neutrophil infiltration was highest 1 day after wounding in both cKO and control mice (5.3B) and remained elevated after 3 days compared to unwounded skin. Although no statistically significant differences in neutrophil density was observed between cKO and control mice at any time point that we examined, a trend toward lower neutrophil density was seen in cKO mice at day 3 compared to controls. These results suggest that loss of keratinocyte VEGFR-1 likely does not significantly alter neutrophil recruitment to wounds.

5.2.4 Loss of keratinocyte VEGFR-1 causes decreased macrophage recruitment to wounds. To further investigate a possible effect on the inflammatory response after healing, macrophage recruitment to wounds was examined in cKO and control mice. Macrophage recruitment was assessed by immunohistochemical staining for the macrophage-specific marker F4/80 in wounds from cKO and control mice (5.4A). Wounds collected at day 5 and day 7 were quantified because a robust infiltration of macrophages in the wounds was observed at these times. Wounds from cKO mice had significantly fewer macrophages after 5 days of healing compared to controls (5.4B). A trend toward lower macrophage density was also observed in wounds collected after 7
Figure 5.3 Loss of keratinocyte VEGFR-1 does not alter neutrophil infiltration during wound healing. cKO and control mice received 3 mm excisional wounds. Wounds were examined at day 1 and 3 for the presence of neutrophils by immunohistochemical staining for the neutrophil marker Ly6G. Representative Ly6G stained sections from control (top) or cKO (bottom) wounds are shown at 20 x magnification, along with unwounded skin (A). Neutrophil density was quantified in each side of the wound edge (B). Bars represent mean neutrophil density +/- S.E.M. (**p<0.01, ***p<0.001 compared to unwounded skin; n = 4-6 per group).
Figure 5.4 Loss of keratinocyte VEGFR-1 causes decreased macrophage recruitment to wounds. cKO and control mice received 3 mm excisional wounds. Wounds were examined at day 3, 5 and 7 for the presence of macrophages by immunohistochemical staining for the macrophage marker F4/80. Representative F4/80 stained sections from control (top) or cKO (bottom) wounds are shown at 10 x magnification, along with unwounded skin (A). Macrophage density was quantified in each side of the wound edge for day 5 and 7 wounds and in unwounded skin (B). Bars represent mean macrophage density +/- S.E.M. (***p<0.001 compared to unwounded skin, †p<0.05 for cKO compared to control mice; n = 8-14 wounds per group, n = 3 unwounded skin samples per group).
days of healing, although the difference did not reach statistical significance (5.4B). Overall, these results suggest that keratinocyte stimulation by VEGF via VEGFR-1 contributes to macrophage recruitment to wounds.

5.2.5 Loss of keratinocyte VEGFR-1 does not alter angiogenesis during wound repair. Angiogenesis is an important process that occurs as part of the proliferative phase of wound healing and previous studies have shown that inhibition of angiogenesis results in impaired healing. Angiogenesis was examined in wounds that had healed for 7 or 14 days by immunohistochemical staining for the endothelial cell-specific marker PECAM-1 (5.5A). A significant increase in vessel density was observed in 7 and 14 day wounds compared to unwounded skin in both cKO and control mice (5.5B). No difference was found in vessel density between cKO and control mice, suggesting that the ablation of keratinocyte VEGFR-1 does not alter the angiogenic response during wound repair.

5.2.6 VEGF levels in wounds are not altered by loss of keratinocyte VEGFR-1. Keratinocytes have been identified as the primary source of VEGF in wounded skin. If keratinocyte VEGF production is affected by VEGF in an autocrine loop during wound repair, then VEGFR-1-deficient keratinocytes could potentially produce lower levels of VEGF. In additional, VEGF has been identified as a chemoattractant for macrophages [126-127, 158], lower overall VEGF levels would help explain impaired macrophage recruitment seen in cKO mice. To determine the levels of VEGF in wounds, VEGF protein was measured by ELISA in homogenates of whole wounds collected from cKO and control mice after 1, 3, 5, 7, or 14 days of healing. As expected, VEGF levels were
Figure 5.5 Loss of keratinocyte VEGFR-1 does not alter angiogenesis during wound repair. cKO and control mice received 3 mm excisional wounds. Wounds were examined at day 7 and 14 for angiogenesis by immunohistochemical staining for the endothelial cell marker PECAM-1. Representative PECAM-1 stained sections from control (top) or cKO (bottom) wounds are shown at 20 x magnification, along with unwounded skin (A). The percent area of PECAM-1-positive staining was quantified in in wound bed and used to estimate blood vessel density (B). Bars represent mean vessel density +/- S.E.M. (*p<0.05, ***p<0.001 compared to unwounded skin; n = 8-14 wounds per group, n = 3 unwounded skin samples per group).
elevated in wounds, with the highest levels observed in 1, 3, and 5 day wounds (5.6). VEGF levels began to fall by day 7 and returned near baseline by day 14. No differences in VEGF levels were found between cKO and control mice at any time point that we examined. These results show that VEGF production is not altered in the wounds of mice with VEGFR-1-deficient keratinocytes.

Figure 5.6 VEGF levels in wounds are not altered by loss of keratinocyte VEGFR-1. cKO and control mice received 3 mm excisional wounds. VEGF protein content was examined in homogenates from day 1, 3, 5, 7, and 14 wounds and unwounded skin by ELISA. Bars represent mean VEGF pg per mg of total protein +/- S.E.M. (**p<0.01, ***p<0.001 compared to unwounded skin; n =5 per group).
5.3 Discussion

Recently, many studies have demonstrated diverse roles of VEGF outside of angiogenesis [132-135, 174]. VEGF receptors have now been identified on a variety of cell types such as monocytes, bone marrow-derived cells, neurons and keratinocytes [132, 151, 166]. VEGF directly impacts neuronal growth cone guidance, monocyte migration and mobilization of bone marrow-derived cells [126, 129-130]. Furthermore, alternative roles for VEGF have been identified in the skin [175]. VEGF has been shown to promote the proliferation, migration and survival of keratinocytes in vitro [132-135]. However, little is currently known about the direct effects of VEGF on keratinocytes during wound healing. In the current study, the direct effects of VEGF on keratinocytes in wound repair were investigated using a conditional knockout approach. By knocking out VEGFR-1 in keratinocytes, mice were generated with keratinocytes that cannot respond to VEGF (cKO mice); therefore, any alterations in healing outcomes could be attributed to the direct effect of VEGF on keratinocytes. Excitingly, we found that VEGF directly affects reepithelialization and keratinocyte-mediated recruitment of macrophages.

Because VEGF has been shown to promote proliferation and migration of several cell types [89, 126, 130, 132, 134], including keratinocytes, we initially hypothesized that VEGF may directly affect reepithelialization of wounds, a process that requires keratinocyte proliferation and migration. Indeed, a significant impairment in reepithelialization was observed in cKO mice compared to controls 5 days after wounding. Other studies examining the effect of VEGF on healing support our results; several studies have demonstrated that VEGF treatment can promote wound closure in both normal and diabetic wounds formation [115-116, 123-125, 167]. Additionally, K5-
VEGF conditional knockout mice, which lack VEGF expression in basal keratinocytes, show a delay in reepithelialization [115]. However, to date, these results have been attributed to the effect of VEGF on angiogenesis, which indirectly affects wound closure. Our results suggest that in addition to effects on angiogenesis, VEGF promotes wound closure by directly signaling through keratinocyte VEGFR-1, identifying a novel mechanism of reepithelialization.

During wound healing, cells produce many soluble factors and communicate with each other in complex ways. Because of this cross talk between cell types, we hypothesized that VEGF may alter keratinocyte functions in ways that could impact additional aspects of wound healing besides reepithelialization. Recruitment of inflammatory cells, including neutrophils and macrophages, was explored. Although a trend toward fewer neutrophils was seen in the wounds of cKO mice, the difference was not statistically significant. However, a significant decrease in macrophage recruitment was observed in cKO mice. Together, these results provide strong evidence that VEGF promotes keratinocyte-mediated recruitment of macrophages. It is possible that VEGF directly induces the production of soluble mediators by keratinocytes that promote the recruitment of inflammatory cells. For example, keratinocytes are known to produce the macrophage chemoattractants MCP-1 and MIP1-α during wound repair [170-171, 176], the production of which could be altered in cKO mice. Future studies will examine the effect of VEGF on keratinocyte cytokine and chemokine production.

The decrease in macrophage recruitment seen in cKO mice could impact healing in several ways due to the diverse functions of macrophages in wound healing. Previous
studies have shown that the depletion of macrophages leads to a decrease in angiogenesis, granulation tissue and collagen synthesis, as well as impaired wound closure [173, 177-178]. Macrophages can influence wound closure by secreting IL-6, a cytokine that can promote keratinocyte proliferation, by producing TGF-β, which activates myofibroblasts and leads to contraction of the wound and by inducing angiogenesis [179-181]. Therefore, it is possible that the differences in reepithelialization that were observed between cKO and control mice could be caused, at least in part, by the differences in macrophage density.

Macrophages express VEGFR-1 and are known to migrate in response to VEGF. Therefore, we reasoned that the decrease in macrophages could also be explained by decreased VEGF levels in the wounds. Keratinocytes are the primary source of VEGF in wounded skin, and it is possible that VEGF could promote the production of additional VEGF via keratinocyte VEGFR-1 in an autocrine loop. To test this possibility, VEGF levels were examined in wounds of cKO and control mice. VEGF was elevated in wounds compared to normal skin as expected; however, no differences were observed in VEGF levels between cKO and control mice at any time point examined, including the time points at which differences in reepithelialization and macrophage recruitment were observed. This suggests that VEGF production is not affected by VEGFR-1 deletion in keratinocytes and that the differences in wound closure and macrophage density between strains are not explained by differences in VEGF levels. These results are supported by the observation that the angiogenic response, which relies heavily on VEGF, is not altered in the wounds of cKO mice.
Overall, these studies suggest that in addition to stimulating angiogenesis, VEGF affects wound healing by acting through VEGFR-1 on keratinocytes. These results demonstrate two previously unknown functions of VEGF in wound repair: direct stimulation of reepithelialization and keratinocyte-mediated macrophage recruitment.
Chapter 6: Conclusions

The skin serves as the barrier to the outside world and, because of this, is vulnerable to injury and to exposure to environmental carcinogens. Non-melanoma skin cancer, which is primarily caused by UV light from the sun, is the most commonly diagnosed type of cancer [13]. Alarmingly, the incidence of NMSC continues to rise, especially among young women [6, 13]. Diabetes rates also continue to climb. Twenty five million people in the US are now living with diabetes and these patients have a 25% life time risk of developing an abnormally healing, chronic wound [57-58]. Diabetic ulcers can lead to extensive hospitalization, limb loss and even death. With 3.5 million new cases of NMSC and 6.5 million chronic wounds diagnosed each year and rates continuing to rise [57-58], it is critical that we understand the underlying mechanism of how the skin responds to environmental stresses and use this knowledge to develop new therapies.

The processes of cutaneous wound healing and skin carcinogenesis share many overlapping characteristics. Both entail a hyperproliferation of epidermal keratinocytes, remodeling of the dermal extracellular matrix, an influx of inflammatory cells and a robust angiogenic response [182-183]. The response of keratinocytes to injury is similar to what occurs during exposure to a carcinogen such a UV light; proliferation is increased along with the production of soluble mediators such as growth factors and pro-angiogenic factors. One important pro-angiogenic factor that is produced by keratinocytes during both situations is VEGF [41, 82]. VEGFR-1 has now been identified on keratinocytes
[131-132], suggesting that VEGF may directly affect wound healing and skin carcinogenesis by directly acting on keratinocytes, in addition to promoting angiogenesis. The studies described in this document examined the direct effects of VEGF on keratinocytes during UV exposure and wound repair.

In Chapter 3, the role of VEGF/VEGFR-1 signaling in the response of keratinocytes following UV exposure was explored in vitro. In general, UV is thought to upregulate VEGF in keratinocytes. Surprisingly, VEGF production was actually inhibited by UV light in both NHEK cells and two SCC cell lines. However, a close examination of the literature reveals a much more nuanced picture. In vivo experiments clearly demonstrate an increase in keratinocyte VEGF following UV [41-43] but in vitro studies in cultured keratinocytes and keratocyte cell lines have been variable [96-97, 184-185]. The results described here from acute UV study in SKH-1 hairless mice in vivo were in agreement with the vast majority of the literature and revealed elevated VEGF levels following UV. These studies highlight the potential for differences between in vitro and in vivo model systems and demonstrate the importance of utilizing multiple experimental systems.

In addition to differences in VEGF production, conflicting results in the effect of VEGF on keratinocyte apoptosis in response to UV light were found between in vitro and in vivo studies. In Chapter 3, NHEK cell and SCC-13 cell apoptosis following UV radiation was not altered by blocking VEGF or VEGFR-1 or treating with exogenous VEGF. However, in Chapter 4, cKO mice, which lack keratinocyte VEGFR-1, were shown to have an increased rate of epidermal cell apoptosis after UV exposure, suggesting that VEGF is a keratinocyte survival factor in vivo. Keratinocytes grown in culture are in a very different
environment than they are in intact skin. They may respond differently in vitro due to differences in proliferation rate or differentiation status, the lack of a 3-D structure and a basement membrane, or perhaps due to the loss of communication with additional cell types that would be present in vivo. Future studies in a 3-D skin model may help to answer these questions.

In Chapters 4 and 5, a conditional VEGFR-1 knockout mouse strain was used to examine whether keratinocytes respond directly to VEGF in both a UV-induced carcinogenesis model and a wound healing model. Excitingly, evidence was found in both models to support the hypothesis that VEGF can directly affect keratinocytes via VEGFR-1 in vivo. cKO mice displayed impaired reepithelialization of wounds, suggesting that VEGF, through VEGFR-1, directly stimulates keratinocyte proliferation or migration. In acute UV studies, keratinocytes of cKO showed an increase in the number apoptotic epidermal cells following UV exposure compared to controls, indicating that VEGF may serve as a survival signal to keratinocytes after UV irradiation. If so, this means that VEGF could play a role in skin carcinogenesis by promoting the survival of UV-damaged keratinocytes that harbor mutations. Indeed, preliminary long term UV-induced carcinogenesis studies suggest that cKO mice may be less susceptible to skin tumor development. Our work suggests that VEGF/VEGFR-1 may be a viable target for future skin cancer prevention and treatment. These studies add support for the use of VEGF as a potential therapeutic agent in chronic wounds, which display deficits in reepithelialization.
In addition to direct effects on keratinocytes, striking alterations in macrophage recruitment in both wound healing and acute UV studies in cKO mice were discovered. Macrophages can play many roles in carcinogenesis; macrophages with an M1 phenotype tend to suppress tumors while more M2-like tumor-associated macrophages can aid with tumor growth [186]. Ongoing long term UV studies will examine the number and type of macrophages recruited to skin tumors in cKO mice to see if a decrease in macrophage recruitment persists long term and to characterize the macrophages that do reach the tumor microenvironment. In wound healing studies, the loss of keratinocyte VEGFR-1 also decreased macrophage recruitment. Macrophages play an important role in wound repair where they clear dead cells, bacteria and debris, release growth factors and cytokines, directly promote angiogenesis and aid in resolution of inflammation [48]. Therefore, a deficiency in macrophages at the wound bed is likely to be detrimental to healing. It remains unclear how VEGF is mediating the recruitment of macrophages via keratinocyte VEGFR-1. Keratinocytes produce many factor that attract and activate macrophages such as MCP-1 and MIP-1α in wound repair [170-171, 176], and IL-1β and TNF-α during UV irradiation [38-39]. The production of these factors will be examined in future studies.

Because VEGF can affect processes in the skin in complex ways, both negative and positive, it is critical to understand how VEGF is affecting each cell type individually. Overall, these studies have broadened our knowledge of keratinocyte biology and have uncovered novel functions of VEGF in the cutaneous response to UV exposure and to injury.
References


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