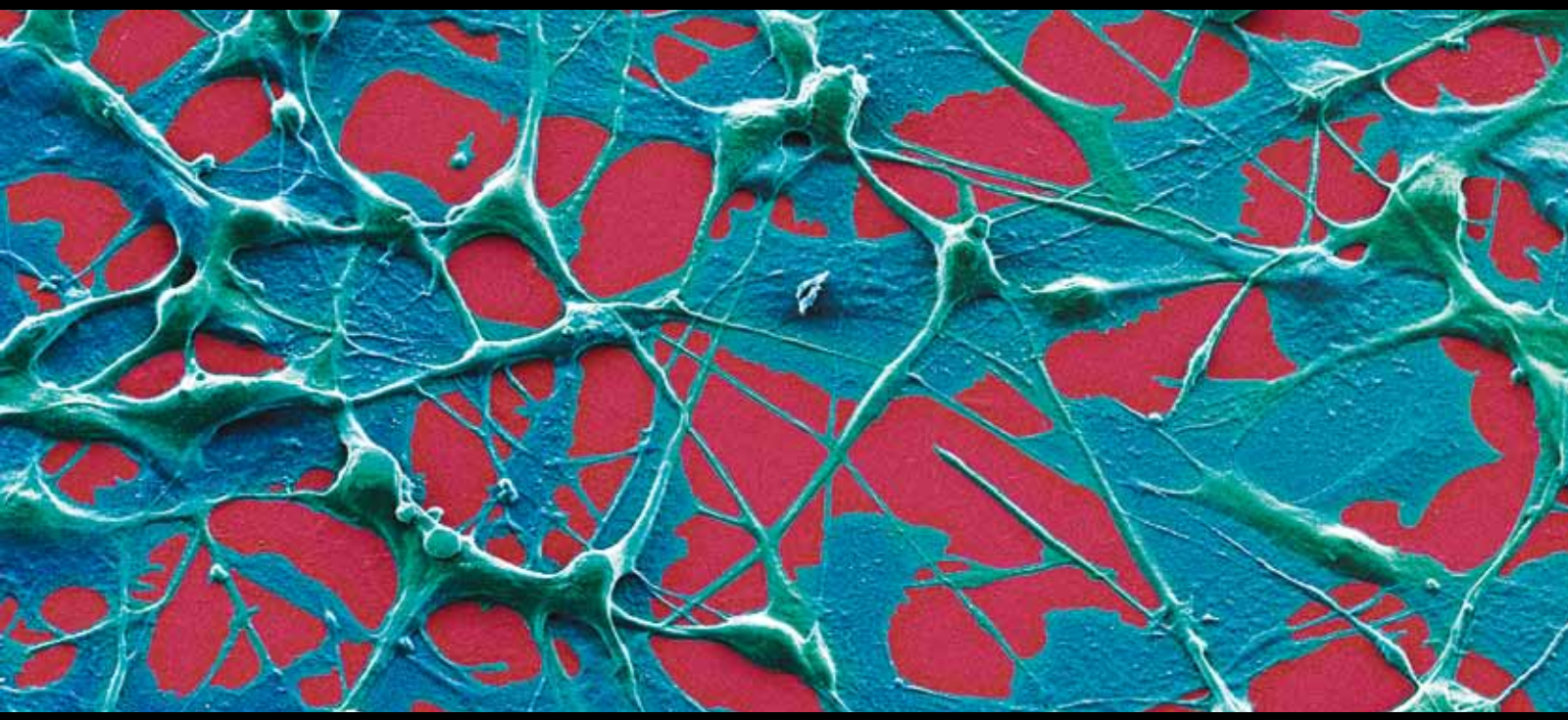
Journal of Skin Cancer

Mouse Models of the Skin: Models to Define Mechanisms of Skin Carcinogenesis

Guest Editors: Deric L. Wheeler, Ajit K. Verma, and Mitchell F. Denning



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Editorial Board

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Editorial

Mouse Models of the Skin: Models to Define Mechanisms of Skin Carcinogenesis

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The multistep model of mouse skin carcinogenesis has facilitated identification of irreversible genetic events of initiation and progression, and epigenetic events of tumor promotion. Mouse skin tumor initiation can be accomplished by a single exposure to a sufficiently small dose of a carcinogen, and this step is rapid and irreversible. However, promotion of skin tumor formation requires a repeated and prolonged exposure to a promoter, and that tumor promotion is reversible. Investigations focused on the mechanisms of mouse carcinogenesis have resulted in the identifications of potential molecular targets of cancer induction and progression useful in planning strategies for human cancer prevention trials. This special issue contains eight papers that focus on mouse models used to study individual proteins expressed in the mouse skin and the role they play in differentiation, tissue homeostasis, skin carcinogenesis, and chemoprevention of skin cancer.

In the paper entitled "*Ap1 transcription factors in epidermal differentiation and skin cancer*," R. Eckert et al. highlight the role of AP1, a transcription factor composed of c-jun and c-fos, that serves as a central node in epidermal keratinocyte survival and differentiation. The authors discuss how AP1 deregulation leads to key steps in driving the development of cancer and how these functions in cancer may be different in epidermal development. Finally, they summarize the various mouse models that have helped elucidate the role of this very interesting molecule.

In the paper entitled "*The role of TGFβ signaling in squamous cell cancer: lessons from mouse models*," A. Glick summarizes the current literature on the role of TGFβ1 in normal tissues and in carcinogenesis. TGFβ1 is a member of a large growth factor family including activins/inhibins and bone morphogenic proteins (BMPs) that have potent growth regulatory and immunomodulatory functions in normal skin homeostasis, regulation of epidermal stem cells, extracellular matrix production, angiogenesis, and inflammation. The author presents a thorough comparison between the role of TGFβ1 in signaling in human HNSCC and cutaneous SCC and the various mouse models that have been developed to elucidate the role this molecule plays in oncogenesis.

In the paper entitled "*Multiple roles for VEGF in nonmelanoma skin cancer: angiogenesis and beyond*," K. Johnson and T. Wilgus overview how vascular endothelial growth factor (VEGF), a potent proangiogenic factor in mouse and human skin tumors, plays a role in the development of nonmelanoma skin cancers. The authors have detailed the use of both transgenic and knockout mice that have provided key clues, primarily alteration of proliferation, survival, and stemness, that have helped elucidate the function of VEGF in carcinogenesis.

In the paper entitled "*Protein kinase Cε, which is linked to ultraviolet radiation-induced development of squamous cell carcinomas, stimulates rapid turnover of adult hair follicle stem cells*," A. Singh et al. report that protein kinase C epsilon (PKCε), a member of the protein kinase C superfamily, plays a critical step in the development of cutaneous SCC induced by repeated exposures to ultraviolet radiation (UV). The authors focus their investigation on how PKCε, using transgenic mice, may modulate the hair follicle stem cell (HSC). The authors report that overexpression of PKCε in the skin, driven by the K14 promoter, leads to a 7-fold increase in the proliferation of the HSC, indicating a rapid turnover of these cells.

In the paper entitled "*Patched knockout mouse models of basal cell carcinoma*," the authors discuss the link Patched (PTCH), the receptor for the hedgehog ligand, in the development of Basal cell carcinoma (BCC), the most common form of human skin cancer. In this comprehensive review, the authors compare conventional and conditional PTCH knockout mouse models to investigate BCC as well as for potential use in preclinical research.

In the paper entitled "*Delineating molecular mechanisms of squamous tissue homeostasis and neoplasia: focus on p63*," K. King et al. focus on summarizing mouse models that have highlighted the importance of p63, a transcription factor that plays an essential role in the development and maintenance of normal stratified squamous epithelium. The authors present that p63 has multiple splice variants and p63 plays a critical role in normal skin biology and neoplastic development.

In the paper entitled "*Role of Stat3 in skin carcinogenesis: insights gained from relevant mouse models*," E. Macias et al. review the role of signal transducer and activator of transcription 3 (Stat3) in skin biology. The authors detail the various transgenic, knockout, and conditional knockout mice that have led to the understanding of STAT3 in normal skin homeostasis, migration, wound healing, and hair follicle growth and maintenance as well as skin carcinogenesis.

In the paper entitled "*Topical curcumin-based cream is equivalent to dietary curcumin in a skin cancer model*," the authors present the first study that compares the use of topical curcumin versus the use of oral curcumin as a chemopreventive strategy for the development of SCC of the skin.

This collection of papers provides an overview of mouse models investigating several intensely studied molecules involved in skin carcinogenesis. We hope the molecular mechanisms revealed in this special issue will enlighten readers and provide them with motivation to continue their research endeavors.

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Review Article

AP1 Transcription Factors in Epidermal Differentiation and Skin Cancer

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AP1 (jun/fos) transcription factors (*c-jun, junB, junD, c-fos, FosB, Fra-1, and Fra-2*) are key regulators of epidermal keratinocyte survival and differentiation and important drivers of cancer development. Understanding the role of these factors in epidermis is complicated by the fact that each protein is expressed, at different levels, in multiple cells layers in differentiating epidermis, and because AP1 transcription factors regulate competing processes (i.e., proliferation, apoptosis, and differentiation). Various *in vivo* genetic approaches have been used to study these proteins including targeted and conditional knockdown, overexpression, and expression of dominant-negative inactivating AP1 transcription factors in epidermis. Taken together, these studies suggest that individual AP1 transcription factors have different functions in the epidermis and in cancer development and that altering AP1 transcription factor function in the basal versus suprabasal layers differentially influences the epidermal differentiation response and disease and cancer development.

1. Introduction

Keratinocytes are the major cell type responsible for the structure of the epidermis. They begin as stem cells in the basal epidermal layer and hair follicles [1–3]. During differentiation, as the cells migrate to the surface, cell division ceases and morphological changes ensue to produce the spinous, granular, transition, and cornified layers. Spinous layer cells are distinguished by the presence of desmosomal connections, whereas granular layer cells are characterized by the presence of granules that contain the products of keratinocyte differentiation. Differentiation of the granular layer cells results in the formation of the transition zone which separates the dead from living epidermal layers. It is in this zone that the cellular constituents are extensively enzymatically remodeled. This remodeling results in the covalent crosslinking of proteins to produce terminally differentiated corneocytes that form the skin surface [4, 5]. Achieving these morphological alterations relies on executing a preset program of differentiation that requires tight regulation of gene transcription [6].

The process of activation and suppression of gene transcription is controlled by a diverse family of regulators called transcription factors. Transcription factors mediate the final steps in the relay of information from the cell surface to the nucleus and the gene. This is accomplished by the interaction of the transcription factor with specific DNA elements that are usually located immediately upstream of the sequence

that encodes the gene. DNA elements are generally a short DNA sequence of 8–20 nucleotides that encode a specific consensus sequence. A host of transcription factors has been implicated in control of epidermal differentiation and function, including activator protein 1 (AP1), AP2, Sp1, POU domain proteins, and CCAAT enhancer binding proteins [7]. AP1 transcription factors are among the most interesting and important regulators in epidermis [7]. Members of this family (c-fos, fosB, Fra-1, Fra-2, c-jun, junB, and junD) are expressed in specific epidermal layers and control multiple key functions [8]. This review focuses on summarizing interesting animal-based studies designed to identify the impact of perturbing AP1 transcription factor function on epidermal homeostasis and cancer.

2. MAPK and AP1 Transcription Factors Are Key Regulators of Keratinocyte Differentiation

The mitogen-activated protein kinases (MAPK) comprise major signaling cascades that regulate differentiation-associated gene expression in epidermis [9–14]. Each MAPK cascade consists of three kinase modulates which include an MEK kinase (MEKK), a mitogen-activate protein kinase/extracellular signal regulated kinase (MEK), and a mitogen-activated protein kinase (MAPK) [15–18]. Activated MEKK phosphorylates MEK which phosphorylates the MAPK. Activated MAPKs phosphorylate a variety of target proteins including transcription factors [10, 19–21]. The most extensively studied MAPKs are the ERK kinases (ERK1, ERK2), the c-jun N-terminal kinases (JNK1, JNK2), and the p38 kinases (p38α, β, δ, and γ). Figure 1 presents a schematic of the p38δ MAPK pathway which regulates expression of differentiation-associated genes during keratinocyte differentiation [7, 11]. The cascade consists of upstream regulator proteins (novel protein kinase c and Ras), an MAPK module (MEKK1, MEK3, and p38δ) and AP1 transcription factors. Activation of this cascade by a differentiation stimulus causes sequential phosphorylation and activation of kinases in the MAPK module which leads to increased AP1 transcription factor level and binding to the DNA response element in the target gene. This leads to increased target gene transcription [10–14, 22].

AP1 transcription factors are key downstream targets of MAPK signaling in keratinocytes [12–14, 22–24]. Activator protein one (AP1) transcription factors include jun (c-jun, junB, junD) and fos (c-fos, FosB, Fra-1, Fra-2) family members [25–28]. They form jun-jun and jun-fos dimers that interact with specific AP1 transcription factor consensus DNA binding elements in target genes to regulate expression. They control keratinocyte proliferation [29–31], differentiation [10, 11, 32], and apoptosis [23, 33] and are important in tumor progression and disease development [9–11, 14, 22, 23, 34–38]. As an example, increased p38δ MAPK activity results in increased AP1 transcription factor level, increased AP1 transcription factor binding to DNA elements on the involucrin promoter, and increased involucrin gene transcription via a scheme similar to that shown in Figure 1

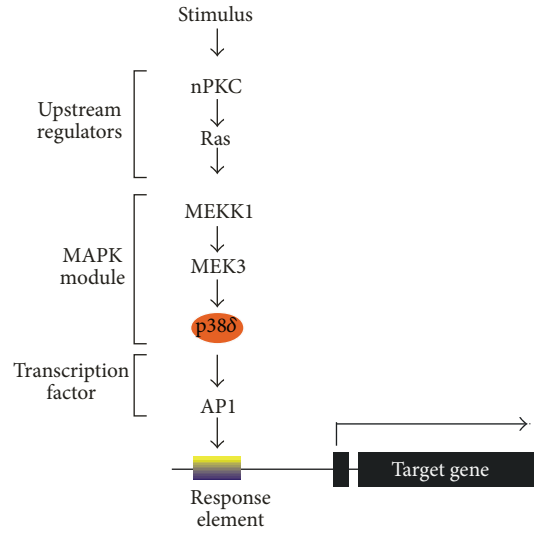

Figure 1: MAPK and AP1 transcription factor control of gene expression. The p38δ MAPK cascade that controls the expression of differentiation-associated genes in epidermis is depicted [10]. The three kinases of the MAPK module include MEKK1, MEK3, and p38δ MAPK. A differentiation stimulus activates upstream regulatory proteins, in this case novel protein kinase c (nPKC) and the Ras small GTPase. These events lead to phosphorylation and activation of MEKK1 which phosphorylates MEK3 which phosphorylates p38δ MAPK. Ultimately p38δ MAPK increases AP1 transcription factor expression and activity and the AP1 transcription factors bind to the response element on the target gene promoter to increase transcription.

[8, 39]. The major AP1 factors that interact with the promoter are JunB, JunD, and Fra-1. Moreover, TAM67, a dominant-negative mutant of c-jun that inhibits the activity of all AP1 transcription factors [40], inhibits p38δ-dependent involucrin promoter activation [13]. MAPK activation by p38δ also results in increased C/EBPα and Sp1 binding to DNA binding sites in the involucrin gene promoter [41–43]. Thus, a PKC, Ras, MEKK1, MEK3 pathway activates p38δ MAPK and p38δ, in turn, acts to increase binding of selected AP1, Sp1, and C/EBP factors to the hINV promoter to increase promoter activity. However, the AP1 transcription factors are the most important family of regulators. In fact, it would be difficult to envision a more important family of transcriptional regulatory proteins in epidermal keratinocytes.

AP1 action in epidermis is complicated for several reasons. First, multiple AP1 family members are expressed in epidermis and form multiple dimer pairs. AP1 transcription factors can theoretically form eighteen different homo- and heterodimers, and work in other systems show that the particular dimer that is formed influences activity. For example, coexpression of c-fos with c-jun, leading to c-fos:c-jun dimer formation, enhances the transforming capacity of c-jun, whereas pairing c-jun with junB inhibits c-jun transforming capacity [44–46]. These differences may be related to the higher DNA binding and transcriptional activity of c-jun:c-fos heterodimer in comparison to c-jun:junB heterodimer [47]. Thus, it is safe to assume that the dimer that is formed

influences activity in differentiating keratinocytes. Second, the expression level of most AP1 family members changes during keratinocyte differentiation [8, 48]. This means that different pairing combinations exist in the basal versus suprabasal layers and that this is likely to drive differences in activity and target gene selection. Third, covalent modification of individual AP1 transcription factors (e.g., phosphorylation) influences activity [49, 50]. For example, c-jun undergoes transient N-terminal phosphorylation as cells exit the G2 phase of the cell cycle, and this state is maintained until the cells complete mitosis [45]. An important lesson from these studies is that the composition of AP1 transcription factors in the tissue and the posttranslational modification state can influence biological activity. The fact that each AP1 transcription factor forms multiple hetero- and homodimers indicates that manipulating the level of one AP1 transcription factor, either by overexpression or knockout, will modify the function of other members. These features must be considered when interpreting the results of studies that alter AP1 transcription factor level or function in epidermis.

3. Animal Models of AP1 Transcription Factor Function

A number of laboratories have used *in vivo* mouse genetic models to study AP1 transcription factor function [34, 51, 52, 52–55]. These include embryonic knockout [54, 56–64], conditional knockout, inducible knockdown, expression of mutant dominant-negative AP1 proteins [65, 65–71], and targeted expression of intact wild-type proteins [72–74, 74–76]. These studies have targeted a variety of tissues, including the epidermis, liver, mammary gland, heart, bone, and blood [77]. The first lesson from these studies is that appropriate AP1 transcription factor expression is required for survival. For example, c-jun knockout mice die at embryonic day E13 due to defects in liver and heart development [78]. Likewise, junB null mice display extraembryonic tissue defects and die at embryonic E9.5 [56]. Fra-1 null mice survive only till embryonic day E9.5, and death is associated with defects in the yolk sac and placenta [54]. JunD knockout mice are born but fail to reproduce due to defects in spermatogenesis and reproduction [64]. These studies indicate that AP1 factors are essential for embryonic survival and are necessary for sustained development and reproduction. This is consistent with a central role for this family of proteins in maintaining tissue and organ homeostasis [77].

AP1 transcription factors also have tissue-specific effects. An *in vivo* example of this is that transgenic re-expression of junB in junB-null embryos rescues the mice from embryonic death. This is associated with normalization of most tissues; however, the junB transgene is silenced by an epigenetic mechanism in the myeloid lineage, and so these mice develop progressive myeloid leukemia [79]. This is also true in the context of tumor formation where AP1 transcription factors can function as oncogenes or tumor suppressors. For example, junD promotes cell survival by protecting cells from p53-dependent senescence and apoptosis [80, 81]. In contrast, JunD can also antagonize ras-mediated transformation [82]. Fra-1 has a complex role in that it enhances breast cancer cell chemosensitivity by driving cancer stem cells from dormancy [83]. In addition, Fra-1 deficient embryonic fibroblasts are resistant to peroxide-induced cell death, presumably because Fra-1 attenuates Nrf2-driven antioxidant responses [84]. Moreover, Fra-1 is increased in breast cancer where it functions as an oncogene to enhance tumor cell migration [85]. Thus, Fra-1 has multiple roles depending upon the tumor type and conditions.

4. AP1 Transcription Factors in Epidermis Knockout and Overexpression Studies

4.1. c-Jun and JunB—an Epidermal Oncogene and a Tumor Suppressor. Altering AP1 transcription factor expression changes epidermal function. Mice in which c-jun is conditionally knocked out in the epidermis develop normal skin, but epidermal growth factor receptor (EGFR) level is reduced in the eyelids leading to open eyes at birth [86]. This mimics the phenotype observed in EGFR- or TNFα-null mice [87–90]. In addition, in the absence of c-jun, the tumor-prone K5-SOS-F transgenic mice develop smaller epidermal papilloma, suggesting that c-jun is required for tumor formation [86], and it has been noted that c-jun expression is increased in tumors, and overexpression of c-jun in an oncogenic Ras background enhances tumor formation [91]. These findings suggest that c-jun functions as an oncogene in keratinocytes.

Mice lacking junB in keratinocytes are born with a normal epidermis. However, the epidermis is not completely normal, as epidermal JunB knockout mice display delayed wound healing [51] and develop systemic lupus erythematosus, an autoimmune disease that influences multiple tissues [92]. This phenotype is associated with increased secretion of epidermis-produced interleukin 6 (IL-6) that is associated with loss of JunB-dependent suppression IL-6 gene expression. IL-6 appears to play an essential role in phenotype development, as the phenotype is alleviated when epidermal JunB-null mice are bred to IL-6 deficient mice [92]. Absence of JunB in the epidermis also results in the release of large quantities of epidermis-derived granulocyte-colony stimulating factor (G-CSF) which is associated with skin ulceration, myeloproliferative disease, and low bone mass [93]. G-CSF appears to be essential for phenotype appearance, as breeding JunB null mice into a G-CSF null background reverses the myeloproliferative phenotype [93]. In addition, simultaneous conditional deletion of c-jun and JunB in the epidermis produces a psoriasis-like phenotype [94]. This is associated with increased production of tumor necrosis factor-alpha (TNFα) and increased epidermal S100A8/S100A9 expression [52]. Chemokine/cytokine production in epidermis presumably recruits immune cells to the epidermis to produce the psoriatic phenotype. Tissue inhibitor of metalloproteinase-3 (TIMP3) level is reduced in junB/c-jun null epidermis. As TIMP3 is an inhibitor of TNFα converting enzyme (TACE), loss of TIMP3 leads to enhanced epidermal TNFα cleavage and release [95]. TNFα is a key regulator in this context, as the biological phenotype can be mitigated by breeding these mice into a TNFα-null background [95]. Moreover,

vascular endothelial growth factor (VEGF) also influences this phenotype, as anti-VEGF antibody treated junB/c-jun null mice show a pronounced reduction of inflammatory cells within the dermis and more normal epidermal differentiation [94]. JunB absence also increases tumor forming potential [91]. Tumor formation in Ras-activated cancer cells is inhibited by overexpression of JunB, an effect that requires the JunB transactivation domain [91]. Moreover, expression of dominant-negative JunB in this model, which inhibits JunB function, increases tumor formation [91].

4.2. c-Fos Acts as an Oncogene in Epidermis. JunB and c-jun are the most heavily studied AP1 transcription factors, but information is also available regarding the role of c-fos. Challenge of v-H-ras positive mice with DMBA (7,12-dimethylbenz[a]anthracene) and TPA (12-O-tetradecanoylphorbol-13-acetate), in the two-stage carcinogenesis protocol, increases skin tumor formation. However, tumor formation is attenuated in the absence of c-fos [34] which is associated with increased p53 expression [96]. The higher than normal level of p53 leads to epidermal tumor cell differentiation and suppression of skin tumor formation, in part due to p53-dependent transcriptional activation of TNFα converting enzyme [96].

4.3. Activating Transcription Factor 2 (ATF2) Suppresses Skin Tumor Formation. Activating transcription factor 2 (ATF2) is a stress-regulated transcription factor, and ATF2 transcriptional activity requires leucine zipper-dependent heterodimerization with members of the AP1 family, including c-jun [97, 98]. Expression of an inactive mutant form of ATF2 (lacking the DNA binding and leucine zipper domains) in the basal epidermis results in reduced tumor formation. When subjected to a two-stage DMBA/TPA skin carcinogenesis protocol, mice expressing the inactive ATF2 display increased tumor formation, and keratinocytes derived from these mice display enhanced anchorage-independent growth [99]. The resulting tumors display enhanced β-catenin and cyclin D1 and reduced Notch1 expression. This is consistent with the observation of reduced ATF2 and increased β-catenin in human squamous and basal cell carcinoma samples [99] and suggests that ATF2 suppresses epidermal carcinogenesis.

5. AP1 Transcription Factors in Epidermis-Dominant-Negative c-Jun (TAM67)

We have hypothesized that AP1 transcription factors perform different functions in the basal (proliferating) versus suprabasal (differentiating) epidermis [11]. However, testing this hypothesis is complicated by the fact that virtually all of the AP1 family members are expressed, at some level, in both the basal and suprabasal compartments [8, 25, 48]. Thus, we sought a model system where we could achieve complete suppression of AP1 transcription factor function in specific epidermal layers. This goal is difficult to achieve using gene knockout strategies, since knockout normally obviates expression of the targeted gene in all epidermal layers. Thus, we turned to targeted expression of dominant-negative c-jun (TAM67) in specific epidermal layers. In our case, we targeted TAM67 expression to the upper epidermal layers to achieve inactivation of AP1 transcription factor function in the suprabasal epidermis [66]. These studies follow a strategy developed by Nancy Colburn and associates where they targeted TAM67 to the basal epidermal layers using the K14 promoter [100]. This strategy has several advantages. First, TAM67 interferes with the function of all AP1 transcription factors [100]. TAM67 forms heterodimers with other AP1 transcription factors and these complexes bind to DNA, but the complexes are not able to activate transcription [100, 101]. Moreover, an early study, using a keratin promoter to drive expression, showed that TAM67 expression reduces TPA-stimulated invasion of mouse 308 cells through matrigel [65]. Further studies show that TAM67 inhibits invasion of human papillomavirus-immortalized human keratinocytes by suppressing AP1 transcription factor and NFκB signaling [102, 103]. These studies suggest that TAM67 is a useful construct for the study of cell function. Second, our use of the involucrin promoter permits targeting of TAM67 to the suprabasal epidermis [104–106] and alleviates problems that are observed with knockout mice where a specific AP1 transcription factor protein is lost from all layers. Third, a basal layer TAM67-targeted mouse model already existed [68, 70, 71, 107, 108] which permitted a direct comparison of the impact of basal versus suprabasal AP1 transcription factor inactivation. We will first discuss the impact of targeted expression of TAM67 in the epidermal basal layer.

5.1. TAM67 in the Basal Epidermis. *In vivo* studies in mouse epidermis show that TAM67-dependent inactivation of AP1 transcription factor function in the basal epidermal layer does not produce obvious changes in keratinocyte proliferation or epidermal or dermal appearance [68, 71, 107]. However, basal layer TAM67 expression does reduce susceptibility of SKH-1 hairless mice to UVB-dependent cancer progression [68, 71, 107]. Both tumor number and size are reduced and this is associated with reduced numbers of cyclin D1 positive cells in the tumors [107]. Expression of the E7 gene from human papillomavirus type 16 in mouse skin induces hyperplasia and enhances tumor promotion, and TAM67 protects mice from E7-enhanced tumorigenesis [70].

Some additional details are known regarding the mechanism of impact of AP1 transcription factor inaction in epidermal cancer cells. TPA treatment induces transformation of JB6/P+ cells. JB6/P+ cells are murine keratinocytes that undergo transformation following treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) [109]. Screening of microarrays from TPA-treated JB6/P+ cells, maintained in the presence or absence of TAM67 expression, revealed that high-mobility group A1 (HMGA1) protein is induced by TPA, and this induction is inhibited by TAM67. Further studies show that knockdown of HMGA1 with siRNA reduces JB6/P+ transformation, which is consistent with HMGA1 being an important AP1 transcription factor target [109]. A similar approach, also using JB6/P+ cells, identified sulfiredoxin as an additional gene that is required for TPA-induced transformation and is suppressed by TAM67 [110].

Sulfiredoxin is important for redox homeostasis and acts to reduce hyperoxidized peroxiredoxins. Cyclooxygenase-2, osteopontin, programmed cell death-4, and Wnt5a are additional proteins that may be important in transformation and have been identified [108, 111, 112]. It is possible that these proteins play a role in reducing tumor formation observed in mice where TAM67 is expressed in the basal layer.

5.2. TAM67 in the Suprabasal Epidermis. A recent study shows that targeted expression of TAM67 in the suprabasal epidermis results in extensive hyperplasia and hyperkeratosis [66]. This is associated with a substantial increase in proliferation of basal layer keratinocytes as measured by increased BrdU incorporation and increased appearance of Ki67-positive cells. This is not due to a direct effect of TAM67 on basal cells, as two different staining methods reveal that the TAM67-FLAG expression is confined to the suprabasal layers. Thus, inactivating suprabasal AP1 transcription factor function appears to feedback on the basal layer in a manner that stimulates basal layer cell division. In addition, differentiation appears to be delayed and incomplete. Consistent with delayed differentiation, keratins K5 and K14, which are normally exclusively expressed in the basal layer, are detected in all epidermal layers, and K6 is expressed in all epidermal layers. K6 is a keratin that is expressed under conditions of hyperproliferation but is not expressed in normal epidermis [66]. Thus, suprabasal TAM67 expression leads to increased basal layer proliferation and delayed differentiation and ultimately results in extensive hyperkeratosis. This is in marked contrast to the finding that targeting TAM67 to the epidermal basal layer using the keratin 14 promoter (K14-TAM67) produces no overt phenotype under resting conditions [71]. We propose that normal differentiation leads to accumulation of signals, generated by suprabasal cells, that suppress basal layer cell proliferation and that inhibiting differentiation opens this feedback loop leading to increased basal keratinocyte proliferation [66].

Because of the hyperproliferative phenotype, it was anticipated that mice expressing TAM67 in the suprabasal epidermis would be more susceptible to tumor formation. This was tested by treating control and suprabasal TAM67 mice with a DNA mutagenic agent, 7,12-dimethylbenz[α]anthracene (DMBA) to produce initiated cells, and then inducing TAM67 expression. Surprisingly, TAM67 expression, and the associated increase in cell proliferation, did not drive tumor formation in DMBA treated mice. This is interesting, because cell proliferation is thought to predispose tissue to enhanced tumor formation [113]. Treatment with carcinogen (7,12-dimethylbenz[α]anthracene, DMBA) followed by tumor promoter (12-O-tetradecanoylphorbol-13-acetate, TPA) is known to cause tumor formation [113]. However, in a protocol where mice were treated with DMBA, followed by treatment with TPA, TAM67 expression reduced tumor formation. The possibility that TAM67 may interfere with the proliferation promoting activity of TPA in the carcinogenesis protocol was considered; however, these experiments suggest that TAM67-expressing epidermis is fully competent to respond to TPA. Taken together, these findings show that inaction of AP1 transcription factor function in the suprabasal epidermis increases epidermal proliferation but reduces carcinogen/tumor promoter-induced cancer development. The underlying mechanism responsible for these surprising observations is under study.

Thus, although the basal and suprabasal targeted TAM67 mice produce very different epidermal phenotypes, these mice share features in common [66, 71]. First, TAM67 basal and suprabasal epidermal mice respond to stress agents (okadaic acid, TPA, etc.) with increased basal cell proliferation, and this response is not reduced when compared to control mice. Second, both strains display a reduced sensitivity to DMBA/TPA induced tumor formation. The fact that inactivating AP1 factor function in the basal or suprabasal epidermis reduces tumor formation, clearly suggest that, on balance, AP1 factors have an essential role in driving tumor formation.

6. Summary

A variety of genetic approaches have been used to study the *in vivo* role of AP1 transcription factors in epidermis. It is clear from these studies that AP1 transcription factors play a key role in controlling differentiation of epidermal keratinocytes and that perturbing this process results in a variety of disease phenotypes including psoriasis and cancer. It is also clear that some AP1 transcription factors function as procancer proteins (e.g., c-jun, c-fos), while others inhibit cancer development (e.g., JunB, ATF2). Additional studies suggest that a host of cytokines and chemokines is involved in generation of the disease and cancer phenotypes that develop when AP1 transcription factor function is perturbed, and these studies suggest that the epidermis can act as an endocrine organ to influence the function of other organs. It also appears that AP1 transcription factors have differing roles in basal and suprabasal epidermis, as inactivation of AP1 transcription factor function in these compartments produces no change (basal targeted TAM67 expression) or hyperproliferation (suprabasal targeted TAM67 expression).

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Research Article

Protein Kinase Cε, Which Is Linked to Ultraviolet Radiation-Induced Development of Squamous Cell Carcinomas, Stimulates Rapid Turnover of Adult Hair Follicle Stem Cells

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To find clues about the mechanism by which kinase C epsilon (PKCε) may impart susceptibility to ultraviolet radiation (UVR)-induced development of cutaneous squamous cell carcinomas (SCC), we compared PKCε transgenic (TG) mice and their wild-type (WT) littermates for (1) the effects of UVR exposures on percent of putative hair follicle stem cells (HSCs) and (2) HSCs proliferation. The percent of double HSCs (CD34+ and α6-integrin or CD34+/CD49f+) in the isolated keratinocytes were determined by flow cytometric analysis. Both single and chronic UVR treatments (1.8 kJ/m^2) resulted in an increase in the frequency of double positive HSCs in PKCε TG mice as compared to their WT littermates. To determine the rate of proliferation of bulge region stem cells, a 5-bromo-2′-deoxyuridine labeling (BrdU) experiment was performed. In the WT mice, the percent of double positive HSCs retaining BrdU label was 28.4 ± 0.6% compared to 4.0 ± 0.06% for the TG mice, an approximately 7-fold decrease. A comparison of gene expression profiles of FACS sorted double positive HSCs showed increased expression of Pes1, Rad21, Tfdp1 and Cks1b genes in TG mice compared to WT mice. Also, PKCε over expression in mice increased the clonogenicity of isolated keratinocytes, a property commonly ascribed to stem cells.

1. Introduction

The multistage model of mouse skin carcinogenesis is a useful system in which biochemical events unique to initiation, promotion, or progression steps of carcinogenesis can be studied and related to cancer formation. 12-O-Tetradecanoylphorbol-13-acetate (TPA), a component of croton oil, is a potent mouse skin tumor promoter [1, 2]. A major breakthrough in understanding the mechanism of TPA tumor promotion has been the identification of protein kinase C (PKC), as its major intracellular receptor [3]. PKC forms part of the signal transduction system involving the turnover of inositol phospholipids and is activated by DAG, which is produced as a consequence of this turnover [3]. PKC represents a family of phospholipid-dependent serine/threonine kinases [3–6]. PKCε is among the six PKC isoforms (α, δ, ε, η, μ, and ξ) expressed in both mouse and human skin [7]. We have reported that epidermal PKCε levels dictate the susceptibility of PKCε transgenic (TG) mice to the development of squamous cell carcinomas (SCC) elicited either by repeated exposures to ultraviolet radiation (UVR) [8] or initiation with 7, 12-dimethylbenz[a]anthracene (DMBA) and tumor promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) [9]. Histologically, SCC in TG mice, like human SCC, is poorly differentiated and metastatic [10].

SCC developed in PKCε transgenic mice is metastatic and originates from the hair follicle [10]. The papilloma-independent carcinomas which develop in PKCε transgenic mice arise from the hair follicle and have increased metastatic potential [10]. The difference in metastatic potential and the different origin of malignancy when compared to WT provided support for the hypothesis that papilloma-independent carcinomas in PKCε TG mice were pathologically distinct from WT mouse carcinomas. Although the papilloma-independent carcinomas appeared to originate from the hair follicle, it was possible that the origin of the tumor was not within the hair follicle. The hair follicle might have been the easiest pathway for invasion. However, this did not appear to be the case because we observed neoplastic cells arising only from the hair follicle and not the epidermis. By harvesting PKCε TG and WT mice after 8 weeks of DMBA + TPA or DMBA + acetone treatments, we identified possible premalignant areas in PKCε transgenic mice as early as 8 weeks after DMBA + TPA treatment. The premalignant lesions originated within the hair follicle [10].

The metastatic potential of a transformed keratinocyte appeared to inversely correlate with the differentiation potential of that keratinocyte in the limited number of tumors studied to date. This conclusion was based on the location of invasion and pathological categorization of PKCε TG mouse carcinomas compared with WT mouse carcinomas. Bulge keratinocytes are located near the sebaceous gland within the hair follicle. Evidence suggests that these cells appear to be the stem or progenitor cells for both the hair follicle and epidermis and, therefore, would be in a less-differentiated state than other epidermal cells [10]. These properties may increase the metastatic potential of these cells. The carcinomas of PKCε TG mice that led to metastases were also less differentiated than carcinomas from WT mice. Evidence suggests that malignant cells invading from the hair follicle were less differentiated and had a higher metastatic potential than cells that invaded from the epidermis. PKCε, when activated either via direct binding to TPA or indirectly by UVR treatment, mediates two potential signals leading to inhibition of apoptosis [11, 12] and induction of cell proliferation.

Epidermal stem cells in the mouse hair follicle are known to be the precursor cells for SCC in the mouse skin [13–17]. Evidence suggests that epithelial stem cells reside in the bulge region [18, 19]. Stem cells, unlike transit amplifying cells, are slowly cycling and thus seem probable target cells. Moreover, stem cells may retain those mutations and pass them on to their progeny [14]. Morris et al. [20] demonstrated that label retaining cells (LRCs) have another property characteristic of potential initiated cells: they could retain carcinogen-DNA adducts. The contribution of follicular and interfollicular stem cells to the induction of skin papillomas and carcinomas was also determined [20]. Both follicular and interfollicular stem cells contributed to the development of papillomas. However, only follicular stem cells were linked to the development of carcinomas.

As a prelude to determine the SCC lineage from HSCs in PKCε TG mice, we compared the responses of PKCε TG and their WT littermates to UVR treatment. We examined the effects on proliferation, turnover, and gene expression profile of HSCs. In this communication, we present for the first time that (1) UVR exposures increased the number of double positive HSCs in TG mice, (2) the percent of double positive HSCs retaining BrdU label in the WT mice was 7-fold more than the TG mice, indicating that the double positive cells in the TG mice cycle at a faster rate, (3) the keratinocytes from PKCε TG mice have higher proliferating potential compared to their WT littermates, and (4) a comparison of gene expression profile of FACS-sorted HSCs showed an increase expression of Pes1, Rad21, Tfdp1, and Cks1b genes in TG mice compared to their WT littermates.

2. Materials and Methods

2.1. Chemicals and Antibodies. BrdU was purchased from Sigma Aldrich (St. Louis, MO, USA). BrdU antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Antibodies used for FACS such as α6-integrin PE-conjugated, CD34 FITC-conjugated antibodies, APC BrdU labeling kit, and propidium iodide were purchased from BD Biosciences (San Jose, CA, USA). BrdU antibody conjugated with Alexa Fluor 647 was procured from Biosciences (Frederick, MD, USA). PCR gene array focused to cell cycle was purchased from SA Biosciences (Frederick, MD, USA).

2.2. Keratinocyte Isolation and Flow Cytometric Analysis. Keratinocytes were harvested as described elsewhere [21]. In each experiment an equal size of skin is excised from the WT and TG mice. Viable cell counts were determined using 0.4% Trypan Blue. Keratinocytes were incubated for 1 hr in the dark at 4°C with PE-conjugated Rat Anti-Human α6-integrin antibody at 10 μL per 10^6 cells and FITC-conjugated rat antimouse CD34 antibody at 2 μg per 10^6 cells (PE-α6-integrin and FITC-CD34 antibodies; BD Biosciences). Keratinocyte preparations were sorted based on α6-integrin+ and CD34 status using a FACS Aria cell sorter (BD Biosciences). Cells were stained with PE-conjugated anti-α6-integrin and FITC-conjugated anti-CD34 antibodies for flow cytometry. A 488 nm laser was used to detect FITC with a 530/30 filter and a 532 nm laser for PE with a 575/25 filter. The nozzle size was 130 nm and the pressure used was 14 p.s.i. The live cell population gate was estimated using forward and side scatter positioning and confirmed with 7AAD staining.

2.3. Keratinocyte Colony Forming Assay. Keratinocytes were harvested from the dorsal skin of 7-8 weeks old PKCε overexpressing mice (TG224 and TG215) and their WT littermates. The skin hairs were clipped and the skin pieces trypsinized for 2 hrs at 32°C. Epidermis was scraped in keratinocyte medium (SMEM) to isolate keratinocyte cells. Three thousand cells per dish were seeded onto irradiated 3T3 cells in 60 mm dishes and cultured for 2 weeks in high calcium medium. For feeder layer, 3T3 cells were cultured in EMEM medium with 10% FBS and 1% Penicillin-streptomycin and irradiated in Cesium Gamma Irradiator at 5000 rad. Irradiated 3T3 cells seeded 10^6 cells/dish to the 60 mm dishes a day before seeding keratinocytes. The clonal culture was grown in William's E

media with 10% FBS and supplements. For counting and measurement of colonies, dishes were fixed with 10% formalin for overnight. After fixation, the cultures were stained with 0.5% rhodamine B for 30 min to visualize colonies. The dishes were rinsed in cold tap water and dried before counting. The colonies were counted and colony size measured using vernier caliper.

2.4. Mice and UVR Treatment. WT and PKCε TG 224 and 215 mice lines (FVB background) described elsewhere [9, 10] were housed in groups of two to three in plastic bottom cages in light-, humidity-, and temperature-controlled rooms; food and water were available *ad libitum*. The animals were kept in a normal rhythm of 12 h light and 12 h dark periods. The UVR source was Kodacel-filtered FS-40 sun lamps (approximately 60% UVB and 40% UVA). UVR dose was measured using UVX-radiometer. Mice were used for experimentation starting at 5 to 6 weeks of age. For 24 hr, 48 hr, and 72 hr time points, the mice were treated for 10 min (2 kJ/m^2) and skin was harvested for keratinocyte isolation after UV exposure. However, for multiple or chronic UVR exposures, mice were exposed to UVR (2 kJ/m^2) three times weekly (Monday, Wednesday, and Friday) or a total of 8 times.

2.5. Detection of BrdU-Labeled Cells in the Hair Follicle Using Flow Cytometric Analysis. To identify the label retaining cells (LRCs), newborn mice (3 days old) were injected subcutaneously with BrdU (50 mg/kg body weight) twice daily for 3 days. There were three mice per group. Mice were sacrificed 3 to 8 weeks after BrdU injection (3, 4, 5, 6, and 8 weeks). Keratinocytes from the epidermis were harvested as described elsewhere [21]. Freshly harvested keratinocytes were incubated with PE-conjugated anti-α6-integrin and FITC-conjugated anti-CD34 antibodies, fixed and stained using the APC BrdU Flow Kit, following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). To prepare BrdU positive control samples for FACS, 5-week-old female mice were injected BrdU (50 mg/kg body weight) intraperitoneally for two days (2 times each). After two days, the spleen and thymus were harvested for BrdU positive cells and used as a positive control.

For cell cycle analysis, freshly harvested keratinocytes were isolated from mouse dorsal skin and stained with PE-conjugated α6-integrin and FITC-conjugated anti-CD34 antibodies. After surface staining, the cells were fixed and then stained overnight with DAPI for cell cycle analysis. Flow cytometric analysis based on α6-integrin, CD34, and BrdU was performed on a LSRII benchtop flow cytometer (BD Biosciences). A multilength ultraviolet laser along with a 450/50 bandpass filter was used to detect DAPI. DAPI was used for DNA staining for live/dead determinations along with cell cycle. For the detection of APC-conjugated anti-BrdU antibody, a 640 nm laser and a 660/20 filter was used.

2.6. Phenotyping and Estimation of the Frequency of CD34+/α6-Integrin+ Stem Cells. The phenotyping assays were acquired on a BD FACSCalibur (BD Biosciences), and BrdU assays were acquired on an LSR II (BD Biosciences) benchtop flow cytometer. Both instruments were calibrated daily by the University of Wisconsin Carbone Cancer Center Flow Cytometry Laboratory staff using the manufacturer's Cytometer Settings and Tracking calibration software. Data were analyzed using FlowJo software version 9.4.3 (Treestar, Ashland, OR, USA). Positive staining and gating strategy were determined by comparison to isotype controls. Dead cells were excluded using 7-aminoactinomycin D (7AAD) staining on FACS Calibur assays or Invitrogen Live/Dead Fixable Violet (FLVD) staining for BrdU assays acquired on the BD LSR II. Data demonstrate frequency of cells in a parent population of live intact cells for α6-integrin and CD34 expression and of α6-integrin+/CD34+/live intact cells for BrdU incorporation.

The frequency of CD34+/α6-integrin+ stem cells represents the percent of CD34+/α6-integrin+/7AAD- cells ("cells" determined by FSC/SSC morphologic gate) in the total 7AAD population. The absolute number of CD34+/α6-integrin+ cells in individual samples was calculated by multiplying frequency of CD34+/α6-integrin+ stem cells by the total number of Trypan-Blue excluding cells in the single cell keratinocyte preparation. The data represent absolute number of CD34+/α6-integrin+ stem cells from the equal size of dorsal skin from WT and TG mice used in the study.

Similarly, for calculation for the absolute count of CD34+/α6-integrin+/BrdU+ cells, the total frequency of CD34+/α6-integrin+/BrdU+/FLDV-cells in total FLVD- was multiplied by the total counts of Trypan-Blue excluding cells in the single cell keratinocyte preparation.

2.7. Immunofluorescence Analysis. To identify the LRCs, newborn mice (3 days old) were injected subcutaneously with BrdU (50 mg/kg body weight) twice daily for 3 days. Mice were sacrificed at 3, 4, 5, 6, and 8 weeks after BrdU injection. Mouse skin was then excised promptly after euthanasia and immediately placed in 10% neutral-buffered formalin for fixation and then embedded in paraffin. Four to five μm sections were cut for immunohistochemistry of BrdU and K15.

For immunofluorescence study extra paraffin was removed using three xylene gradient washes followed by alcohol gradient (95%, 90%, 70%, 50%, and 30%) for 10 min each. The slides were washed with Milli-Q water, and then 1XPBS. The antigen retrieval was done using antigen unmasking solution as per the protocol (Vector Laboratories). The blocking process was done in normal goat and normal horse serum for 1 hr at room temperature (RT). After blocking, primary antibodies (Keratin-15 monoclonal from Neomarkers, CA, dilution 1 : 30) and BrdU (Santa Cruz, dilution 1 : 50) were incubated to tissue section on the slides for overnight. Tissue sections were incubated with their secondary antibodies for 1 hr at RT such as Alexa-Fluor 488-Donkey antimouse IgG (H + L) and Alexa-Fluor 594-Donley antirat IgG (H + L) for k15 and BrdU from Invitrogen, respectively. After incubation with secondary antibody slides were washed three times with 1XPBS, mounted with DAPI, and observed under the fluorescent microscope (Vectra).

2.8. PCR Array and Real-Time PCR. TG mice and their WT littermates were exposed to UV once (2 kJ/m^2), and 24 hrs after UV treatment mice were sacrificed and the dorsal skin removed for keratinocyte isolation. The cells were stained with fluorescent conjugated CD34 and α6-integrin antibodies and sorted. The cells were sorted into 5 mL tubes containing 0.5 mL of heat inactivated, chelated, fetal bovine serum. The collected cells were then spun down and placed into 300 mL of RNAprotect Cell Reagent (Qiagen; Valencia, CA, USA). RNA was isolated from double positive HSCs using SA Biosciences RT^2 qPCR grade isolation Kit (SA Biosciences, Frederick, MD, USA). 250 ng RNA was used for first-strand cDNA synthesis with the SA Biosciences RT^2 FirstStrand Kit. The resulting cDNA was used in the SA Biosciences Cell Cycle Gene Array according to the manufacturer's instructions.

The real-time expression primers of PKCε in the study were selected from Origene website and further checked in NCBI primer blast for their primer-specific details such as proper target binding and amplification product (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The RNA was isolated from FACS-sorted keratinocytes using Qiagen RNeasy mini kit. The samples were treated with DNAse to remove the DNA contamination using Qiagen RNase-free DNase set (Qiagen). For cDNA synthesis SuperScript First-Strand Synthesis Kit (Invitrogen) was used as per the manufacturer' protocol.

Briefly a total of 50 μL reaction mixture consisted of 25 μL; 2X FastStart Universal SYBR Green master (ROX) mix, 30.0 μM (1 μL) forward and reverse primers, and PCR grade water, and 50–100 ng of cDNA (5–10 μL) was used. Final volume of the reaction was adjusted with RNase-free water provided with the kit. The PCR was set up as per instrument protocol in MyiQ Biorad machine. A cycle to threshold (Ct) value was assigned automatically at the beginning of the logarithmic phase of real-time PCR. Finally, differences in Ct value of control (mouse Gapdh) and stem cell samples were used to determine the relative gene expression or fold changes of the PKCε.

3. Results

3.1. Hair Follicle Stem Cells and Clonogenicity of Epidermal Keratinocytes Isolated from PKCε Overexpressing Mice and Wild-Type Littermates. To determine the basal levels of double positive HSCs (CD34+/α6-integrin+) in untreated WT, TG224, and TG215 mice, freshly harvested keratinocytes were labeled with CD34 and α6-integrin antibodies and analyzed by flow cytometry for their total frequency. We observed higher frequency and absolute count of total HSCs in TG215 mice compared to WT and TG224 mice (Figures 1(a) and 1(b)). Furthermore, we determined the effects of PKCε on the clonogenicity of keratinocytes. Clonogenicity is an intrinsic property of adult stem cells. In this experiment (Figures 1(c) and 1(d)), an equal number of isolated keratinocytes from WT, TG224, and TG215 mice were seeded onto the irradiated 3T3 (fibroblast) cells and left for two weeks. We observed increased colony formation in keratinocytes isolated from TG224 and TG215 mice compared with their WT littermates indicating more proliferative potential (Figures 1(c) and 1(d)). Notably, the colonies greater than 2 mm and total numbers of colonies were higher in TG215 mice compared to their WT littermates (Figure 1(d)).

3.2. UVR Treatment Stimulates Putative Hair Follicle Stem Cell Proliferation. The cell surface markers CD34 and α6-integrin mark mouse hair follicle bulge cells, which have attributes of stem cells, including quiescence and multipotency. We determined the effects of UV treatment on total number of HSCs. In this experiment, PKCε TG and WT mice were exposed to a single or chronic UV doses (1.8 kJ/m^2, Monday, Wednesday, and Friday). At the indicated times after last UV exposure, mice were sacrificed and the number of putative HSCs was determined by flow cytometric analysis. The total frequency as well as absolute count of double positive HSCs were increased at 48 and 72 hr in TG224 after-UV exposure (Figures 2(a) and 2(b)) and 24, 48, and 72 hr in TG215 (Figures 2(c) and 2(d)) compared to their WT littermates. As shown in Figures 3(a) and 3(b), chronic UV exposures also showed an increase in total double positive HSCs in both TG224 and TG215 mice compared to their WT littermates.

3.3. PKCε TG Mice Have Increased Turnover of Putative HSCs. To determine the proliferation rates of bulge region stem cells in WT and TG mice, a 5-bromo-2$'$-deoxyuridine labeling (BrdU) experiment was performed. In this experiment, three-day-old neonatal mice were injected with 50 mg/kg of BrdU in PBS twice daily for three days. At 3, 4, 5, 6, and 8 weeks after BrdU injections, mice were sacrificed and the dorsal skin excised for keratinocyte isolation. Immunohistochemistry results revealed that, at 3-week time point, BrdU labeling was prominent in bulge region of hair follicle, interfollicular epidermis, and sebaceous glands of TG 224, 215, and their WT littermates (Figures 4(a)–4(c)). However, at later time points (6 and 8 weeks), BrdU labeling was decreased in TG mice compared to WT mice and localized to bulge region only (Figures 4(d)–4(h)). It is interesting to note that even after 3 weeks, some cells of interfollicular epidermis are able to retain the BrdU label (Figures 4(a)–4(c)). Moreover, dual immunofluorescence staining of BrdU and k15 indicates the colocalization of BrdU with k15 expressing cells in the stem cell-specific compartment, that is, bulge (Figures 4(i)–4(k)).

We further evaluated the cell cycle pattern in sorted double positive HSCs in TG 215 and their WT littermates at 8 weeks after-BrdU injection. The percent of BrdU-labeled cells was different in WT and TG215 mice. In the WT mice, the percent of double positive cells maintaining BrdU label was 28.4 ± 0.6% compared to 4.0 ± 0.06% for the TG, an approximately 7-fold decrease (Figure 5(a)).

We further determined the turnover of HSCs in WT and TG 224 mice. In this experiment, we analyzed the BrdU retaining double positive HSCs in isolated keratinocytes from WT and TG mice. BrdU retaining cells were analyzed at 4, 5, and 8 weeks after-BrdU injections. The frequency of BrdU retaining double positive HSCs was at 4 weeks (WT = 0.246%, TG = 0.0807%), 5 weeks (WT = 0.0364%, TG = 0.00337%), and 8 weeks (WT = 0.167%, TG = 0.008%).

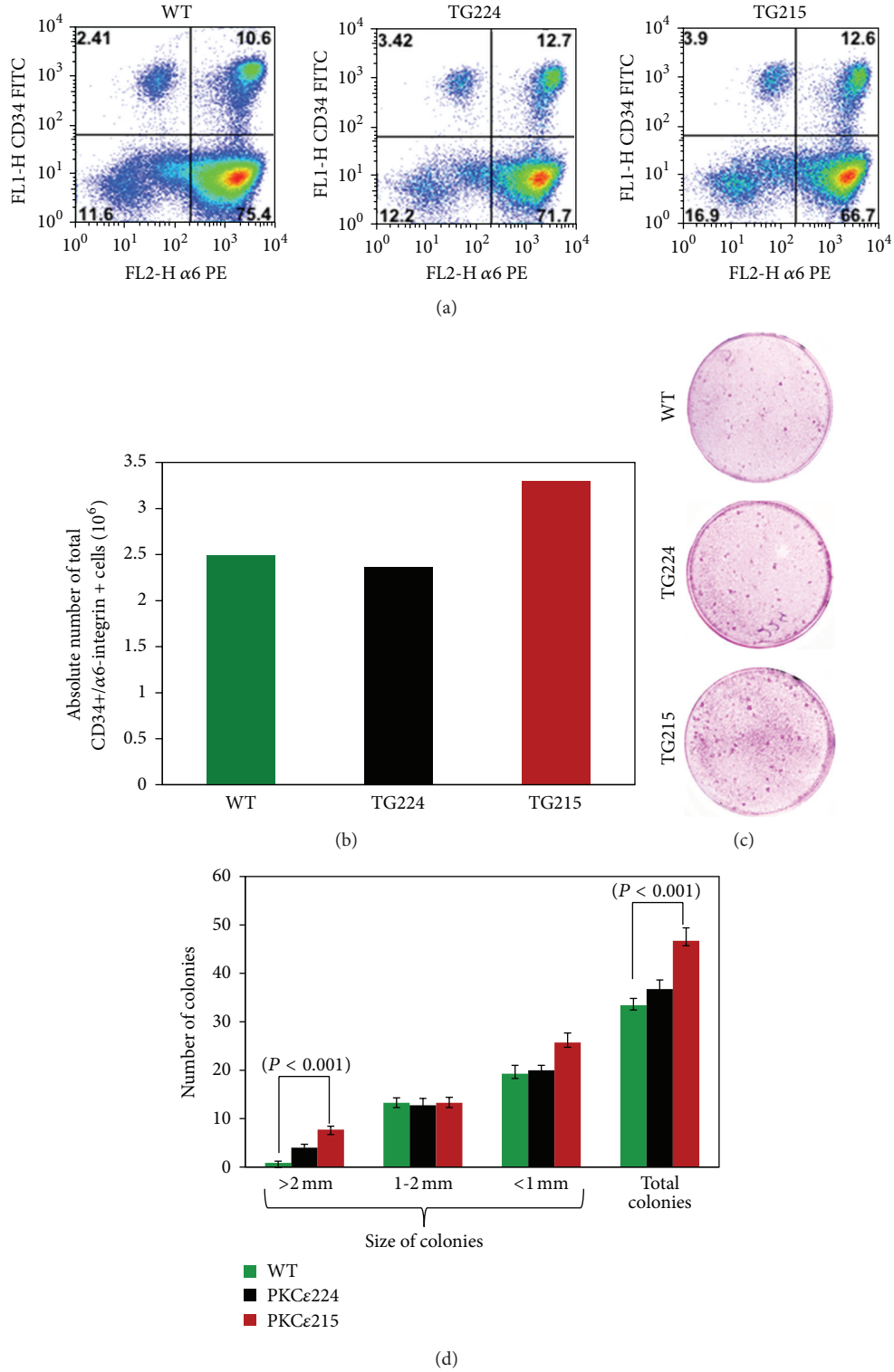

Figure 1: Hair follicle stem cells and clonogenicity of epidermal keratinocytes isolated from PKCε overexpressing mice and wild-type littermates. (a) is showing the representative gating of epidermal stem cell population in a dot plot from untreated WT, TG224, and TG215 mice. In each dot plot, the upper right quadrant is representing the CD34+/α6-integrin+ (double positive HSCs) stem cell population. Each value in the histogram is an average of FACS analysis of triplicate samples from keratinocytes pooled from two mice. (b) represents the total frequency of CD34+/α6-integrin+ keratinocytes in untreated indicated mice. (c) and (d) Clonogenicity of epidermal keratinocytes. Briefly, the keratinocytes from 7-8 weeks old indicated that mice were harvested using SMEM harvesting medium. Irradiated 3T3 cells seeded at density 10^6 cells/dish to the 60 mm dishes a day before seeding keratinocytes. For feeder layer, irradiated 3T3 cells were cultured in EMEM medium with 10% FBS and 1% penicillin-streptomycin. Equal numbers of keratinocyte cells (3000 cells/dish) were seeded for each type of mice and cultured with William's E media for 2 weeks. For counting and measurement of colonies, dishes were fixed with 10% formalin and stained with 0.5% rhodamine B. (c) Keratinocyte colonies. Shown are the representative dishes of adult keratinocyte colonies from PKCε TG mice and their WT littermates. (d) Quantitation of colonies. The colonies were counted and colony size measured by using vernier caliper (Figure 1(c)). Each value is the mean ±SE of colonies from 4–7 dishes.

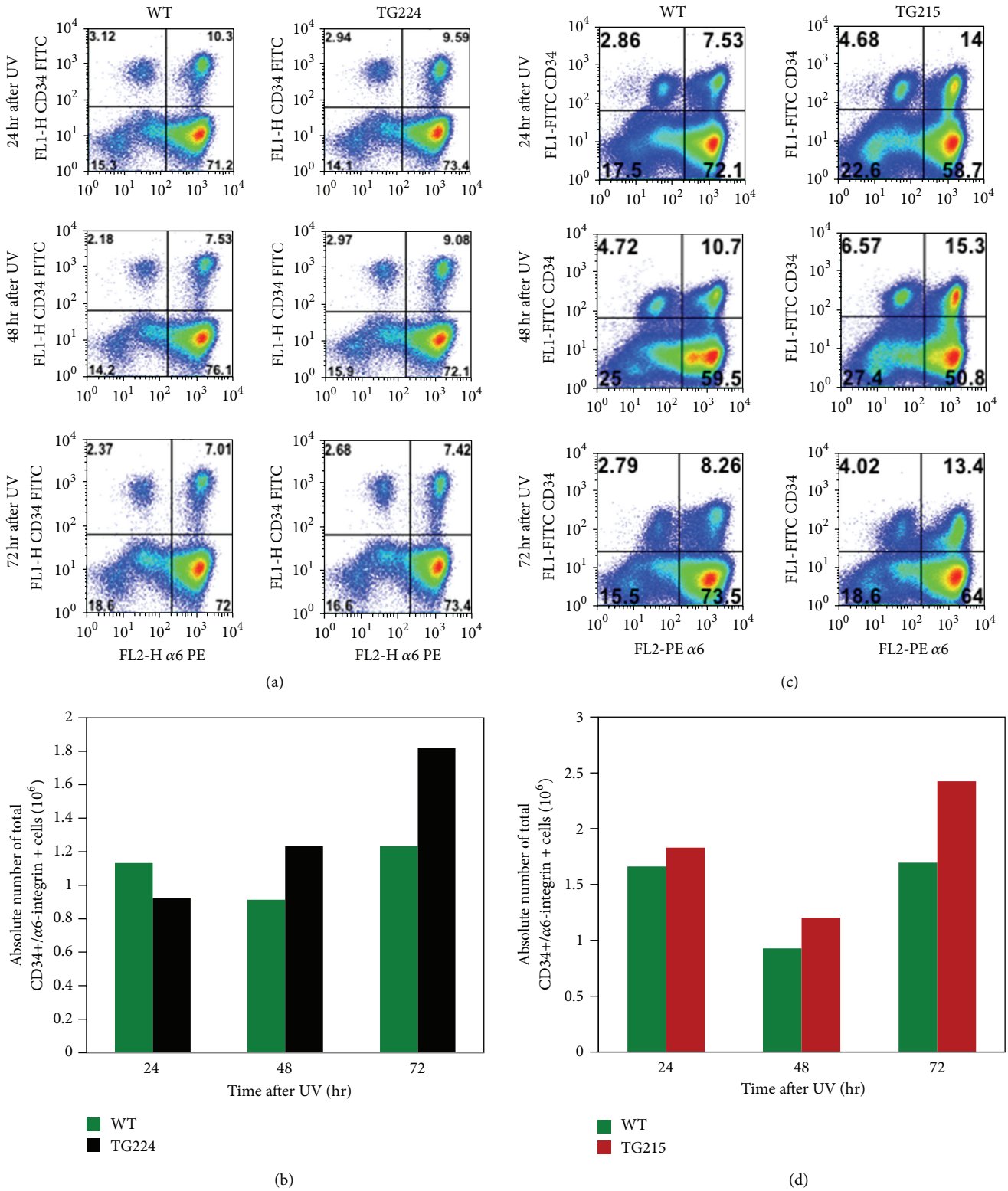

Figure 2: Effects of single UV exposure on live epidermal stem cell population determined by flow cytometric analysis. PKCε overexpressing TG and their WT littermates were exposed once to UV (1.8 kJ/m^2). At the indicated times after UV, mice were sacrificed and the dorsal skin removed for keratinocyte isolation as previously described [21]. (a) and (c) Percent distribution of FACS-sorted keratinocytes following UV exposure of the indicated mice at the indicated times after UV exposure. (b) and (d) Frequency of total double positive HSCs (CD34+/α6-integrin+) in TG224, TG215, and their WT littermates at the indicated times after single UV exposure.

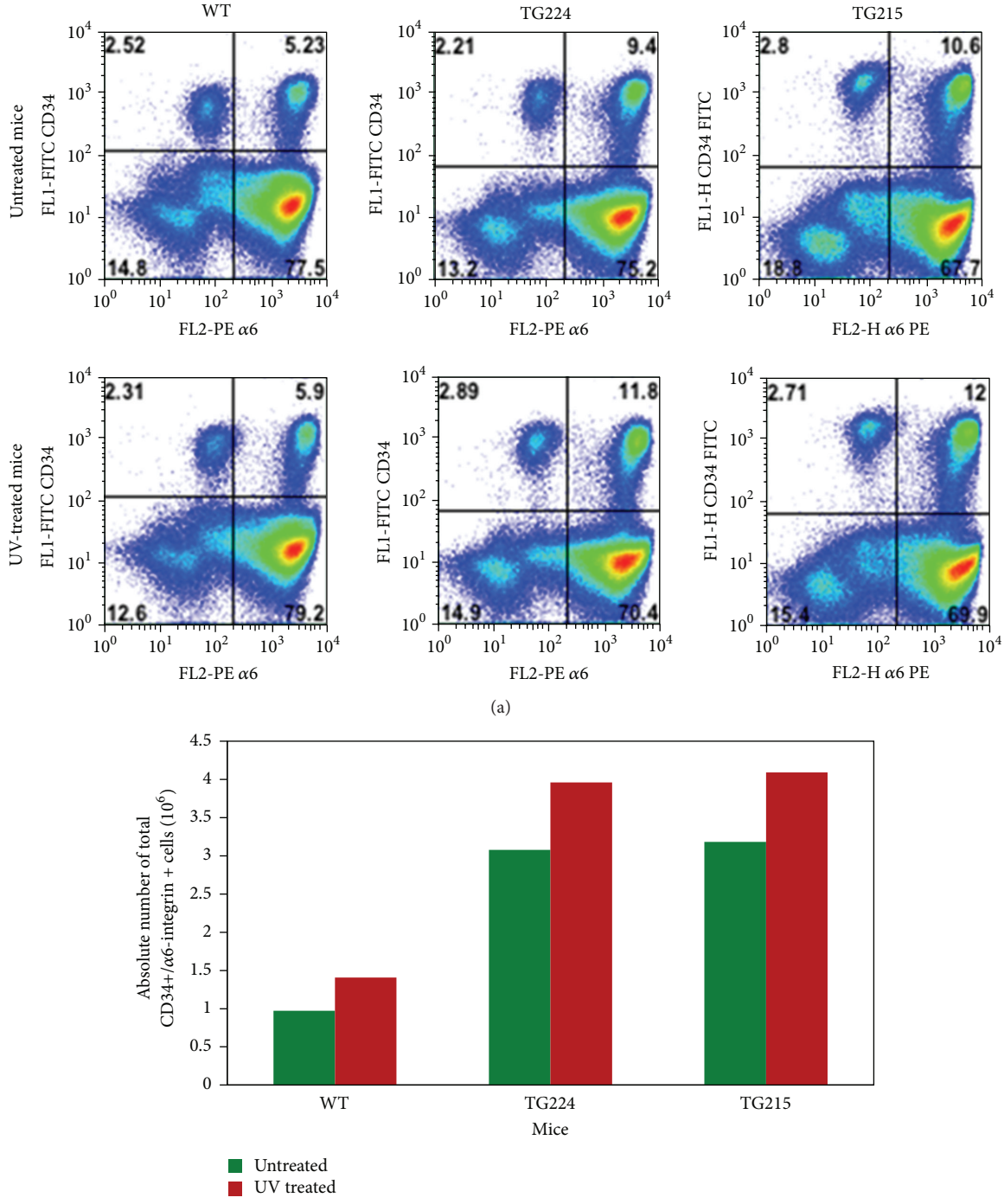

Figure 3: Effects of chronic UV exposures on live epidermal stem cell population determined by flow cytometric analysis. PKCε overexpressing TG and their WT littermates were given total eight UV exposures (1.8 kJ/m^2, Monday, Wednesday, and Friday). At 24 hr after the last UV exposure, mice were sacrificed and the dorsal skin removed for keratinocyte isolation. (a) Percent distribution of FACS-sorted keratinocytes following UV exposures of the indicated mice. (b) Frequency of total double positive HSCs (CD34+/α6-integrin+) keratinocytes in UV-treated mice.

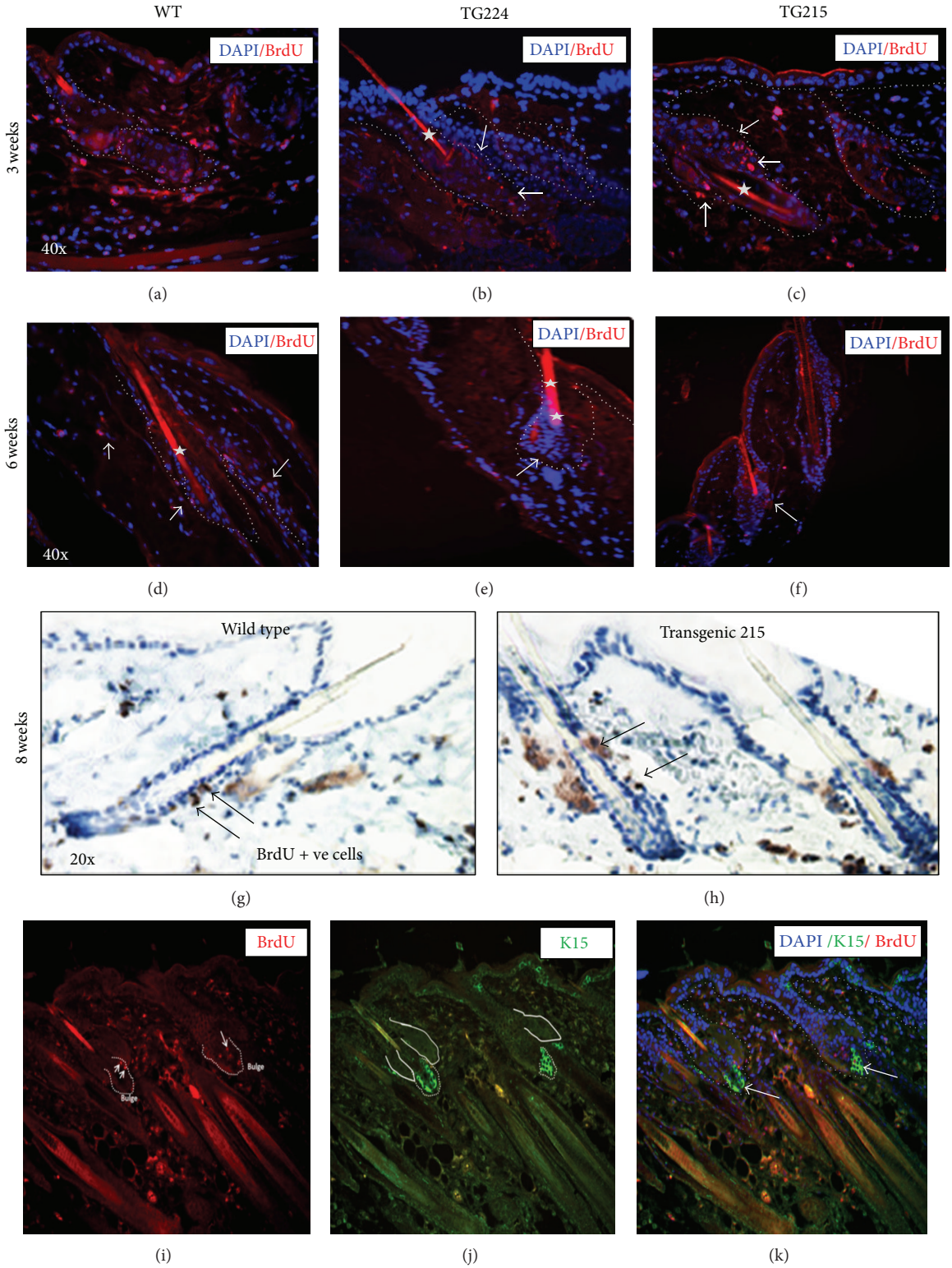

Figure 4: Detection of label retaining cells (LRC) by immunostaining using antibody to BrdU: to identify the LRCs, newborn mice (3 days old) were injected subcutaneously with BrdU (50 mg/kg body weight) twice daily for 3 days. Mice were sacrificed at 3, 6, and 8 weeks after BrdU injection. Shown are the representative photographs of BrdU-labeled cells from paraffin-fixed skin sections from WT and TG mice. The white arrow points to BrdU positive cells in the bulge region of hair follicle. In all the figures, the asterisk is the autofluorescence of the hair shaft. The incorporation and retention of BrdU are shown in the various parts of hair follicle at 3, 6, and 8 weeks in the indicated mice (a)–(h). (i)–(k) are showing the dual labeling of BrdU and k15 expressing cells in the bulge region of hair follicle.

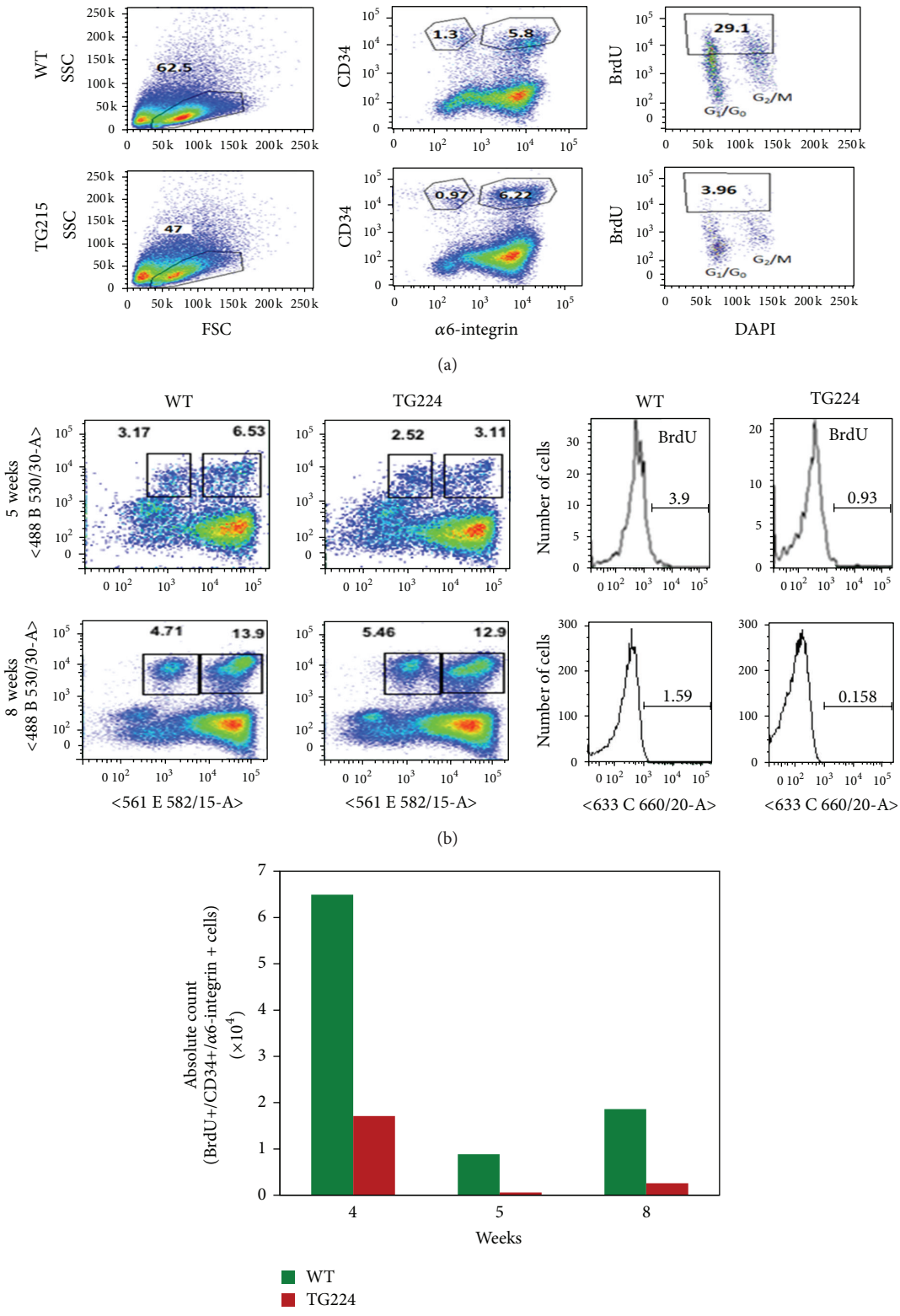

Figure 5: PKCε overexpressing transgenic mice have increased turnover of HSCs as determined by BrdU retaining double positive HSCs (CD34+/α6-integrin+). To identify LRCs, newborn mice (3 days old) were injected subcutaneously with BrdU (50 mg/Kg body weight) twice daily for 3 days. Mice were then sacrificed at 8 weeks after the last BrdU injection. There were four mice per group. (a) Representative gating of cell populations for side scatter (SSC) versus forward scatter (FSC). (b) Representative gating of cell populations at 5 and 8 weeks in the indicated mice. (c) Frequency of total BrdU-labeled double positive (CD34+/α6-integrin+) keratinocytes in TG 224 and their WT littermates at 4, 5, and 8 weeks.

Table 1: List of differentially expressed genes in CD34+/α6-integrin+ stem cells from PKCε TG mice. Overexpression of PKCε in the epidermis results in increased expression of genes linked to cell transformation, invasion, and metastasis.

Serial number	Gene name	Upregulated (↑) genes/fold change	Serial number	Gene name	Downregulated (↓) genes/fold change
(1)	Pescadillo	(3.2) ↑	(8)	Ccnf	(0.06) ↓
(2)	Tfdp-1 (Transcriptional factor)	(2.2) ↑	(9)	Cdkn1a (p21)	(0.5) ↓
(3)	Rad21	(1.8) ↑	(10)	Pkd-1	(0.5) ↓
(4)	Nfatc1	(1.7) ↑	(11)	Taf10 (TafII30)	(0.5) ↓
(5)	Cks1b	(1.6) ↑	(12)	Sfn	(0.6) ↓
(6)	Ak1	(1.3) ↑	(13)	Sumo1	(0.7) ↓
(7)	Itgb1	(1.2) ↑	(14)	RAN	(0.8) ↓

There was also a decrease in total frequency as well as absolute count of BrdU retaining double positive HSCs in TG224 mice compared to WT littermates (Figures 5(b) and 5(c)).

3.4. PKCε mRNA Levels in FACS-Sorted Keratinocytes. We first analyzed the percent distribution of CD34+, CD34+/α6-integrin+ (double positive HSCs), α6-integrin+, and CD34−/α6-integrin- (double negative) in TG224, TG215, and their WT littermates. The α6-integrin+ cells were 70.9%, 62.7%, and 54.8% in WT, TG224, and TG215 mice, respectively. The CD34+ cells were 2.8%, 4.0%, and 3.1% in WT, TG224, and TG215 mice, respectively (Figure 6(a)). We analyzed the PKCε mRNA expression levels in FACS-sorted keratinocytes (Figure 6(b)). The higher expression of PKCε was recorded in double positive HSCs of TG215 mice compared to WT and TG224 (Figure 6(b)).

3.5. PKCε Transgenic Mice Have Increased Expression of Genes Linked to Cell Transformation, Invasion, and Metastasis. A possibility explored that an increased turnover of HSCs in TG mice may be the result of changes in specific genes. In this experiment (Table 1), the effect of UV on cell cycle-related genes in double positive HSCs of TG and WT was determined using a focused cell cycle PCR array. Double positive HSCs of TG215 and their WT littermates were sorted out. A comparison of gene expression profiles of double positive HSCs is shown in Table 1. A 1.7- to 3.2-fold increase in the expression of Pes1, Rad21, Tfdp1, and Cks1b genes was observed in TG215 mice compared to their WT littermates. However, downregulation of Ccnf, Cdkn1a (p21), pkd-1, and Taf10 was observed in TG215 mice as compared to WT littermates.

4. Discussion

Chronic exposure of Sun's UV radiation is linked to the development of human SCC, a metastatic nonmelanoma skin cancer [22]. We found using a novel PKCε TG mouse model that the PKCε levels in epidermis dictate the susceptibility of transgenic mice to the induction of SCC by UV [8]. PKCε TG mice, when exposed to UV (2 kJ/m^2 thrice weekly), elicited 3-fold increased SCC multiplicity and decreased tumor latency by 12 weeks. PKCε overexpression in mice suppressed UV-induced sunburn (apoptotic) cell formation and enhanced both UV-induced hyperplasia and levels of specific cytokines (tumor necrosis factor α (TNFα), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor, and interleukin six (IL-6)), implying inhibition of apoptosis and promotion of preneoplastic cell survival [8, 23]. Additionally, PKCε may impart sensitivity to UVR carcinogenesis via its association with Stat3, a transcriptional factor that is constitutively activated in both mouse and human SCC [24]. We now present that PKCε-mediated susceptibility to UV carcinogenesis may involve stimulation of putative HSCs proliferation possibly mediated by PKCε and other specific genes linked to the cell cycle regulation.

The epidermis undergoes a continual renewal throughout life, and the process is facilitated by various stem cell localized in both interfollicular epidermis and other specialized stem cell niches such as bulge. The skin stem cells present in different compartment of hair follicles respond differently to various signals mediated by their microenvironment [25]. Interestingly, multiple skin stem cell populations exist in the epidermis and play an important role during the process of controlled proliferation and differentiation (reviewed in [26]). The major stem cell population of hair follicle includes interfollicular label retaining cells (LRCs), double positive HSCs (CD34+/α6-integrin+), Mts24+ cells, Blimp1, Nestin, Lgr5+, and Lgr6+ cells (reviewed in [26]). However, the bulge region of hair follicle is considered as the major niche for keratinocyte stem cells [27, 28]. Particularly, the CD34+/α6-integrin+ cells are slow cycling and colocalize with LRCs and confined to bulge region of hair follicles. In terms of their colony forming ability (clonogenicity), CD34+ cells make larger colonies compared to CD34− cells [16, 29]. Keratinocytes isolated from PKCε overexpressing TG have higher frequency of double positive HSCs (CD34+/α6-integrin+) and clonogenicity than their WT littermates (Figure 1).

UV treatment resulted in a modest increase in total double positive HSCs in both TG224 and TG215 mice compared to their WT littermates (Figure 2). UV treatment in TG mice as compared to WT mice leads to constitutive activation of Stat3, increased Stat3-DNA binding [24], and increased

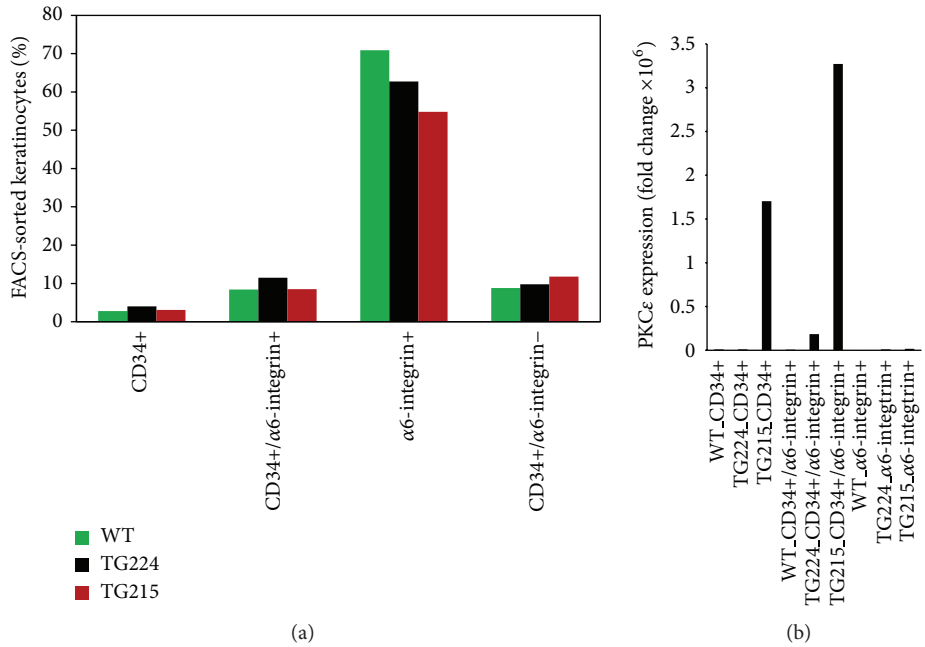

Figure 6: Distribution and expression of PKCε in FACS-sorted keratinocytes from WT, TG224, and TG215 mice. (a) FACS-sorted keratinocytes. The keratinocytes were harvested from 5 weeks old wild-type and TG mice and incubated with the CD34 and α6-integrin florescent antibodies. After labeling, the cells were washed twice, filtered, and sorted for CD34+, α6-integrin+ cells, CD34+/α6-integrin+, and CD34−/α6-integrin-cells. (b) PKCε expression. The RNA was isolated from the sorted cell, followed by cDNA preparation, and then real-time PCR using SYBR Green double-strand DNA binding dye. After real-time PCR Ct values were calculated and analyzed for expression. All the expression values shown in the figures are relative to their mouse Gapdh internal control.

expression of TNFα and G-CSF [8]. Results from genetic experiments indicate that both Stat3 and TNFα are linked to UV-induced development of SCC. It yet remains to be proven that PKCε downstream components Stat3 and TNFα directly affect the proliferation of putative HSCs.

We observed less BrdU retaining double positive HSCs cells in TG mice compared to WT (Figure 3). These results indicate rapid turnover of double positive HSCs cells in TG mice. Evidence indicates that at least two types of cell population exist: the slow cycling designated as stem cells and rapidly cycling cells as transit amplifying cells [30–32]. Rapid turnover of double positive HSCs cells in TG mice may be the effect of overexpression of PKCε in TG mice and its associated cytokines such as TNFα and G-CSF [33]. Additionally, the level or percentage of BrdU retention in the stem cell populations is not consistently uniform. BrdU retention is influenced by mice age, time of labeling, and site of labeling [34]. This may be the possible explanation that the amount of BrdU retained varies in different repeat experiments (Figure 3). However, BrdU retaining double positive HSCs cells were consistently less in TG mice compared to WT.

An analysis of focused cell cycle cDNA array revealed up- and downregulation of specific genes. The genes found to be overexpressed in double positive HSCs in TG215 were Pescadillo, Tfdp-1, Rad21, Nfatc1, Cks1b, AK1, and Itgb1. The gene Tfdp-1 is found to be overexpressed in SCC [35–38]. Interestingly, Nfatc1 gene is found to be overexpressed in many cancers, and its loss is linked with constant hair cycling and no quiescence [39]. Nfatc1 is also responsible for the balance between the quiescence and proliferation stage of skin stem cells [40]. Other overexpressed genes, Pescadillo, Rad21, Cks1b, Ak1, and Itgb1, are also linked with the process of carcinogenesis (Table 1).

5. Conclusion

In summary, we present for the first time an association of PKCε with HSCs, the SCC precursors [20]. PKCε overexpression in mice increased the clonogenicity of isolated keratinocytes, a property commonly ascribed to stem cells. Both single and chronic UV-treatments resulted in an increase in the frequency of double positive HSCs in PKCε TG mice as compared to their WT littermates. In TG mice, HSCs cycle at a faster rate as compared to wild-type mice. A comparison of gene expression profiles of FACS-sorted double positive keratinocytes isolated from UV-treated WT and TG mice indicated increased expression of Pes1, Rad21, Tfdp1, and Cks1b genes in TG mice linked to cell transformation, invasion, and metastasis of cancer cells.

It is believed that the skin stem cells are the major targets of carcinogen [15, 41]. However, the identification and the precise location of cancer initiating cells in cutaneous SCC is not clear. Furthermore, the role of nonstem cell cannot be overlooked during the process of carcinogenesis. It has been observed that the differentiated, nondividing epidermal cells with activated MAPK kinase 1 and inflammatory infiltrate can initiate benign tumor formation [42]. Interestingly,

the differentiated keratinocytes can reenter into active cell cycling, dedifferentiating, and acquiring the stemness [43]. In future it will be interesting to study the link of double positive HSCs and other skin stem cell populations, along with other inflammatory signals in UV-induced Squamous cell carcinoma.

Abbreviations

BrdU: 5-Bromo-2′-deoxyuridine
DAG: Diacylglycerol
DMBA: 7,12-Dimethylbenz[a]anthracene
FACS: Fluorescence assisted cell sorting
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
HSCs: Hair follicle stem cells
LRCs: Label retaining cells
PKCε: Protein Kinase C epsilon
SCC: Squamous cell carcinoma
TNFα: Tumor necrosis factor alpha
TPA: 12-O-Tetradecanoylphorbol-13-acetate
UVR: Ultraviolet radiation.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Delineating Molecular Mechanisms of Squamous Tissue Homeostasis and Neoplasia: Focus on p63

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Mouse models have informed us that p63 is critical for normal epidermal development and homeostasis. The p53/p63/p73 family is expressed as multiple protein isoforms due to a combination of alternative promoter usage and C-terminal alternative splicing. These isoforms can mimic or interfere with one another, and their balance ultimately determines biological outcome in a context-dependent manner. While not frequently mutated, p63, and in particular the ΔNp63 subclass, is commonly overexpressed in human squamous cell cancers. *In vitro* keratinocytes and murine transgenic and transplantation models have been invaluable in elucidating the contribution of altered p63 levels to cancer development, and studies have identified the roles for ΔNp63 isoforms in keratinocyte survival and malignant progression, likely due in part to their transcriptional regulatory function. These findings can be extended to human cancers; for example, the novel recognition of NFκB/c-Rel as a downstream effector of p63 has identified a role for NFκB/c-Rel in human squamous cell cancers. These models will be critical in enhancing the understanding of the specific molecular mechanisms of cancer development and progression.

1. Introduction

p53 is a tumor suppressor that is upregulated and activated across organ systems as a tissue protective stress response mechanism [1]. *p63* is a member of the p53 gene family which also includes *p73*. In contrast to p53, both p63 and p73 exhibit cell-type-specific expression patterns and exert tissue-specific functions [2, 3]. Relevant to this review, p63 plays an essential role in the development and maintenance of normal stratified squamous epithelium. All *p53* family members encode multiple protein isoforms that act in overlapping or opposing manners both within and across family members. Given the complexity of the p53 family and the potential for the different family members to mimic or interfere with each other, the balance of p53 family isoforms in a given cellular context can impact the biological outcome. In this review, we highlight how information derived from mouse models has provided insight into molecular mechanisms of normal keratinocyte growth regulation and human cancer pathogenesis. In particular, we focus on the *p63* gene, the role of its gene products in normal epidermal development and homeostasis, and how dysregulation of p63 protein expression, which is tightly controlled under normal conditions, contributes to squamous carcinogenesis, not only of the skin, but also in other squamous epithelial cancers such as those of the head and neck.

2. Overview of p63 Structure/Function

Members of the p53 family were identified based on shared homology within their major functional domains: transactivation (TA), DNA binding (DBD), and oligomerization (OD); and exist as multiple protein isoforms due to a combination of alternate promoter usage and alternative splicing [4, 5]. Use of alternative promoters gives rise to isoforms of two classes: TA and ΔN. The TAp63 and TAp73 isoforms possess a transactivation domain with homology and function similar to that of p53, while the ΔNp63 and ΔNp73 isoforms

lack this domain and can act to block TAp53-, TAp63-, and TAp73-mediated transcription [4] via the mechanisms discussed below. However, this does not imply that ΔNp63 isoforms lack transcriptional activation activity as alternate transactivation domains have been described both within the N-terminus of the ΔNp63 isoforms [6, 7] and in exons 11 and 12 of the C-terminus (transactivation domain 2 (TA2)) [8]. Further analysis has suggested that the second region is unlikely to be an independent activation domain [7]. Refined mapping studies indicate that this domain instead serves to modulate transcriptional activities associated with ΔNp63 isoforms [7]. Correspondingly, many positive transcriptional targets of ΔNp63 have been identified, which are discussed in this review.

All TA and ΔNp63 isoforms contain the DBD and OD domains but differ at the C-termini. This additional complexity is conferred on these proteins due to C-terminal alternative splicing, which in the case of p63 gives rise to TA and ΔN subclasses of p63α, β, γ, δ, and ε isoforms [4, 9] (Figure 1). Of these isoforms, α is the longest and contains a sterile alpha motif (SAM) protein-protein interaction domain [10] and a transcriptional inhibition domain (TID) [11]. The TID comprises 2 subdomains, one of which binds and masks the TA domain of TAp63α and the other, which is subject to sumoylation resulting in decreased intracellular p63α concentration and correspondingly to decreased activity [11–13]. Degradation of p63α is also promoted by the E3 ubiquitin ligase ITCH via ubiquitylation at the N-terminal border of the SAM domain of the p63α isoforms [14]. Regarding the other p63 C-terminal splice variants, exon 13 is spliced out of the β-isoform, which thus also lacks the SAM and TID domains. Both the α and β isoforms of p63 contain a phosphodegron motif utilized by Fbw7 E3 ubiquitin ligase in MDM2-mediated degradation [15]. The γ, δ, and ε isoforms all truncate shortly after the oligomerization domain, with each containing a unique C-terminal sequence [9]. Thus, all three isoforms lack the SAM and TID domains (Figure 1).

Like p53, the p63 and p73 proteins function as tetramers via their oligomerization domains. The oligomerization domains of p63 and p73, due to the presence of an additional α-helix, are more similar to one another than to that of p53 [16]. p63 and p73 were not observed to interact with p53 through their oligomerization domains but strongly interact with one another through this domain, with the p63/p73 heterotetramers exhibiting enhanced stability over homotetramers [16]. While p53 does not interact with p63/p73 through the oligomerization domain, WTp53 has been shown to target ΔNp63α for caspase-mediated degradation via interactions between the DNA binding domain of each protein [17], and mutated p53 has been shown to interact with the core DNA binding domains of p63 and p73, thereby impairing DNA binding and transactivation [18].

DNA binding is an area in which p53 family members can mimic or compete with each other. While p63 has been shown to bind to p53 responsive consensus sequences, distinct p63 responsive elements have also been identified [19–21]. It has been reported that the global DNA binding pattern of p73 does not differ from that of p63, but intensity of binding at given sites does vary depending on the cell type profiled [22]. This suggests that competition between the homo- and heterotetramers of different isoforms of p63 and p73, which are subject to cellular context, may define site occupancy. Beyond interactions with one another via their oligomerization domains or their DNA binding domains, and their competition at DNA response elements, p53 family members have been shown to be involved in feedback loops with one another that impact expression levels [23, 24]. Thus, at many levels dysregultion of any one family member may impact the fine balance that is involved in maintaining normal epidermal homeostasis.

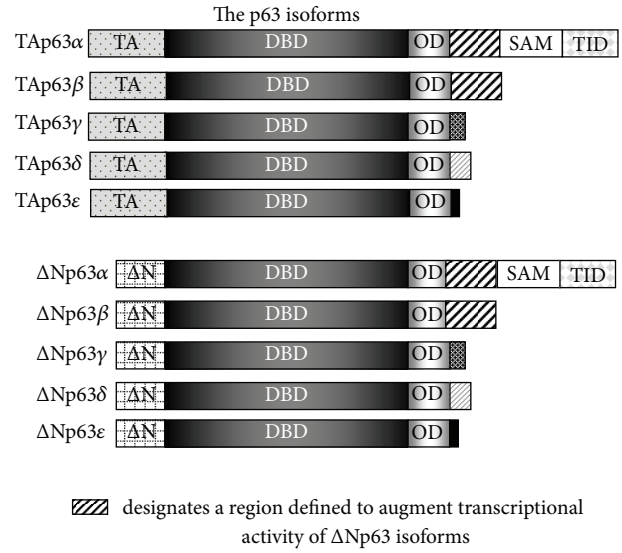

Figure 1: Use of alternative splicing gives rise to p63 isoforms of 2 subclasses: TAp63 and ΔNp63. Within each of these subclasses, C-terminal alternative splicing gives rise to α, β, γ, δ, and ε isoforms. The isoforms share homology in certain protein domains: TA (transactivation domain), ΔN, DBD (DNA binding domain), OD (oligomerization domain), SAM (sterile alpha motif domain), and TID (transactivation inhibition domain).

3. p63 and Normal Skin Biology

p63 is critical for normal epidermal morphogenesis [3, 25]. In the mature epidermis, the predominant p63 isoform expressed is ΔNp63α, and expression of this isoform is associated with the proliferative compartment [26]. Expression of this isoform is critical for the maintenance of the mature epidermis [27]. However, it is also the ΔNp63α isoform that is overexpressed in many squamous cell cancers [28]. As a starting point to understand how overexpression of a single isoform with ensuing disruption of the balance of p53 family members might contribute to squamous cancer pathogenesis, it is important to first understand the role of p63 both in normal epidermal morphogenesis and in homeostasis of the mature epidermis. Significant insight has been obtained through the use of mouse models outlined below.

3.1. Role of p63 in Mouse Models of Epidermal Morphogenesis and in Human Ectodermal Dysplasias

3.1.1. Mouse Models of Epidermal Morphogenesis. The criticality of p63 to normal epidermal development was highlighted by simultaneously published mouse models lacking functional *p63* [3, 25]. The mice were developed using distinct molecular strategies and upon gross phenotypic examination appeared similar; however, in-depth analysis of the epidermal phenotype gave rise to alternate hypotheses as to the role of p63 in epidermal development: epidermal progenitor cell maintenance versus commitment to stratification. In the mice developed by Yang et al., exons 6–8 corresponding to p63's DBD were replaced with the neomycin resistance gene [3]. In these mice, patches of disorganized epithelial cells positive for late markers of keratinocyte differentiation and negative for keratin 5 were evident, suggestive of a role for p63 in maintaining epidermal progenitor cells. Mice generated by Mills et al. were derived using an insertional gap repair mechanism [25]. Two strains generated by this approach, Brdm1 (truncating within exon 6) and Brdm2 (truncating after exon 10), appeared macroscopically identical, and thus, the strains were not distinguished in subsequent experiments in the seminal paper. Microscopic analysis of these mice revealed a layer of flattened cells expressing keratin 14 at low levels with no evidence of stratification or differentiation marker expression, suggestive of a failure to commit to a stratified epidermis. While no mRNA transcripts were detected from these mice by northern blotting, the transcript in the Brdm2 mouse model, which truncates after exon 10 [25], could in theory give rise to shortened ΔNp63 transcripts, similar to those described by Mangiulli et al. [9]. A recent recharacterization of a line of Brdm2 mice by Wolff et al. [29] revealed patches of keratinizing epidermis expressing truncated p63 at levels similar to wild type with stratification overlaying hair follicles. Based on further studies in embryos, the authors proposed these patches to be remnants of a more developed E15 epidermis 3–5 layers thick containing terminally differentiated epithelium that was transient in nature due to mechanical stress at birth, and suggested that the Brdm2 mice were equivalent to p63α/β knockout mice [29]. This observation and ensuing studies generated much controversy, as to whether the recharacterized mice were the same as those used by others or if perhaps a spontaneous genetic event might be at play [29–33], which to date remains unresolved.

Studies of the p63-deficient mouse lines provided strong evidence for the critical nature of the *p63* gene; however, interpretation of p63 function is confounded by the existence of multiple p63 protein isoforms. Therefore, single isoform knock-in mouse models have been developed on a *p63* null background to elucidate the role of specific p63 isoforms. These models have also generated controversy. Reconstitution of different p63 isoforms in the Brdm2 mice using tissue-specific inducible mouse models generated by separate groups gave rise to opposing conclusions as to the role of ΔNp63 in initiating stratification of simple epithelium [34, 35]. In one model, TAp63α, but not ΔNp63α, was found to drive stratification and keratin 5/keratin 14 expression of the simple lung epithelium [34], while in the other model, ΔNp63α or ΔNp63β caused stratification and keratin 5/keratin 14 expression in the simple lung epithelium [35]. With respect to the epidermis, differential results were also obtained by these two groups. In the first model, keratin 14-driven expression of TAp63α resulted in a severely hyperplastic epidermis exhibiting delayed differentiation [34], and based on their data the authors concluded that TAp63α is the initiating switch for epidermal stratification. In contrast, in the second model, expression of ΔNp63α or ΔNp63β under the control of the keratin 5 promoter did not result in complete restoration of epithelial integrity, but it did result in several areas of stratified epidermis, which expressed differentiation markers, indicating that the ΔNp63α and ΔNp63β can act to initiate stratification [35]. Further support for ΔNp63α as an initiator of stratification comes from other genetic complementation studies in which ΔNp63α or TAp63α, both under the keratin 5 promoter, was introduced into the *p63*(−/−) mice from Yang et al. [3, 36]. In these studies, ΔNp63α was able to partially restore the epidermal basal layer, but not differentiation marker expression, whereas TAp63α reconstitution resulted in a phenotype similar to *p63*(−/−) mice [36]. Reconstitution of a combination of ΔNp63α and TAp63α resulted in a more complete epidermis formation containing patches with a more organized structure that expressed markers of differentiation [36]. It is possible that differences with respect to the differentiation status of the epidermis generated by reconstitution of ΔNp63α in the later two studies could be due to the mouse model used, but in contrast to the first model discussed, partial epidermal restoration by ΔNp63α is a common feature of both.

Finally, subclass-specific knockout mice have been developed as a means of exploring functions attributable to the TAp63 or ΔNp63 subclasses in the presence of wild-type expression levels of the opposing subclass. Germline ablation of TAp63 did not impact normal epidermal morphogenesis in the presence of ΔNp63 isoforms [37]. In contrast, mice in which ΔN exon was replaced with GFP appeared phenotypically similar to *p63*(−/−) mice, and, like the *p63*(−/−) mice generated by Yang et al. [3], retained only disorganized patches of keratinocytes expressing terminal markers of differentiation [38]. However, in contrast to the mice generated by Yang et al. [3], these mice coexpressed keratin 5 along with the markers of terminal differentiation. Furthermore, expression of the basal transcription factor AP-2α indicated that in the absence of ΔNp63, basal patches can form, but these were observed to have decreased Ki67 staining. Taken together, this is suggestive of a role for ΔNp63 during epidermal morphogenesis in both progenitor cell maintenance and in epidermal commitment, closing the gap between the original interpretations of the pan-p63 mouse models [38].

3.1.2. p63 and Human Genetic Syndromes. In humans, heterozygous mutations in *p63* are linked to genetic syndromes that include ectodermal dysplasia as part of the disease phenotype [39]. Distinct phenotypes are associated with

mutations in specific p63 domains, providing clues to structure/function relationships. For example, ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome is associated with mutations in the SAM domain [40], while ectodermal dysplasia and cleft/lip palate (EEC) syndrome is associated with mutations within the DBD [41, 42]. Not all of the syndromes include skin involvement. Of the syndromes, skin involvement is most pronounced and severe in AEC, which is linked to missense mutations in the SAM domain [40, 43] and therefore implicates the α isoforms.

Unlike normal skin where ΔNp63 expression is associated with the basal proliferative compartment, in AEC patient skin samples, nuclear p63 expression extends beyond the basal layer to the terminally differentiating cells and is accompanied by coexpression of differentiation markers [40]. *In vitro* studies have shown that SAM domain mutations, as seen in AEC, block interaction between p63α and mRNA splicing/processing proteins critical to direct splicing of FGFR-2 to the isoform required for normal epithelial differentiation [44]. Characterization of the AEC L514F ΔNp63α mutant in stable cell lines revealed that activation of a cryptic splice site due to loss of these interactions resulted in production of a C-terminally truncated form of ΔNp63α exclusively located in the nucleus and resistant to proteosome degradation [45]. Mouse models have helped to further define the contribution of this genetic alteration to the phenotype of this syndrome. A knock-in mouse model developed for the L514F mutation recapitulates the expected AEC phenotype including hypoplastic and fragile skin with a transient reduction in proliferation during embryonic development [46]. Skin fragility in these mice, and in humans with AEC syndrome, was associated with altered desmosome gene expression mediated by mutant p63 [47]. To gain insight into further pathways impacted, intact and eroded AEC syndrome skin and normal skin were compared by microarray analysis. The findings revealed changes in expression of genes associated with epidermal adhesion, skin barrier formation, and hair follicle biology, all consistent with the clinical presentation [48]. Thus, SAM domain mutations highlight the importance of p63 and in particular ΔNp63α to normal epidermal morphogenesis/homeostasis.

3.2. p63 in Normal Epidermal Homeostasis. Maintenance of normal epidermal homeostasis involves mediation of processes including proliferation, differentiation, stem cell maintenance, senescence, viability, and cell adhesion. Evidence suggests that each of these is impacted by p63 protein expression (Figure 2). *In vivo,* in the adult human epidermis, p63 is highly expressed in the basal cells with proliferative potential [26] and is downregulated in the suprabasal layers [4]. *In vitro* depletion of p63 in human regenerating organotypic cultures resulted in hypoproliferation and a lack of stratification and differentiation [49]. These effects were found to be mainly due to the ΔNp63α isoform. In a mouse model, specific knockdown of ΔNp63α in the mature epidermis resulted in severe skin fragility with erosion [27]. A multitude of studies, primarily *in vitro*, focused on the downstream targets mediated by ΔNp63α have shed light on the network of target genes implicated in these ΔNp63α-mediated biological processes. While an extensive cataloguing of all of these studies is beyond the scope of this review, some of these studies, with a focus on those performed in keratinocytes, are discussed here to highlight the potential impact of dysregulated ΔNp63α on signaling pathways that may be assessed using mouse models of the skin.

3.2.1. Cell Cycle Regulation. Numerous examples serve to illustrate how perturbation of ΔNp63 expression could result in altered biological outcome. *In vitro*, in developmentally mature murine keratinocytes, we and others demonstrated that ΔNp63α is associated with maintenance of proliferative capacity [49–53]. Mimicking overexpression of ΔNp63α seen in squamous cell carcinomas blocks the normal growth arrest and induction of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ in response to elevated Ca^{2+} conditions [50, 51] and correspondingly suppresses the differentiation markers keratin 10 and filaggrin, but not keratin 1. The α-tail of ΔNp63 is required for its suppressive effect on differentiation but not for the aberrant growth arrest response [51], which may be mediated at least in part by transcriptional repression of $p21^{WAF1}$ by binding of ΔNp63α to its promoter [52]. Regulation of $p21^{WAF1}$ is further impacted by crosstalk between p63 and Notch 1, whereby Notch 1 is negatively regulated by ΔNp63α in cells of high renewal potential but synergizes with ΔNp63α during early differentiation to induce keratin 1. Subsequently, Notch 1 downregulates ΔNp63α to permit the expression of the late differentiation marker involucrin [54]. This context-dependent crosstalk is implicated in maintaining the balance between keratinocyte growth arrest and differentiation.

In addition to the factors regulating $p21^{WAF1}$ described above, a balance exists between ΔNp63α and many other target genes involved in proliferation and differentiation that are critical for maintenance of or for the switch between the states. For example, the cell cycle inhibitor PTEN is negatively regulated by ΔNp63α. Depletion of either ΔNp63α or PTEN alone had opposite effects on colony growth in colony forming assays, but depletion of both ΔNp63α and PTEN at the same time had no impact, implying the balance between the two is critical to biological outcome [55]. Another example focuses on the epidermis of mice with mutant IRF6, which is hyperproliferative and fails to undergo differentiation [56]. This has been attributed to a failure of a feedback loop with ΔNp63α that controls ΔNp63α expression, thereby regulating the switch between proliferation and differentiation. In this feedback loop, IRF6 is a direct transcriptional target of ΔNp63α, which when upregulated induced proteasome-mediated degradation of ΔNp63α allowing for keratinocytes to exit the cell cycle [57]. In addition to regulation of levels of ΔNp63α impacting biological outcome as exemplified by the previous two examples, Runx1, a transcription factor involved both in keratinocyte proliferation and differentiation, is directly differentially regulated by ΔNp63α in proliferating versus differentiating keratinocytes by binding to distinct DNA binding sites on the Runx1 promoter. This represents

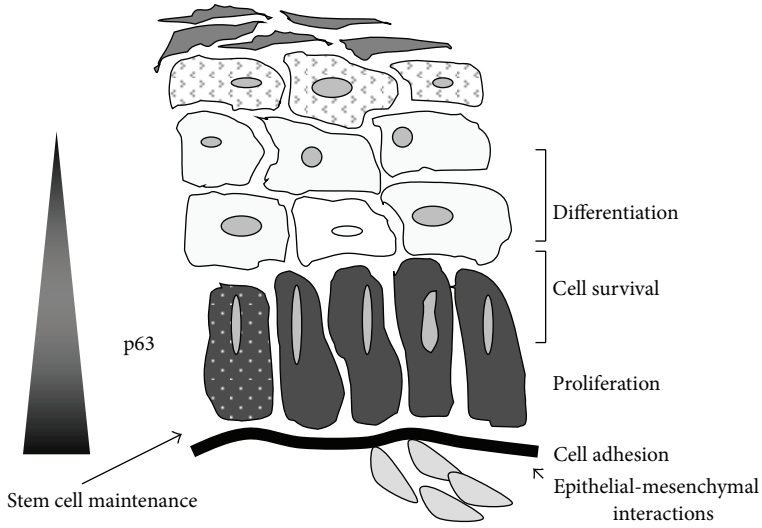

Figure 2: p63 impacts multiple biological endpoints involved in normal epidermal homeostasis. Overexpression of ΔNp63α impacts pathways that can contribute to cancer development.

a different mechanism of regulation [58], however, one that also could be perturbed by altered expression of ΔNp63α.

3.2.2. Differentiation. The mouse models described in Section 3.1.1 support a role for ΔNp63α not only in the maintenance of epidermal progenitor cells, but also, in the commitment to stratification. At a molecular level, ΔNp63α synergizes with Notch 1 to induce keratin 1 expression during differentiation, and the ΔNp63α target gene *IKKα* is necessary for epidermal differentiation [54, 59–63]. However, overexpression of ΔNp63α in primary murine keratinocyte cultures blocks expression of keratin 10 and filaggrin, but not keratin 1 [50] implying that a fine balance in levels of ΔNp63α is required for complete differentiation. Some examples of transcription factors which based on *in vitro* studies are thought to interplay with ΔNp63α during differentiation follow.

Basally expressed keratin 14 is a known direct transcriptional target of ΔNp63α [64]. The transcription factor Skn1a (Oct11) blocks ΔNp63α induction of the keratin 14 promoter and promotes keratin 10 upregulation [65]. There is also evidence for ΔNp63α both blocking and inducing transcription factors that promote differentiation in a cell-context-dependent manner. For instance, ΔNp63α directly represses high-mobility group box protein 1 (HBP1), a transcription factor necessary for stratification of organotypic cultures [66]. In contrast, in differentiating keratinocytes ΔNp63α induces ZNF750, a transcription factor that is required for terminal epidermal differentiation [67]. Interestingly, ZNF750 is bound by ΔNp63α in both proliferating and differentiating keratinocytes, but expression is only induced in differentiating cells, suggesting that additional cofactors are involved for distinct biological endpoints. The contribution of cofactors is further exemplified by the case of Alox12, a granular layer protein important for epidermal barrier formation, which is induced by ΔNp63α only in differentiating keratinocytes [68].

ΔNp63α expression can be regulated at the transcriptional level as well as by altered protein stability, as noted above. Another means of controlling levels of ΔNp63α is by microRNAs, short RNA molecules that act as posttranscriptional regulators. They recognize seed sequences in the 3′UTR and serve to block protein translation or decrease RNA stability. Such an interaction was identified as part of a feedback loop between p63 and iASPP, an inhibitory member of the apoptosis stimulating protein of p53 family, critical for epidermal homeostasis [69]. In this loop, iASPP is a direct transcriptional target of p63 that positively regulates ΔNp63 via the repression of miRs 754-3p and 720 to allow for proliferation. Blocking iASPP expression allows for differentiation via upregulation of miRs 754-3p and 720, which downregulate ΔNp63α. Other examples include miR203, which directly targets p63 through its 3′UTR for degradation and promotes differentiation by restricting proliferative potential and promoting cell cycle exit [70]. miRs are also regulated by ΔNp63α. miR-34a and miR-34c, associated with cell cycle withdrawal, are negatively regulated by ΔNp63α [71]. In contrast, miR17, miR20b, miR30a, miR106a, miR143, and miR 455-3p are positively regulated by p63 and critical for the onset of keratinocyte differentiation via modulation of the MAPKs [72].

3.2.3. Epidermal-Dermal Interface and Adhesiveness and Viability. Adhesiveness and cell viability are two additional properties positively impacted by ΔNp63α. Epidermal-specific knockdown of ΔNp63 in mature keratinocytes in mice resulted in impaired differentiation and compromised basement membranes [63]. In an *in vitro* model, *Fras1*, which encodes for an extracellular matrix protein, was identified as a ΔNp63α regulated gene important for maintaining the epidermal-dermal interface integrity [63]. To maintain this

interface, p63 prevents nonepidermal gene expression in keratinocytes via positive regulation of bone morphogenetic protein- (BMP-) 7 [73]. The importance of ΔNp63 in maintaining epithelial-mesenchymal crosstalk was highlighted by the discovery of *interleukin-1 α (Il-1α)* as a p63 target gene. IL-1α induces growth factors in fibroblasts that can bind to receptors on the basal keratinocytes to promote proliferation [74]. Cell-cell adhesiveness was found to be mediated by p63 via Perp, which is a critical desmosomal component for cell-cell adhesion in normal development and in wound healing [75, 76]. With respect to apoptosis, the proapoptotic protein, IGFBP3, is directly repressed by ΔNp63 in both normal and SCC cells [77]. Similarly, downregulation of p63 in primary human foreskin keratinocytes was found to induce apoptosis and to reduce both β1 and β4 integrin expression [78], linking adhesiveness with viability.

Taken together, the data presented in Sections 3.2.1–3.2.3 demonstrate that the network of genes regulated by ΔNp63α is large and perturbation of the balance between ΔNp63α and members of this network could have adverse biological consequences.

3.3. Stem Cell Maintenance and Senescence. The proliferative lifespan of cells is limited by replicative senescence during which the cells permanently withdraw from the cell cycle, yet remain viable [79, 80]. This phenomenon is associated with the normal ageing process of renewable tissues such as the epidermis. p63 has been proposed as a marker of human epidermal keratinocyte stem cells that is downregulated when keratinocytes become transient amplifying cells [81]. Consistent with these data and the hypothesis that epidermal progenitor cell exhaustion occurs in *p63*(−/−) mice, depletion of p63 in immature human epidermal keratinocytes resulted in reduced clonal growth [82]. Regulation of replicative senescence in human epidermal keratinocytes involves miRs-138, 181a, 181b, and 130b which promote senescence by targeting ΔNp63α and Sirt1 for degradation. However, in a feedback loop, these miRs are themselves targets of negative regulation by ΔNp63α [83]; thus, overexpression of ΔNp63α could also perturb senescence.

Mouse models provide support for a role for p63 in the maintenance of stem cell proliferative capacity. Characteristics of accelerating ageing were noted in *p63*(+/−) mice observed for extended periods generated by two groups using the mice developed by both Yang et al. and Mills et al. [3, 25, 84, 85]. Germline or somatic *p63* depletion under control of the keratin 5 promoter gave rise to enhanced senescence marker expression [84], suggesting a role for p63 in the negative regulation of senescence. Indeed, overexpression of ΔNp63α in primary mouse keratinocytes overcame replicative senescence in association with delayed and diminished induction of INK4/p16 and Arf/p19 [86]. Consistent with these findings, crossing of *p63*(−/−) mice developed by Yang et al. [3] with *INK4/p16*(−/−) or *Arf/p19*(−/−) mice was able to partially rescue the proliferation and differentiation defects observed in *p63*(−/−) mice [87], reinforcing a role for p63 in blocking senescence. While these mice display reepithelialization, skin from *p63*(−/−) mice crossed with *Ink4a/p16*(−/−) or *Arf/p19*(−/−) mice is fragile and easily detachable, suggestive of defective adhesion, which also can be attributed to p63.

In the mouse models described above, all p63 isoforms were knocked down. A TAp63-specific knockdown mouse model that supports a role for TAp63 in adult stem cell maintenance was generated by crossing TAp63 floxed mice with germline-specific promoter cre or keratin 14-cre mice [37]. *TAp63*(−/−) mice exhibited signs of premature ageing. Interestingly, overexpression of ΔNp63α under control of the keratin 14 promoter resulted in a phenotype similar to that reported in mice lacking TAp63 [88]. Skin-derived precursor (SKP) cells are multipotent precursor cells derived from the dermis that can differentiate into mesodermal and neural cells [89]. In the *TAp63*(−/−) mice, SKP cells proliferate more rapidly than wild-type SKP cells, and thus, undergo senescence more rapidly. As adult stem cell populations are not immortal, this enhanced proliferation in *TAp63*(−/−) cells would be expected to lead to stem cell exhaustion, which is associated with accelerated ageing.

4. p63 and Neoplasia

4.1. Observational Studies of Human Tumors. The *p53* tumor suppressor gene is commonly mutated in human cancer [1]. Due to the similarity of the TAp63 isoforms with p53, it was hypothesized that mutation of p*63* could provide a mechanistic explanation for tumors in which *p53* was not mutated. It was found, instead, that mutation of *p63* is a rare event in human cancer cell lines [90], but that p63 overexpression is seen in human squamous cell cancers including esophageal squamous cell carcinoma [91, 92], nasopharyngeal carcinoma [93], and squamous cell carcinoma of the skin [94, 95]. Overexpression of the ΔNp63 protein in primary squamous cell carcinomas (SCCs) of the head, neck, and lung correlates with amplification of the p63 gene locus, which occurs frequently in these cancers [28, 96]. While there is agreement that ΔNp63α is overexpressed in lung SCCs, conflicting results have been published as to whether this correlates with prognosis [96, 97].

In squamous cell carcinomas of the skin, a significant increase in p63 expression, both in terms of intensity and distribution, is seen relative to normal skin, as the proliferative fraction is expanded in tumors [26, 95]. Examination of skin lesions ranging from keratoacanthoma to a grade IV spindle cell carcinoma revealed very strong p63 immunoreactivity in grade 3 SCC with decrease in a single grade IV spindle SCC. In these tumors, carcinoma *in situ* was characterized by p63 immunoreactivity in all layers [94]. While ΔNp63α was shown to be the most overexpressed isoform in squamous cell tumors, careful characterization of the TA and ΔN isoforms from different tissue and tumor types revealed that individual isoforms are differentially expressed in the neoplastic transformation of different tissue types [98], implying specific contributions of the isoform expressed in a context-dependent manner. While ΔNp63α is overexpressed in primary skin tumors, expression of TAp63 is not a common event but has been reported to be downregulated relative to normal

skin using PCR-based methods [99]. It is clear that ΔNp63α is overexpressed in skin SCCs, however, whether it actively plays a role in tumor formation or is a bystander has been unclear. Further insight into this question has been gained by *in vitro* and *in vivo* studies, as discussed below.

4.2. In Vitro/Molecular Studies with Human Cancer Cell Lines and Primary Keratinocytes

4.2.1. Impact on Signaling Pathways. In particular, *in vitro* studies in SCC cells have provided insight into the potential signaling pathways impacted by p63 dysregulation in squamous cell carcinoma. As discussed previously, IRF6 is involved in a negative feedback loop with ΔNp63α that is necessary for the downregulation of ΔNp63α seen with differentiation [57], and an appropriate balance between these factors is required for the switch between proliferation and differentiation in the normal epidermis [100]. Correspondingly, expression of IRF6 was found to be strongly downregulated in human SCC [101]. Reexpression of IRF-6 in the context of primary human keratinocytes expressing both ΔNp63α and a mutant v-ras 12 oncogene was found to abolish the ability of ΔNp63α to promote colony growth and restore oncogene induced senescence [101], supporting a role for IRF6 in regulating ΔNp63α as part of its tumor suppressor function. In other studies, ΔNp63α has been shown to upregulate Hsp70, a protein colocalized with ΔNp63 in primary SCCs of the head and neck (HNSCCs) that is associated with proliferation and viability of HNSCC [102]. Likewise, accumulation of β-catenin in the nucleus and activation of downstream signaling pathways common to many cancers are induced by ΔNp63α in HNSCC cells [103].

Consistent with a role in promoting adhesion, ΔNp63 is negatively regulated by the epithelial-to-mesenchymal transition (EMT) promoting transcription factors snail and slug, and this association is observed in primary human cervical, head and neck, and esophageal SCCs. This decrease in ΔNp63α is associated with increased migration in SCC cell lines [104]. ΔNp63α also physically sequesters YB-1, a positive translational mediator of snail, thereby preventing both enhanced snail activity and YB1's function in actin cytoskeleton reorganization, both of which lead to cancer cell migration and invasion [105]. Another direct transcriptional target of ΔNp63α is the vitamin D receptor (VDR) [106], which is induced by multiple p63 isoforms. Downregulation of VDR expression results in increased cell migration of A431 epidermoid carcinoma cells, which can be rescued by ΔNp63α or VDR [107]. A role for ΔNp63α in preventing metastasis is further supported by the finding that antagonism of ΔNp63α by mutant-p53/Smad complex allows TGF-β to convert from a tumor suppressor role to a role in promoting metastases [108]. In line with this, knockdown of p63 in squamous cancer cell lines, in which the predominant isoform expressed was ΔNp63, led to an increase of mesenchymal and neural markers and upregulation of genes associated with invasion and motility [109].

Based on the data, it is enticing to contemplate that ΔNp63α plays a role in cancer development by promoting proliferation and viability at earlier stages, while it may need to be downregulated during progression to allow for the necessary enhanced motility, invasiveness, and EMT [110] that allow metastases to form.

4.2.2. Altered Responsiveness to Genotoxic Stress. ΔNp63α can impact cellular response to genotoxic stress. A mouse model in which ΔNp63 was overexpressed under control of the loricrin promoter showed that downregulation of ΔNp63 is required for UVB-induced apoptosis of the epidermis [111]. Mechanistically, degradation of ΔNp63α in keratinocytes exposed to apoptotic doses of UV was shown to be mediated by p38 MAPK, which phosphorylates ΔNp63α. This led to its detachment from p53-dependent promoters and results in apoptosis induction [112]. Consistent with this report, occupancy of binding sites involved in cell cycle arrest and apoptosis switched following adriamycin or UV treatment of human epidermal keratinocytes from ΔNp63α to p53 occupancy, which would be expected to result in increased apoptosis or cell cycle arrest [113].

Many therapeutic agents used in cancer treatment promote genotoxic stress as a means to reduce or control tumor growth. Expression of high levels of ΔNp63α predicts responsiveness of primary HNSCC to platinum-based therapies [114]. Upon exposure to cisplatin, ΔNp63α is proteosomally degraded via stratifin-mediated nuclear export and Rack1 targeting [114, 115]. An interaction between the p63 proteins and the NF-κB pathway also plays a role in responsiveness to chemotherapeutics. In JHU-022 oral cavity SCC cells, IKKβ, a known activator of RelA, promotes ΔNp63α degradation in response to cisplatin [116]. In this cell line, cisplatin treatment resulted in a physical interaction between RelA and ΔNp63α that abrogates ΔNp63α mediated $p21^{WAF1}$ promoter repression and targets ΔNp63α for proteosomal degradation [117]. The presence of c-Abl, which has been implicated as an oncogene, in HNSCC cells treated with cisplatin stabilizes ΔNp63α expression. This stabilization of ΔNp63α leads to enhanced cell viability [118], which could be anticipated to result in clinical consequences.

Survival of HNSCC cells that overexpress ΔNp63α is dependent on the presence of ΔNp63α, which functions by blocking TAp73-driven apoptosis both via promoter binding and physical interaction with p73 in a p53-independent manner [119]. TAp73 and ΔNp63α are engaged in a feedback loop involving miR-193a-5p, which is repressed by ΔNp63α and activated by TAp73 and targets the p73 UTR. Cisplatin treatment results in ΔNp63α degradation and TAp73-mediated activation of miR-193a-5p, limiting TAp73's pro-apoptotic effects and chemosensitivity [23]. Reimplantation in the presence or absence of a miR-193a-5p antagomir of disaggregated cells from primary mouse SCCs generated by a chemical carcinogenesis protocol revealed that knockdown of this miR resulted in reduced tumor formation and enhanced chemosensitivity [23], indicating that a strategy targeting both ΔNp63α and miR-193a-5p might be more effective in this scenario. HNSCC cells can circumvent the requirement for ΔNp63α expression for survival by the overexpression of Bcl2 [119]. In addition to blocking p73 to promote survival of

HNSCC, ΔNp63α associates with histone deacetylase 1 and 2 forming an apoptotic transcriptional repressor complex. This complex is sensitive to breakdown by cisplatin and HDAC inhibitors, in the presence of low, but not high, levels of endogenous Bcl-2 indicating once again that the context of the tumor impacts the success of chemotherapy [120]. Unlike the case of TAp73 described above, in HaCaT cells in response to chemotherapy, ΔNp63α is involved in an antiapoptotic feedback loop in which it, as well as mutant p53, induces ΔNp63α [24]. Thus, response to genotoxic stress is another biological endpoint that can be impacted by dysregulated ΔNp63α.

4.3. Modeling Human Cancers in Mouse to Assess the Contribution of p63 to Neoplasia. Cancer arises as a multistep process that can be reiterated in well-established mouse models in a controlled fashion [121]. Results of the studies presented above suggest that TAp63 would harbor tumor suppressor properties and overexpressed ΔNp63 would harbor oncogenic properties. In this section, we highlight the use of mouse models to dissect out how altered p63 levels contribute biologically to prevention or development of cancer, either alone or in altered balance with other family members or other oncogenic pathways. Approaches discussed utilize mice with a heterozygous null mutation in *p63* on a background of wild type, *p53(+/−)* and/or *p73(+/−);* TA-isoform-specific knockout mice; and mouse models where the elevated levels of ΔNp63α observed in human SCC are mimicked in cultured keratinocytes and transplanted to nude mice.

4.3.1. TAp63 as a Tumor Suppressor. The potential role for physiological levels of p63 acting as a tumor suppressor with respect to spontaneous tumor development was explored by two groups in the context of alteration of other p53 family members [85, 122]. In a mouse model in which the *p63* genotype was contributed by mice developed by Yang et al. [3], mice heterozygous for a null mutation in both *p63* and *p73* displayed a higher incidence of spontaneous tumor formation relative to wild-type mice. Furthermore, mice heterozygous for *p53*, *p63*, and *p73* developed a higher incidence and formed more aggressive tumors than mice heterozygous for the *p53* null mutation alone. These findings suggest that p63 and p73 share a tumor suppressor role as has been long established for p53 [1, 85]. In the absence of additional genetic mutations, these *p63*(+/−) mice developed squamous cell carcinomas (10%), adenomas (15%), and histiocytic sarcomas (20%) at 10%, 15%, and 20% greater rates, respectively, than wild type. In contrast, in a study using mice with a p63 genotype contributed by the mice developed by Mills et al. [25], *p63*(+/−)/*p53*(+/−) mice were found to be less prone to spontaneous tumors than p53 +/− mice alone. Additionally, these *p63*(+/−) mice were shown to have decreased susceptibility to chemically induced carcinogenesis, suggesting that p63 does not contribute a tumor suppressor activity in cancer. To date, this controversy remains unresolved.

In the mouse models described above, all p63 isoforms were targeted. TAp63-subclass-specific knockdown mice allow distinction between the TA and ΔN subclass properties. Following observation for 2.5 years, an enhanced incidence of carcinoma, including SCC of the skin and sarcoma development, was observed in *TAp63*(+/−) and *TAp63*(−/−) mice relative to wild-type mice [123], again supporting a tumor suppressive role for TAp63. It was noted that tumors from the *TAp63*(+/−) and *TAp63*(−/−) mice were highly metastatic, and at a mechanistic level TAp63 was found to positively regulate Dicer, a protein critical for miR processing, and miR 130b. Reexpression of both Dicer and miR130b in *TAp63*(−/−) MEFs decreased invasiveness of these cells, suggesting that TAp63's tumor suppressor role could be mediated at least in part through Dicer and miR130b [123]. As mentioned previously, miR130b targets ΔNp63α for degradation [83].

4.3.2. Overexpressed ΔNp63α Facilitates Tumor Progression. The mouse models described above focused on the TAp63 isoforms and were performed at wild-type or decreased levels of endogenous p63. However, overexpression of ΔNp63α is a common event in squamous cancers. Two independent studies have used similar approaches to mimic this overexpression with the goal of examining the *in vivo* functional consequences of ΔNp63α overexpression in the epidermis. Results from both lab groups support a contributory role for ΔNp63α in the cancer phenotype with mechanistic distinctions. In studies performed in our laboratory, wild-type primary murine keratinocytes were transduced with retrovirus encoding a v-rasHA oncogene in combination with a lentivirus encoding either a control GFP construct or ΔNp63α and grafted onto the dorsum of nude mice in combination with primary dermal fibroblasts [86]. This model allows growth of normal keratinocytes as well as benign and malignant tumor phenotypes in the graft site. Mice were observed up to a month following cell grafting for tumor formation. No lesions were observed in graft sites following transplantation of keratinocytes expressing only GFP or ΔNp63α alone. Grafting of keratinocytes expressing v-Ras^{HA} + GFP resulted, as expected, in the formation of well differentiated papillomas, while grafting of keratinocytes expressing v-Ras^{HA} + ΔNp63α resulted in 100% malignant conversion to carcinoma [86]. Although elevated levels of ΔNp63α alone are insufficient to confer a tumor phenotype *in vivo*, we found that ΔNp63α blocks oncogene-induced senescence by inhibiting p16^{ink4a}/p19^{arf} pathways and cooperates with oncogenic v-Ras^{HA} to enhance malignant conversion *in vivo*. This study supports a contributory role for ΔNp63α in cancer pathogenesis and a mechanistic link to cell survival by overriding oncogene-induced senescence through inhibition of p16^{ink4a} and p19^{arf}, key mediators of cellular senescence.

Using a similar approach, Keyes et al. [124] demonstrated that overexpressing ΔNp63α in keratinocytes in the presence of oncogenic ras resulted in growth of malignant carcinomas following subcutaneous injection. In this study also, the malignant phenotype was associated with a bypass of oncogene-induced senescence. Overexpression of ΔNp63α was further shown to enhance stem-like proliferation of keratinocytes and maintain survival of the keratin 15-positive stem cell population. Furthermore, chromatin-remodeling protein Lsh was identified as a new target of ΔNp63α and as

an essential mediator of senescence bypass. Although $p19^{arf}$ was not detectable in the tumors derived from ras/ΔNp63α keratinocytes in this study, an *in vitro* component of the study indicated that $p16^{ink4a}$ and $p19^{arf}$ were not reduced during the initial stages of senescence bypass. Therefore, contrary to our study, it was proposed that the initiating events through which ΔNp63α inhibits senescence do not occur via $p16^{ink4a}$/$p19^{arf}$ pathways. Although the difference in $p16^{ink4a}$/$p19^{arf}$ between these two studies may be due to the different time courses used, it further indicates the complexity of the pathways interacting with p63 family members and underscores the need for additional studies to understand the role of p63 and its downstream effectors in tumorigenesis and senescence.

An oncogenic role for ΔNp63 is further supported by studies in a mouse model containing a dominant negative 14-3-3σ mutation (*Er*/+). 14-3-3σ, a protein associated with keratinocyte differentiation, is a direct target for ΔNp63α repression in undifferentiated human epidermal keratinocytes [52]. Treatment of *Er*/+ mice on a *p63*(+/+) background with a two-stage carcinogenesis protocol resulted in the formation of tumors in which ΔNp63α was strongly expressed, while loss of function of an endogenous allele of p63 in this context, which generated (*Er*/+/*p63*+/−) mice, resulted in reduced sensitivity to this protocol, suggestive of cooperation of ΔNp63α in Ras/14-3-3σ-induced tumorigenesis [125].

A Role for NF-κB/c-Rel in ΔNp63α-Mediated Carcinogenesis. In a transcription factor profiling exercise, we identified activation of NF-κB in keratinocytes following the overexpression of ΔNp63α. The NF-κB family comprises 5 members functioning as hetero- and homodimers [126]. Only NF-κB/c-Rel was found to be modulated by ΔNp63α under these conditions, with nuclear accumulation of phosphorylated c-Rel but none of the other NFκB subunits enhanced in the presence of overexpressed ΔNp63α. NF-κB is associated with multiple human diseases, including cancer, for which therapeutics targeting its constitutive NF-κB activation are under development [127, 128]. Of the five family members, RelA, p50, and c-Rel subunits have been implicated in the maintenance of normal epidermal homeostasis [129, 130], and in SCC, the RelA/p50 heterodimer has been shown to promote or repress malignancy in a context-dependent manner [131, 132]. Nuclear c-Rel expression is associated with both solid breast tumors and hematopoietic malignancies [133]; however, it had not previously been investigated in SCC of the skin. The increase in nuclear c-Rel accumulation seen with elevated ΔNp63α levels was found to be critical to the ability of ΔNp63α overexpressing keratinocytes to proliferate under conditions that normally induce growth arrest [134]. Mechanistically, this is correlated with a physical interaction between ΔNp63α and c-Rel on the promoter of the $p21^{WAF1}$ gene in these cells, both *in vitro* and *in vivo*, which represses $p21^{WAF1}$ expression. These findings extended to primary human HNSCC, in which we found that ΔNp63α and c-Rel colocalized in the nuclei throughout the tumor sections, as opposed to a more restricted expression in normal tissue.

In an extension of these studies, Lu et al. characterized a dynamic mechanism whereby c-Rel, ΔNp63α, and TAp73, which are coexpressed in the nuclei of a subset of HNSCC cell lines, control expression on binding sites including $p21^{WAF1}$, Noxa, and Puma [135]. Exposure of HNSCC cell line cultures to TNF-α to mimic inflammation in the tumor environment was found to induce nuclear accumulation of c-Rel. In the absence of this stimulus, ΔNp63α was found to physically interact with TAp73. Similar to our results in untreated primary keratinocytes upon overexpression of ΔNp63α, a physical interaction between endogenous ΔNp63α and c-Rel was observed following TNF-α treatment in these HNSCC cell lines. Interestingly, under conditions of c-Rel overexpression, the interaction between ΔNp63α and TAp73 was blocked and TAp73 was translocated to the cytoplasm. This suggests that c-Rel displaces TAp73. ChIP assays indicated that this regulation occurred on the promoters of genes involved in growth arrest and apoptosis, resulting in their downregulation [135].

5. Summary/Future Directions: Challenging *In Vitro* Findings in *In Vivo Models*

Primary murine cell cultures and *in vivo* murine models have been instrumental in elucidating the multistep nature of carcinogenesis [121] and in challenging the role of specific genetic alterations, such as those observed in *p63*, in cancer pathogenesis [86, 124]. The mouse models described in this review have provided us with a clear picture of the importance of p63 to normal epidermal development and homeostasis and have highlighted the roles for specific p63 isoforms in neoplasia. However, given the complexity of the p63 family members, their interactions, and the context-specific manner in which they can exert their effects, much remains to be defined.

Mouse models with molecular alterations that allow targeting of specific gene products will be indispensible in deepening our understanding of and resolving controversy related to the role of *p63* both in normal tissue and in disease. Once a pathway has been implicated, primary cultures from mouse epidermis can be readily manipulated to express or eliminate a particular protein presumed active upstream or downstream to assess the impact. Applied in combination with keratinocytes from genetically altered mice, both *in vitro* and *in vivo* findings can be challenged further for the consequences of the alterations. However, it is important to remember that all models have limitations, and a deeper understanding of the role of p63 in normal epidermal homeostasis and neoplasia will ultimately be derived from an iterative process involving *in vitro* observations in primary cells, cell lines and primary tumors, *in vivo* queries of these findings, and reexamination of the outcomes in the context of human tumors. Mouse models comprise a critical component of this process.

As an example, the observation in primary mouse keratinocytes that c-Rel acts downstream of ΔNp63α in modulating keratinocyte growth regulation led to a further novel observation that c-Rel levels are enhanced in primary

HNSCC of humans and links this protein accumulation to altered NFκB/c-Rel activity in human head and neck squamous cell cancer cells [134]. The requirement for c-Rel in these cancers can be tested by modulating c-Rel and ΔNp63α independently using lentiviral gene transduction followed by grafting. Long-term overexpression of ΔNp63α has been shown to support sustained high levels of nuclear c-Rel expression, and c-Rel shRNA lentiviruses are capable of depleting c-Rel in keratinocytes for extended time (unpublished observations). Assessing the impact of these modulations *in vivo* will clarify the interplay between these alterations and their relevance to cancer development and progression.

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Review Article

Role of Stat3 in Skin Carcinogenesis: Insights Gained from Relevant Mouse Models

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Signal transducer and activator of transcription 3 (Stat3) is a cytoplasmic protein that is activated in response to cytokines and growth factors and acts as a transcription factor. Stat3 plays critical roles in various biological activities including cell proliferation, migration, and survival. Studies using keratinocyte-specific Stat3-deficient mice have revealed that Stat3 plays an important role in skin homeostasis including keratinocyte migration, wound healing, and hair follicle growth. Use of both constitutive and inducible keratinocyte-specific Stat3-deficient mouse models has demonstrated that Stat3 is required for both the initiation and promotion stages of multistage skin carcinogenesis. Further studies using a transgenic mouse model with a gain of function mutant of Stat3 (Stat3C) expressed in the basal layer of the epidermis revealed a novel role for Stat3 in skin tumor progression. Studies using similar Stat3-deficient and gain-of-function mouse models have indicated its similar roles in ultraviolet B (UVB) radiation-mediated skin carcinogenesis. This paper summarizes the use of these various mouse models for studying the role and underlying mechanisms for the function of Stat3 in skin carcinogenesis. Given its significant role throughout the skin carcinogenesis process, Stat3 is an attractive target for skin cancer prevention and treatment.

1. Introduction

Signal transducers and activators of transcription (Stats) are proteins that are activated by extracellular signaling proteins, such as growth factors, cytokines and various peptides [1]. Stats can also be activated via nonreceptor tyrosine kinases (e.g., src and abl) [1]. Cell-surface-mediated receptor activation causes the phosphorylation of tyrosine kinases, such as Janus-associated-kinase (Jak), which provides docking sites for the src homology (SH2) domain, enabling the binding and subsequent phosphorylation of Stats. The reciprocal interaction between the SH2 domains of two phosphorylated Stat monomers results in the formation of a functional Stat dimer. By this process, the Stat proteins are recruited to Jaks and are phosphorylated at their critical tyrosine residues. The phosphorylated Stats dimerize, translocate to the nucleus, and drive transcription of their target genes (Figure 1) [2].

While activation of Stats downstream of ligand-induced receptor activation is linked to differentiation and growth regulation functions, constitutive activation of Stats is often associated with deregulated cell growth [1]. There are seven different Stat proteins, Stat1 through Stat6, including two isoforms of Stat5 (Stat5a and Stat5b). Stat1, Stat3, Stat4, Stat5a, and Stat5b all form homodimers. In addition, Stat1, Stat2, and Stat3 can form heterodimers. Phenotypic analysis of genetically targeted mouse models for individual Stat genes has aided in delineating their biological roles. Interestingly, of all the Stat proteins only deletion of Stat3 leads to embryonic lethality [3]. Stat3 was originally identified as an IL-6-dependent transcription factor that promotes acute phase gene expression [4, 5]. However, subsequent studies have shown Stat3 activation by various cytokines, growth factors, and hormones [1]. In addition to its role in numerous cellular functions, there is strong evidence correlating Stat3

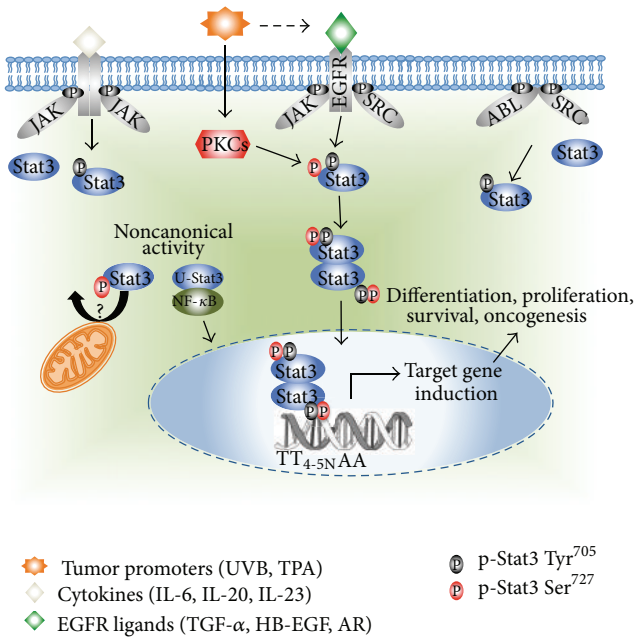

FIGURE 1: Pathways associated with Stat3 activation. Stat3 is activated downstream of receptor tyrosine kinases (e.g., EGFR), cytokine receptors via associated Janus family kinases (JAKs) (e.g., IL-6 receptor), and nonreceptor-associated tyrosine kinases (e.g., c-src). Tumor promoters such as TPA and UVB activate Stat3 in keratinocytes primarily via the EGFR. Activation of PKCs by tumor promoters leads to the processing of membrane-bound proforms of EGFR ligands such as heparin-binding EGF (HB-EGF) by matrix metalloproteinases (MMPs). In addition, PKCs associate with and phosphorylate Stat3 at Ser^{727}, which is necessary for maximal Stat3 transcriptional activity. Furthermore, transcriptional induction of cytokines and EGF ligands can lead to autocrine stimulation and sustained Stat3 phosphorylation. After phosphorylation, STAT3 dimerizes and translocates to the nucleus, where Stat3 dimers directly regulate gene expression of transcriptional targets including Bcl-x_L, cyclin D1, c-myc, Twist and Survivin. STAT3-mediated regulation of target gene expression is involved in various cellular functions including cell differentiation, proliferation, survival, and oncogenesis. Stat3 can also act through noncanonical signaling pathways. In this regard, unphosphorylated Stat3 (U-Stat3) can drive gene expression of a subset of genes that are different from p-Stat3 dimers in an NF-κB-dependent and independent manner. In addition, p-Stat3 Ser^{727} can translocate into the mitochondria and influence mitochondrial respiratory chain activity. These noncanonical Stat3 signaling pathways have protumorigenic roles in certain cell/tissue types; however their role in epithelial carcinogenesis has not been evaluated.

activation and cancer. Stat3 is found constitutively activated in cells transformed by the oncogenes v-Src and v-Abl, as well as in various human cancers, including hematologic, pancreas, breast, head and neck, and prostate cancer [6, 7]. Although there is substantial data in the literature on the protumorigenic effects of Stat3, there have been reports that activation of Stat3 can have an opposite, tumor-suppressive role (e.g., PTEN wt versus null gliomas) [8] and that activated Stat3 is associated with better prognosis in leiomyosarcoma and human papillary thyroid carcinoma [9, 10].

The mouse skin model of multistage carcinogenesis has been used for over 60 years and is one of the most well-established *in vivo* models for studying the stepwise and chronological development of epithelial tumors [11, 12]. Multistage chemical carcinogenesis in this model can be subdivided into three stages: initiation, promotion, and progression. The initiation step involves application of a subcarcinogenic dose of a carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA), which induces mutations in gene(s) through metabolism to reactive diol-epoxide metabolites and their subsequent covalent binding to DNA forming DNA adducts. The Ha-ras gene is a primary target of DMBA in this model and is routinely found mutated at codon 61 (A to T mutation) in tumors generated by initiation with polycyclic aromatic hydrocarbon [11]. Subsequently, the process of tumor promotion is accomplished by the repeated application of a tumor-promoting agent, most commonly the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). TPA treatment induces epidermal proliferation and clonal expansion of initiated cells. Lastly, the tumor progression stage occurs stochastically and leads to the conversion of skin papillomas to squamous cell carcinomas (SCCs) [11–14]. In this model of skin carcinogenesis, Stat3 is activated very early in the epidermis following treatment with different classes of tumor promoters, including TPA, okadaic acid, and chrysarobin [15]. In addition, Stat3 is upregulated and constitutively activated in skin papillomas and SCCs generated by the two-stage protocol. The primary mechanism for activation of Stat3 in mouse keratinocytes exposed to tumor promoters is through activation of epidermal growth factor receptor (EGFR) (Figure 1) [15], although other pathways may also contribute to its activation during tumor promotion. Given the unique embryonic lethal phenotype of Stat3 targeting, tissue-specific knockout and inducible knockout strategies using the Cre-*loxP* system have been used to elucidate the role(s) of Stat3 in skin biology and skin carcinogenesis [16]. Loss-of-function studies have been complimented by a Stat3 mouse model expressing a constitutively activated Stat3 protein (Stat3C) under the control of the keratin 5 promoter (K5.Stat3C transgenic mice). Studies from this comprehensive set of skin-specific knockout and transgenic Stat3 mouse models have shown that Stat3 plays a major role in skin carcinogenesis. Herein, we review the use of these mouse models and the insights gained regarding the role of Stat3 in skin carcinogenesis.

2. Skin-Specific Deletion of Stat3 Reveals Roles in Wound Healing and Hair Cycle

Due to the embryonic lethality of $Stat3^{-/-}$ mice, mice with conditional deletion of Stat3 in the skin were generated by crossing $Stat3^{flox/-}$ mice with a transgenic line expressing Cre recombinase under the control of the human keratin 5 promoter (K5.*Cre*) [17, 18]. The K5 promoter drives gene expression in the basal cell layer of the epidermis and follicular keratinocytes [19]. Thus, expression of Cre recombinase under control of K5 allowed for deletion of the Stat3 gene throughout the epidermis and the outer root sheath of hair

follicles [18, 19]. Unlike Stat3$^{-/-}$ mice, K5.*Cre* × Stat3$^{flox/-}$ mice were viable, developed normally, and exhibited normal skin at a young age. However, Stat3 disruption in these mice impaired keratinocyte migration during the wound healing process both *in vivo* and *in vitro*. In addition, Stat3 loss compromised mainly the second anagen phase of the hair cycle [17]. Thus, older mice developed a sparse hair coat and also developed spontaneous skin ulcers due to impaired wound healing. From these studies it was concluded that Stat3 was not involved in skin morphogenesis but that it plays a significant role in skin remodeling through its effects on wound healing and the hair cycle [17, 20].

3. Skin-Specific Deletion of Stat3 Reveals Roles in both the Initiation and Promotion Stages of Two-Stage Chemical Carcinogenesis

Various forms of skin wounding are known to promote skin tumors in mice [11]. As mentioned above, studies using mice with a keratinocyte-specific deletion of Stat3 revealed a significant role for Stat3 in skin wound healing. Therefore, further studies were conducted to examine the possible role of Stat3 in skin carcinogenesis. For these studies, mice containing both floxed Stat3 alleles (i.e., Stat3$^{flox/flox}$ mice) were used to reduce the severity of the wounding defect seen in mice harboring one floxed allele and one null allele (i.e., Stat3$^{flox/-}$ mice) in the original studies [17]. Stat3$^{flox/flox}$ mice were crossed with K5.Cre mice to produce an epidermis-specific deficiency Stat3 [21]. Thus, use of this skin-specific Stat3-deficient mouse model demonstrated that Stat3 was absolutely required for development of skin tumors using the two-stage DMBA-TPA protocol. In this regard, loss of Stat3 completely suppressed the development of skin papillomas. Further studies using these mice revealed that Stat3 deficiency sensitized keratinocytes to DMBA-induced apoptosis both *in vivo* and *in vitro* [21]. Interestingly, Stat3-deficient keratinocytes that underwent apoptosis after DMBA treatment *in vivo* were localized in the bulge region adjacent to label-retaining cells (LRCs) [21]. The proximity of DMBA-sensitive cells to LRCs raised the possibility that the loss of Stat3 induced a loss of initiated keratinocyte stems cells in this model (discussed in more detail below). These data suggested that Stat3 played a role during the initiation stage of skin carcinogenesis through its ability to regulate genes involved in keratinocyte survival during the process of tumor initiation.

The repeated treatment with TPA after initiation is used to induce epidermal proliferation and clonal expansion of initiated cells that harbor Ha-ras mutations, which ultimately leads to development of premalignant papillomas [11, 12]. Loss of Stat3 in K5.Cre × Stat3$^{flox/flox}$ mice resulted in a significant reduction of epidermal hyperproliferation (assessed by bromodeoxyuridine (BrdU) labeling index) compared to control littermates following TPA treatment [15, 21]. Mechanistic studies showed that recovery of cell cycle regulatory proteins cyclin D1 and cyclin E was delayed and c-myc expression was persistently downregulated in the epidermis of K5.Cre × Stat3$^{flox/flox}$ mice after topical treatment with TPA in comparison to control mice. Thus, constitutive deletion of Stat3 in the basal layer of epidermis inhibited TPA-induced epidermal hyperproliferation during tumor promotion. Additional studies investigating the role of Stat3 in clonal expansion of initiated cells during promotion were conducted using the TG.AC mouse model. TG.AC transgenic mice express a fusion protein of the activated v-Ha-Ras oncogene and the mouse zeta-globin gene [29]. In this model expression of activated v-Ha-Ras replaces the initiation step (DMBA treatment) of the two-stage chemical carcinogenesis protocol. Promotion in TG.AC mice with TPA results in development of multiple papillomas that progress to SCCs [29]. Inhibition of Stat3 using an oligonucleotide decoy targeting Stat3 inhibited TPA-induced papilloma formation in TG.AC mice, confirming that Stat3 is required for the clonal expansion of initiated cells during the promotion phase of two-stage skin carcinogenesis [21]. Moreover, intratumoral injection of Stat3 decoy caused regression in preexisting skin papillomas [21]. Collectively, these studies using K5.Cre × Stat3$^{flox/flox}$ mice indicated that Stat3 is required for survival of keratinocytes that have accumulated DNA damage during initiation with DMBA and that initiated keratinocytes harboring Ha-ras mutations require Stat3 for proliferation and clonal expansion during tumor promotion with TPA.

4. Inducible Stat3 Deficiency Using K5.CreER^{T2} × Stat3$^{flox/flox}$ Mice Directly Confirms a Role for Stat3 in Both the Initiation and Promotion Stages of Skin Carcinogenesis

While the studies using K5.Cre × Stat3$^{flox/flox}$ mice provided strong evidence that Stat3 was involved in both the initiation and promotion stages of skin carcinogenesis, more direct evidence was obtained using an inducible system where Stat3 could be deleted in a temporal manner. Thus, intercross of Stat3$^{flox/flox}$ mice with a transgenic mouse expressing a tamoxifen inducible *Cre* (i.e., Cre-ER^{T2}) gene under the control of the K5 promoter (K5.CreER^{T2} mice) provided a temporally controlled and inducible epidermis-specific Stat3-deficient mouse model [24, 30]. Using this mouse model, temporal disruption of Stat3 at the time of initiation resulted in an increased number of apoptotic cells following DMBA treatment [24]. In a two-stage carcinogenesis experiment, tamoxifen treatment prior to DMBA treatment of inducible Stat3-deficient mice significantly delayed tumor onset and reduced the number of papillomas per mouse [24]. Similarly, inducible deletion of Stat3 prior to each TPA treatment during the tumor promotion stage delayed tumor onset and tumor multiplicity. Mechanistic studies confirmed that deletion of Stat3 using this inducible system led to reduced levels of survival proteins such as Bcl-x$_L$ and S-phase proteins such as cyclin D1, cyclin E, and c-myc supporting the earlier observations using K5.Cre × Stat3$^{flox/flox}$ mice.

The availability of an inducible Stat3 knockout model also allowed deletion of Stat3 in skin papillomas generated by the DMBA-TPA protocol. In this regard, deletion of Stat3 in skin papillomas by i.p. injection of tamoxifen inhibited subsequent growth of these tumors. Collectively, these studies using an inducible Stat3 knockout system provided direct evidence for a role of Stat3 in both the initiation and promotion stages of skin carcinogenesis. Furthermore, these studies showed that deletion of Stat3 reduced the levels of both survival proteins and cell cycle proteins involved in G1 to S-phase transition. Finally, the use of these models demonstrated the requirement for Stat3 activation for continued growth of skin papillomas.

5. Stat3C Transgenic Mice Reveal a Novel Role for Stat3 in Skin Tumor Progression

Constitutive activation of Stat3 is observed in a variety of human tumors [6, 7] as noted above. This persistent activation can be recapitulated experimentally by substituting residues A661 and N663 for cysteine residues, allowing for cysteine-cysteine sulfhydryl bonds between Stat3 monomers and the formation of Stat3 homodimers without the phosphorylation of Tyr^{705} [31]. This form of Stat3, referred to as Stat3C, was initially shown to transform mouse and rat fibroblasts as demonstrated by anchorage-independent growth in soft agar and formation of tumors when these cells were injected into nude mice [31]. Further study of the role of Stat3 in skin carcinogenesis was facilitated by the generation of mice that express this constitutively active/dimerized form of Stat3 targeted to the proliferative compartment of epidermis using the bovine K5 promoter (i.e., K5.Stat3C transgenic mice) [27]. K5.Stat3C transgenic mice did not develop spontaneous tumors but did exhibit a mild hyperproliferative epidermis and developed spontaneous psoriatic skin lesions with age [28]. In addition, K5.Stat3C mice showed an increased BrdU labeling index after TPA treatment compared to nontransgenic littermates. Expression of Stat3C in the basal compartment of the epidermis significantly protected keratinocytes from DMBA-induced apoptosis [27]. In a DMBA-TPA skin carcinogenesis protocol, K5.Stat3C mice developed skin tumors in greater number and with a shortened latency compared to nontransgenic littermates. Notably, 100% of skin tumors that developed in K5.Stat3C transgenic mice bypassed the premalignant (papilloma) stage and initially developed as carcinoma *in situ*. Histological and immunohistochemical analyses revealed that these tumors were highly vascularized and poorly differentiated, and invasion into surrounding dermal/mesenchymal tissue was observed at a very early stage. Expression of K10, filaggrin, and E-cadherin was completely lost in skin tumors from K5.Stat3C transgenic mice by 20 weeks [27]. Thus, expression of a constitutively active form of Stat3 significantly increased the rate of tumor progression in this model system. This effect of Stat3 was associated with increased expression of Twist, a transcription factor known to regulate genes involved in epithelial-mesenchymal transition (EMT) [27, 32]. Thus, use of this unique mouse model led to the discovery that Stat3 plays an important role not only in the initiation and promotion stages of skin carcinogenesis but also during the progression stage.

6. Deletion of Stat3 in Bulge Region Keratinocyte Stem Cells

Keratinocyte stem cells (KSCs) located in the bulge region of hair follicles are self-renewing cells that provide transit-amplifying cells necessary for hair regrowth and skin homeostasis [33]. In addition, KSCs in the bulge region are believed to be target cells for tumor development in two-stage chemical carcinogenesis of mouse skin [34, 35]. The K15 promoter has been reported to be specifically active in the bulge region of the murine hair follicle and has been used to characterize KSCs in the bulge region. For loss-of-function studies of bulge region KSCs, Morris [35] generated K15.CrePR1 transgenic mice. Cre-PR1 is a fusion protein that consists of Cre recombinase and a truncated form of the progesterone receptor that binds to the progesterone antagonist RU486 but not to endogenous progesterone [35, 36]. To further investigate the role of Stat3 in bulge region keratinocytes during multistage skin carcinogenesis, Dae et al. [25] utilized the K15.CrePR1 transgenic mouse model in combination with $Stat3^{flox/flox}$ mice. Using this inducible model, Stat3 deletion at the time of initiation in bulge region keratinocytes led to a significant reduction in tumor incidence and multiplicity (~80% reduction in papilloma formation). The K15.CrePR1 inducible system is not 100% efficient and the small number of papillomas obtained from these knockout mice stained positive for Stat3. This data indicated that Stat3 is absolutely necessary for tumor development, since the remaining Stat3-positive KSCs were selected during tumor promotion. In addition, DMBA treatment led to a significant increase in the number of apoptotic keratinocytes in the bulge region of the knockout mice. FACS analysis showed that there was a reduction in the percentage of bulge region KSCs that were positive for CD34 and α6-integrin in the knockout mice compared to the control mice 24 hours after DMBA treatment. Furthermore, the α6+CD34+ population from K15.CrePR1 × $Stat3^{flox/flox}$ mice showed a reduction of the signature Ha-ras codon 61 A^{182} to T mutation induced by topical application of DMBA [25]. Hence, Stat3 status influenced survival of DNA-damaged KSCs, since the absence of Stat3 led to increased apoptosis of bulge region KSCs following treatment with DMBA ultimately leading to reduced number of mutated cells available for clonal expansion during tumor promotion [25]. These data suggested that Stat3 plays an important role in the behavior of bulge region KSCs during the initiation step of skin tumor development by the two-stage chemical carcinogenesis protocol.

7. Stat3 in UVB-Induced Skin Carcinogenesis

The availability of both loss-of-function and gain-of-function mouse models for Stat3 in skin keratinocytes facilitated further study of this important molecule in skin carcinogenesis mediated by ultraviolet B (UVB) exposure. UV radiation, and in particular UVB exposure, is the major risk factor

for nonmelanoma skin cancer in humans [37]. Following exposure to UVB, the level of phosphorylated Stat3 (p-Stat3) is initially decreased, followed by a significant increase at later time points in the mouse epidermis. The levels of Stat3 target genes, such as cyclin D1, Bcl-x_L, and c-Myc, followed the changes in activated Stat3 in response to UVB irradiation [38]. Epidermis-specific Stat3-deficient mice were found to be very sensitive to UVB radiation as revealed by a higher number of sunburned and apoptotic cells following irradiation with UVB [22]. On the other hand, the epidermis of K5.Stat3C mice was significantly resistant to UVB-induced apoptosis [23]. Furthermore, additional studies showed that protection against UVB-induced apoptosis in Stat3C transgenic mice was not due to impaired DNA damage response. Instead, the status of Stat3 influenced the survival of cells containing UVB-induced DNA photoproducts, including those cells located in the bulge region of the hair follicles through regulation of antiapoptotic genes such as Bcl-x_L [23, 26]. In line with these observations, overexpression of Stat3C in K5.Stat3C mice enhanced UVB-induced skin carcinogenesis (both incidence and tumor multiplicity) compared to the wild-type controls [23]. In contrast, Stat3-deficient mice were resistant to UVB skin carcinogenesis compared to wild-type controls [23]. Thus, based on these studies Stat3 appears to play a strikingly similar role in both chemical and UVB-mediated skin carcinogenesis.

8. Studies Using Other Mouse Models Support an Important Role of Stat3 in Skin Carcinogenesis

As noted above Bcl-x_L is one of several antiapoptotic proteins regulated by Stat3 [39]. Deletion of Stat3 in keratinocytes leads to a concomitant and dramatic reduction in levels of Bcl-x_L [24]. To study the functional role of Bcl-x_L in skin carcinogenesis, skin-specific Bc-x_L-deficient mice were generated. In this model, Bcl-x_L expression is disrupted in the basal compartment of mouse epidermis using the bovine K5 promoter to drive expression of Cre recombinase (i.e., K5.Cre × Bcl-$x_L^{flox/flox}$ mice). A significant increase in apoptosis induced by either UVB irradiation or DMBA treatment was observed in the epidermis of Bcl-x_L-deficient mice. Furthermore, an increase in apoptotic cells was noted in hair follicle keratinocytes, including those located in the bulge region. Cell proliferation was not affected by Bcl-x_L deficiency following exposure to either UVB or TPA. Bcl-x_L-deficient mice were more resistant than wild-type controls to skin tumor development with delayed onset and reduced number of tumors using either UVB complete carcinogenesis or the DMBA/TPA two-stage regimen. Moreover, Bcl-2, Mcl-1, and survivin protein levels were increased in the epidermis of Bcl-x_L-deficient mice in the absence of stimuli. Furthermore, levels of these antiapoptotic proteins were also high in skin tumors from Bcl-x_L-deficient mice that developed in response to either UVB or two-stage carcinogenesis protocols. Collectively, these studies demonstrated that Bcl-x_L plays a role early in skin carcinogenesis through its antiapoptotic functions to enhance survival of keratinocytes, including bulge region KSCs, following DNA damage. These data also demonstrated that one of the antiapoptotic genes known to be regulated by Stat3 was likely mediating at least some of its action during the initiation stage of multistage skin carcinogenesis. However, deletion of Bcl-x_L did not fully recapitulate the actions of Stat3 deletion during initiation indicating that other Stat3-regulated genes are also likely involved in the action of Stat3.

It is also noteworthy that Stat3 function appears to be necessary for epidermal hyperplasia and susceptibility to skin tumor formation in other transgenic mouse models. In this regard, Stat3 has been implicated in proteins kinase c epsilon (PKCε) mediated UV skin carcinogenesis. Stat3 Tyr^{705} and Ser^{727} phosphorylation is increased in K5. PKCε transgenic epidermis after UV irradiation compared to controls. Like Stat3C, overexpression of PKCε in mouse skin (K5.PKCε) inhibited apoptosis, promoted cell survival, and induced development of SCCs in UVB skin carcinogenesis experiments [40–42]. These studies show an interaction between PKCε and Stat3 that leads to Stat3 activation by phosphorylation at Ser^{727}. Phosphorylation at Ser^{727} is necessary for full transcriptional activity of Stat3 *in vivo* and may aid in the development of SCCs in K5.PKCε transgenic mice [43]. Collectively, these studies demonstrate that Stat3 plays a critical role in the development of UVB-induced skin tumors through its effects on both survival and proliferation of keratinocytes [23].

In other studies, overexpression of human papillomavirus 8 (HPV8) in mouse skin using the K14 promoter (i.e., K14.HPV8 transgenic mice) leads to epidermal hyperplasia and the development of spontaneous SCCs [44]. These tumors exhibited an increased level of p-Stat3 Tyr^{705} [44]. In a two-stage carcinogenesis bioassay, loss of a single Stat3 allele reduced HPV8-mediated epidermal hyperplasia and skin tumorigenesis [44]. Loss of Stat3 also produced similar effects in a mouse model of basal cell carcinoma [45]. In this model, SmoM2 expression in the basal epidermal layer was also driven by the K14 promoter (i.e., K14.SmoM2 transgenic mice). Epidermal-specific knockout of Stat3 in the K14.SmoM2 transgenic mice significantly reduced SmoM2-mediated epidermal hyperplasia and tumor development [45]. These studies further demonstrate the importance of Stat3 in skin carcinogenesis.

9. Stat3 and Human Skin Cancer

Activation of Stat3 appears to play an important role in the development of human nonmelanoma skin cancer. Stat3 is found constitutively activated in UVB-induced SCCs from both mouse and human skin [22]. Increased p-Stat3 expression in human SCCs and basal cell carcinomas (BCCs) in comparison to normal skin has been observed in various retrospective studies [46–49]. This increased expression of p-Stat3 is inversely associated with cellular differentiation, with expression in poorly differentiated SCCs being significantly higher than in well-differentiated SCCs [46, 47]. A positive correlation between p-Stat3 expression and depth of tumor invasion, but not tumor size, was also observed [46, 47].

Furthermore, like SCCs derived from K5.Stat3C transgenic mice, human SCCs display a negative correlation between the expression of p-Stat3 and E-cadherin [27, 46]. Stat3-mediated downregulation of E-cadherin, in conjunction with Stat3-mediated induction of twist as observed in K5.Stat3C-derived SCCs, may promote EMT and contribute to the metastatic potential of SCCs. These data suggest that Stat3 plays an important role in the development and progression of human SCCs.

Head and neck cancers are a group of biologically similar cancers that stem from the lip, oral cavity, nasal cavity, paranasal sinuses, pharynx, and larynx. In this group of cancers, it is estimated that 90% are SCCs or head and neck squamous cell carcinoma (HNSCC), which originate from the mucosal lining (epithelium) in these tissues [50]. HNSCCs are very similar histologically to SCCs of the skin and both share a number of similar molecular alterations. In particular, as observed in cutaneous SCCs, preclinical and clinical studies have implicated Stat3 in the development and progression of HNSCCs [51–54]. In head and neck tumor tissue, Stat3 is found upregulated and constitutively activated (phosphorylated) and has a positive correlation with poor prognosis [51, 54]. Preclinical studies have shown that HNSCC cell lines stably transfected with a constitutively active STAT3 construct expressed elevated levels of STAT3 target genes, including Bcl-x_L and cyclin D1, leading to increased proliferation *in vitro* and more rapid tumor growth rates *in vivo* [55]. In addition, targeting Stat3 increases tumor cell apoptosis and decreased Bcl-x_L expression in a head and neck xenograft model [53]. In addition, reducing Stat3 activity by targeting upstream proteins has shown promise in HNSCC preclinical and clinical studies. In this regard, inhibiting EGFR-Stat3 pathway in 4-nitroquinoline-1-oxide (4-NQO-) induced murine model of oral carcinogenesis by erlotinib, a small molecule inhibitor of EGFR, inhibited development of preneoplastic lesions and oral tumors by approximately 70% with a concomitant decrease of Stat3 levels in erlotinib-treated mice [56]. Similarly, primary human oral cavity squamous cell cancers showed reduced levels of both EGFR and p-Stat3 after treatment with erlotinib compared to pretreated paired tissue [57]. Moreover, combined molecular targeting of Stat3 sensitizes cells to radiotherapy and small molecule chemotherapeutic agents *in vitro* [58, 59]. Together, these data have provided the basis for targeting Stat3 in HNSCC in a clinical setting (discussed below).

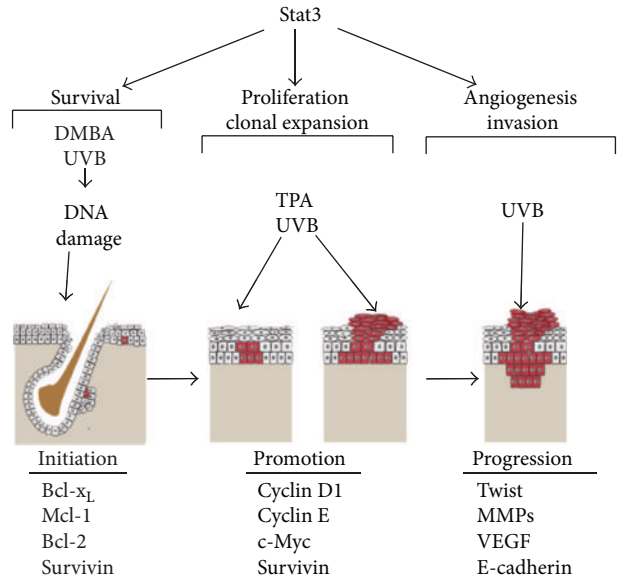

Figure 2: From studies in loss-of-function and gain-of-function mouse models, Stat3 has been shown to have a critical role in all three stages of skin carcinogenesis whether induced by chemicals (using the standard DMBA-TPA protocol) or by complete carcinogenesis with UVB. During the initiation stage, Stat3 aids in the survival of DNA-damaged keratinocyte stem cells (bulge region and possibly those in the interfollicular epidermis) induced by DMBA or UVB-irradiation by upregulation of prosurvival proteins such as bcl-x_L, bcl-2, mcl-1, and survivin. Clonal expansion of initiated cells (promotion) is carried out through repeated treatment with TPA or UVB. In this stage, Stat3-mediated induction of cell cycle regulatory proteins (e.g., cyclin D1, cyclin E, c-myc, and survivin) is necessary for keratinocyte proliferation, epidermal hyperplasia, and development of papillomas/premalignant lesions. Progression or conversion of papillomas to SCCs is denoted as a downward invading lesion that traverses into the dermal compartment. Stat3 plays a role in the progression stage by regulating genes involved in angiogenesis and invasion (e.g., VEGF, MMPs, twist, E-cadherin).

10. Perspectives and Future Directions

Collectively, the comprehensive set of skin-specific gain- and loss-of-function mouse models described above have revealed that Stat3 plays a critical role in all three stages of skin carcinogenesis induced by either chemical exposure or UVB irradiation (see Table 1 for a listing of these mouse models). Stat3 is required for survival of DNA-damaged KSCs and the proliferation necessary for clonal expansion of initiated cells during tumor promotion. In addition, Stat3 also appears to have a role in driving malignant conversion of skin tumors. Although UVB is a complete carcinogen, it possesses both initiating and promoting activity that can be distinguished experimentally. From the available data, Stat3 appears to play a very similar mechanistic role during UVB-mediated skin carcinogenesis. Figure 2 summarizes our current state of knowledge regarding Stat3 function in skin carcinogenesis.

The current data also suggest that targeting Stat3 activation may provide an effective strategy for both the prevention and treatment of skin cancer. However, targeting Stat3 directly has proven to be difficult and this molecule has largely seemed "undruggable" [60]. Many approaches have been taken to target Stat3 activity, including inhibition of upstream proteins such as receptor and nonreceptor tyrosine kinases, targeting of Stat3 SH2 domains to prevent phosphorylation/dimerization, inhibition of Stat3 DNA-binding activity, and inhibition of nuclear import [61]. Until recently, inhibition of upstream regulatory proteins has made the most progress in clinical trials [62]. Phase 0 (University of Pittsburgh, Pittsburgh, PA) and phase 1 clinical trials (MD Anderson Cancer Center, Houston, TX, USA and Otsuka Pharmaceuticals, Princeton, NJ, USA) evaluating

Table 1: Mouse models for evaluating Stat3 function in skin carcinogenesis.

Mouse model	Skin phenotype	Susceptibility to skin carcinogenesis	References
K5.Cre × $Stat3^{flox/-}$	(i) Defective wound healing (ii) Defective hair cycle from 2nd anagen onward	Not tested	[17]
K5.Cre × $Stat3^{flox/flox}$	No visible phenotype	Reduced susceptibility to both DMBA-TPA and UVB carcinogenesis	[21–23]
K5.$CreER^{T2}$× $Stat3^{flox/flox}$	No visible phenotype	Reduced susceptibility to both tumor initiation with DMBA and tumor promotion with TPA; UVB not tested	[24]
K15.CrePR1 × $Stat3^{flox/flox}$	No visible phenotype	Reduced susceptibility to tumor initiation by DMBA; UVB not tested	[25]
K5.Cre × Bcl-$x_L^{flox/flox}$	No visible phenotype	Reduced susceptibility to both DMBA-TPA and UVB carcinogenesis	[26]
K5.Stat3C	(i) Enlarged blood vessels in skin at birth (ii) Sparse hair coat (iii) Increased skin vascularization in adult mice (iv) Hypervascularization in response to mild wounding (e.g., tape stripping) (v) Develop scaly, hyperkeratotic lesions on tail (psoriasis) (vi) No spontaneous skin tumors	Enhanced susceptibility to DMBA-TPA and UVB skin carcinogenesis Enhanced progression of skin tumors to SCCs	[22, 23, 27, 28]

Stat3 inhibitors have recently been completed. In addition, a phase 1 (Isis Pharmaceuticals, San Diego, CA, USA) and an observational clinical trial (New York University, New York, NY, USA) are in the recruiting stages. The completed phase 0 clinical trial evaluated the safety of an oligonucleotide decoy targeting Stat3 by intratumoral injection in patients with HNSCC [63]. Interestingly, a single intratumoral injection with a Stat3 decoy showed decreased Stat3 target gene expression of cyclin D1 and Bcl-x_L in HNSCC biopsies [63]. Moreover, by circularizing the 15-base pair oligonucleotide with two hexaethylene glycol linkages, Sen and colleagues were able to inhibit tumor growth in preclinical mouse xenografts via systemic administration [63]. These studies offer promise for expanded phase 1 clinical trials in HNSCC patients and a wide range of malignancies that are dependent on Stat3 activation, including nonmelanoma skin cancers.

Recent evidence suggests a novel role for Stat3 in mitochondrial respiration, presumably via its interaction with electron transport chain components [64] and see again Figure 1. Mitochondrial localized Stat3 (referred to as mitoStat3) is necessary for Ha-ras-mediated transformation of mouse embryonic fibroblasts independent of Stat3 nuclear activity or tyrosine phosphorylation [65]. In addition, unphosphorylated Stat3 (U-Stat3), which was previously thought to be an inactive protein, has recently been shown to regulate gene transcription through a mechanism distinct from that of tyrosine-phosphorylated Stat3 dimers [66–68]. U-Stat3 has been shown to interact with nuclear factor-κB (NF-κB) (Figure 1) and regulate genes with κB elements, but it can also induce a cohort of genes through an NF-κB-independent mechanism [67]. Expression of U-Stat3 enhances hepatocellular carcinoma (HCC) formation in Ras-transformed $p19^{ARF-/-}$ hepatocytes [67, 69]. Whether mitoStat3 or U-Stat3 plays a role in epithelial carcinogenesis is unclear at the present time. Future studies should also evaluate these potential mechanisms and will likely require the development of additional mouse models. In addition, targeting or exploiting these noncanonical activities associated with Stat3 for both prevention and treatment strategies may also be warranted.

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Review Article

The Role of TGFβ Signaling in Squamous Cell Cancer: Lessons from Mouse Models

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TGFβ1 is a member of a large growth factor family including activins/inhibins and bone morphogenic proteins (BMPs) that have a potent growth regulatory and immunomodulatory functions in normal skin homeostasis, regulation of epidermal stem cells, extracellular matrix production, angiogenesis, and inflammation. TGFβ signaling is tightly regulated in normal tissues and becomes deregulated during cancer development in cutaneous SCC and many other solid tumors. Because of these diverse biological processes regulated by TGFβ1, this cytokine and its signaling pathway appear to function at multiple points during carcinogenesis with distinct effects. The mouse skin carcinogenesis model has been a useful tool to dissect the function of this pathway in cancer pathogenesis, with transgenic and null mice as well as small molecule inhibitors to alter the function of the TGFβ1 pathway and assess the effects on cancer development. This paper will review data on changes in TGFβ1 signaling in human SCC primarily HNSCC and cutaneous SCC and different mouse models that have been generated to investigate the relevance of these changes to cancer. A better understanding of the mechanisms underlying the duality of TGFβ1 action in carcinogenesis will inform potential use of this signaling pathway for targeted therapies.

1. Pathogenesis of Squamous Cell Carcinoma

Nonmelanoma skin cancer including both basal cell carcinoma and squamous cell carcinoma is the most frequent cancer among Caucasian populations, with incidence rates matching all other cancers combined in these groups [1]. Although exposure to ultraviolet radiation from the sun is the major risk factor for cutaneous squamous cell carcinoma (SCC), other risk factors also include chronic inflammation, and wounding, as well as exposure to arsenic, tobacco, and coal tar products [2]. The multistage mouse skin carcinogenesis model has been instrumental in defining the basic biology of SCC development in the skin and other epithelia. Mice are treated once with a carcinogen such as the polycyclic aromatic hydrocarbon dimethylbenz[a]-anthracene (DMBA) followed by 20 weekly applications of a nonmutagenic agent such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) that provides a microenvironment and proliferative stimulus that favors clonal outgrowth of initiated keratinocytes. Benign papillomas representing clonal outgrowths of keratinocytes with initiating mutations in the *Hras1* arise within 10–15 weeks. These are largely exophytic hyperplastic and hyperkeratotic lesions that retain the stratified organization of the normal epidermis and retain expression of normal differentiation markers such as keratin 1 and keratin 10. Many of these lesions are also promoter dependent, and if the stimulus is removed, tumor regression occurs. In the most benign lesions proliferation is confined to the basal layer as in the normal epidermis. Tumor progression in this model is associated with focal loss of keratin 1 and 10, expression of keratin 13, a keratin not normally expressed in the epidermis, expansion of the proliferative compartment, and changes in integrin expression. At the genetic level, tumor progression is associated with trisomy of chromosomes 6 and 7, loss of heterozygosity at the *Hras1* locus, followed by amplification of the mutated ras gene, and increasing aneuploidy [3]. Many additional genetic changes and signaling pathways that have been identified in this model are important for tumor promotion and progression and applicable to development of human SCC [4, 5]. In the typical 2-stage model using inbred strains such as SENCAR A which are highly sensitive to tumor promoters, most papillomas do not convert to SCC.

A number of studies have documented the existence of subpopulations of papillomas with differing potential for malignant progression, and at early time points, this is reflected in distinct patterns of gene expression [6, 7].

2. TGFβ1 Signaling Pathway Overview

From its initial identification as a major negative regulatory pathway for epithelial cell proliferation, Transforming growth factor-beta (TGFβ1) and its signaling pathway has been identified as a critical regulator of cancer development and progression in humans and in many experimental cancer models in mice [8, 9]. The cell surface receptor for TGFβ1 is a complex of TGFβ1 type I and type II transmembrane receptors (TβRI and TβRII), both of which are serine threonine kinases. Binding of TGFβ1 to TβRII recruits TβRI into a heterotetrameric complex resulting in phosphorylation and activation of the cytoplasmic domain of TβRI by TβRII kinase (Figure 1). This activates the kinase activity of the TβRI towards its substrates the R-(receptor activated) Smads which for TGFβ1 and activin are Smad2 and Smad3. Smad1, 5, and 8 are R-Smads activated by BMP and its specific transmembrane receptors. Once phosphorylated, Smad2 or Smad3 form a complex with the co-Smad, Smad4, and translocate to the nucleus to regulate TGFβ responsive genes, through either specific Smad-binding elements, other suppressive elements or through interaction with other transcription factors [10, 11]. TGFβs can also activate members of the mitogen-activated protein (MAP) kinase signalling molecules, including JNK, p38, ERKs, and the PI3 K/AKT pathway [9].

There are a number of mechanisms for downregulating or inhibiting TGFβ signaling, including phosphatases which dephosphorylate Smad2 and 3 and attenuate signal strength [12, 13] inhibitory or I-Smads, Smad6, and Smad7 which block type I receptor phosphorylation of R-Smads [14, 15] and also recruit Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2 ubiquitin ligases to cause degradation of the type I receptor and Smads [16]. Other ubiquitin ligases such as the HECT (homologous to the E6-accessory protein C-terminus)-type E3 ubiquitin ligases are also important in regulating Smad levels [17]. Additional cell surface coreceptors, predominantly betaglycan, and endoglin modulate TGFβ1 family members binding to their signaling receptor [18, 19]. There are three TGFβ's: TGFβ1, β2, and β3, with similar but not identical receptor affinities and biological activity, and distinct patterns of expression [20]. All bioactive TGFβ's are 25 Kd disulfide-linked homodimers generated from the C-terminal 112 amino acids of the primary translation product (390 amino acids for TGFβ1) [21]. Production of bioactive TGFβ is also a complex process. TGF-β1 is secreted as a biologically inactive molecule called the small latent complex (SLC) that is unable to bind to its receptor [22]. The SLC consists of the active cytokine noncovalently linked to its propeptide called the latency-associated peptide (LAP) [22]. Additional proteins known as latent TGFβ-binding proteins LTBP-1, 3, and 4 form disulfide bonds with the LAP to generate the large latent complex [23]. The LTBPs are structurally similar and part of the fibrillin protein family, an extracellular matrix protein. TGFβs are secreted as a complex termed the large latent complex (LLC) in which the LTBP is covalently bound to the TGFβ propeptide, and on secretion, the (LLC) may be covalently linked to the extracellular matrix (ECM) [24]. Several mechanisms for the activation of latent TGFβ complexes are known, and a diverse group of activators, including proteases, thrombospondin-1, the integrin $\alpha_v\beta_6$, reactive oxygen species (ROS), and low pH can activate TGFβ [23–25].

3. Alterations in TGFβ1 Pathway in Human SCC

A number of immunohistochemical and mutational analysis studies have been done in human SCC to determine what changes in the TGFβ signaling pathway are associated with tumor development. A number of studies in human head and neck SCC (HNSCC), cutaneous, and cervical SCC have been done by IHC with both increase and decrease relative to adjacent normal tissue reported. In studies with largest sample siz,e the results support a decrease in TGFβ1 expression in HNSCC and cervical SCC [26–29], while other studies have shown an increase in TGFβ1 expression in human cutaneous SCC [30]. It is not clear if the tumors with elevated ligand expression represent a distinct subset of tumors, but we and others have linked decreased or loss of TGFβ1 expression with increased risk for malignant progression in the 2-stage skin carcinogenesis model [31, 32]. A number of different mutations in both the type I and type II receptors with distinct biological properties have been identified in HNSCC, but these are present at low frequency (up to 10%) in human SCC. In contrast, downregulation of expression of either receptor is much more frequently observed in up to 60% of tumor samples (see Xie and Riess, 2011, for comprehensive review) [33]. Only a handful of mutations in the Smad2 or Smad4 genes have been identified in human SCC, and none for Smad3. However, loss of heterozygosity (LOH) has been observed in Smad4; LOH occurs in 30–50% of HNSCC and esophageal SCC tumors and cell lines [34–37]. In a sample of 36 HNSCC Smad4 mRNA levels were reduced by about 50% compared to normal control mucosa in 86% of tumors, and Smad4 protein was reduced or not detected similarly [35]. Similarly in a sample of 85 human skin SCC, Smad4 and Smad2 proteins were each absent in 70-% of the tumors relative to normal skin, with Smad2 loss observed in 100% of poorly differentiated tumors. A similar reduction in Smad2 and Smad4 mRNA levels in poorly differentiated tumors was also observed [38]. In two large tissue array studies of HNSCC (170 and 340 samples), 18.5% had no detectable expression of phospho-Smad2, 40% had no detectable phospho-Smad3 (indicating likely downregulation of the pathway), and 12% did not express Smad4 [39, 40]. Among 198 patients with survival information, those with pSmad2/pSmad3 negative tumors had a better overall survival rate compared to those with pSmad2-positive SCC [40]. It is not clear whether the wide variance in percentage of skin or HNSCC exhibiting loss of Smad immunostaining represents differences in patient

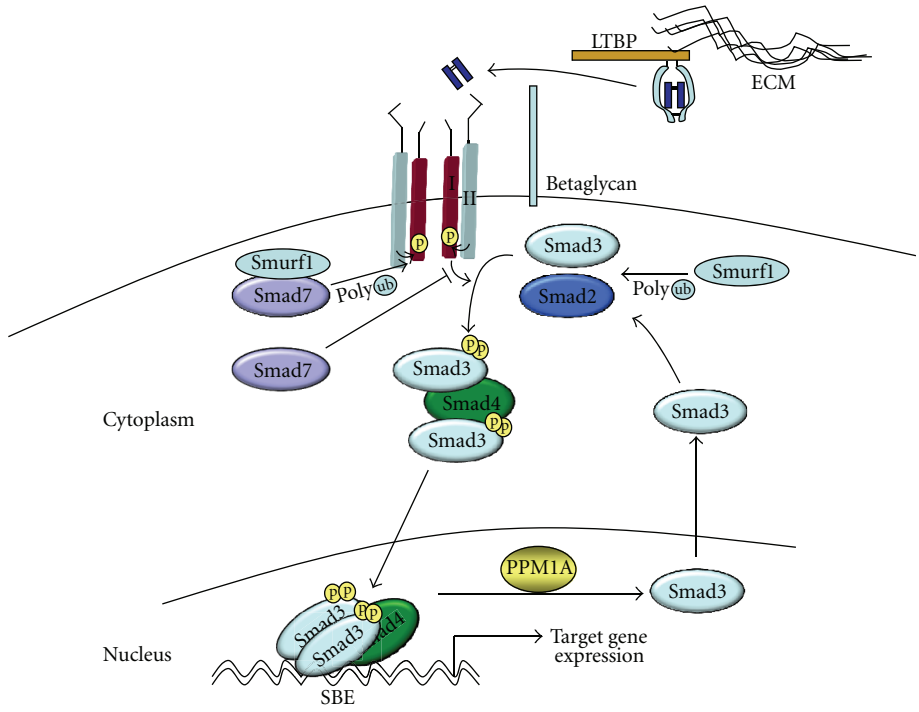

Figure 1: Schematic of TGFβ1 signaling pathway and its regulation. TGFβ1 is secreted and sequestered in the extracellular matrix as a biologically inactive complex composed of the TGFβ1 peptide linked to the latency-associated peptide (LAP) and a member of the latent TGFβ-binding protein (LTBP) family. Activation of latent TGFβ1 allows binding of active peptide dimer to TβRII and formation of a heterotetrameric receptor complex Between TβRI and TβRII. Coreceptors such as betaglycan act to enhance TGFβ binding to its receptors. TβRII, phosphorylates the cytoplasmic domain of TβRI and activates its serine-threonine kinase activity towards the R-Smads, Smad2, or Smad3, Phosphorylation of an R-Smad for allows complex formation with Smad4 and translocation to the nucleus, where binding to SBE target sites in gene promoters activates transcription with many other cofactors. Dephosphorylation of R-Smads by Smad phosphatases such as PPM1A attenuate signaling and cause Smads to recycle to the cytoplasm. Smad7 can block type I receptor phosphorylation of R-Smads and in conjunction with E3 ubiquitin ligases such as Smurf1 cause polyubiquitination and degradation of TβRI. Smurf1 and similar proteins have also been implicated in degradation of R-Smads.

population or methodology. Nevertheless, loss of components of the TGFβ1-signaling pathway represent a significant component of HNSCC and cutaneous SCC pathogenesis.

4. Mouse Models of Altered TGFβ1 Signaling in Skin Cancer

4.1. TGFβ Receptors. Mice expressing a dominant negative *Tgfbr-2* (delta-TβRII) transgene in the basal and suprabasal epidermis exhibited a hyperkeratotic and thickened skin at birth, with increased basal and suprabasal proliferation and altered differentiation [41]. Primary keratinocytes from these mice were resistant to TGFβ1-induced growth inhibition as expected [41]. In a 2-stage chemical carcinogenesis experiment with the delta-TβRII mice, benign papillomas appeared 2 weeks earlier than in control nontransgenic mice, and there was a 2-fold increase in tumors from 4 per mouse to 8 per mouse in the delta-TβRII mice [42]. While many papillomas that arise in the 2-stage model are promoter dependent, and regress when promotion is stopped, papillomas that formed in delta-TβRII mice did not regress when TPA promotion was stopped but progressed rapidly to squamous cell carcinoma [42] (Table 1). This suggests that suppression of TGFβ1 signaling converts benign tumors from promoter dependent to promoter-independent lesions, a characteristic of tumors at high risk for malignant conversion. Surprisingly, TPA treatment alone induced papilloma formation suggesting that inhibition of TGFβ1 signaling in some cells could act as an initiating event. Tumors from the delta-TβRII mice exhibited altered cell cycle regulation and reduced expression of TGFβ1 regulated cyclin-dependent kinase inhibitors such as $p15^{ink4b}$, $p21^{waf1}$ and p27, but no evidence for chromosome instability [43]. Additionally tumors that formed in mice with blocked TGFβ1 signaling had increased neovascularization and changes in expression of positive regulators of angiogenesis including vascular endothelial growth factor (VEGF) and TGFβ1 and reduced expression of the angiogenesis inhibitor thrombospondin-1 [42]. However, it is not clear if these are direct effects of inactivation of TGFβ signaling or simply reflective changes of a more progressed tumor phenotype.

Using a complimentary approach several groups have generated tissue-specific conditional knockouts of the type 2 and type 1 receptor. Deletion of *Tgfbr2* in the epidermis

Table 1: Skin and oral carcinogenesis studies with mouse models of TGFβ1 signaling.

Signaling component	Mouse model	Study details	Phenotype	Reference
		TGFβ1 overexpression		
TGFβ1 ligand	K6-TGFβ1*, K10-TGFβ1 DMBA/TPA	Constitutive and inducible suprabasal expression	Suppressed papilloma formation, increased malignant conversion and spindle cell carcinoma	Cui et al., 1996 [44]
	Loricrin-TGFβ1 gene switch DMBA/TPA	Long-term expression in papillomas	Increased EMT, invasion, and metastasis	Weeks et al., 2001 [45]
	K5rTA x tetOTGFβ1 DMBA/TPA	Short-term expression in papillomas	Growth arrest, regression, and tumor inflammation	Mohammed et al., 2010 [46]
		TGFβ1 knockdown		
	Tgfb1+/− versus *Tgfb1*+/+ DMBA/TPA	Germline *Tgfb1* heterozygote	Reduced papillomas in TGFβ1+/−, increased malignant conversion	Pérez-Lorenzo et al., 2010 [47]
	Tgfb1−/−; v-*Ras*^{Ha} xenotransplantation	Skin grafts of PMEK onto athymic mice	SCC with TGFβ1−/−, papilloma with TGFβ1+/− and +/+	Glick et al., 1994 [48]
TβRI	DMBA/TPA pharmacological inactivation	Topical SB431542 during TPA promotion	Reduced papilloma, increased conversion	Mordasky Markell et al., 2010 [49]
	DMBA/TPA pharmacological inactivation	Systemic LY2109761 during TPA promotion	Increased malignant phenotype of SCC	Connolly et al., 2011 [50]
	K14Cre^{ER} x *Tgfb1*$^{fl/fl}$ DMBA	deletion of TβRI in oral mucosa	Accelerated HNSCC with AKT activation	Bian et al., 2009 [51]
	K14Cre^{ER} x *Tgfb1*$^{fl/fl}$ x *Pten*$^{fl/fl}$	deletion of TβRI and PTEN in oral mucosa	Accelerated HNSCC	Bian et al., 2012 [52]
TβRII	Loricrin-Δ*Tgfbr*2	Epidermal expression of dominant negative type II receptor	Reduced tumor latency, increased SCC	Go et al., 1999 [42] Go et al., 2000 [43]
	K5Cre^{Pr1} x *Tgfbr*2$^{fl/fl}$ DMBA or x K-Ras^{12D}	Oral mucosa deletion of TβRII	HNSCC only with DMBA or K-Ras	Lu et al., 2006 [53]
	K14-Cre x *Tgfbr*2$^{fl/fl}$	Epidermal deletion of TβRII	No skin tumors, spontaneous anogenital SCC	Guasch et al., 2007 [54]
	K14-Cre x *Tgfbr*2$^{fl/fl}$	v-Ras^{Ha} xenotransplantation	Aggressive SCC	Guasch et al., 2007 [54]
R-Smads	K5Cre^{Pr1} x *Smad*2$^{fl/fl}$ DMBA/TPA	Basal/stem cell deletion of Smad2 in epidermis	Increased tumors accelerated more aggressive SCC	Hoot et al., 2008 [38] Hoot et al., 2010 [55]
	MMTV-Cre x *Smad*4$^{fl/fl}$	Epidermal deletion of Smad4	Hair follicle defects spontaneous SCC	Qiao et al., 2006 [56]
	K5Cre^{Pr1} x *Smad*4$^{fl/fl}$	Deletion of Smad4 in oral mucosa	Spontaneous HNSCC w/genomic instability increased inflammation normal and tumor tissue	Bornstein et al., 2009 [35]
	Smad3−/− DMBA/TPA	germline Smad3 null	Suppressed tumor formation, resistance to TPA	Li et al., 2004 [57]
	Smad3−/−; v-Ras^{Ha}	Primary mouse keratinocyte skin grafts	Progression to SCC	Vijaychandra et al., [58]
I-Smads	Smad7 + v-Ras^{Ha} Smad6 + v-Ras^{Ha}	Primary mouse keratinocyte skin grafts	Smad7: rapid progression to SCC Smad6: papilloma	Liu et al., 2003 [59]
TGFβ1/TβRII	TGFβ1 gene switch x Δ*Tgfbr*2 DMBA/TPA	Inducible expression of TGFβ1 in papillomas with inhibition of TGFβ receptor	Suppressed EMT in papillomas, increased metastasis	Han et al., 2005 [30]

*Unless otherwise indicated TGFβ1 transgene used was TGFβ1$^{S223/S225}$ constitutively active mutant
fl/fl: floxed alleles.
Δ: truncation of cytoplasmic domain generating dominant negative receptor.
DMBA/TPA indicates 2-stage chemical carcinogenesis protocol.
Cre^{ER}: tamoxifen-inducible Cre recombinase.
Cre^{Pr1}: rU486 inducible Cre recombinase.

and oropharyngeal epithelium with an inducible Keratin 5 (K5)-Cre by itself only caused slight epithelial hyperplasia after one year. However, when crossed onto mice expressing a K-Ras transgene or when *Tgfbr2* mice were initiated with DMBA, the development of SCC was greatly accelerated and some SCC became metastatic [53]. Similar to results with the DN-TβRII there was increased expression of TGFβ1 in the head and neck tumors that developed which correlated with increased inflammation and angiogenesis [53]. Deletion of *Tgfbr2* with a Keratin 14 (K14)-Cre transgene also had only mild effects on the epidermis, with increased epidermal proliferation balanced by increased apoptosis [54]. However, skin grafts of Ha-Ras^{V12} retrovirus transduced *Tgfbr2* null keratinocytes rapidly developed into large, aggressive tumors. Thus, loss of TGFβ signaling reset epidermal homeostasis but did not by itself cause significant precancerous changes in the epidermis, but facilitates rapid malignant progression in the presence of oncogenic Ras. In contrast invasive SCC developed spontaneously in the anogenital epithelium, which also expresses K14, and this is likely due to the elevated basal proliferation and turnover in this tissue [54].

Similar observations were made using K14-CreER mice to drive an inducible conditional deletion of the *Tgfbr1* gene, although the focus in these studies was epithelia of the oral cavity [51]. Again in the absence of initiating mutations, deletion of *Tgfbr1* did not result in tumor formation, but with DMBA treatment HNSCC developed in approximately half of the mice, preceded by enhanced proliferation and decreased apoptosis in basal epithelial cells and activation of the PI3-kinase/AKT pathway [51]. In a recent followup study from this group, conditional deletion of both *Tgfbr1* and the tumor suppressor phosphatase and tensin homologue (PTEN) which inhibits the PI3-kinase/AKT pathway leads to rapid development of SCC with near complete penetrance. These tumors exhibit expansion of the putative cancer stem cell compartment, escape from senescence and an immunosuppressive inflammatory tumor microenvironment [52]. Taken together these results clearly show the tumor suppressor function of both type I and type II TGFβ receptors, although the inactivation of this signaling pathway by itself does not appear to be enough to cause tumor formation. However, it is not clear why overexpression of the truncated dominant negative type II receptor has such profound effects by itself on epidermal homeostasis while deletion of either type I or type II has relatively mild effects. One possibility is that the truncated type II receptor is able to interact with and inhibit function of other type I receptors for members of the TGFβ superfamily such as activin receptors, and this exaggerates the effect on epidermal hyperproliferation. Although speculative, interactions between TGFβ and activin receptors have been described in endothelial cells [60], and epidermal-specific deletion of activin receptor type 1B causes epidermal hyperproliferation along with significant hair cycle defects [61]. Although by itself inactivation/loss of either TGFβ receptor does not cause tumor formation, cooperation with either a RAS oncogene or activation of the PI3-kinase/AKT pathway through PTEN loss generates SCC in squamous epithelia.

4.1.1. Pharmacological Inactivation of TGFβ Receptors. A number of small molecule inhibitors of TβRI and related serine threonine kinases have been developed [62, 63] and been shown in a number of different cancer models to block TGFβ responses in tumor cell lines and in cells in the tumor stroma [64, 65]. Two studies have been published using ALK5 inhibitors in the mouse skin carcinogenesis model. In the first, FVB/*n* mice were initiated with DMBA, and the ALK5 inhibitor SB431542 was applied topically during tumor promotion. Mice that were treated with TPA and SB431542 developed significantly fewer papillomas than TPA alone, but those tumors that did form had a higher frequency of conversion to SCC. SB431542 treatment blocked TPA-induced Smad2 phosphorylation in keratinocytes and dermal cells, and TPA-induced skin inflammation, suggesting that the induction of TGFβ1 by TPA [66] and subsequent activation of signaling in keratinocytes and stromal cells is critical for tumor outgrowth, possibly through effects of TGFβ1 on inflammatory gene expression [49]. Early papillomas that did form under conditions of inhibited TGFβ signaling, however, had elevated intratumor inflammatory infiltrates and reduced expression of squamous differentiation, markers, similar to SCC. A subsequent *in vitro* study also provided evidence that pharmacologic inhibition of ALK5 with SB431542 induced terminal differentiation in primary mouse keratinocytes expressing an inducible oncogenic human H-*RAS*^{V12G} transgene [67], and this could be an additional mechanism for suppression of papilloma formation. In a second chemical carcinogenesis study, mice were placed on systemic LY2109761, a potent inhibitor of both TβRI and TβRII, during tumor promotion. While in this study, the effect was seen on tumor incidence or latency, the SCC that formed under conditions of sustained type I/type II kinase inhibition had elevated levels of pSmad2 and appeared resistant to the drug and expressed markers of a more aggressive and invasive phenotype [50]. While it is not clear how topical versus systemic inhibition of TGFβ signaling may differentially affect tumor formation, taken together these data suggest that subpopulations of initiated keratinocytes may respond differently to inhibition of TGFβ signaling either within themselves or the tissue microenvironment. One population appears to require TGFβ signaling for clonal expansion in response to TPA, while in the other inhibition of TGFβ, it signaling appears to promote outgrowth and more rapid progression, possibly selecting for premalignant cells with pathway activation via a distinct mechanism.

4.2. Smads

4.2.1. Smad2. In a 2-stage chemical carcinogenesis study, *Smad2*+/− mice had accelerated skin tumor formation that was characterized by moderately differentiated SCC with local invasion [68]. Mice with a keratinocyte-specific *Smad2* deletion exhibited accelerated formation and malignant progression of chemically induced skin tumors compared with WT mice, and the Smad2−/− tumors were poorly differentiated and exhibited epithelial to mesenchymal transition (EMT) characterized by reduced E-cadherin expression

[38]. In addition, these tumors were angiogenic and this was associated with epithelial overexpression of HGF and endothelial activation of the HGF receptor c-Met [55]. Both increased Snail and HGF expression in *Smad2*−/− tumors was directly linked to a switch from Smad2 repressive activity to increased binding of Smad4 to transcriptional coactivators at the Snail and HGF promoters [38, 55]. This study also provided evidence for a correlation of Snail and HGF expression in human SCC, where Smad2 expression was lost compared to Smad2 positive tumors. These studies contrast significantly with an earlier analysis of the role of Smad2 in conversion of murine squamous cell carcinoma to spindle cell carcinoma cell phenotype. Spindle cell carcinoma are a highly undifferentiated and invasive tumor type in the epidermis thought to result in part from an EMT of SCC cells, dependent on TGFβ1 signaling [69, 70]. Overexpression of Smad2 in SCC cells in the context of elevated H-Ras causes EMT to a spindle cell phenotype and increases invasiveness and metastasis [71]. Although the conflict may arise from the analysis of Smad2 function in the context of the intact epidermis versus cell lines it is also possible that long-term loss of Smad2 in the epidermis causes compensatory mechanisms that generate the same phenotype as Smad2 overexpression. Nevertheless, loss rather than overexpression phenocopies human skin cancer [38]. However, it remains to be determined how the mouse model fits with observed increased survival of patients with pSmad2/pSmad3 negative HNSCC relative to to those with pSmad2-positive SCC [40]. Deletion of Smad2 in papillomas or SCC or conditional overexpression of Smad2 would help resolve these issues.

4.2.2. Smad3. In two chemical carcinogenesis studies using Smad3+/− and Smad3−/− mice, it was found that in contrast to Smad2 deletion, Smad3+/− mice developed fewer tumors compared to wild-type controls [68]; *Smad3*−/− mice also developed fewer papillomas than wildtype controls and did not progress to SCC [57]. Additionally, *Smad3*−/− epidermis and keratinocytes were significantly resistant to the proliferative and proinflammatory effects of TPA, suggesting that Smad3 is critical for tumor promotion by TPA [57]. In contrast to these whole animal knockout studies, when *Smad3*−/− keratinocytes were transduced with a v-*Ras*^{Ha} oncogene and skin grafted onto athymic mice, they rapidly progressed to SCC, while wildtype controls formed benign papillomas as expected from previous studies [58]. v-*Ras*^{Ha}-transduced *Smad3*−/− keratinocytes were less sensitive to TGFβ1-induced growth arrest *in vitro* and were able to escape Ras-induced senescence, that is mediated in part through upregulation of TGFβ1 expression and signaling [72]. Overexpression of Smad3 but not Smad2, accelerated senescence in v-*Ras*-^{Ha}-transduced wildtype keratinocytes and rescued the senescence defect in *Smad3*−/− keratinocytes [58]. The ability of TGFβ1 to induce growth arrest and senescence in v-*Ras*^{Ha} keratinocytes was linked to the induction of p16ink4a and p19ARF, and this was dependent on intact Smad3 [73]. These results suggest that Smad3 does indeed function as a tumor suppressor in keratinocytes, and these cells are not inherently resistant to malignant conversion. However it is clear that Smad3 function in keratinocytes or other resident or infiltrating cells in the skin are critical for tumor promotion, further studies with epidermal specific deletion of Smad3 will provide insight as to the lack of SCC formation in *Smad3*−/− mice.

4.2.3. Smad4. In two models of epidermal-specific Smad4 deletion, the mice exhibited progressive hair-loss due to defects in hair follicle cycling, and the majority developed spontaneous development of SCC within 1 year [56, 74]. Tumors were characterized by altered expression of TGFβ1-regulated cell cycle genes including c-Myc, p21, and p27. Significantly, *Smad4*−/− tumors exhibited inactivation of PTEN and activation of AKT [56], and codeletion of the Smad4 and PTEN resulted in accelerated hair loss and skin tumor formation [74]. Similar results in HNSCC suggest that activation of AKT is a critical event in tumorigenesis mediated by inactivation of the TGFβ1-signaling pathway.

4.2.4. I-Smads. Transgenic mice in which Smad7 was targeted to the basal layer of the skin with a keratin 5 promoter exhibited hyperproliferation in the skin and other stratified epithelia, but these animals died within 10 days after birth [75]. More recently, an inducible Smad7 transgenic has been developed, and in these animals, induction of Smad7 during wounding enhanced keratinocyte proliferation and accelerated reepithelialization through effects on keratinocyte migration and stromal cells in the wound [76]. Glick and colleagues used retroviruses to coexpress Smad7 or Smad6 in primary mouse keratinocytes with v-*Ras*^{Ha} oncogene retroviruses and transplanted these cells onto athymic mice using a skin grafting system [59]. Skin grafts of keratinocytes transduced with v-*Ras*^{Ha} alone generated papillomas as expected, as did v-*Ras*^{Ha} and Smad6. In contrast skin grafts of v-*Ras*^{Ha}- and Smad7-transduced keratinocytes rapidly progressed to SCC [59]. These results demonstrate that Smad7 inhibition of TGFβ1 signaling can drive progression of Ras oncogene expressing primary keratinocytes but BMP signaling and Smad6 inhibition of BMP signaling do not play a significant role in progression in this model.

4.3. Non-Smad-Signaling Pathways. Many different non-Smad-signaling pathways downstream of the TGFβ receptor with likely impact on various aspects of the cancer phenotype have been identified using cultured cells [77]. Yet, the importance of this as a component of TGFβ1 signaling in tumor formation and progression *in vivo* has been more difficult to prove simply because these pathways are activated by many upstream-signaling molecules, and appear to synergize with Smad pathways to generate maximal biological responses [78–80]. The most clearcut evidence for importance of non-Smad signaling by TGFβ receptors in a cancer phenotype comes from analysis of TGFβ1 mediated EMT. TGFβ associated kinase 1 (TAK1) is a MAPK kinase kinase (MAPKKK) family member that is important for TGFβ-induced activation of the p38 MAPK pathway (Yamaguchi et al. 1995), although it can also activate other pathways such as NFkB and JNK. In NMuMG, mouse mammary epithelial cells

knockdown of TRAF6, a key intermediate between TβRI and TAK1, blocked the ability of TGFβ1 to induce EMT, but had no effect on Smad-dependent responses [81]. TGFβ1 can also induce EMT through activation of the PI3Kinase/Akt/mTOR pathway, and this has been studied in both the murine mammary gland NMuMG cells and human HaCaT keratinocytes [82, 83]. While inhibition of mTORC1 in these cells with rapamycin did not block TGFβ1-induced EMT [83], inhibition of TGFβ1-induced activation of mTORC2 did block EMT [84]. Recent reviews provide more detailed analysis of non-Smad signaling pathways and potential impact on cancer [77, 85] and potential targets for inhibition of TGFβ1 driven invasion and metastasis. However, direct demonstration that these pathways are specifically activated by TGFβ *in vivo* is a significant challenge.

4.4. TGFβ Ligand. Although there are three distinct TGFβ family members, TGFβ1, β2, and β3 all of which have been detected in skin and skin tumors, nearly all mouse models have focused on TGFβ1. Both TGFβ2 and TGFβ3 null mice have been generated, and these have distinct developmental defects that lead to perinatal lethality [86, 87]. No skin targeted knockouts of these genes or overexpression models have been developed that would specifically allow determination of a distinct role in carcinogenesis. Increased levels of TGFβ1 occurs in primary keratinocytes expressing oncogenic v-Ras^{Ha} [88], and TPA and other tumor promoters rapidly induce TGFβ1 expression in the suprabasal layers of the epidermis [66, 89]. TPA also induces expression of TβRII in normal epidermis [90]. Thus TGFβ1 expression is likely elevated in the microenvironment surrounding an expanding clone of initiated keratinocytes. Overexpression of TGFβ1 in the epidermis blocks TPA-induced hyperplasia and papilloma formation [90] and *Tgfb1*−/− keratinocytes transduced with a v-Ras^{Ha} retrovirus rapidly form SCC in athymic mouse skin grafts, while *Tgfb1*+/+ keratinocytes develop only benign papillomas [48]. Similarly, benign papillomas with a high risk progression phenotype exhibit reduced expression of TGFβ1 [31, 32]. In contrast to these studies *Tgfb1*+/− mice develop fewer chemically induced benign tumors than *Tgfb1*+/+ mice, although the tumors formed in *Tgfb1*+/− mice had a higher frequency of malignant conversion [47]. TPA-induced proliferation was reduced in *Tgfb1*+/− skin and in tumors that formed in *Tgfb1*+/− mice. Surprisingly while TPA-induced inflammation was exaggerated in *Tgfb1*+/− skin, tumors formed in *Tgfb1*+/+ mice had increased tumor inflammation, and this was paralleled by elevated proinflammatory cytokine expression in v-Ras^{Ha}-transduced *Tgfb1*+/+ keratinocytes compared to *Tgfb1*+/− keratinocytes [47]. These results suggest that within the local microenvironment of the initiated keratinocyte physiological levels of TGFβ1 function in either an autocrine or paracrine way to enhance tumor outgrowth but act to suppress malignant progression.

Several transgenic mouse models overexpressing either active or latent TGFβ1 in the basal layer of the skin exhibit an inflammatory infiltrate coupled with angiogenesis and hyperproliferation [91, 92]. It is possible that elevated TGFβ1 by itself acts as a tumor promoter, although this has not been directly demonstrated. More likely the effect may be indirect through the actions of inflammatory cytokines produced by infiltrating immune cells which could counteract the growth inhibitory effects of TGFβ1 on initiated cells [44, 45]. Lesions that develop in mice overexpressing TGFβ1 have high levels of proinflammatory cytokines and chemokines similar to Th1 inflammatory diseases such as psoriasis [91], and the pattern of gene expression in inflamed skin is similar but not identical to that of psoriasis [93], where TGFβ1 is also overexpressed in lesional keratinocytes and sera [94, 95]. Expression of TGFβ1 in the oral mucosa also caused a similar inflammatory and angiogenic response [96]. Thus, in this context, TGFβ1 overexpression appears to provoke a chronic inflammatory response, although is not yet clear if the inflammatory infiltrate is similar to that following TPA treatment or wounding. Nevertheless, the hyperproliferation is likely due to either downregulation of TGFβ1-signaling components or secondary factors produced by the inflammatory cells that can stimulate keratinocyte proliferation. The psoriasis-like inflammation that develops in TGFβ1 overexpressing mice however does not appear dependent on T cells [97] or the IL17/IL23 axis [98]. TGFβ1 is chemotactic for certain innate immune cells, such as macrophages [99] mast cells [100, 101], and neutrophils [102] and it is possible that directs effects of TGFβ1 on innate immune cells recruitment to the skin is responsible for the inflammatory phenotype. We have shown recently that as early as 2 days after elevation of TGFβ1 in the epidermis there is an increased numbers of $B220^+$ plasmacytoid dendritic cells (pDCs), $Langerin(CD207)^+$ dermal dendritic cells and $CD11b^+$ and $CD11b^-$ dermal DCs (dDCs) concomitant with increased expression of CD86, a maturation marker in skin-draining lymph nodes (LNs). This was accompanied by increased T cell activation in the LN and an increased contact hypersensitivity responses to topical DNFB. In addition there was a significant influx of plasmacytoid and dermal dendritic cells into the skin following TGFβ1 induction [103], and pDCs have been strongly linked to the initiation of chronic inflammation in psoriasis [104]. We observed a similar influx of DC into papillomas expressing TGFβ1, although these were not characterized as completely [46]. Other studies have shown that overexpression of TGFβ1 in xenotransplanted human SCC lines traps dendritic cells within the tumor [105, 106] thereby allowing escape from antitumor immunity. These results suggest that activation of skin DC by TGFβ1 is linked to its proinflammatory function in normal skin and this may have significant consequences for the function of this cytokine in skin carcinogenesis.

In contrast to the suppressive effects of TGFβ1 overexpression on papilloma formation [45, 90], continuously elevated levels of TGFβ1 appear to promote formation of highly undifferentiated spindle carcinoma [90], and 15 weeks of TGFβ1 overexpression in benign papillomas lead to increased invasiveness and metastases [45]. These results support the concept that has been studied *in vitro* in detail that TGFβ1 can cause an EMT-like phenotype in SCC cells. However it is not clear if the *in vivo* studies represent selection for more malignant cells under the influence of high

tissue levels of TGFβ1, since short-term expression of TGFβ1 in benign papillomas causes significant tumor regression coupled with a neutrophilic and T cell infiltrate into the tumors [46]. To examine whether TGFβ1 signaling in tumor cells was required for suppression of EMT and metastasis, Wang and colleagues made compound transgenic mice expressing an inducible TGFβ1 and delta-TβRII transgenes. Here, TGFβ1 overexpression in late-stage papillomas with wildtype Type II receptor did not inhibit proliferation but increased metastasis and EMT. TGFβ1-induced EMT was blocked by the delta-TβRII transgene, but metastasis was not [30]. Tumors overexpressing TGFβ1 with blocked TGFβ1 signaling had greater metastasis than tumors with each transgene alone, although some non-Smad pathways of TGFβ1 signaling appeared to be intact in the compound transgenic tumors. Thus, it appears that TGFβ1-mediated EMT is a tumor cell autonomous effect, but metastasis induction may involve changes in the tumor microenvironment or altered TGFβ1 signaling in tumor cells.

4.4.1. Coreceptors and Binding Proteins. These proteins regulate interaction of TGFβ1 with receptors and control extracellular levels of active TGFβ1 and so are considered here. Although endoglin is expressed primarily on vascular endothelial and smooth muscle cells, it has been detected in normal mouse and human epidermis, in both hair follicles and basal layer of the interfollicular epidermis [107]. Endoglin exists as a membrane bound form but is shed from the membrane at late stages of tumor progression in spindle cell carcinoma [108]. The role of endoglin in skin carcinogenesis was determined using *Eng*+/+ and *Eng*+/− mice [107]. *Eng*+/− mice had significantly reduced numbers of benign papillomas but the tumors that did form were largely SCC and spindle-cell carcinoma. Knockdown of endoglin in transformed keratinocyte cell lines not only enhanced TGFβ1 signaling, induced growth arrest and suppressed tumor formation, but also caused EMT, invasiveness and conversion to spindle cell carcinoma [108]. Expression of endoglin in a spindle cell carcinoma line suppressed Smad phosphorylation and tumorigenicity [108]. These results suggest that endoglin acts to downmodulate TGFβ1 signaling in keratinocytes, and generating results similar to the TGFβ1+/− mice [47], during tumor progression enhances TGFβ1 signaling, EMT, and progression to spindle-cell carcinoma.

Activation of latent TGFβ1 is a complex process that is critical for maintenance of normal tissue homeostasis and rapid release of bioactive TGFβ1 in response to signals that disrupt the normal tissue microenvironment. LTBP-1 is covalently linked to the propepeptide region of TGFβ1 and secreted from cells as the large latent complex. To determine the role of LTBP-1 in TGFβ1 function, Rifkin and colleagues generated mice in which cysteine 33 in both propeptide chains was mutated to serine to prevent disulfide bond formation with LTBP-1 [109]. These animals phenocopied *Tgfb1*−/− mice [110], although with a less severe phenotype suggestive of a hypomorphic state due to reduced active TGFβ1 levels. In addition to the multiorgan inflammation, absence of epidermal Langerhans cells and shortened lifespan, these animals also spontaneously developed stomach, rectal, and anal tumors [109]. While these mice did not develop skin cancers, this model illustrates the critical nature of latent TGFβ1 activation for generating sufficient TGFβ1 in the microenvironment for normal tissue homeostasis.

5. Conclusions

The role of TGFβ1-signaling pathway in the pathogenesis of SCC and other cancers is complex due to the diverse biological processes that are regulated by TGFβ1 and the cell type and context dependence of specific responses. Nevertheless, sufficient studies have been done to make some general conclusions. First, inactivation or diminution of pathway activity represents a significant component of human SCC pathogenesis, whether by receptor mutation, loss of receptor expression as measured by reduced receptor or pSmad2 levels, or loss of Smad4 expression. However, the mouse models suggest that except for Smad4, inactivation of the TGFβ pathway by itself is not sufficient for tumorigenesis, despite alterations in tissue homeostasis. It may be that this stems from the centrality of Smad4 in multiple TGFβ1 superfamily-signaling pathways. Further, the mouse models suggest that Smad2 and Smad3 function in carcinogenesis may be distinct, but this also may depend on what tissue compartment function is inactivated. It remains to be determined whether epidermal specific Smad3 deletion will have similar or distinct effects on cancer development as the Smad2 epidermal null. While the ability of TGFβ1 pathway inactivation to collaborate with oncogenic Ras has been shown in multiple studies, the finding that PI3-kinase/AKT is activated in tumors from two different models of pathway inactivation, that PTEN deletion cooperates with TGFβ1 pathway inactivation for tumorigenesis, and that parallel changes occur in human SCC suggests that the interaction of these two pathways is important for SCC pathogenesis and deserves further analysis. It is an accepted paradigm that long-term expression of TGFβ1 promotes a more malignant phenotype, and this is certainly born out by in vitro studies of TGFβ1-treated SCC cells and elevated expression of TGFβ1 in mouse and human cancers where pathway inactivation occurs. Nevertheless, the animal models suggest that increased expression in benign tumors or during the course of cancer induction selects for cells with a more aggressive, metastatic phenotype. The observation that this is enhanced when receptor signaling is blocked suggests that other pathways are activated in the tumor cells or that effects of TGFβ1 on the tumor microenvironment predominate, where elevated TGFβ1 leads to significant inflammation. Finally, although nearly all of these studies have been done in the chemical carcinogenesis model, for cutaneous cancer at least, it is not clear if alterations in TGFβ1 signaling would impact UV-induced skin cancer in the same way. Research on TGFβ has been one of many surprises. It is certain that many surprises remain in the years ahead.

Abbreviations

DMBA: 7,12-Dimethylbenz(a)anthracene
EMT: Epithelial-to-mesenchymal transition

HNSCC: Human head-&-neck squamous cell carcinoma
IHC: Immunohistochemistry
SCC: Squamous cell carcinoma
Tgfbr-1: Type I TGF-β receptor gene
Tgfbr-2: Type II TGF-β receptor gene
Tgfb1: Murine transforming growth factor beta gene
TGFβ: Transforming growth factor-β
TPA: 12-tetradecanoyl-phorbol-13-acetate
TβRI: Type I TGF-β receptor
TβRII: Type II TGF-β receptor.

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Research Article

Topical Curcumin-Based Cream Is Equivalent to Dietary Curcumin in a Skin Cancer Model

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Skin squamous cell carcinoma (SCC), the most common cancer in the USA, is a growing problem with the use of tanning booths causing sun-damaged skin. Antiproliferative effects of curcumin were demonstrated in an aggressive skin cancer cell line SRB12-p9 ($P < 0.05$ compared to control). Topical formulation was as effective as oral curcumin at suppressing tumor growth in a mouse skin cancer model. Curcumin at 15 mg administered by oral, topical, or combined formulation significantly reduced tumor growth compared to control ($P = 0.004$). Inhibition of pAKT, pS6, p-4EBP1, pSTAT3, and pERK1/2 was noted in SRB12-p9 cells post-curcumin treatment compared to control ($P < 0.05$). Inhibition of pSTAT3 and pERK1/2 was also noted in curcumin-treated groups *in vivo*. IHC analysis revealed human tumor specimens that expressed significantly more activated pERK ($P = 0.006$) and pS6 ($P < 0.0001$) than normal skin samples. This is the first study to compare topical curcumin to oral curcumin. Our data supports the use of curcumin as a chemopreventive for skin SCC where condemned skin is a significant problem. Prevention strategies offer the best hope of future health care costs in a disease that is increasing in incidence due to increased sun exposure.

1. Introduction

The American Cancer Society estimates that 1–1.3 million cases of nonmelanoma skin cancer (NMSC) will be detected annually. Cutaneous SCC accounts for nearly 20% of all skin cancers, and excluding melanoma, 75% of all deaths attributed to skin cancers [1]. Unlike the more prevalent basal cell carcinoma (BCC), SCC is an aggressive tumor that metastasizes with a frequency as high as 12.5% [2]. Prevalence is common in fair complexion Caucasians with lower reported rates in individuals with darker complexions including Asians and Africans. Cutaneous SCC of the face often metastasizes to parotid lymph nodes, which can be detrimental to the facial nerve during treatment and nodes in the neck, as the head and neck are rich in lymphatic networks. Treatment for NMSC may include cryotherapy, electrosurgery, topical 5-fluorouracil, photodynamic therapy, imiquimod, and radiation therapy; however, surgical intervention is the primary treatment modality. When treated early, the five-year cure rate is greater than 90% [3]. NMSC recurrence varies from 8–16%, second lesion recurrence rates are as high as 75% within the first two years and 95% within five years [3]. This suggests a window of opportunity for chemopreventive agents to delay or prevent a recurrence or metastatic spread. Lymph node metastasis in NMSC varies from 0.1 to 28%, with a resulting

mortality from 50–75% [4]. Overall five-year survival rates for regional lymph node metastasis are 25–35% [3, 5–7] and less than 20% at ten years [1]. Early cancer detection offers the best window of opportunity for treatment. Early stage skin cancer has a high cure rate, whereas advanced stage cutaneous SCC often develops resistance to chemotherapy. Therefore, research has focused on developing these novel chemopreventive agents to delay or prevent cutaneous SCC formation.

Curcumin, an extract from the Indian spice turmeric, has been investigated in a variety of human cancers including pancreatic, prostate, breast, and head and neck cancer. The first published report demonstrating the topical use of curcumin in cancer reported a sustainable reduction in lesion size and pain [8]. Curcumin has antioxidant, anti-inflammatory, antiangiogenic and anticarcinogenic activity, although its clinical use is limited by low bioavailability [9].

More recently, several studies have examined curcumin's effect in inhibiting skin carcinogenesis. Additionally, numerous reports have identified signaling pathways related to epidermal growth factor receptor (EGFR) that are essential to formation and progression of cutaneous malignancy. The MTOR and MEK/ERK signaling cascades are two of the most well-studied pathways [10]. In a prior study by our group [11] we subcutaneously injected immunodeficient mice with SRB12-p9 skin SCC and demonstrated that curcumin administered by oral gavage significantly inhibited tumor growth and downregulated pS6, a well-established downstream biomarker of the MTOR and MEK/ERK pathways. Curcumin's anti-carcinogenic effects have been linked to inhibition of the MEK/ERK signaling pathway in breast carcinogenesis, and researchers continue to explore these potential biomarkers in other cancers [12]. However, ERKs activity in cutaneous malignancy is not well defined in the literature. Hence, we wanted to determine if topical curcumin was as efficacious as oral curcumin in a SCC skin xenograft model and elucidate the pathways down-regulated by curcumin as potential biomarkers for future chemopreventive studies with our topical curcumin cream. In addition, we wanted to observe the potentially additive effects of topical application and oral dosing. We also wished to explore whether the MEK/ERK pathway is overexpressed in human cutaneous SCC and BCC in the hope of identifying a novel intracellular target at which curcumin may act to inhibit tumorigenesis. We hypothesized that pERK and its downstream target pS6 would be overexpressed in cutaneous skin cancers given its role in promoting cellular proliferation in aggressive malignancy. Identifying intermediate endpoints is necessary to assess intervention results for primary cancer prevention and address problems with feasibility posed by large patient numbers, length of study, and cost when cancer occurrence or recurrence is an endpoint [13].

2. Materials and Methods

2.1. Curcumin. Curcumin C3 Complex (>98% pure) was obtained from Sabinsa Corp. *In vivo* studies were conducted with curcumin (15 mg) suspended in vehicle (100 μL corn oil) for oral gavage feeding or suspended in a vanishing cream paste (15 mg/100 μL cream) for topical administration provided by our study compounding pharmacist (DB).

2.2. Cell Lines and Xenografts. The human skin SCC cell line SRB12-p9 was derived by single-cell cloning from aggressive skin SCC SRB12 cells (a gift from Dr. Reuben Lotan, Department of Thoracic Head and Neck Medical Oncology, University of Texas M.D. Anderson Cancer Center in 2003) and was cultured as described [14]. This cell line was chosen due to its sensitivity to curcumin as evidenced in cell culture studies. DNA was isolated from the cell lines using a commercially available DNA purification kit (Qiagen). DNA sample was sent to Genetica (Cincinnati, OH, USA), and the cell line was validated by DNA profiling.

2.3. Cell Proliferation. 2,000 SRB12-p9 cells per well were seeded in triplicate onto 96 well plates in complete media at 37°C with 5% CO_2. After adherence, cells were treated with curcumin (0–40 μM) for 0–72 hours. Cell viability was measured using MTS (Promega).

2.4. Subcutaneous HNSCC Xenograft Model. Studies were conducted in accordance with the Declaration of Helsinki (1964) and in compliance with Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee guidelines. Animals housed in a barrier facility were maintained on a normal diet *ad lib*. Forty 6–8-week-old Severe Combined Immunodeficiency (SCID) mice were shaved and pretreated with either 0 mg (corn oil), 15 mg curcumin by oral gavage, 15 mg curcumin topical paste, or combined 15 mg oral gavage and 15 mg curcumin topical paste once daily for 3 days prior to squamous cell carcinoma xenograft injection ($n = 10$ per group). Mice were then injected subcutaneously with 1×10^6 SRB12-p9 cells suspended in sterile PBS (Day 0). All mice continued daily treatment with either 0 mg or 15 mg curcumin by gavage, topical, or both, and tumors were measured daily with digital calipers. Xenograft tumors did not form in one animal per group and were excluded ($n = 9$ per group). Tumor volume (mm^3) was calculated using the following formula: $(0.52 \times \text{length}^2 \times \text{width})$. Body weight was measured daily, and mice were monitored for adverse effects from the experiment. Daily oral gavage and tumor volume measurement continued through day 29, at which time tumors were harvested after the mice were anesthetized with isoflurane and sacrificed. *Ex vivo* tumor volume was calculated using the following formula: $(4/3\pi 0.5 \times \text{length} \times 0.5 \times \text{width} \times 0.5 \times \text{height})$. The study pathologist (FA) measured maximum skin thickness, including the stratum corneum but not the granular layer.

2.5. ELISA. Pooled serum from mice ($n = 3$/group) was analyzed by enzyme-linked immunosorbent assay (ELISA, BD Bioscience) according to the manufacturer's instructions, to assess expression of human and murine IL6. Samples were analyzed in duplicate for IL-6 expression with a spectrophotometric plate reader.

2.6. Immunohistochemical Analysis of Molecular Markers in Skin Squamous Cell Carcinoma. Tumors harvested on day 29 were embedded in paraffin, sectioned, and H&E stained for confirmation of squamous cell carcinoma presence by our study pathologist (FA). Tumors ($n = 3$ per group) were then stained with phospho-ERK (cell signaling, Thr202/Tyr204; 1 : 600) and phospho-STAT3 (cell signaling, Tyr705; 1 : 200) as previously described [15, 16]. Subcellular localization was determined by immunofluorescence. Paraffin sections of tumors with overlying mouse skin were probed with pERK1/2 and pSTAT3 antibodies (Cell Signaling) followed by an Alexa-546-labeled secondary antibody.

Human actinic keratosis, skin SCC, and BCC paraffin-embedded blocks were sectioned and stained with phospho-p44/42 MAPK (ERK 1/2) rabbit monoclonal antibody (Thr202/Tyr204, 1 : 600) and phospho-S6 ribosomal protein rabbit monoclonal antibody (Ser235/236, 1 : 100) as previously described [17–19] and read by our study pathologist (FA). Specimens were scored based on the intensity of antibody nuclear and cytoplasmic staining in each slide, with absence of staining scored as a [0], weak or focal staining scored as a [+], and strong staining with a [++].

2.7. Western Blot Analysis. Soluble proteins extracted from SRB12-p9 cell lysates treated with 0 μM or 20 μM curcumin for 24 hours or xenograft tumors were analyzed by western blot as previously described [19]. Proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and analyzed with ImageQuant TL7.0 (GE Healthcare) software ($n = 6$/group). The following antibodies from cell signaling were used: AKT (1 : 200), phospho-AKT (Ser^{473}; 1 : 100), S6 ribosomal protein (1 : 500), phospho-S6 ribosomal protein ($Ser^{235/236}$; 1 : 500), STAT3 (1 : 200), phospho-STAT3 (Tyr705; 1 : 200), 4EBP1 (1 : 200), phospho-4EBP1 (Ser^{65}; 1 : 200), ERK1/2 (1 : 200), phospho-ERK1/2 (Thr^{202}/Tyr^{204}; 1 : 200), and actin (1 : 3500).

2.8. Patient Tissue Samples and Controls. All BCC and SCC tissue samples were obtained from patients recently diagnosed with nonmelanoma skin cancer of the face or neck, after obtaining approval by the institutional review board and obtaining informed consent from all subjects. Patients were treated primarily with surgical resection at Louisiana State University Health Shreveport and the Overton Brooks Veterans Administration Hospital from 2009 to 2011. Formalin-fixed, paraffin-embedded tissue blocks were obtained from 27 BCC tissue samples, 4 Actinic Keratosis (AK) tissue samples, and 17 SCC tissue samples (from 16 SCC patients). Normal human skin samples were surgically obtained from uninvolved adjacent skin in patients undergoing resection for skin cancer. Total of 25 normal (noncancer) skin samples were analyzed in the study. Several 5 μm slides were cut from each tissue block, and one slide was stained with hematoxylin and eosin (H&E) and reviewed by a pathologist to confirm pathologic findings and assess surgical margins. All other slides were used for immunohistochemical staining.

2.9. Statistics Applied for the Analysis. Proliferating cell percentages were compared using one-way analysis of variance (ANOVA). One-way ANOVA was also used to determine significant differences in skin thickness and the differences between individual treatment groups. A Tukey's multiple comparison as a *post hoc* test was performed to evaluate differences between treatment groups. Tukey's *post-hoc* testing, Chi-square test for independence, or Fisher's exact probability test was used to determine the ability of pERK and pS6 expression to correlate with cutaneous SCC, differentiate tumor types from normal skin and BCC, and determine if there was a significant difference between pERK and pS6 staining and the different types of histologic cutaneous lesions. Paired t-test was used to determine significant difference in biomarker expression by western blot analysis.

3. Results

3.1. Growth Inhibitory Effects of Curcumin In Vitro and In Vivo. To determine whether a skin SCC cell line is sensitive to curcumin, a cell proliferation assay was performed on SRB12-p9 SCC cell line. Curcumin's growth inhibitory effects in the aggressive skin cancer cell line (SRB12-p9) were noted as early as day 2 at 20 μM ($P < 0.05$) curcumin compared to control. Curcumin treatment at doses 20 μM and 40 μM was significantly effective in inhibiting the proliferation of SRB12-p9 cells compared to control on days 2 and 3 ($P < 0.05$; Figure 1(a)).

Curcumin appears to inhibit growth compared to control in SRB12-p9 xenograft tumors after tumor cells had a chance to engraft (Figure 1(b)). There was a significant effect for curcumin treatment ($F(3, 96) = 11.58$, $P < 0.001$) in suppressing growth of the SRB12-p9 xenograft tumors. Tukey's *post hoc* comparisons of the four groups indicate tumor volume from the gavage group ($M = 44.55$, 95% CI [35.77, 53.77]) and the combined group ($M = 88.81$ CI [71.73, 105.89]) was significantly smaller than the control group tumor volume ($M = 191.35$, 95% CI [127.12, 255.59]), $P < 0.001$. The topical group ($M = 130.66$, 95% CI [95.29, 166.04]) tumor volume was also statistically smaller than the control group tumor volume ($P = 0.02$). There was no difference between the gavage group tumor volume and the topical group tumor volume ($P = 0.19$).

Because invasive tumors could give inaccurate measurements and overlying skin could influence *in vivo* tumor measurements, we also measured tumors *ex vivo* and measured skin thickness (Figure 1(c)). There was a significant effect of curcumin on *ex vivo* tumor volume ($F(3, 32) = 5.49$, $P = 0.004$). Tukey's *post hoc* comparisons of the four groups indicate that the tumor volumes from the gavage group ($M = 72.06$, 95% CI [37.78, 106.35]), topical group ($M = 195.82$, 95% CI [71.59, 320.05]), and combined group ($M = 152.32$, 95% CI [101.048, 203.60]) were significantly smaller than the control ($M = 416.77$, 95% CI [161.48, 672.06]), $P < 0.001$, $P = 0.006$ and 0.02, respectively. There was a significant effect for curcumin treatment on tumor mass ($F(3, 32) = 5.79$, $P = 0.003$), where the gavage group ($M = 0.043$, 95%

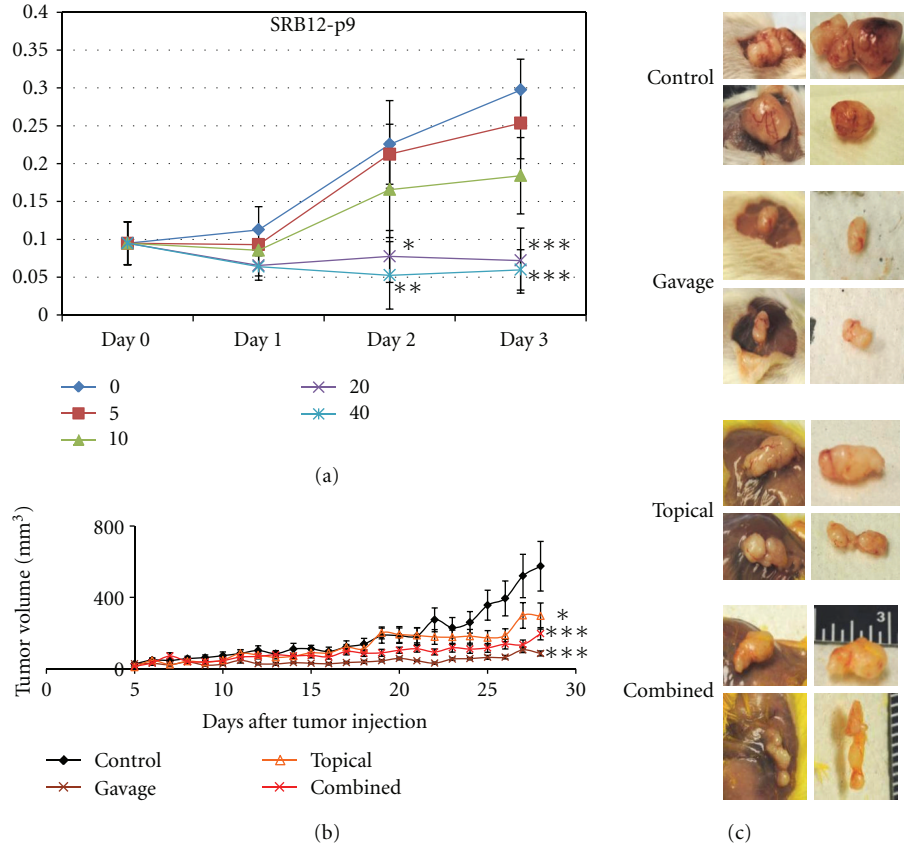

Figure 1: Curcumin inhibits skin SCC cell growth *in vitro* and *in vivo*. (a) Cell proliferation of the aggressive skin cancer cell line SRB12-p9 after treatment with 0–40 μM curcumin. $^{*}P < 0.05$ versus control group; $^{**}P < 0.01$ versus control group; $^{***}P < 0.001$ versus control group. (b) Mice were pretreated with the indicated dose of curcumin for 3 days prior to injection with 1×10^6 SRB12-p9 tumor cells in the dorsal region (day 0) and continued receiving daily curcumin treatment (9 mice per group, mean tumor volume ± SD). Tukey's *post hoc* test: $^{*}P < 0.05$ versus control group; $^{***}P < 0.001$ versus control group. (c) Representative images of xenograft tumors at harvest and *ex vivo* from the indicated treatment groups.

CI [0.02, 0.07]), topical group ($M = 0.112$, 95% CI [0.041, 0.184]), and combined treatment group tumors ($M = 0.076$, 95% CI [$M = 0.050$, 0.101]) were significantly smaller than that of the control group ($M = 0.244$, 95% CI [0.09, 0.39]) tumors, $P < 0.001$, $P = 0.02$, and 0.003, respectively. There was no difference in skin thickness in mice treated with curcumin by gavage, topical, and combined groups compared to the control group ($P = 0.73$).

3.2. Curcumin's Effects on Signaling Pathways. We next evaluated curcumin's effects on signaling pathways in the aggressive skin cancer cell line (SRB12-p9) *in vitro*. Using a concentration that significantly inhibited cell growth (20 μM), there was significant inhibition of pAKT, pS6, p-4EBP1, pSTAT3, and pERK1/2 (Figure 2). As can be seen in Figure 2 there was about twofold inhibition in the phosphorylation of the aforementioned markers in SRB12-p9 cells after curcumin treatment.

We next evaluated curcumin's effects on signaling pathways in xenograft tumors using western blot analysis (Figure 3). Among the tested biomarkers an inhibition of pERK1/2 was noted in the curcumin-treated groups, whereas inhibition of pSTAT3 was only noted in the combined curcumin group (Figure 3(a)).

As western blot analysis involves homogenization of total tumor tissue, such as stroma and infiltrating host inflammatory cells, we also evaluated curcumin's effects on signaling pathways by immunohistochemistry, which can distinguish nonviable and nontumor components, such as stroma, that are not included in the scoring of the biomarker analyzed. IHC results revealed strong positive pERK staining throughout tumors in the control group and weaker, focal staining in the curcumin-treated tumors (Figure 3(b)). Immunofluorescence confirmed curcumin's effects on pERK and a shift in the subcellular localization of the activated state of STAT3 in the topical group compared to the control group (Figure 3(c)). Curcumin is known for its anti-inflammatory effects. Therefore, we evaluated its effects on the inflammatory marker IL6 in all curcumin treatment groups using pooled serum samples. The levels of soluble

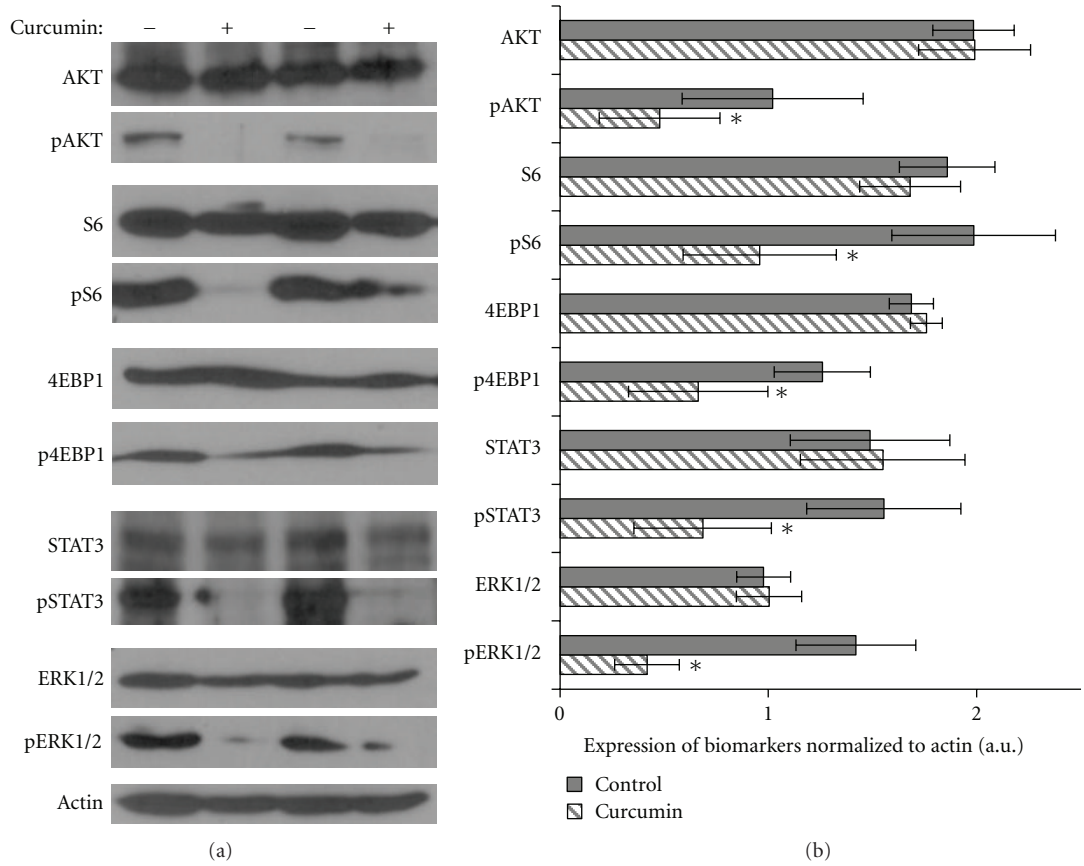

Figure 2: Curcumin's effects on AKT/MTOR and ERK pathways *in vitro*. (a) Western blot of SRB12-p9 tumor cells treated with (+) or without (−) 20 μM curcumin for 24 hours and probed with the indicated antibody. Representative Western blots for two analyzed sets are shown. (b) Band densities of indicated biomarkers ($n = 6$) were quantified using ImageQuant software and normalized to actin protein level. Data presented as Mean ± SE. * Indicates $P < 0.05$ versus vehicle-treated control. A significant inhibition of expression of the following biomarkers was observed: pAKT ($P = 0.0368$); pS6 ($P = 0.0182$); p4EBP1 ($P = 0.0098$); pSTAT3 ($P < 0.0001$); pERK1/2 ($P = 0.0313$). a.u.: arbitrary units.

IL6 were the lowest in the topical curcumin group, while curcumin did not affect IL6 levels in the gavage or combined groups (Figure 3(d)).

3.3. Patient Characteristics. Patient demographics and clinical characteristics are summarized in Table 1. Tissue samples from 46 male patients and 4 female patients were analyzed. Age ranged from 39 to 93 with a mean age of 66 ± 14 years. There was no difference in age between the groups by ANOVA ($F = 1.272, P = 0.29$). The large majority of patients were white, except for one African American patient with albinism. Nonmelanoma skin cancers analyzed were excised from the external nasal skin (14), cheeks (14), ears (9), scalp and forehead (13), neck, chin, and lip (6). No skin site was overrepresented in analysis.

3.4. IHC Analysis of Patient Tissues. The presence and intensity of pERK and pS6 staining in all SCC, BCC, and normal tissue samples were compared (Table 2). All SCC specimens ($n = 17$, 100%) stained positive for phosphorylated ERK, while only 10 of 27 (37%) BCC samples stained positive. Although all the normal skin samples stained weakly positive (grade 1+) for activated pERK in the stroma, palisading cells, and epithelium ($n = 24$, 100%), significantly more SCC specimens showed strong staining with pERK (grade 2+) than normal skin ($P = 0.0028$, Table 2 and Figure 4). However, the majority of BCC specimens (17/27, 63%) showed no pERK staining ($P < 0.0001$ compared to normal skin).

Most specimens containing SCC ($n = 13$, 81%) and BCC ($n = 16$; 64%) showed strong staining (grade 2+) for activated pS6, while all the analyzed normal skin specimens ($n = 8$; 100%) demonstrated negative pS6 staining. Tumor specimens expressed significantly more activated pS6 than normal skin samples (1+ score and above; $P < 0.0001$; Figure 4). Skin cancer type significantly predicted intensity of pERK staining, as SCC tumors stained more intensely for pERK than the background stroma in normal skin and BCC tumor cells ($P < 0.0001$; Figure 4). When pERK expression was analyzed and compared to other demographic factors, the variance in pERK expression scores correlated significantly with tumor type, $R^2 = 0.25$, $P = 0.0007$. Patient age ($P = 0.85$) and gender ($P = 0.35$) did not explain the variance in pERK staining.

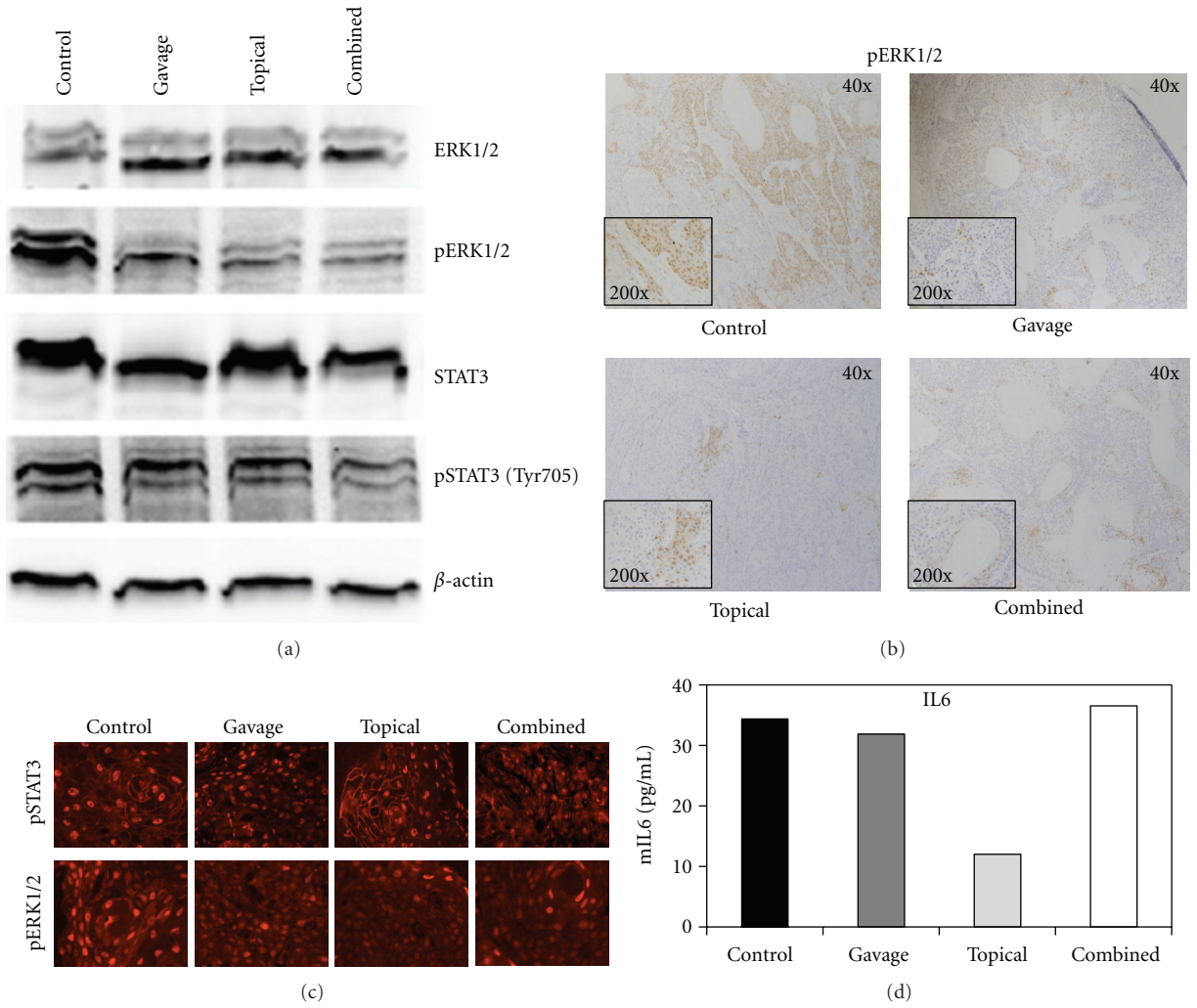

Figure 3: Curcumin's effects on the ERK pathway *in vivo*. (a) Western blot of pooled xenograft tumors (n = 6/group) of the indicated antibody. (b) The presence and intensity of pERK staining (brown) in the control group compared to the presence and intensity of pERK staining in the curcumin-treated xenograft tumors. (c) Representative IHC staining of SRB-12 p9 WT cell tumor xenografts. Paraffin sections of tumors were probed with STAT3 phospho-Tyr705 (pSTAT3, top row) or ERK1/2 phospho-Thr202/Tyr204 (pERK, bottom row), followed by an Alexa546-labeled secondary antibody (400x). (d) IL-6 ELISA of pooled mouse serum (n = 3/group) in duplicate.

4. Discussion

Identifying consistent intracellular biomarkers at which a potential chemopreventive may act is essential prior to initiating clinical trials. As curcumin acts on many different biomolecular targets in a variety of different cell types it is important to determine if curcumin directly affects either a few major downstream biomarkers or a multiplicity of downstream targets which may serve to explain curcumin's varying effects in different cell types. Aberrant signaling through the epidermal growth factor receptor (EGFR) plays a major role in cutaneous skin cancer progression. EGFR inhibitors have been used for SCC therapy to downregulate aberrant EGFR signaling with little change in overall survival [20], possibly due to compensating mutations downstream of EGFR. One of these signaling pathways is PI3 K/AKT that plays a role in skin carcinogenesis and in chemotherapy resistance [21].

Activated Ras/Raf signaling has also been implicated in a small percentage of SCC [22] and can lead to activation of the MAPK pathway. Ras/Raf gain-of-function can occur through activation of ERK1 and ERK2, which are constitutively active in 70% of malignant melanoma due to RAS or BRAF activating mutations [2]. Activated ERK1/2 is rarely seen in normal skin specimens but is shown in all cases of SCC with a positive association with the degree of malignancy and proliferative activity of SCC [23]. In this study, Zhang et al. looked at 10 well-differentiated and 10 poorly differentiated skin SCC cases. Another study looked at activated ERK in 101 human head and neck squamous carcinoma specimens [24]. Therefore, inhibiting ERK may be a promising approach in targeted cutaneous skin SCC therapy. Having previously

Table 1: Clinical and demographic patient characteristics.

	Total	Normal*	AK	SCC	BCC	*P* value
Gender**						
Male	46	21	4	14	25	0.77***
Female	4	4	0	2	2	
Race						
White	49	24	4	15	27	0.57***
African American	1	1	0	1	0	
Age						
<60	17	13	1	7	7	
60–70	14	7	1	3	9	0.44***
>70	19	5	2	6	11	
Skin site						
Nose	14	6	2	2	8	
Cheeks	14	7	0	8	6	
Ear	9	5	1	1	5	0.71***
Scalp and forehead	13	5	1	5	5	
Other	7	4	0	2	3	

* Normal skin samples were surgically obtained from uninvolved adjacent skin in patients undergoing resection for skin cancer.
** Some patients had more than one type of cancer and are counted in both groups.
*** No significant difference in number of males and females, race, age, or skin site distribution per group by Fisher's exact test.

Table 2: Summary of pERK and pS6 IHC staining in normal (noncancer), AK, BCC, and SCC skin samples.

	[0]	[1+]	[2+]	*P* value*	Total
			pERK staining		
Normal Skin	0	24	0		24
AK	0	4	0	1.0000	4
BCC	17	5	5	<0.0001	27
SCC	0	11	6	0.0028	17
			pS6 staining		
Normal Skin	8	0	0		8
AK	2	0	2	0.0909	4
BCC	5	4	16	<0.0001	25
SCC	1	2	13	<0.0001	16

* Compared to normal skin by Fisher's exact test. *P* values for overall comparison are shown. See text for a subset analysis.

determined curcumin's growth inhibitory effects in skin SCC [11], we sought to determine whether these effects were similar to our observations in upper aerodigestive head and neck SCC (HNSCC) where curcumin inhibited the AKT/MTOR pathway through rapid curcumin-dependent inhibition of MTOR's downstream target pS6 and 4EBP1 phoshorylation [18].

In this study we found significant and complete inhibition of SRB12-p9 cell proliferation after treatment with curcumin at a dose 20 μM or higher (Figure 1(a)) suggesting a highly potent anticarcinogenic effect of curcumin in skin cancer. Additionally, we found that the inhibitory effect of curcumin on skin cancer proliferation was associated with inhibition of AKT/mTOR and ERK signaling (Figure 2).

In our *in vivo* study, curcumin paste was formulated to penetrate human skin epidermis and dermis. However, given the thin nature of mouse skin, topical curcumin penetration was much greater such that curcumin possibly did not remain in the epidermis for a prolonged period, leading to prolonged contact with the cancer cells. The irritant nature of the cream caused the skin overlying the tumor to thicken, although this was not statistically significantly different from control ($P = 0.73$). The SRB12-p9 cell line is invasive in this model [25], producing inaccurate tumor caliper measurements due to the inability to account for the portion of the tumor that invaded into the abdominal wall. Therefore, the *ex vivo* tumor weight provided a more accurate tumor size endpoint. In human skin, SCC emerges directly from the epidermal layer, unlike in our xenograft model, where tumor is encapsulated under the epidermis. We therefore anticipate a more pronounced tumor-suppressive effect of topical curcumin in humans.

The SRB12-p9 xenograft cells were more sensitive to curcumin-induced cell death and apoptosis than the surrounding normal mouse skin and grew at a much slower rate in the presence of curcumin, whether topical or systemic,

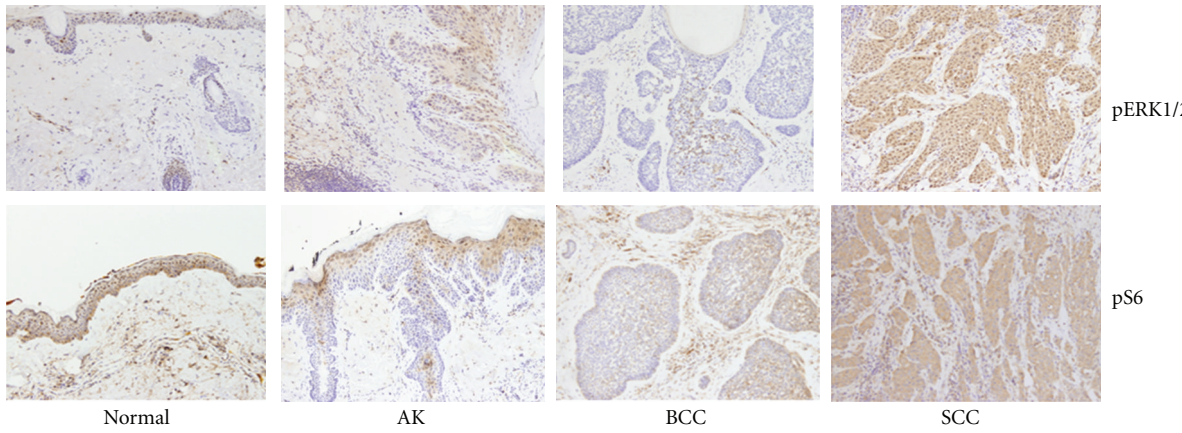

Figure 4: IHC analysis of pS6 and pERK expression in patients with negative (blue) staining and strong positive (brown) staining of tumor cells with pERK and pS6. Normal patient skin samples with minimal background staining and normal appearing cells. Representative actinic keratosis (AK) patient samples showing weak, cytoplasmic staining. Representative BCC patient samples with negative (blue) staining and few scattered positive (brown) staining of tumor cells. Representative SCC patient samples with strong positive (brown) nuclear and cytoplasmic staining with pERK and pS6. Note that the stroma stains positive (brown) in BCC, whereas the tumor stains negative (blue).

compared to control. IL6 plays a central role in regulating the inflammatory response [25]. Because IL-6 may contribute to angiogenesis and metastasis [26], inhibition of IL-6 with topical curcumin suggests a mechanism of chemoprevention. Although curcumin has previously been shown to inhibit IL-6 in HNSCC cell lines [27], this is the first skin cancer model investigating curcumin's inhibition of systemic IL-6. The present study demonstrates that topical curcumin reduces skin SCC tumor growth, and this effect might be explained, by the inhibition of IL-6.

In this study we demonstrated significant inhibition of several biomarkers of the AKT/mTOR pathway as well as STAT3 and ERK1/2 in SRB12-p9 cells after treatment with 20 μM of curcumin. In our *in vivo* experimentation, we observed inhibition of pERK in the curcumin-treated tumors and inhibition of pSTAT3 in the combined curcumin group. However, tumor heterogeneity and degree of dysplasia can often confound immunohistochemistry results, depending on where in the lesion the biopsy was taken. Therefore, it is important to develop serum biomarkers that can be obtained with a simple blood draw. As curcumin is a well-known anti-inflammatory agent, we measured its effects on pooled serum of treated mice and noted a decrease in IL-6 in the topical group compared to the control group. We observed that systemic curcumin did not cause a decrease in serum IL-6 levels. However, only three mice in each group were analyzed, and it is possible that statistically significant differences in IL6 levels could be detected upon analysis of greater numbers of mice in the topical and combined curcumin-treated groups compared to control mice.

As curcumin slowed progression of aggressive skin SCC xenografts and inhibited pERK expression, the ERK pathway may prove to be a key biomarker in developing topical pharmaceutical agents that prevent skin SCC tumor growth or recurrence. We observed that the overall reduction in pERK staining in the curcumin-treated tumors was not cell autonomous but rather manifested as an expansion in areas of very low or no expression, such that focal regions of intense staining remained. Alternatively, control tumors had smaller regions of low staining and a higher number of intensely staining areas. This indicates that a global reduction of pERK staining was achieved with curcumin treatment, rather than a complete shutdown. [23] confirmed that phosphorylated ERK is overexpressed in patient skin SCC in a Caucasian population, which further supports our findings and suggests that pERK may be a useful chemoprevention biomarker.

Chronic inflammation is linked to both cancer and angiogenesis. The anti-inflammatory properties of curcumin may contribute to its potential as an effective chemopreventive agent. However, curcumin's systemic anti-inflammatory effects (reduced serum IL-6 levels) were more pronounced in topical curcumin group compared to gavage. Given these findings, it was unexpected that tumor growth was inhibited more effectively in the gavage group than in the topical group. However, there was no statistically significant difference in tumor volume between the two treatment groups. Despite this data, we speculate that local anti-inflammatory activity of topically applied curcumin contributes significantly to its chemopreventive activity, circumventing its poor systemic bioavailability.

As curcumin continues to be explored as a chemopreventive and therapeutic agent for skin cancer treatment, establishing defined biomarkers upon which curcumin acts to inhibit tumorigenesis is essential. The ERK pathway is an important protein kinase signaling cascade involved in cellular proliferation and is activated in carcinogenesis. In this study, activated pERK expression significantly increased in SCC compared to the less aggressive BCC and AK. As curcumin has been shown to inhibit activated ERKs in carcinogenesis, the present data suggests that components of the ERK pathway may prove to be key biomarkers for curcumin chemopreventive efficacy in cutaneous SCC.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Multiple Roles for VEGF in Non-Melanoma Skin Cancer: Angiogenesis and Beyond

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Vascular endothelial growth factor (VEGF) is known to play a critical role in the development of non-melanoma skin cancers. VEGF is a potent pro-angiogenic factor and it is elevated in mouse and human skin tumors. The use of transgenic and knockout mice has shown that VEGF is essential for tumor development in multiple models of skin carcinogenesis and, until recently, the mechanism of action has been primarily attributed to the induction of angiogenesis. However, additional roles for VEGF have now been discovered. Keratinocytes can respond directly to VEGF, which could influence skin carcinogenesis by altering proliferation, survival, and stemness. *In vivo* studies have shown that loss of epidermal VEGFR-1 or neuropillin-1 inhibits carcinogenesis, indicating that VEGF can directly affect tumor cells. Additionally, VEGF has been shown to promote tumor growth by recruiting macrophages to skin tumors, which likely occurs through VEGFR-1. Overall, these new studies show that VEGF carries out functions beyond its well-established effects on angiogenesis and highlight the need to consider these alternative activities when developing new treatments for non-melanoma skin cancer.

1. Introduction

Non-melanoma skin cancer (NMSC) is the most commonly diagnosed type of cancer. Over 2 million patients are treated for these cancers each year in the USA alone [1], resulting in nearly $1.5 billion total direct costs annually [2]. Unlike many other types of cancer, the rates of NMSC continue to rise [3], indicating the need to increase research and identify new, more effective therapies. 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 [1, 4]. There are two main types of NMSC: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCCs account for about 80% of skin cancers [3] and although these tumors are rarely metastatic, patients have a high risk of developing additional tumors within 5 years of diagnosis [5]. SCCs make up roughly 16% of all skin cancers [3] and are typically more aggressive than BCCs, posing a higher risk for metastasis and leading to approximately 2,500 deaths annually [1]. 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 [6]; however, certain populations such as transplant patients are at an even greater risk [7, 8].

Angiogenesis, the growth and expansion of the vasculature, is an important process in the growth and metastasis of many cancers, including NMSC [9]. Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor and several studies have established a critical role for VEGF in skin cancer [10]. VEGF transgenic and conditional knockout mice subjected to skin carcinogenesis protocols, such as the well-established two-stage chemical carcinogenesis model [11, 12], have demonstrated that VEGF promotes skin carcinogenesis through the induction of angiogenesis [13, 14]. Additionally, several recent studies have now uncovered direct effects of VEGF on keratinocytes and skin tumor cells. These studies have suggested that in addition to enhancing angiogenesis, VEGF may promote skin carcinogenesis by altering the survival, proliferation, or stemness of keratinocytes and tumor cells in an autocrine manner

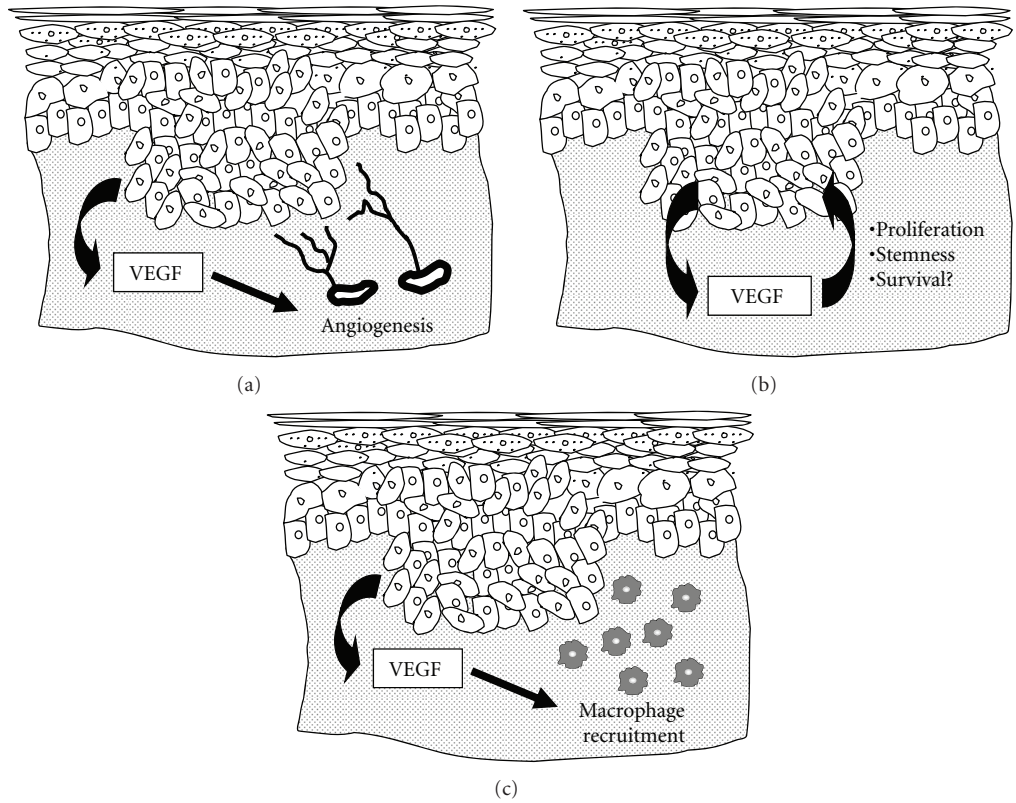

Figure 1: Tumor-promoting actions of VEGF in skin carcinogenesis. Epithelial tumor cells produce large amounts of VEGF in the skin, as depicted by the arrow on the left side of each panel. Traditionally, VEGF has been recognized only for its ability to stimulate angiogenesis through paracrine actions on endothelial cells (a). However, additional functions of VEGF have now been described. Recent studies have suggested that VEGF can affect epithelial cells in an autocrine manner by stimulating proliferation, maintaining stemness, or possibly by promoting survival (b). Additionally, macrophages can be recruited to skin tumors by VEGF through paracrine mechanisms. These macrophages are capable of producing an array of mediators that can support the growth of tumor cells in the skin.

[15–18]. Furthermore, immune cells such as macrophages can respond to directly VEGF [19, 20] and recent studies indicate that VEGF recruits macrophages to skin tumors [21]. This review will highlight our current knowledge of the angiogenic and newly discovered non-angiogenic activities of VEGF that contribute to non-melanoma skin cancer, which are summarized in Figure 1.

2. Angiogenesis and VEGF

Angiogenesis is a key process in the growth and spread of many cancers, including skin cancer. 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 [22]. Because of this, tumor angiogenesis has been an attractive and promising therapeutic target [23]. 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" [24]. When pro-angiogenic signals outweigh anti-angiogenic signals, it allows for capillary sprouting through the proliferation and migration of endothelial cells. Eventually, the newly formed vessels supply the tumor with oxygen and nutrients required for continued growth. Many pro-angiogenic factors have been identified and characterized, including basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), placental growth factor (PlGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF).

VEGF-A (referred to as VEGF throughout this article) is a 45 kDa heterodimeric heparin-binding protein belonging to the family of vascular endothelial growth factors. At least 5 splice variants of VEGF have been identified in humans, including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ [25, 26]. VEGF binds to three known receptors: VEGF receptor-1 (VEGFR-1), VEGF receptor-2 (VEGFR-2), and neuropilin-1 (NRP-1) [27–29]. 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 [30]. NRP-1 is a single pass transmembrane protein that binds semaphorins as well as some isoforms of VEGF [31]. NRP-1 functions as a coreceptor for the VEGFRs, enhancing their activity [32]; however, NRP-1 may be able to signal independently of VEGFRs in response to VEGF, particularly in tumor cells [33]. VEGF is well characterized as a potent inducer of angiogenesis and functions as a survival factor and mitogen for endothelial cells [34, 35]. In general,

VEGF is expressed at low levels by epidermal keratinocytes and is upregulated during many pathological processes such as wound healing, psoriasis, and skin carcinogenesis [36–38]. VEGF production by keratinocytes can be induced by many stimuli including hypoxia, transforming growth factor-α, keratinocyte growth factor, UV radiation, and the tumor promoter 12-O-tetradecanoylphorbol-13 acetate (TPA), while VEGF production is inhibited by the transcription factor Fra-1 [39–46].

3. VEGF and Angiogenesis in Skin Tumors

Strong evidence has demonstrated that VEGF plays an important role in skin carcinogenesis. In human skin, VEGF is expressed at low levels in normal epidermis, with more differentiated epidermal cell layers generally expressing more VEGF than less differentiated epidermal cells [47–49]. Several studies have confirmed that VEGF levels are elevated in tumor cells compared to normal epidermal cells using immunohistochemistry and *in situ* hybridization techniques [47–49]. Tumor cells of human BCCs tend to show weak VEGF expression [47, 48, 50] with positive tumor cells predominantly localized to the invading margin [50]. In contrast, SCCs, which are typically more aggressive than BCCs, display more intense and widespread staining, with higher expression in tumor cells localized near infiltrating inflammatory cells [47, 50]. Furthermore, VEGF expression is elevated in poorly differentiated SCCs compared to well differentiated tumors [50]. Vessel density is also high in SCCs, especially in late-stage SCCs, compared to normal skin, actinic keratoses, BCCs, or early-stage SCCs [48, 49].

In mice, acute exposure to tumor promoters such as TPA or UV light causes upregulation of VEGF and induction of angiogenesis in the skin [38, 51–53]. VEGF expression patterns in murine models of skin carcinogenesis mimic what is observed in human tumors. VEGF is low in murine skin and increases stepwise during tumorigenesis [38]. A functional role for VEGF in skin tumor angiogenesis has been demonstrated through the use of transgenic and conditional knockout mice. Both K6-VEGF and K14-VEGF transgenic mice which overexpress VEGF in epidermal keratinocytes show elevated blood vessel density in the skin and in skin tumors compared to controls [13, 14, 54]. VEGF transgenic mice are also more susceptible to two-step chemical carcinogenesis [13, 14]. In addition to containing a larger number of blood and lymphatic vessels both within and surrounding skin tumors, K14-VEGF mice develop chemically-induced tumors more rapidly and also have a dramatically higher incidence of metastasis than controls [14]. Conversely, conditional K14-VEGF knockout mice have reduced blood vessel density in tumors and are much more resistant to chemical carcinogenesis [55].

VEGF also plays a role in UV-induced skin carcinogenesis. In addition to inducing papillomas and SCCs, UV exposure increases VEGF levels and neovascularization in the skin [52, 53, 56]. Inhibition of VEGF in the skin with compounds such as epigallocatechin-3-gallate (ECGC) and myricetin leads to a decrease in angiogenesis and a reduction in the number of UV-induced skin tumors [56–58].

Evidence from orthotopic skin tumor models has also shown a link between VEGF, angiogenesis, and tumor development [59, 60]. SCC-13 cells transfected with VEGF form invasive, highly vascularized tumors when injected subcutaneously or intradermally into nude mice [59]. Similarly, tumors arising from a malignant HaCaT cell line, which produce large amounts of VEGF, initiate angiogenesis more quickly and to a larger degree than HaCaT cell lines which form benign tumors [60]. Furthermore, treatment with VEGFR-2 blocking antibodies reduces endothelial cell proliferation and vessel density in tumors derived from the malignant cell lines to levels comparable to benign cell lines. In addition, VEGFR-2 antibody treatments reduce tumor growth and invasiveness, suggesting that VEGF promotes tumor growth by inducing angiogenesis. Taken together, the evidence from human tumors and animal models demonstrate that VEGF is critical for the development, growth, and spread of skin tumors, and these findings have been largely attributed to the promotion of angiogenesis by VEGF.

4. Autocrine Roles for VEGF in Skin Carcinogenesis

Although dermal cells such as macrophages, fibroblasts, and other cell types are known to produce VEGF, epidermal keratinocytes are believed to be the principle source of VEGF in the skin [36, 45, 55, 61]. In addition to stimulating angiogenesis through its actions on endothelial cells, recent evidence has demonstrated that VEGF can have direct effects on keratinocytes. Several groups have now identified VEGF receptors on keratinocytes, suggesting the possibility of autocrine VEGF signaling. Currently, there is some disparity in the exact receptor profiles that have been described. Some studies have identified VEGFR-1, VEGFR-2, and NRP-1 on keratinocytes [18, 62]; however, others do not detect VEGFR-2 [15–17]. Our lab has shown that VEGF induces the proliferation of cultured primary human keratinocytes through VEGFR-1 [15] and this finding has been confirmed by others in murine keratinocytes [17]. VEGF has also been shown to induce the migration of primary keratinocytes *in vitro* [63]. Additionally, VEGFR-1 is expressed in mouse and human skin tumor cells and in squamous cell carcinoma cell lines [17], suggesting that VEGF could affect tumor cells directly.

Autocrine functions for VEGF in keratinocytes and skin tumor cells have also been suggested by recent functional studies performed *in vivo* [16, 17]. Lichtenberger et al. utilized various conditional knockout mice to uncover a direct role of VEGF in skin carcinogenesis using the K5-SOS model, in which the ras activator Son of Sevenless is constitutively activated in the epidermis [17]. In this model, K5-SOS mice develop skin tumors spontaneously and tumors can be induced rapidly by wounding the skin [17, 64]. Keratinocytes were shown to overexpress VEGF in the K5-SOS model, and K5-specific deletion of VEGF reduced tumor development in these mice. Loss of keratinocyte VEGF also lead to a decrease in vessel density and a decrease in tumor cell proliferation, and VEGF was able to enhance keratinocyte proliferation *in vitro*. Because VEGFR-1 expression was detected in murine and human skin cells, epidermal VEGFR-1 was deleted

in K5-SOS mice. A reduction in papilloma development and tumor cell proliferation was observed in conditional VEGFR-1 knockout mice compared to controls, while blood vessel density was unaffected. VEGFR-1 knockdown in SCC tumor cell lines was also shown to slow proliferation. Together, these studies establish a direct role for VEGF in skin carcinogenesis, wherein VEGF stimulates tumor cell proliferation through VEGFR-1.

Interestingly, an autocrine loop between VEGF and NRP-1 has also been discovered. Using the two-stage chemical skin carcinogenesis model, Beck et al. recently reported an effect of VEGF on $CD34^+$ cancer stem cells (CSCs) [16]. $CD34^+$ tumor cells were shown to express higher levels of VEGF than $CD34^-$ tumor cells or normal keratinocytes [16]. Epidermal overexpression of VEGF increased the pool of $CD34^+$ CSCs, while inhibition of VEGFR-2 activity with DC101 or conditional deletion of VEGF in the epidermis reduced the CSC pool and diminished CSC proliferation, in addition to reducing the number of established tumors. Interestingly, VEGF-overexpressing CSCs were found to have high levels of NRP-1. Conditional deletion of NRP-1 completely blocked tumor formation in the chemical carcinogenesis model compared to control mice which all developed papillomas. In addition, when conditional NRP-1 knockout mice were crossed with VEGF transgenic mice, VEGF was unable to promote tumor growth, even though efficient tumor angiogenesis was still observed. Overall, the results suggest that epithelial cell-derived VEGF regulates CSCs in an autocrine manner.

In addition to affecting epithelial cell proliferation and stemness, a recent study suggested that VEGF may also directly affect keratinocyte survival *in vitro*.Zhu et al. showed that exposure to UV light, the primary causative agent of NMSC, increased the expression of VEGFR-1, VEGFR-2, and NRP-1 in primary normal human keratinocytes *in vitro* and in human epidermis *in vivo* [18]. VEGFR upregulation was found to be a result of UV-induced oxidative stress. UV exposure also resulted in activation of VEGFR-1 and VEGFR-2. Interestingly, VEGF was able to protect keratinocytes from apoptosis following exposure to moderate ($300\,J/m^2$) but not high ($700\,J/m^2$) doses of UV. Activation of VEGFR-2, but not VEGFR-1, was responsible for the observed increase in keratinocyte survival. Although these results will need to be confirmed *in vivo*, they suggest that VEGF could function as a survival factor for keratinocytes following UV exposure.

5. Paracrine Roles for VEGF in Skin Carcinogenesis

In addition to endothelial cells, some immune cells also express VEGF receptors, supporting the idea that VEGF can have paracrine effects that are not related to its pro-angiogenic activity. For example, monocytes and macrophages express VEGFR-1 and VEGF has been shown to be a chemoattractant for these cells [19, 20]. Tumor-associated macrophages, particularly M2 macrophages, are believed to promote tumor growth and invasion and well as angiogenesis [65, 66]. Recently, Linde et al. used an orthotopic tumor model in which control or VEGF-transfected HaCaT cells were injected subcutaneously into mice [21]. VEGF-driven HaCaT tumors were larger, more vascular, more invasive, and had higher numbers of infiltrating M2 macrophages compared to control tumors. Depletion of macrophages reversed the effects of VEGF overexpression, indicating that VEGF was influencing tumor development by affecting macrophages. In this model, VEGF stimulated the recruitment of macrophages to the tumors but was not sufficient to polarize them. Additional tumor- and macrophage-derived IL-4 and IL-10 were responsible for M2 polarization. These studies indicate that in addition to promoting angiogenesis, VEGF can influence skin carcinogenesis by recruiting immune cells.

6. Conclusions

Strong evidence has established a critical role for VEGF in the development of non-melanoma skin cancers. VEGF is produced by the skin in response to tumor-promoting agents such as TPA and UV light, and skin tumors are known to express elevated levels of VEGF. In mouse studies, VEGF increases angiogenesis and tumor growth, while the loss of VEGF inhibits skin carcinogenesis. To date, these findings have been primarily attributed to the potent pro-angiogenic effects of VEGF. However, the presence of VEGF receptors on non-endothelial cell types, such as keratinocytes and macrophages, has expanded our view of the potential functions of VEGF. Indeed, new evidence suggests that VEGF can impact skin carcinogenesis by directly affecting keratinocytes, tumor cells, and immune cells. While there is no doubt that VEGF plays an important role in skin carcinogenesis, more work is required to characterize the various mechanisms by which VEGF contributes to this process and to understand the relative importance of each of these pathways. Further studies will have to be carried out to determine whether these newly described alternative functions of VEGF can be targeted to treat NMSC.

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Review Article

Patched Knockout Mouse Models of Basal Cell Carcinoma

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Basal cell carcinoma (BCC) is the most common human tumor. Mutations in the hedgehog (HH) receptor Patched (PTCH) are the main cause of BCC. Due to their high and increasing incidence, BCC are becoming all the more important for the health care system. Adequate animal models are required for the improvement of current treatment strategies. A good model should reflect the situation in humans (i.e., BCC initiation due to *Ptch* mutations on an immunocompetent background) and should allow for (i) BCC induction at a defined time point, (ii) analysis of defined BCC stages, and (iii) induction of BCC in 100% of animals. In addition, it should be easy to handle. Here, we compare several currently existing conventional and conditional *Ptch* knockout mouse models for BCC and their potential use in preclinical research. In addition, we provide new data using conditional $Ptch^{flox/flox}$ mice and the $K5\text{-}Cre\text{-}ER^{T+/-}$ driver.

1. General Aspects and Current Therapies of BCC

1.1. Epidemiology. BCC is a tumor of the skin and the most prevalent cancer in the Western world. Its incidence is increasing worldwide. Retrospective studies show that the increase in mainland Europe is approximately 1/100,000 persons per year and even 6/100,000 in the UK [1]. It is estimated that the lifetime risk of developing BCC for a child born in 1994 is 28% to 33% [2] and that young people will suffer more and more from this tumor [3]. Risk factors for BCC formation are exposure to ultraviolet radiation (UV) or ionizing radiation (IR), immunosuppression, or a genetic predisposition [4]. Due to their high and increasing incidence, BCC are becoming an important issue for the health care system [5]. In some countries, the cost of care for BCC and other nonmelanoma skin cancers comprises 9% of the costs for all cancers [6].

1.2. Histology. BCC are usually well differentiated and the tumor cells appear histologically similar to basal cells of the epidermis. BCC can be subdivided into two subgroups that show either an indolent or an aggressive growth behavior. The indolent-growth variants comprise nodular/micronodular and superficial BCC. These subtypes occur in 21%/15% and 17% of cases, respectively, and thus are the most common BCC variants [7]. Whereas nodular BCC consist of nests of basaloid cells in the dermis, superficial BCC are characterized by numerous small tumor nests attached to the undersurface of the epidermis by a broad base. The more aggressive tumors are less frequent and include infiltrative, metatypical, morpheaform, or sclerosing BCC (for review see [8]). Although BCC very rarely metastasize, they can result in local tissue destruction due to invasion into deeper layers of the skin, thereby causing significant morbidity [9].

1.3. Molecular Pathogenesis of BCC. BCC are thought to be caused by uncontrolled activation of the hedgehog (HH) signaling pathway. In the majority of cases, this is due to inactivating mutations in the HH receptor and tumor suppressor gene *PTCH*. *PTCH* mutations in BCC were first observed in basal cell nevus syndrome (also known as nevoid basal cell carcinoma syndrome or Gorlin-Goltz syndrome), which is a rare familial autosomal-dominant disorder that predisposes the affected individual to developing this tumor. Only a minority of BCC are caused by activating mutations in Smoothened (SMO) (reviewed in [9]).

PTCH normally acts as an inhibitor of HH signaling by repressing the function of SMO. Binding of the HH ligand to PTCH or inactivating *PTCH* mutations suspend this inhibition, which allows activation of SMO and results in the formation of activator forms of the GLI zinc finger transcription factors GLI2 and GLI3. Activation of GLI2 and GLI3 leads to transcription of *GLI1*. Thus, the expression level of *GLI1* is considered as a reliable indicator of the pathway's activity. Another HH target is *PTCH* itself, which regulates its expression in a negative feedback (reviewed in [10]). Indeed, nearly all BCC express *GLI1* and *PTCH*, which demonstrates the important role of aberrant HH signaling in these tumors [11].

Several other signaling pathways are presumably involved in BCC tumorigenesis. Mutations of the tumor suppressor gene *p53* have been shown in 40% of sporadic BCC [12] and were correlated with aggressive behavior [13–15]. In addition, activation of canonical Wnt/β-catenin signaling seems to play a role in specific histological BCC subtypes. These subtypes include early stages of superficial BCC [16], pilomatricoma (a tumor of the hair follicle [17]) as well as infiltrative BCC variants [18, 19]. Indeed, nuclear β-catenin is found in infiltrative BCC and in superficial BCC [18], but only rarely in human nodular BCC [17, 18, 20]. BCC also express activated AKT [21]. Finally, EGFR signaling seems to be an essential *in vivo* requirement in HH-driven BCC because EGFR signaling cooperates with the HH pathway to induce genes (e.g., *JUN*, *SOX9,* and *FGF19*) critical for the determination of the oncogenic BCC phenotype [22].

Growth and progression of human BCC is also highly influenced by the tumor microenvironment. For example, tumor-associated macrophages are able to enhance the invasive phenotype and angiogenesis [23]. Furthermore, α-smooth muscle actin positivity of peritumoral fibroblast tends to be greatest in infiltrative tumor areas [24]. In addition, stromal cells of BCC produce high levels of *Gremlin1*, which is a factor stimulating BCC growth by antagonizing bone morphogenic protein-mediated repression of cell proliferation [25, 26]. Finally, EGFR ligands are increased in the tumor stroma [27], which may influence tumor intrinsic EGFR signaling (see above).

However, whereas all the above-mentioned factors may influence the susceptibility to BCC or the BCC phenotype, deregulation of HH signaling is the central abnormality in all these tumors and seems to play the major role in its formation [9].

1.4. Conventional Treatment Options of BCC. Surgical excision is currently by far the most commonly used treatment of BCC. However, surgery can result in permanent tissue damage and scarring, which is unwanted especially in facial areas. In addition, surgery may be problematic if the tumor is localized around the eye, mouth, or in close vicinity of the cartilage of the nose and ears [9]. This has led to less invasive treatment strategies such as photodynamic therapy or application of imiquimod-containing creams. Photodynamic therapy refers to a technique in which the tumor is treated with a photosensitizing chemical in a cream and is exposed to light several hours later [28]. Imiquimod is an immune response modifier, which stimulates the Toll-like receptor 7 and increases the activity of natural killer cell, macrophages, and the proliferation and differentiation of B lymphocytes [29]. Another option is cryotherapy, which destroys the skin lesion by application of extreme cold such as compressed nitrous oxide [30]. Another agent for topical application is 5-fluorouracil (5% cream), which leads to tumor necrosis. Among the drawbacks of the latter agent is the limited tissue penetration [31].

Although these therapies are associated with moderate morbidity, the outcome is still considered to be unspecific. In addition, these treatments sometimes have side effects such as pain, scarring, and local skin reactions [31]. Together, these data show that the availability of a simplified and more effective treatment would contribute to lower the costs related to this tumor.

1.5. Targeting the HH Signaling Pathway in BCC. The knowledge about the genetic and molecular events involved in BCC pathogenesis has enormously contributed to the establishment of new treatment options. Very successful have been strategies specifically targeting HH signaling. The first small-molecule inhibitor of the HH pathway was the naturally occurring compound cyclopamine that inhibits SMO activity by direct binding [32]. Within the last few years, more potent SMO inhibitors have been developed and are currently being tested in phase I and II clinical trials [33]. Recently, the SMO antagonist vismodegib (Erivedge, GDC-0449) has been approved by the FDA for the treatment of metastasizing and locally destructive BCC [34, 35]. However, although vismodegib shows both remarkable therapeutic and preventive efficacy, the cumulative toxicity of this agent has led to discontinuation of therapy in a substantial fraction of patients [35, 36]. Therefore, it will be necessary to develop strategies that ameliorate some of the common toxicities of this drug [35].

2. Mouse Models of BCC for Preclinical Studies

The establishment of new treatment strategies requires adequate animal models. An ideal model should allow for analysis or modulation of molecular events associated with tumor initiation or tumor progression. It should also permit to evaluate antitumor therapies useful to prevent, inhibit, or even to induce regression of BCC *in vivo*. To fulfill these requirements, an ideal animal model should allow for analysis of BCC that have reached a defined BCC stage after their initiation in 100% of animals.

Hitherto, several murine BCC models exist. These include *Ptch* knockout mice and mice overexpressing Hh, oncogenic Smo, Gli1 or Gli2 specifically in the skin using the keratin (K) 5, 6, or 14 promoters. Depending on the gene and the targeted cell type, the skin tumor subtypes range from follicular hamartoma and trichoepithelioma to nodular or invasive BCC [16, 37–45]. In addition, allografts from BCC-bearing $Ptch^{+/-}$ $p53^{-/-}$ mice or from Shh transgenics can be grown in scid mice [43, 46]. Finally, the cell line ASZ001 generated from a BCC of an irradiated *Ptch* heterozygous

mouse (see below) has been successfully implanted into nude mice and used to study the effects of the EGFR-inhibitor afatinib [22].

Since most human BCC arise due to *PTCH* mutations and since the stromal microenvironment plays an important role in formation and progression of this tumor (see section "*Molecular pathogenesis of BCC*"), immunocompetent *Ptch* mutant mice certainly represent the closest model to the human condition.

3. *Ptch* Knockout Mouse Models for BCC

3.1. Spontaneous Ptch Mutations in Mice. Two spontaneous *Ptch* mutant animals have been described. The spontaneous recessive mutation "mesenchymal dysplasia" (mes) is caused by a deletion of 32 bp in the C-terminal cytoplasmic domain of *Ptch* [47]. $Ptc1^{mes/mes}$ mice are viable and show increased proliferation and hyperplasia of the basal cell layer [48]. However, in spite of these skin anomalies $Ptc1^{mes/mes}$ mice do not develop BCC even after exposure to radiation [49].

The *Ptch1D11* is a mutation caused by an aberrant recombination event while producing a *Ptch* null allele for the generation of $Ptch^{neo12/+}$ mice (see below). The $Ptch1^{D11}$ locus presumably results in a weak *Ptch* allele [50]. $Ptch1^{D11/D11}$ animals are sterile, but otherwise appear normal [50].

3.2. Conventional Ptch Knockout Mice. So far, two different conventional *Ptch* knockout mouse models for BCC have been described. These are the $Ptch^{neo12}$ and $Ptch^{neo67}$ strains, in which exons 1 and 2 or exons 6 and 7, respectively, are deleted in the germline [51, 52]. Homozygous $Ptch^{neo12/neo12}$ and $Ptch^{neo67/neo67}$ embryos die around embryonic day 9.5 due to heart and neural tube closure defects. Heterozygous $Ptch^{neo12/+}$ and $Ptch^{neo67/+}$ animals survive and show increased susceptibility to spontaneous formation of rhabdomyosarcoma, medulloblastoma, and tiny epidermal hyperproliferations. To induce BCC, $Ptch^{neo12/+}$ animals are usually exposed to UV three times per week for up to several months [53–55]. After 12 months of chronic UV exposure, all $Ptch^{neo12/+}$ mice develop lesions with histologic features of human BCC. Of these lesions, 44% can be classified as superficial, 13% have histologic features of nodular or infiltrating human BCC and 43% have features of trichoblastoma [53]. Chronic UV exposure also results in macroscopic tumors. Of these visible tumors, approximately 20% are BCC or trichoblastomas (tumors with follicular differentiation that share many histologic features with BCC), 30% are squamous cell carcinoma (SCC) or keratoacanthomas (SCC-like tumors), and 50% are fibrosarcomas or fibromas [53].

The situation is somewhat different when inducing BCC-like lesions by IR. As shown by Aszterbaum et al. [53], a single dose of 1–4 Gy applied at 2 months of age results in microscopic trichoblastoma-like tumors in all $Ptch^{neo12/+}$ mice after 1 year. Another study performed by Mancuso et al. revealed that a dose of 3-4 Gy applied to adult $Ptch^{neo67/+}$ mice at the age of 2-3 months leads to nodular BCC-like lesions in 21–47% of animals, and in infiltrative lesions in 5–12% [45]. IR exposure never results in fibrosarcomas or SCC [45, 53]. Particularly BCC in the IR-induced model further progress into an aggressive phenotype [45]. Immune surveillance was not impaired in either model [53].

On the molecular level, formation of IR-induced nodular BCC requires *Ptch* heterozygosity in conjunction with mutations in other molecules such as *p53* [45]. Moreover, the progression into an aggressive phenotype seems to be associated with biallelic loss of *Ptch* [45]. This might be different from human BCC, which in most cases lack aggressiveness [56] and which frequently show loss of heterozygosity at the *PTCH* locus on chromosome 9q22 already at the nonaggressive stage [57–59]. Thus, it remains to be resolved whether loss of the wildtype *Ptch* allele in irradiated mice indeed triggers BCC aggressiveness or whether it is just a secondary event due to general irradiation-induced genomic instability.

These differences to human BCC and the fact that BCC in irradiated *Ptch* heterozygous mice develop at undefined time points and in indefinite areas of the exposed skin render this animal model may complicate the examination of early molecular processes involved in the initiation of BCC. However, these mice are a great tool to evaluate new treatment options of microscopic, macroscopic, and aggressive BCC that are caused by *Ptch* mutations along with additional irradiation-dependent mutations. Indeed, irradiated $Ptch^{neo12/+}$ knockouts have been used in several preclinical studies (Table 1), which are described in the following section.

To study the effects of the Hh inhibitor cyclopamine [60], BCC have been induced in $Ptch^{neo12/+}$ animals by UV exposure 3 times per week from age 6 to 32 weeks. After this time, approximately 50% of the mice had developed one or more macroscopic BCC. For the following 20 weeks the animals were treated with cyclopamine that significantly reduces tumor growth [60]. Regression of microscopic BCC after Hh inhibition has also been shown in skin punches of UV-irradiated $Ptch^{neo12/+}$ mice, which were kept in cell culture for 6 days and treated for the last 4 days with the small molecule inhibitor of Hh signaling CUR61414 [61].

$Ptch^{neo12/+}$ animals have also been used to analyze the antitumoral effects of α-difluoromethylornithine (DFMO) [62]. DFMO is a potent inhibitor of cutaneous ornithine decarboxylase, which is expressed in BCC and is known to promote tumor formation [69, 70]. To analyze its antitumoral effects, $Ptch^{neo12/+}$ animals were irradiated with UV 3 times per week for 32 weeks [62]. Thereafter, the tumor-bearing animals obtained DFMO in the drinking water for 20 weeks. The results show that DMFO reduced the number of visible BCC and diminished BCC-like microscopic lesions. Furthermore, a reduction of *Ptch*, *Gli1*, *Gli2,* and *Gli3* expression in nontumor-bearing skin of these animals was evident [62].

A fourth study analyzed the antitumoral activity of the retinoid tazarotene [55]. Retinoids are ligands of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) and show tumor-suppressive capacity in several tumor entities [71]. Tazarotene was topically applied to the skin of 1.5 or

Table 1: *Ptch* knockout mouse models for preclinical BCC treatment studies.

BCC model	Mode of BCC induction	Treatment	Reference
$Ptch^{neo12/+}$	UV	Cyclopamine	[60]
$Ptch^{neo12/+}$ skin punches	UV	CUR61414	[61]
$Ptch^{neo12/+}$	UV	α-difluoromethylornithine	[62]
$Ptch^{neo12/+}$	IR/UV	Tazarotene	[55]
$Ptch^{neo12/+}$	UV	Tazarotene, ATRA, AGN195813, AGN194204, AGN194310	[63]
$Ptch^{neo12/+}$	IR/UV	Celecoxib, sulindac, MF-tricyclic	[64]
$Ptch^{neo12/+}$	UV	Green/black tea	[54]
$Ptch^{neo12/+}$ *K14-Cre-ER p53fl/fl*	IR and conditional p53 ablation	CUR61414	[65]
$Ptch^{neo12/+}$ *K14-Cre-ER p53fl/fl*	IR and conditional p53 ablation	Itraconazole	[66]
$Ptch^{neo12/+}$ *K14-Cre-ER p53fl/fl*	IR and conditional p53 ablation	Vitamin D3	[67]
$Ptch^{flox/flox}ERT2^{+/-}$	conditional *Ptch* ablation	Calcitriol	[68]

UV: ultraviolet radiation; IR: ionizing radiation.

2.5 months old $Ptch^{neo12/+}$ mice for 5 consecutive days/week. Two weeks after onset of treatment, that is, at the age of 2 or 3 months, BCC were induced by exposure to UV (3 times/week) or IR (once), respectively. In order to examine the growth of microscopic BCC, skin biopsies of the UV-treated group were taken at the ages of 7, 9, and 11 months, whereas those of the IR-treated group were taken at the age of 10 months. Tazarotene treatment reduced the number and size of microscopic BCC after UV or IR exposure and also prevented formation of macroscopic BCC in IR-exposed animals at the age of 16 months [55]. A follow-up study showed that tazarotene also inhibited the number and size of preexisting microscopic BCC lesions [63]. For this purpose, animals were exposed to IR at 2 months of age and the tazarotene treatment was started 5 months later for additional 5 months. Efficacy was also shown for other retinoid-related agents such as ATRA (pan-RAR agonist), AGN195813 (RARα agonist), or AGN194204 (pan-RXR agonist), however, to a lesser extent [63].

In a next study, the antitumoral effects of cyclooxygenases (COX) inhibitors have been analyzed [64]. COX inhibitors belong to nonsteroidal anti-inflammatory drugs, which are thought to prevent the formation of SCC in humans [72]. The COX inhibitors sulindac (nonspecific COX inhibitor), MF-tricyclic (COX2-specific inhibitor) or celecoxib (COX2-specific inhibitor) were administered starting at the age of 6 weeks and BCC were induced 2 weeks later by exposure to UV (3 times/week, continued until the age of 12 months) or IR (once). At the age of 9 months, the burden of microscopic BCC was reduced by 35% in celecoxib-treated animals and by 50–60% in sulindac- or MF-tricyclic-treated mice [64].

In just another study, $Ptch^{neo12/+}$ mice were utilized to assess the effect of tea on BCC formation [54]. The rational for this experiment was the observation that green tea may protect against photocarcinogenesis [73]. Green or black tea was added to the drinking water of $Ptch^{neo12/+}$ mice beginning from the age of 46 days. UV exposure (3 times/week) was started 2 weeks later. However, neither number nor size of BCC was reduced 5 or 7 months after initial UV exposure [54].

$Ptch^{neo12/+}$ mice were also used to analyze the effects of itraconazole, vitamin D3, or CUR61414. Similar to CUR61414, the antifungal compound itraconazole and vitamin D3 derivatives have been shown to inhibit Hh signaling, probably by interaction and inhibition of SMO [61, 66, 74]. In order to accelerate BCC carcinogenesis in these studies, $Ptch^{neo12/+}$ mice were crossed to *K14-Cre-ER p53fl/fl* mice and *p53* was deleted in the $Ptch^{neo12/+}$*K14-Cre-ER p53fl/fl* offspring at the age of 6 weeks by injection of 100 μg tamoxifen on 3 consecutive days. Two weeks later, the animals were exposed once to IR. This resulted in visible BCC at the age of 5-6 months. CUR61414 was applied topically twice daily to BCC on the dorsal skin 5 days a week for up to 42 days. This decreased the tumor size by 60%, which was accompanied by inhibition of *Gli1* expression in tumor tissue [65]. The same was shown for itraconazole. When applied orally twice daily for 18 days, itraconazole led to a significant suppression of tumor growth, which was reversible after drug withdrawal [66]. The treatment with vitamin D3 was also effective. Although an impact on tumor size has not been mentioned by the authors, vitamin D3 blocked proliferation and Hh signaling in visible BCC when applied topically 5 days/week for 30 days [67].

Altogether, these data show that conventional *Ptch* knockout mice are an extremely valuable tool to analyze the efficacy of new anti-BCC drugs. Still there might be

a few drawbacks when using irradiated $Ptch^{+/-}$ animals. First, the onset of tumor formation is variable, with tumors arising at different time points and different sites after exposure to radiation [45]. This may complicate studies, which address the use of drugs in small precursors as opposed to progressed tumors. Second, due to the mode of BCC induction by exposure to IR or UV, the molecular mechanisms responsible for BCC formation are probably very heterogeneous. This heterogeneity may also be reflected by the spectrum of skin tumor, which comprises nodular, superficial as well as infiltrative BCC subtypes, trichoblastomas, and also SCC [45, 53]. These characteristics of irradiated $Ptch^{+/-}$ mice may hamper the evaluation of new treatment strategies designed for targeting specific BCC subtypes.

3.3. Conditional Ptch Knockout Mice. With respect to timing of BCC initiation and to investigate defined BCC stages, conditional *Ptch* knockout mice may be a more suitable model. Conditional knockouts also allow for induction of the *Ptch* mutation in specific cell lineages, which is important when seeking for, for example, the identification of the cell of origin of BCC [44, 75].

To our knowledge five different conditional *Ptch* knockout mouse strains have been generated up to date. Of these, only one has been used in a preclinical study targeting BCC [68].

3.3.1. Conditional Ptch Knockout Mice Targeting Exons 1, 2, or 3 of the Ptch Gene. In $Ptch^{neo/neo}$ mice, exon 3 is flanked by *loxP* sites [76]. The deletion of exon 3 is expected to lead to a premature stop codon and thus to a truncated *Ptch* protein. Indeed, embryos with a homozygous deletion of *Ptch* exon 3 display developmental defects and die at embryonic day 9.5. This is similar to conventional *Ptch* knockouts, in which the homozygous germline mutation results in embryonal lethality between embryonic day 9.0 and 10.5 [51, 52]. In adult $Ptch^{neo/neo}$ mice, BCC can be induced with tissue-specific Cre drivers. For example, BCC arise in conditional $Krt6aCrePtch^{neo/neo}$ mice after activation of the Krt6a promotor by topical application of retinoic acid (RA) [44]. Expression of Krt6aCre results in a loss of *Ptch* in 40% of interfollicular basal cells and outer root sheath cells of multiple hair follicles. Within 4 weeks, 25% of animals develop basal cell invaginations and after 12 additional weeks 100% of mice suffer from BCC, which show high Hh signaling activity. However, since the *Krt6a* promotor is also permanently active in the companion cell layer, untreated $Krt6aCrePtch^{neo/neo}$ mice develop epidermal hyperproliferations by 9 to 12 months and suffer from hair loss. These hyperproliferations are associated with hair follicles or sebaceous glands and do not progress to BCC.

BCC in $Ptch^{neo/neo}$ mice can also be induced using the skin-specific *K14Cre* or the *MxCre* mouse. The latter strain is transgenic for a Cre recombinase controlled by the interferon-inducible promoter *Mx.1*. Besides heamatopoetic cells, liver, spleen, kidney, lung, gastric epithelium, and other tissues [77], the *Mx.1* promotor is also active in basal cells of the skin [78]. $K14Ptch1^{\Delta/\Delta}$ mice (derived from a cross of *K14Cre* and $Ptch^{neo/neo}$ mice) develop BCC within 3-4 weeks after birth. In $MxPtch1^{\Delta/\Delta}$ animals, BCC occur 8–10 weeks after activation of the MxCre by intraperitoneal injection of the immune stimulator polyinosinic-polycytidylic acid (poly(I:C)) on 3 consecutive days. However, due the widespread activity of the *Mx.1* promotor, activation of MxCre in $Ptch^{neo/neo}$ mice also ablates *Ptch* in other organs. This result in B- and T-cell defects, thymic atrophy, increased numbers of myeloid progenitors, and loss of osteoblasts [78]. Due to these defects, this model is rather unsuitable for preclinical studies using anti-BCC drugs.

In addition to $Ptch^{neo/neo}$ mice, other conditional *Ptch* knockout mouse models targeting *Ptch* exons 1, 2, or 3 exist. In the $Ptch1^{c/c}$ mouse model, the *Ptch* exon harboring the first ATG of the *Ptch* gene and exon 2 are flanked by *loxP* sites [79]. According to the provided data and to the precise nomenclature (see [80]), the exon containing the first ATG equates exon 1B. Therefore, the floxed region in the $Ptch1^{c/c}$ mouse model additionally contains the alternative first *Ptch* exons 1 and 1A [80]. After Cre-mediated excision of these exons, the resulting $Ptch1^{\Delta loxP/\Delta loxP}$ embryos display the same phenotype as homozygous embryos derived from conventional knockouts. However, embryonic and neonatal lethality is also observed in some $Ptch1^{c/c}$ mice, which probably results from *Ptch* misexpression due to the insertion of a *lacZ* gene.

The $Ptch1^{c/c}$ mouse model is similar to a third $Ptc1^{F1\text{-}2m}$ conditional mouse model described by Taniguchi and colleagues, which likewise allows for the ablation of the exons 1B, 1, 1A, and 2 [81]. Finally, one recent publication described *Ptch1neo(fl)Ex2(fl)* mice, which develop BCC-like lesions after activation of the Cre recombinase K5Cre*PR1 by RU486 or of Lgr5-EGFP-IHRES-creER^{T2} by tamoxifen [75]. Although not explicitly mentioned by the authors, we assume that exon 2 is targeted in *Ptch1neo(fl)Ex2(fl)* animals.

Due to alternative splicing of *Ptch* exons, the above-mentioned animal models may be somewhat leaky when it comes to a complete deletion of all *Ptch* transcript variants. As shown by Shimokawa et al. [80], the first *Ptch* exons 1B, 1 and 1A as well as exons 2–5 can be subjected to alternative splicing. Furthermore, an alternative first exon 1C exists, which is located more than 9 kb upstream of exon 2 and can be spliced into exon 2 or 3 of the *Ptch* transcript [80]. This has also been shown by Nagao et al. [82, 83], who used a different numbering for the alternative first exons and who named the most upstream exon 1A [82, 83]. Although the role of the various *Ptch* splice variants is not completely understood, they are expressed in specific tissues and can modulate Hh signaling to various extents [80, 82, 83].

According to Nagao et al., *Ptch* exons 6 to 9 are not subjected to alternative splicing [83]. Therefore, targeting this region is beneficial in order to obtain a complete loss of regular *Ptch* transcripts.

3.3.2. Conditional Ptch Knockout Mice Targeting Exons 8 and 9 of the Ptch Gene. We have recently described

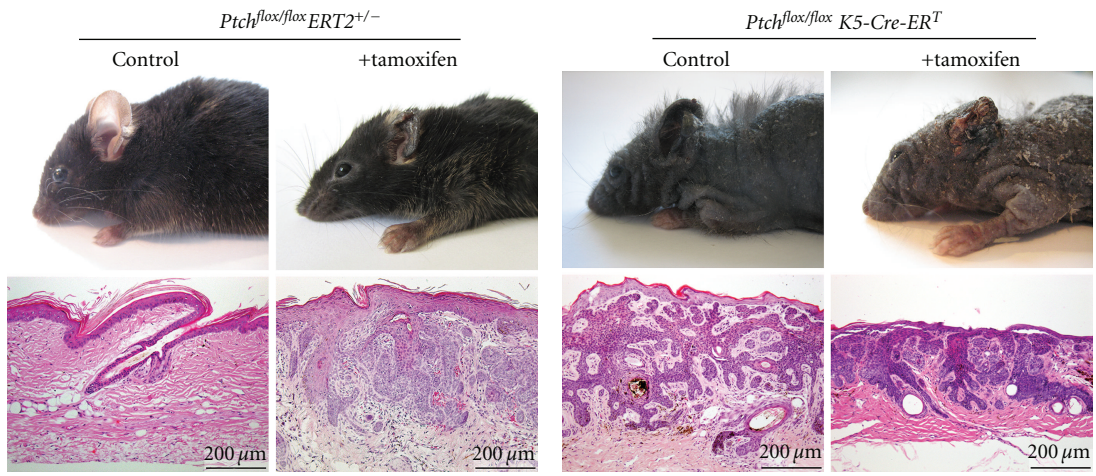

Figure 1: Features of BCC in $Ptch^{flox/flox}ERT2^{+/-}$ and $Ptch^{flox/flox}$K5-Cre-ER^T mice. $Ptch^{flox/flox}$ animals were bred with the mouse lines *ERT2* or K5-Cre-ER^T. The respective $Ptch^{flox/+}ERT2^{+/-}$ and $Ptch^{flox/+}$K5-Cre-ER^T mice were backcrossed to $Ptch^{flox/flox}$ mice to obtain $Ptch^{flox/flox}ERT2^{+/-}$ and $Ptch^{flox/flox}$K5-Cre-ER^T mice. ERT2 or K5-Cre-ER^T was activated by one intramuscular (i.m.) injection of 100 μg tamoxifen as described recently [20, 85], or by intraperitoneal injections of 1 mg tamoxifen (10 μg/μL in sterile ethanol/sun flower oil 1 : 25) for 5 consecutive days (see [88]), respectively. Genotyping of the $Ptch^{flox}$, $Ptch^{del}$, *ERT2,* and K5-Cre-ER^T alleles was performed as described recently [20, 84, 85]. All mice used in the study were handled in accordance with the German animal protection law. (a) shows the appearance and histology of skin from control and tamoxifen-treated $Ptch^{flox/flox}$ $ERT2^{+/-}$ mice and (b) shows that of control and tamoxifen-treated $Ptch^{flox/flox}$K5-Cre-ER^T mice.

$Ptch^{flox}$ knockout mice (available at http://www.jax.org/: *B6N.129-Ptch1*^{tm1Hahn}*/J*, Stock 012457) permitting the conditional ablation of exons 8 and 9 by introduction of *loxP* sites into the introns 7 and 9. $Ptch^{flox/flox}$ mice are born at the expected Mendelian ratio and are viable and fertile. Neither the *loxP* sites nor the neomycin resistance cassette in intron 9 disturb the normal splicing of the *Ptch* mRNA derived from the $Ptch^{flox}$ allele [84]. As reported by our group, the excision of exons 8 and 9 can be carried out very effectively, thereby generating the $Ptch^{del}$ allele [20, 84–86]. This results in an aberrant *Ptch* transcript with exon 7 spliced into exon 10 and leads to a frameshift and a premature stop codon. The postulated truncated protein consists of 341 instead of 1093 aminoacids and lacks the sterol sensing domain, the second extracellular loop, and the C-terminus. Due to the lack of appropriate *Ptch* antibodies, we were not able to detect this protein, but the phenotype of $Ptch^{del/del}$ embryos indicates a complete loss of *Ptch* function. Indeed, all homozygous $Ptch^{del/del}$ mutants die before embryonic day 10 *in utero*. $Ptch^{del/+}$ mice survive and develop malformations at incidences similar to those observed in conventional *Ptch* knockout mice on the same genetic background [20, 85].

For the induction of BCC, $Ptch^{flox/flox}$ mice can be crossed to *Rosa26CreERT2* mice (hereafter *ERT2* mice) that express a tamoxifen-inducible Cre recombinase under the control of the ubiquitously active *ROSA26* promoter [87]. Activation of ERT2 by a single intramuscular (i.m.) injection of 100 μg tamoxifen results in BCC in 100% of animals. Microscopically, BCC precursors are visible 45 days after tamoxifen induction. The tumors are fully developed by day 90 (Figure 1(a)) [85]. After that time, the tumors start to regress, which is becoming obvious 200 days after tamoxifen-treatment [20]. All BCC in this model have features of the human nodular subtype and are noninvasive. As indicated by abundant *Gli1* and *Ptch* expression, they are characterized by strong Hh signaling activity [20, 85]. The tumors develop preferentially on ears and tails and are very rarely detected in hairy skin. The reason for this preference is unknown but may involve a better blood circulation in ears and tails, resulting in elevated tamoxifen concentrations after i.m. application (discussed in [85]).

This shows that in $Ptch^{flox/flox}$ $ERT2^{+/-}$ mice BCC can be induced very easily and reliably by one single injection of tamoxifen. Furthermore, in this model, where all mice show full developed BCC 90 days after activation of ERT2, specific antitumor treatments can be commenced at specific time points after tumor induction and at a defined age of the animals.

As indicated in Table 1, we recently examined the antitumor effects of calcitriol, which is the physiologically active form of vitamin D3 [68]. Calcitriol treatment was started either immediately or 60 days after BCC initiation. The treatment was continued until day 90, when all mice were sacrificed. The study revealed that BCC growth was significantly inhibited in mice treated from days 0 to 90, but not in those treated from days 60 to 90 [68]. These data show that conditional $Ptch^{flox/flox}$ $ERT2^{+/-}$ mice are particularly useful to study the preventive or curative effects of a specific

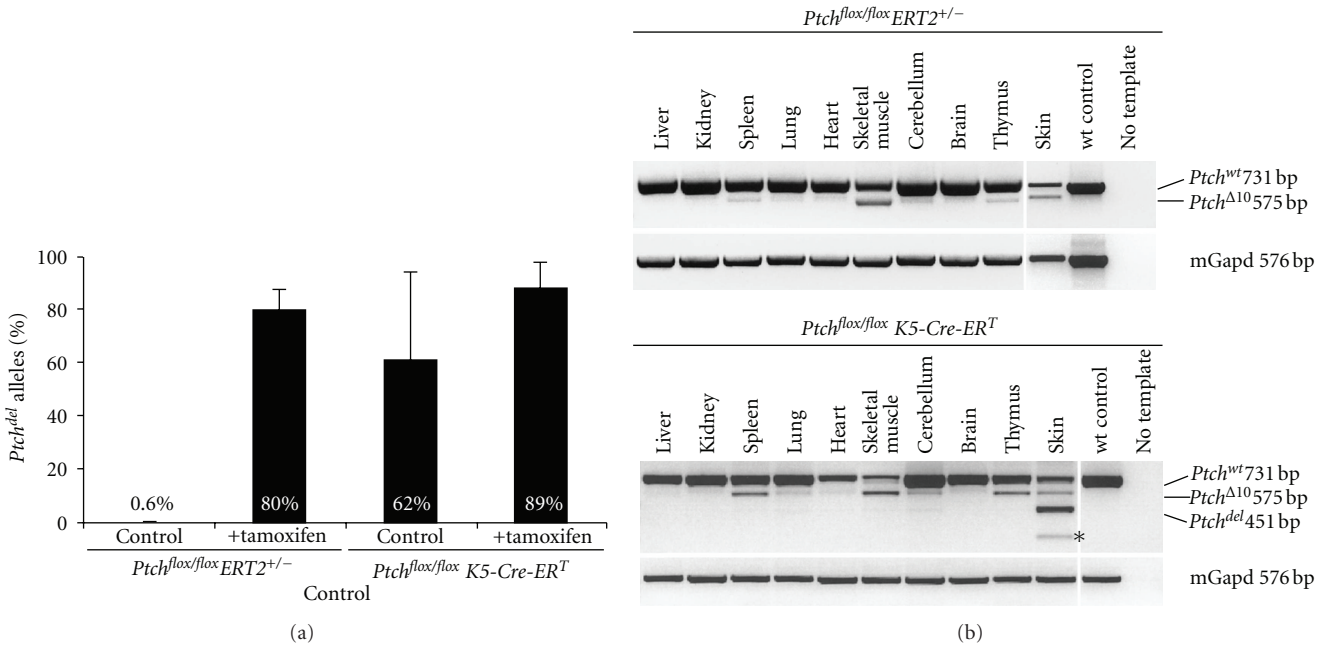

Figure 2: *Ptch* recombination and expression in $Ptch^{flox/flox}ERT2^{+/-}$ and $Ptch^{flox/flox}K5\text{-}Cre\text{-}ER^T$ mice. (a) The efficiency of *loxP* recombination at the *Ptch* locus in DNA derived from tail skin from untreated and tamoxifen treated mice was determined by allele-specific real-time PCR as described in [85]. (b) *Ptch* transcripts in different tissues were analyzed by RT-PCR. The transcripts derived from the $Ptch^{flox}$ and the $Ptch^{del}$ locus (the latter equates to the floxed *Ptch* locus after Cre-mediated excision) were analyzed by semiquantitative RT-PCR as described in [85]. In the skin, untreated $Ptch^{flox/flox}ERT2^{+/-}$ mice only expressed $Ptch^{wt}$ transcripts and the normally occurring $Ptch^{\Delta 10}$ transcript lacking exon 10. In contrast, untreated $Ptch^{flox/flox}K5\text{-}Cre\text{-}ER^T$ mice expressed $Ptch^{del}$ transcripts and $Ptch^{del}$ transcripts lacking exon 10 (asterisk) in the skin.

Table 2: BCC formation in $Ptch^{flox/flox\,flox}ERT2^{+/-}$ and $Ptch^{flox/flox}K5CreERT^{+/-}$ mice.

Genotype	Age at tamoxifen application	*n*	Age range (days)	Mice with BCC	Healthy
$Ptch^{flox/flox\,flox}ERT2^{+/-}$	—	13	56–293	0	13
*$Ptch^{flox/flox\,flox}ERT2^{+/-}$	42–56 days	10	93–365	10	0
$Ptch^{flox/flox}K5CreERT^{+/-}$	—	14	87–172	12	2
$Ptch^{flox/flox}K5CreERT^{+/-}$	—	14	200–246	14	0
$Ptch^{flox/flox}K5CreERT^{+/-}$	55–82 days	9	132–170	9	0

*Data already published in [85].

anticancer drug. This is due to the reliable BCC initiation and progression to early precursors (after 45 days) and fully developed (after 90 days) tumors.

Although the $Ptch^{flox/flox}ERT2^{+/-}$ BCC model is very easy to handle (i.e., application of one single dose of tamoxifen) and is solid with respect to induction of a specific BCC subtype (i.e., 100% of animals develop the nodular subtype 90 days after BCC induction), it also may have some disadvantages. Due to the ubiquitous expression of ERT2, the i.m. application of even a low dose of tamoxifen may cause *Ptch* deletion in other cell lineages or tissues. Even though we have not found any evidence for *Ptch*-ablation in other organs than the epidermis and the injected muscle [20, 85], we now have crossed $Ptch^{flox/flox}$ animals to $K5\text{-}Cre\text{-}ER^T$ mice, which express the tamoxifen-inducible ER^T selectively in cells of the basal layer of the skin [89–92]. Activation of ER^T by 4-hydroxy-tamoxifen (the active metabolite of tamoxifen) is ~10-fold less efficient than that of ERT2 [89]. Therefore, a cumulative dose of 5 mg has been used to activate $K5\text{-}Cre\text{-}ER^T$ in 10-weeks-old $Ptch^{flox/flox}$ $K5\text{-}Cre\text{-}ER^T$ animals. Untreated $Ptch^{flox/flox}$ $K5\text{-}Cre\text{-}ER^T$ mice served as controls. All $Ptch^{flox/flox}K5\text{-}Cre\text{-}ER^T$ mice developed BCC on tails and ears after tamoxifen injection (Figure 1(b)). They also suffered from BCC in hairy skin. Importantly, BCC also developed in $Ptch^{flox/flox}$ $K5\text{-}Cre\text{-}ER^T$ untreated control mice. Some control mice even suffered from complete hair loss (Figure 1(b)). Histological examination revealed that 86% of the controls (12/14) have developed BCC at the age of 87–172 days (Table 2). After 200–246 days, all control mice have developed tumors even without $K5\text{-}Cre\text{-}ER^T$ activation (Table 2, Figure 1(b)). The leakiness of $K5\text{-}Cre\text{-}ER^T$ was also demonstrated on molecular level. Thus, the amount

of recombined *Ptch* alleles in DNA isolated from skin derived from untreated controls was up to 83% ($n = 5$, mean 62%). This was almost identical to tamoxifen-treated $Ptch^{flox/flox}$ *K5-Cre-ER*^{T} animals (amount of recombined *Ptch* allels 91%, $n = 6$) (Figure 2(a)). Consistent with these data, untreated $Ptch^{flox/flox}$ *K5-Cre-ER*^{T} mice also showed high expression of $Ptch^{del}$ transcripts in the skin (Figure 2(b)).

This is considerably different to untreated $Ptch^{flox/flox}$ $ERT2^{+/-}$ mice, which do not develop any skin tumors within up to 293 days ($n = 13$) (Table 2), and which do not show recombination at the floxed *Ptch* locus in the absence of tamoxifen. Thus, whereas the recombination is 89% in tamoxifen-treated mice ($n = 4$), it is only 0.6% in untreated $Ptch^{flox/flox}$ $ERT2^{+/-}$ animals ($n = 7$) and $Ptch^{del}$ transcripts were never detected in any of the examined tissues (Figure 2(a)).

Although BCC in both the $Ptch^{flox/flox}$ $ERT2^{+/-}$ and $Ptch^{flox/flox}$ *K5-Cre-ER*^{T} model are identical based on histology and also at the level of Hh signaling activity (i.e., BCC of both models express *Gli1* and *Ptch*), K5-Cre-ER^T is highly leaky resulting in BCC formation even without Cre activation. Therefore, *K5-Cre-ER*^{T} should not be used in combination with $Ptch^{flox/flox}$ mice if exact timing of BCC induction is of interest. However, since leakiness of CreER lines can differ between conditional mouse strains [93] it remains to be elucidated whether K5-Cre-ER^T leakiness is also seen in other conditional *Ptch* models.

4. Conclusion

BCC is the most common cancer in humans. Due to their high and increasing incidence, the improvement of current treatment options and the development of new treatment approaches are of great importance. Based on the essential role of HH signaling in formation of BCC, targeting this pathway is currently being put forward (for a review on 36 HH inhibitory compounds see [94]). The preclinical evaluation of these anti-BCC drugs requires good animal models. General requirements for such a model are a close relationship to the human situation (i.e., BCC caused by *Ptch* mutations on an immunocompetent background), reliable induction of BCC, defined BCC growth, and easy handling.

We have compared several *Ptch* knockout mouse models suitable for preclinical studies. So far, most studies have been conducted in UV- or IR-exposed conventional heterozygous *Ptch* knockout mice. Whereas UV-exposure leads to both superficial and nodular BCC and several other tumors, IR-exposure results in nodular and infiltrative BCC. Although the UV- or IR-related BCC models are valuable tools to analyze the antitumoral response of BCC, the responsiveness of defined BCC stages (i.e., early-stage or fully developed) or subtypes (e.g., nodular or superficial) in these models is hard to analyze due to heterogeneous BCC growth. For this purpose, conditional inactivation of *Ptch* by inducible and cell-specific Cre drivers may be advantageous. Five different conditional *Ptch* knockout mouse strains are currently available. However, preclinical studies on anti-BCC drugs have only been carried out in one of them. As revealed by this study, conditional *Ptch* ablation indeed enables the investigator to accurately induce BCC at a defined time point. In addition, conditional *Ptch* ablation results in a homogeneous BCC histology, which may be due to omission of irradiation. Therefore, conditional *Ptch* knockout mice are a valuable tool to study the curative or preventive effects of a certain drug on defined BCC subtypes and stages.

Abbreviations

Ptch: Patched
BCC: Basal cell carcinoma.

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