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Role of IKK α in skin squamous cell carcinomas

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Abstract

Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are two major types of skin cancer derived from keratinocytes. SCC is a more aggressive type of cancer than BCC in humans. One significant difference between SCC and BCC is that SCC development is generally associated with cell dedifferentiation and morphological changes. When SCC is converted to spindle cell carcinoma, the latest stage of cancer, the tumor cells change to a fibroblastic cell morphology (epithelial-to-mesenchymal transition) and lose their differentiation markers. Recently, several laboratories have reported altered I κ B kinase α (IKK α) protein localization, downregulated IKK α , and *IKK α* gene deletions and mutations in human SCCs of the skin, lung, esophagus, and neck and head. In addition, IKK α reduction promotes chemical carcinogen- and ultraviolet B-induced skin carcinogenesis, and IKK α deletion in keratinocytes causes spontaneous skin SCCs, but not BCCs, in mice. Thus, IKK α emerges as a *bona fide* skin tumor suppressor. In this article, we will discuss the role of IKK α in skin SCC development.

Keywords

carcinogenesis; EGFR; gene mutations; IKK α ; keratinocyte differentiation and proliferation; squamous cell carcinoma; TGF- β

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A signature phenomenon: lack of terminal differentiation in the epidermis associated with IKK α loss in mice

IKK α /IKK/NF- κ B in skin development of mice

When the conserved helix-loop-helix (HLH) ubiquitous kinase (CHUK) was shown to specifically phosphorylate serines 32 and 36 of I κ B α , a major NF- κ B inhibitor, it was named I κ B kinase α (IKK α) or IKK1 [1–3]. I κ B α binds to NF- κ B proteins in the cytoplasm and masks the nuclear translocation signal of NF- κ B components, thereby blocking NF- κ B translocation to the nucleus [4]. NF- κ B refers to a group of transcription factors that regulate the expression of many genes encoding proteins which are broadly involved in immune and inflammatory responses, cell death, cell-cycle regulation, cell proliferation and cell migration. IKK α , IKK β , and IKK γ (NF- κ B essential modulator [NEMO]) form the IKK complex that phosphorylates I κ Bs [5,6]. The phosphorylation induces I κ B α protein degradation through the S26 proteasome ubiquitination machinery, allowing the freed NF- κ B to move to the nucleus, functioning as a transcription factor. IKK α and IKK β contain a kinase domain, a leucine zipper (LZ), and a HLH motif, and form homodimers and heterodimers through their motifs [3,7]. The two are highly conserved serine/threonine kinases and share many kinase substrates. However, IKK β shows a stronger kinase activity for I κ B α than IKK α does. IKK γ is a regulatory subunit. Given the similarities of IKK α and IKK β function *in vitro*, they were expected to have similar physiological activities.

Genetic studies have shown that mice deficient in RelA (p65, a major NF- κ B component), IKK β , or IKK γ die from a liver cell death-induced hemorrhage during embryonic development [8–13]. Depleting tumor necrosis factor receptor 1 (TNFR1) rescues the phenotype in these mice (TABLE 1) [8,14–17], indicating that IKK β and IKK γ are upstream activators of p65 in the antiapoptotic TNFR-associated pathway. No defects have been reported in embryonic skin development from the *p65*, *Ikk γ* , and *Ikk β* knockouts that have been rescued by depleting TNFR1. Unexpectedly, *Ikk α* ^{−/−} mice complete embryonic development to full-term [18–20]. Newborns look like pupa with shiny and unwrinkled skin. Owing to the severely impaired skin development, *Ikk α* ^{−/−} mice lose body liquid, dry quickly, and die soon after birth. Depleting TNFR1 does not affect the phenotype of *Ikk α* ^{−/−} mice [21]. Thus, IKK α plays a distinct role from other IKK subunits and NF- κ B during embryonic skin development.

Lack of terminal differentiation as a signature phenotype in the epidermis of *Ikk α* ^{−/−} mice

Skin presents the largest organ in the human body, and it is composed of dermal and epidermal layers. The epidermal barrier shields the internal organs of the body. The stratified epidermis is composed of various cells, more than 90% of which are keratinocytes, and is divided into the basal and suprabasal layers [22]. The basal epidermal keratinocytes are capable of proliferating. The suprabasal epidermis includes stratum spinosum, granulosum, lucidum and corneum, and each layer represents dissimilar differentiating keratinocytes. Keratinocytes lose their nuclei and die to give rise to a skin barrier when arriving at the top epidermal layer. Thus, the barrier formation is a consequence of epidermal keratinocyte maturation. Keratinocytes express special keratins and filaments at different differentiation

stages, such as keratin 5/14 (K5/14) for basal keratinocytes, K1/10 for intermediately differentiating keratinocytes, and loricrin and filaggrin for terminally differentiating keratinocytes [22]. Because terminally differentiated keratinocytes peel off layers of skin, new keratinocytes are constantly required. Thus, maintaining skin homeostasis requires balanced differentiation and proliferation. Disrupting this balance can lead to skin diseases [23,24]. The epidermis of *Ikka*^{-/-} mice exhibits a strikingly increased thickness, which is associated with significantly increased BrdU signals, an S-phase cell cycle indicator, in both the basal and suprabasal epidermal layers [18,25]. The electron microscope shows a lack of granulosum and corneum keratinocytes in the epidermis of *Ikka*^{-/-} mice [18]. Immunostaining demonstrates that both the *Ikka*^{-/-} basal and suprabasal epidermis express mitotic cell markers and basal epidermal markers K5/14, and that the suprabasal epidermis expresses intermediate differentiation markers K1/K10 and involucrin, but lacks terminal differentiation markers loricrin and filaggrin [18,25]. These results indicate that IKKα is essential for embryonic skin development and that the loss of terminally differentiated keratinocytes is a signature phenotype in the epidermis of *IKKα*^{-/-} mice.

IKKα is a master regulator for keratinocyte terminal differentiation & proliferation

Mouse models

Sil and colleagues have shown that transgenic IKKα driven by a K14 promoter rescues the epidermal phenotype in *Ikka*^{-/-}/*K14-IKKα* mice [26]. Subsequently, Liu *et al.* demonstrated that transgenic IKKα produced from the K5 promoter also rescues the epidermal phenotype in *Ikka*^{-/-}/*K5-IKKα* mice [23]. However, transgenic IKKα driven by a truncated loricrin promoter, which is active at a low level in the basal and supra-basal epidermis at a later stage of embryonic development, fails to rescue *Ikka*^{-/-} mice [27,28]. Therefore, early expression of IKKα is required for epidermal formation during embryonic development. Furthermore, a nonphosphorylated IκBα, mutated at serines 32 and 36 (mlκBα) and overexpressed in the basal epidermis, affects skin homeostasis and induces skin tumors [29,30]. IκBα is the major substrate of IKKα kinase [2]. Thus, if IKKα function in skin development requires phosphorylation of IκBα, kinase-inactive IKKα should mimic the phenotype of mice overexpressing mlκBα. Sil *et al.* rescued the epidermal phenotype of *Ikka*^{-/-} mice using transgenic kinase-inactive IKKα [26]. Both knock-in mice expressing kinase-inactive IKKα mutated at serine 178 and 180 within the kinase ATP-activating loop (*Ikka*^{AA/AA}), or mutated at lysine 44, an ATP-binding site (*Ikka*^{AA/AA}), developed normal skin [31,32]. Therefore, IKKα kinase is not essential for embryonic skin development. These results indicate that the function of IKKα in skin development is independent of NF-κB. Furthermore, depleting TNFR rescues the skin phenotypes of transgenic mlκBα mice, but does not rescue the skin phenotypes (*IKKα*^{-/-} mice and *Ikka*^{F/F}/*K5. Cre* mice. Thus, IκBα is not an IKKα target in regulating skin development [20,23,33].

Cell culture system

It has been reported that there is a Ca^{2+} gradient from the basal layer to the top terminally differentiating epidermis [34]. Cultured keratinocytes mimic the journey of keratinocyte differentiation in the epidermis [35]; they undergo spontaneous terminal differentiation and the enlarged keratinocytes peeling off from keratinocyte colonies express terminal differentiation markers. Ca^{2+} induces wild-type (WT) keratinocytes to undergo terminal differentiation, however, primary cultured *Ikka*^{-/-} keratinocytes fail to undergo terminal differentiation and fail to respond to Ca^{2+} -induced terminal differentiation [25,36–38]. There are no enlarged terminally differentiating keratinocytes in IKK α -null cultures; IKK α ^{-/-} keratinocytes form larger cell colonies than WT keratinocytes do [25]. Keratinocytes isolated from *Ikka*^{F/F/K5.Cre} mice act as *Ikka*^{-/-} keratinocytes in culture [23].

Reintroduction of WT or kinase-inactive IKK α can induce terminal differentiation in *Ikka*^{-/-} keratinocytes, but IKK α mutants with mutations in the LZ or HLH motif, IKK β , I κ B α and/or p65 do not induce terminal differentiation [25]. The LZ or HLH motifs have been shown to be required for the formation of IKK α and IKK β homodimer and heterodimers [7]. IKK β is irrelevant to keratinocyte terminal differentiation, whereas mutations in the LZ or HLH of IKK α inactivate the function of IKK α in regulating keratinocyte differentiation and proliferation [23,25]. Thus, the IKK α homodimers may be essential for IKK α function in the formation of the epidermis. In addition, a mutant IKK α that is not able to move to the nucleus fails to induce *Ikka*^{-/-} keratinocytes to undergo terminal differentiation [26]. IKK α is found in the nucleus of differentiated keratinocytes and shows a nuclear localization in the normal epidermis of humans and mice [28,39,40]. These results indicate that nuclear IKK α homodimers regulate epidermal development, keratinocyte differentiation, and Ca^{2+} -induced cell differentiation.

Terminally differentiating epidermal keratinocytes produce increased keratin, filaments and other special barrier proteins that build up the skin barrier. Thus, terminally differentiating keratinocytes and undifferentiated keratinocytes are different in many aspects, such as cellular components and morphology. The gene expression profile from cultured keratinocytes shows that IKK α -null keratinocytes lack a group of claudin tight junction proteins that are required to form tissue barriers, compared with WT cultured keratinocytes [23]. Furthermore, repeated epilation (Er) mice, which carry a mutation in the C-terminal region of the *14-3-3 σ* gene [41,42], develop an epidermis lacking terminally differentiating keratinocytes (FIGURE 1) [43]. Reintroduction of IKK α does not induce filaggrin expression and terminal differentiation in cultured Er keratinocytes (FIGURE 1). This indicates that the IKK α -induced filaggrin and loricrin are a consequence of the keratinocyte differentiation process, whereas IKK α does not directly regulate the expression of these terminal differentiation markers. Therefore, nuclear IKK α acts as a master regulator controlling a cascade of genes activated during the maturation process of keratinocytes, leading to the formation of the epidermis. In addition, our unpublished data show that, although primary cultured keratinocytes isolated from both *Ikka*^{-/-} and Er mice are not able to undergo terminal differentiation, *Ikka*^{-/-} keratinocytes form larger cell colonies than Er keratinocytes do. This suggests that IKK α possesses the additional activity of regulating keratinocyte proliferation compared with Er keratinocytes.

Although cell autonomic driving forces, such as the overexpression of Ras in the basal or suprabasal epidermis, are able to prevent cell differentiation or reverse differentiating keratinocytes to proliferating status [44,45], microenvironment is another important element influencing skin homeostasis, and skin disease development. We have shown that the growth medium from cultured WT keratinocytes, but not from *Ikka*^{-/-} keratinocytes, can induce *Ikka*^{-/-} keratinocytes to undergo terminal differentiation [25]. The diffusible protein molecule in the conditional medium is termed keratinocyte differentiation inducible factor (kDIF) [25]. It appears that differentiating keratinocytes may function to maintain a differentiation-inducing environment that contributes to skin homeostasis.

Evidence: IKK α deficiency is associated with the development of human squamous cell carcinomas

Skin squamous cell carcinomas (SCCs) and basal cell carcinomas are two major skin tumors derived from keratinocytes. Basal cell carcinomas rarely metastasize. Skin SCCs can progress to a highly aggressive cancer, metastasize to other organs and cause death. The epithelial cells expressing basal keratins are able to develop SCCs in many organs of humans and mice. These SCCs show a structure similar to the stratified epidermis, containing basal cells and suprabasal cells; they express basal and intermediate differentiation markers, and some even express terminal differentiation markers. The differentiation markers are lost with tumor progression. Thus, the amount of dedifferentiation is generally associated with the degree of severity of SCCs.

The human *Ikka* gene is located at 10q24.31. Gene deletions or loss of heterozygosity within the 10q22–10q26 region have been frequently reported in human cancers [46]. The National Cancer Institute's Cancer Genome Anatomy Project has revealed chromosomal deletion at this position in SCCs of the nasal and oral cavities, larynx, lung and vagina [101]. Mutations of *Ikka* have been frequently identified in several types of human cancer [47]. By sequencing, we identified heterozygous point mutations and deletions in the exon 15 of the *Ikka* gene from human skin SCCs. [28]. These nucleotide substitutions cause missense and nonsense mutations, and deletions cause frameshifts. Some mutations, detected from several tumors, may represent hot spots. We also examined 114 human skin SCCs using immunohistochemical staining and found that a proportion of poorly differentiated human skin SCCs expressed significantly reduced IKK α [28].

Squamous cell carcinoma is the most common neoplasm of the oral cavity. The loss of epithelial phenotypes in the process of carcinoma progression correlates with clinical outcome. Maeda *et al.* [48] demonstrated that IKK α is expressed in the nucleus of the basal cells of normal oral epithelium, but is not detected or marginally detected in 32.8% of carcinomas. IKK α immunoreactivity significantly decreased in poorly differentiated carcinomas ($p < 0.05$) and conversely correlated with the long-term survival of patients ($p < 0.01$). Four out of 64 patients showed allelic/biallelic loss of *Ikka* and 63% of the patients had microsatellite instability and a hypermethylated *Ikka* promoter. These results suggest that oral carcinomas exhibiting genetic instability and promoter hypermethylation

downregulate the expression of IKK α , which is associated with disease progression toward an unfavorable prognosis.

Marinari *et al.* have also found that IKK α nuclear staining is strongly reduced in tumors versus the normal epithelium in 245 skin, pharynx, esophagus and lung SCCs, and in 39 controls [40]. IKK α is downregulated in 78% of skin SCCs and in 82% of SCCs derived from other stratified epithelia such as lung, esophagus and oral cavity/larynx. The amount of IKK α correlates with clinical stage, is highest in well-differentiated tumors and lowest in high-grade, poorly differentiated primary SCCs. In the SCCs with little nuclear staining, cytoplasmic IKK α was frequently observed. Furthermore, Marinari *et al.* demonstrate that nuclear IKK α is progressively lost at the boundary zone of the hyperplastic/dysplastic epidermis (actinic keratose), and that, *in situ*, SCCs convert to invasive SCCs, suggesting that IKK α down-regulation and relocalization are associated with tumor progression [40]. Descargues *et al.* demonstrated a nuclear expression pattern of IKK α in normal human epidermal cells [39]. In addition, Marinari *et al.* [40] and Moreno-Maldonado *et al.* [49] consistently show that IKK α induces differentiation in human HaCaT keratinocytes, and demonstrate a close relationship between IKK α levels and histological variants of human epidermal SCCs.

In summary, IKK α is mainly localized in the nucleus of normal epidermal keratinocytes but moves to the cytoplasm in SCCs. Downregulation of IKK α is associated with human SCC dedifferentiation and progression, and genomic instability, but is conversely associated with the prognosis of this disease. These associations highlight the critical role that IKK α plays in suppressing the development of human SCCs and reveal that the *Ikka* gene is a target during skin tumorigenesis. This suggests that IKK α deficiency may be a critical driving force in SCC development.

Mouse models exhibit IKK α as a *bona fide* skin tumor suppressor

Chemical and ultraviolet B (UVB) carcinogenesis are the two most popular mouse models used to study tumor initiation, progression, and metastasis in the skin. In the classical two-stage chemical carcinogenesis protocol, chemical carcinogen 7,12-dimethylbenz(α)anthracene (DMBA) activates oncogenic H-Ras by inducing *Ras*-activating mutations (V61, CAA $\rightarrow$ CTA; V12, GGA $\rightarrow$ GGC) and tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) expands the Ras-initiated cells [50–53]. Overexpressed active Ras promotes cell proliferation and transformation and prevents keratinocytes from undergoing terminal differentiation [54]. UVB is a carcinogen that can mutate the tumor suppressor gene *p53* at an early stage of UVB carcinogenesis and causes inflammation and suppression of normal immune function [55]. UVB also induces cell death and cell cycle arrest, which limit UVB-induced damage. Both DMBA/TPA and UVB induce papillomas and carcinomas that resemble human SCCs. Most human SCCs contain *p53* mutations and increased Ras activity [56–58]. Skin cells can carry *p53* mutations for many years before the onset of skin cancer. Caulin *et al.* demonstrate that *p53* mutation and K-ras^{G12D} cooperate to initiate skin tumors, and to promote progression and metastasis [59]. Thus, the two pathways are relevant to human skin cancer.

Chemical carcinogenesis

We have shown that *Ikka*^{+/-} mice develop twice as many papillomas and 11-times as many carcinomas than *Ikka*^{+/+} mice in a DMBA/TPA-induced carcinogenesis setting [60]. Most *Ikka*^{+/-} carcinomas and half of the *Ikka*^{+/-} papillomas show loss of heterozygosity of *Ikka* and express reduced levels of IKK α . Thus, IKK α loss promotes tumor progression. The carcinomas derived from *Ikka*^{+/-} mice are poorly differentiated, indicating a correlation between IKK α loss and the expansion of poorly differentiated tumor cells in SCCs. Furthermore, random mutations in the *Ikka* gene were detected in papillomas and carcinomas developed from both *Ikka*^{+/-} and *Ikka*^{+/+} mice [60]. We found that increased levels of mutations in IKK α isolated from DMBA/TPA-induced skin tumors correlated with the loss of IKK α 's ability to induce keratinocyte terminal differentiation in culture [23]. These results suggest that the *Ikka* gene is a relevant target of chemical carcinogens and that IKK α function is important for preventing skin tumors. IKK α reduction also promotes TPA-induced angiogenic and ERK activity, and this increases TPA-induced TNF- α , IL-1, EGF, heparin-binding EGF (HB-EGF), amphiregulin (AR), and VEGF-A levels in the skin [60]. Interestingly, increased IKK kinase activity is observed in carcinomas that have lost IKK α . Whether increased IKK activity contributes to tumor progression remains to be tested. In addition, we observed a reduction of 14–3–3 σ levels, a G2/M cell cycle checkpoint in response to DNA damage, and nucleophosmin, which is important for genomic stability in carcinomas compared with papillomas [32,61–64]. Overall, IKK α reduction provides a growth advantage in conjunction with Ras activity and also promotes the expression of various cytokines, angiogenesis and genomic instability during skin carcinogenesis.

On the other hand, transgenic L α .IKK α mice develop fewer malignant carcinomas and metastases compared with WT mice in a DMBA/TPA-induced carcinogenesis setting [28]. Interestingly, the endogenous IKK α levels are higher in carcinomas developed from L α .IKK α mice than in carcinomas developed from WT mice. In addition, we found that overexpressed IKK α represses TPA- and Ras-induced VEGF-A expression by regulating their promoter activity in keratinocytes, and that it promotes TPA-induced terminal differentiation in the skin. Thus, IKK α affects the microenvironment surrounding tumor sites, which regulates tumor development in mice.

Ultraviolet B carcinogenesis

Ultraviolet B induces twice as many skin tumors in *Ikka*^{+/-} than in *Ikka*^{+/+} mice [65]. The tumor latency is significantly shorter and tumors are much larger in *Ikka*^{+/-} than in *Ikka*^{+/+} mice. UVB-induced signature p53 mutations at the early initiation stage increase dramatically in *Ikka*^{+/-} skin, but UVB-induced cell death is significantly reduced in *Ikka*^{+/-} skin compared with *Ikka*^{+/+} skin. In addition, NF- κ B DNA-binding and ERK activity, TNF- α , IL-1, IL-6, MCP-1/CCL2 levels and infiltrating macrophages increase in *Ikka*^{+/-} compared with *Ikka*^{+/+} skin following UVB treatment. The increased NF- κ B activity and p53 mutations may contribute to decreased apoptosis. Therefore, IKK α reduction promotes UVB carcinogenesis via multiple mechanisms.

Spontaneous skin tumors in mice lacking IKK α in keratinocytes

The *Ikk α* floxed mice with IKK α deletion using K5.Cre (*Ikk α ^{F/F}/K5.Cre*) develop epidermal hyperplasia and die within 3 weeks of birth [23]. Inducible IKK α deletion causes epidermal hyperplasia and spontaneous skin papillomas and carcinomas in adult *Ikk α ^{F/F}/K5.CreER* and *Ikk α ^{F/F}/K15.CrePR1* mice. We have found elevations in EGFR and ERK activity, EGF and HB-EGF levels and the expression of ADAM sheddases (e.g., ADAM9, 10, 12, 17 and 19) that cleave EGF and HB-EGF precursors to generate their active soluble forms [66], in the IKK α -null epidermis in comparison with the WT epidermis [23]. These molecules can be activated in an autocrine loop of EGFR/ERK/EGF/HB-EGF/ADAM signaling. Thus, reintroduction of IKK α , and the inactivation of EGFR, and/or EGFR inhibitors, blocks IKK α loss-induced epidermal hyperproliferation and skin tumors in mice. Interestingly, depleting TNFR1 does not affect the phenotype of epidermal hyperplasia in *Ikk α ^{F/F}/K5.Cre* mice (although we did not test whether depleting TNFR1 affects IKK α deletion-induced skin tumors) [23]. Therefore, EGFR signaling is crucial for IKK α -associated skin homeostasis and skin tumor development.

Mechanism of IKK α -induced keratinocyte differentiation & suppression of skin tumor development

The observation that IKK α deficiency is a driving force in skin tumor development in animal models is fully supported by the observed association of IKK α deficiency with human SCC occurrence. During skin tumorigenesis, IKK α deficiency induces keratinocyte dedifferentiation, increased proliferation, angiogenesis, inflammation, metalloproteinase expression, increased mutagenesis and reduced apoptosis. Several reports have investigated the mechanism by which IKK α regulates keratinocyte differentiation and proliferation. Since SCC is a type of malignancy closely associated with changes in tumor cell differentiation and proliferation, we focus on discussing these issues below.

A loop of EGF receptor/Ras/ERK/EGF receptor ligands

Oncogenic H-Ras antagonizes Ca⁺⁺-induced terminal differentiation and promotes keratinocyte proliferation and transformation [67]. DMBA initiates skin tumors by inducing activating H-Ras mutations [44]. EGFR signaling is upstream of Ras and leads to the activation of ERK and the transcription of genes encoding proteins that regulate cell proliferation. Reduction of EGFR restores keratinocyte differentiation in keratinocytes overexpressing Ras [68]. EGFR is frequently activated in human SCCs [69]. Thus, the EGFR/Ras pathway is important for keratinocyte differentiation, regulation of proliferation and skin tumor development. We have demonstrated that IKK α loss elevates Ras, EGFR and ERK activity, as well as EGF and HB-EGF levels, in keratinocytes, indicating that IKK α represses this loop of EGFR/Ras/ERK/EGF ligands. The introduction of IKK α , a dominant negative form of RasN17, EGFR inhibitor and/or ERK inhibitor reduces Ras, EGFR, ERK activities and growth factor levels, while also repressing cell proliferation and inducing terminal differentiation in IKK α -null keratinocytes [23]. Thus, IKK α regulates keratinocyte proliferation and differentiation via the EGFR/Ras/ERK/EGF-ligand loop (FIGURE 2). We used the chromatin immunoprecipitation (ChIP) assay to demonstrate that

IKK α binds to the promoter regions of *EGF*, *HB-EGF*, *AR* and *ADAMs*, and that these binding levels correlated with the decreased expression of corresponding molecules [23]. Treatment with EGF or TPA reduces IKK α binding to these promoters and elevates expression of these genes. Increased mutation levels in *Ikk α* reduce IKK α binding to the EGF promoter, which correlates with elevated EGF expression in keratinocytes. Thus, nuclear IKK α activity suppresses expression of EGF, HB-EGF and AR (FIGURE 2). The loss of IKK α increases the expression of EGFR ligands, resulting in excessive EGFR, Ras and ERK activation. As previously mentioned, inactivating EGFR induces differentiation, represses proliferation and prevents skin tumors in mice lacking IKK α in the epidermis. The nuclear function of IKK α in regulating the EGFR-mediated loop is consistent with the observed IKK α nuclear localization in normal epidermal keratinocytes [39,40,48]. In addition, treatment with TPA or EGF reduces the binding of IKK α to the promoters of growth factors and elevates the expression of these growth factor genes, indicating that IKK α represses the EGFR/Ras/ERK loop via the suppression of the transcription of genes that encode the EGFR ligands (EGF and HB-EGF). These results suggest that increasing nuclear IKK α levels may compensate for the activity of TPA or EGF in reducing IKK α binding to the promoters of genes encoding growth factors, thereby preventing amplified EGFR/Ras/ERK activity and cell proliferation but maintaining the status of cell differentiation, as we have shown that overexpression of IKK α in the epidermis inhibits carcinogen-induced skin carcinogenesis in mice [28]. On the other hand, the IKK α found in the cytoplasm of SCCs [40] may have a different function. We have also observed increased levels of IKK α in the cytoplasm in some human SCCs [LIU AND HU, 2010, UNPUBLISHED DATA]. In addition, we previously demonstrated that transformed IKK α -null keratinocytes were not able to respond to an IKK α -mediated differentiation signal [38]. Therefore, the altered IKK α (even elevated IKK α levels) may lose its normal function in those tumor cells.

TGF- β /c-Myc pathway

TGF- β acts as a tumor suppressor at the early stage of tumor development and affects cell proliferation, differentiation and inflammation [70]. c-Myc, a transcription factor, forms dimers with Max (c-Myc/Max) in order to regulate gene transcription [71]. Many targets of c-Myc are crucial for keratinocyte proliferation and differentiation in the skin, and these molecules crosstalk with TGF- β signaling via Smads [72]. Max dimer protein 1 (Mad1) and its relatives, including Mad2, Mad3, Mad4 and Ovo-like 1 (Ovo1) can also form heterodimers with Max (Max/Mad). The Max/Mad dimers compete with c-Myc/Max dimers for the consensus elements on the promoters of c-Myc's targets [71]. Thus, Mad proteins antagonize the c-Myc/Max dimer-induced function in regulating cell differentiation and proliferation [71,73,74]. Descargues *et al.* [39] and Marinari *et al.* [40] have shown that IKK α forms a complex with Smad2 or Smad3 on the promoters of *Mad* genes following treatment with TGF- β . IKK α loss downregulates Mad1, Mad2, Mad3, Mad4 and Ovo1 expression and elevates c-Myc activity in keratinocytes. Therefore, IKK α plays an important role in mediating TGF- β signaling to antagonize c-Myc activity (FIGURE 2). Excessive c-Myc activity prevents cell cycle exit, thereby preventing cell differentiation [75]. By contrast, Mad1 and Ovo1 are associated with cell differentiation [71]. Descargues *et al.* [39] and Marinari *et al.* [40] further show that TGF- β -induced keratinocyte differentiation

requires IKK α via Smad2 or Smad3. IKK α loss results in a failure to mediate TGF- β -led differentiation signaling in keratinocytes, but instead elevates c-Myc activity, which contributes to the promotion of cell proliferation. In addition, the G1/S cell cycle checkpoint, p21, is a TGF- β -induced gene [40]. Again, IKK α -null keratinocytes fail to induce the expression of p21 following TGF- β stimulation, suggesting that IKK α -null keratinocytes are not able to respond to TGF- β -mediated cell cycle arrest and cell differentiation. In the SCC environment, TGF- β is elevated, however, TGF- β may fail to exert its tumor suppressor activity in tumor cells lacking IKK α or expressing mutated IKK α , owing to the fact that *Ikk α* is a major target of mutagenesis in tumorigenesis. Thus, IKK α is crucial for TGF- β -induced tumor suppressor activity during skin tumorigenesis.

Microenvironment: differentiation/inflammation/angiogenesis

The nuclear IKK α mediates two intrinsic pathways: the suppression of EGFR/Ras/ERK/EGFR-ligand loop and the promotion of TGF- β -mediated c-Myc antagonists in regulating keratinocyte proliferation and differentiation. At skin lesion sites, keratinocytes, dermal cells and infiltrating leukocytes produce many growth factors, cytokines and chemokines. IKK α provides a surveillance function to limit the impact of these molecules on skin homeostasis. In addition, IKK α directs the production of kDIF, maintaining an environment that induces cells to differentiate. IKK α represses VEGF-A expression, inhibiting the formation of tumor promoter-induced micro-blood vessels. IKK α downregulates the metalloprotein ADAMs that activate various growth factors and cytokines (FIGURE 2). However, IKK α reduction or loss removes these protections, further amplifying these responses in a loop and eventually cause skin diseases.

Future perspective

Tumor development requires progression through multiple stages via distinct mechanisms. In the future, it will therefore be important to determine the specific cell types that initiate tumors in the absence of IKK α . As stem cells are likely to be the targets, it is necessary to verify these targets and further determine whether the molecular events that alter stem cell-related genes contribute to the maintenance of the properties of the progenitor cells that prevent keratinocyte differentiation and promote cell proliferation in the absence of IKK α . We have not identified the crucial mechanisms by which IKK α loss promotes tumor progression or how genomic instability affects the neoplastic transformation of tumor-initiating keratinocytes [60]. We also do not know how nuclear IKK α regulates gene transcription in detail. We have observed elevated IKK activity in those carcinomas [60]; however, whether IKK activity promotes tumor progression remains to be tested. If IKK activity is important for tumor progression, we will need to determine why IKK activity is elevated in carcinomas and how IKK α reduction elevates the expression of cytokines. In addition, tumor development is subject to immune surveillance *in vivo*. We still need to determine the effect of IKK α -associated immune responses on skin inflammation and tumor development. IKK α deficiency has been detected in SCCs from different organs in humans. Thus, it is important to determine whether IKK α deficiency is involved in other types of SCCs in mice. Together, these studies will not only provide the mechanisms of IKK α -

associated tumor development, but also provide new insight into therapeutic targets for preventing and treating skin cancer.

In addition, although this review mainly discusses skin SCCs, Greenman *et al.* also found the mutations of *IKKα*, (*CHUK*) in 210 diverse human cancers including breast, colorectal, gastric, glioma, lung, ovarian, renal, melanoma and acute lymphoblastic leukemia (TABLE 2) [47]. A new study demonstrates that the human lethal syndrome with multiple fetal malformations similar to those observed in *Ikka*^{-/-} mice is linked to a point mutation in the *Ikka* gene that leads to the loss of *IKKα* (*CHUK*) in two affected fetuses [76]. This is the first report for *IKKα* associated with human genetic (autosomal recessive) disease. On the other hand, *IKKα* has been suggested as a tumor promoter [77,78]. Thus, whether *IKKα* functions differently in different types of tumors and in other human diseases remains to be determined.

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Website

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Executive summary

IKK α loss causes epidermal hyperplasia during mouse embryonic development

- *Ikk α* ^{-/-} newborn mice are malformed and die soon after birth,

IKK α ^{-/-} epidermis lacks terminally differentiated keratinocytes

- Body liquid of IKK α ^{-/-} newborn mice is lost very quickly owing to impaired epidermis formation.

IKK α is a master regulator for keratinocyte terminal differentiation & proliferation

- Cultured wild-type (WT) keratinocytes spontaneously undergo terminal differentiation.
- Cultured IKK α -null keratinocytes are not able to undergo terminal differentiation, instead, they continuously proliferate. Thus, IKK α -null keratinocytes form larger cell colonies than WT keratinocytes do.

Altered IKK α localization, downregulated IKK α & *Ikk α* mutations & deletions are reported in multitypes of human squamous cell carcinomas

- Mouse squamous cell carcinomas (SCCs) resemble human SCCs.

Elevated IKK α level in the epidermis inhibits development of skin carcinomas & metastases induced by chemical carcinogens in mice

- IKK α antagonizes chemical carcinogen-induced mitogenic and angiogenic activities, and represses tumor progression and metastases during skin carcinogenesis in mice.

IKK α reduction promotes chemical carcinogen- or ultraviolet B-induced skin carcinogenesis in mice

- *Ikk α* ^{+/-} mice develop two-times more papillomas and 11-times more carcinomas than *Ikk α* ^{+/+} mice do in DMBA/TPA-induced skin carcinogenesis.
- Most of the *Ikk α* ^{+/+} carcinomas and half of the *Ikk α* ^{+/-} papillomas lose the remaining WT allele of *Ikk α* .
- *Ikk α* mutations are detected in DMBA/TPA-induced papillomas and carcinomas.
- *Ikk α* ^{+/-} mice develop two-times more skin tumors than *Ikk α* ^{+/+} do in ultraviolet B-induced skin carcinogenesis.
- Ultraviolet B irradiation induces more p53 mutations and fewer apoptotic cells in *Ikk α* ^{+/-} skin than in *Ikk α* ^{+/+} skin.

IKK α deletion in keratinocytes induces spontaneous skin papillomas & carcinomas

- IKK α deletion elevates EGFR, Ras and ERK activity and elevates levels of EGF, HB-EGF, AR and ADAMs.

- IKK α represses transcription of genes encoding EGF, HB-EFG, AR and ADAMs in keratinocytes.
- Reintroduction of IKK α or dominant negative RasN17 represses IKK α -null keratinocyte proliferation and induces keratinocytes to terminal differentiation.
- Reintroduction of IKK α , or inactivation of EGFR, prevents skin tumor development.
- IKK α induces Mad1, Mad2, Ovol1 and c-Myc antagonists through TGF- β -mediated Smad2/3 pathway in regulation of keratinocyte differentiation.

Figure 1. Keratinocytes isolated from Er (*er/er*) mice lack terminal differentiation marker filaggrin but express IKKα.

(A) Photos of WT, Er (*er/er*) and *Ikkα^{-/-}* newborns stained with toluidine blue. The result indicates that the impaired skin of *er/er* and *Ikkα^{-/-}* mice is not able to prevent water penetration. (B) Western blot compares expression of IKKβ, IKKγ and IKKα in the skin of *Ikkα^{-/-}*, WT and *er/er* newborn mice. (C) Western blot compares terminal differentiation marker filaggrin levels in cultured *er/er* and *Ikkα^{-/-}* keratinocytes.

+IKK α : Overexpressed HA tagged IKK α (HA-IKK α) including WT, C-terminal 80-amino acid deletion (C80); C: Control; KA: Kinase-inactive forms; WM: WT-keratinocyte culture medium (kDIF); WT: Wild-type.
Reproduced from [24].

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Figure 2. Nuclear IKKα function in regulating keratinocyte proliferation and differentiation.

Arrows indicate positive directions and lines present the inhibitory effect. DNA symbols represent promoter regions of genes. EGF/TPA and TGF-β in bold represent exogenous stimuli for cells.

ADAM: A disintegrin and metalloprotease; TPA: 12-0-tetradecanoylphorbol-13-acetate.

Table 1.

Skin phenotypes in IKK mice.

Mouse genotype	Embryonic development	Rescue	Postnatal phenotypes	Ref.
<i>IKKα^{-/-}</i>	Die soon after birth; epidermal hyperplasia; epidermis lacking terminally differentiated keratinocytes	Tg-K14.IKKα; Tg-K14.IKKα-KA; Tg-K5.IKKα; not <i>Tnfr^{-/-}</i>	-	[18–21,23,26]
<i>IKKα^{+/-}</i>	Normal	-	Develop two-times more papillomas and 11-times more carcinomas; LOH in <i>IKKα</i> +/- skin carcinomas; mutations in papillomas and carcinomas	[60]
<i>IKKα^{flf}</i> × K5.Cre [♀] × K14.Cre [♀]	Germline knockout phenotypes; die soon after birth	Tg-K5.IKKα; not <i>Tnfr^{-/-}</i>	-	[23]
<i>IKKα^{flf}</i> × K5.Cre [♂] × K14.Cre [♂]	Normal	Tg-K5.IKKα; <i>Egfr^{+/-}</i>	Die within 3 weeks after birth; epidermal hyperplasia	[23]
<i>IKKα^{flf}</i> × K5.CreER × K15.CrePR1 × MMTV.Cre	Normal		Epidermal hyperplasia; spontaneous skin papillomas and carcinomas	[23]
Tg-K5.IKKα	Normal	-	Normal	[23]
Tg-Lori.IKKα	Normal	-	Normal and inhibit carcinomas and metastases induced DMBA/TPA	[28]
<i>IKKα</i> AA/AA	Normal	-	Normal	[31]
<i>IKKα</i> KA/KA	Normal	-	Unpublished	[32]
<i>IKKβ^{-/-}</i>	Embryonic death	<i>Tnfr^{-/-}</i> normal		[8,9,17]
<i>IKKβ^{flf}</i> × K14.Cre	Normal	<i>Tnfr^{-/-}</i> normal	Epidermal hyperplasia; inflammation	[17]
Tg-K5.IKKβ	Normal	-	Epidermal hyperplasia; inflammation	[79]
<i>IKKγ^{-/-}</i>	Embryonic death	<i>Tnfr^{-/-}</i> normal	-	[10–12]
<i>IKKγ^{flf}</i> × K14.Cre	Normal	<i>Tnfr^{-/-}</i> normal	Epidermal hyperplasia; inflammation	[15]
<i>IKKγ^{flf}</i> × K14.CreER	Normal	<i>Tnfr^{-/-}</i> normal	Epidermal hyperplasia; inflammation	[15]

- : Not examined

♀ : Female

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σ ; Male

DMBA: 7,12-dimethylbenz(a)anthracene; LOH: Loss of heterozygosity; Tg: Transgenic; TPA: 12-O-tetradecanoylphorbol-13-acetate.

Table 2.

Mutations in IKK α (CHUK) identified from human cancer.

Sample	Count	cDNA annotation	Protein change	Type
Breast C	1	717A>G	P239P	Silent
Ovarian C	1	376A>T	S126C	Missense
Multiple	3	1503G>A	G501G	Silent
Multiple	4	1341A>G	G447G	Silent
Multiple	6	464T>C	V155A	Missense
Multiple	9	148C>T	L50L	Silent
Multiple	17	1458C>T	S486S	Silent
Multiple	105	802G>A	V268I	Missense

Results obtained from examining 518 protein kinase genes in 210 diverse human cancers including acute lymphoblastic leukemia, breast, colorectal, gastric, glioma, lung, ovarian, renal and melanoma. C: Cancer.

Data taken from [47].