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PKK Suppresses Tumor Growth and is Decreased in Squamous Cell Carcinoma of the Skin

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Abstract

Non-melanoma skin cancer (NMSC) represents the most common cancer in the United States. Squamous cell carcinoma (SCC) of the skin is a sub-type of NMSC that shows a greater potential for invasion and metastasis. The current study identifies the Protein Kinase C-associated Kinase (PKK), which is also known as the Receptor-Interacting Protein Kinase 4 (RIPK4), as a suppressor of tumor growth in SCC of the skin. We show that expression of PKK is decreased in human SCC of the skin compared to normal skin. Further, suppression of PKK in human keratinocytes leads to increased cell proliferation. Use of RNA interference to reduce PKK expression in keratinocytes leads to an increase in S phase and in proteins that promote cell cycle progression. Consistent with the results obtained from cell culture, there is a dramatic increased tumorigenesis after PKK knockdown in a xenotransplant model and in soft agar assays. The loss of tumor suppression involves the NF-κB and p63 pathways. NF-κB is inhibited through inhibition of IKK function and there is increased nuclear TP63 activity after PKK knockdown. This study opens new avenues both in the discovery of disease pathogenesis and for potential treatments.

Keywords

Squamous Cell Carcinoma; Signal Transduction; Human Cancer

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Author Contributions

B.P., L.C., D.O., C.A., J.Z, K.G. performed experiments. B.P., L.C, D.O., E.G., A.P., S.I. contributed to experimental design and data analysis. B.P., E.G. and L.C. performed manuscript writing.

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Introduction

Non-melanoma skin cancer (NMSC) represents the most common group of cancers in the United States. There are more than 3.5 million cases per year (Rogers *et al.*, 2010). Moreover the incidence has been increasing (Karia *et al.*, 2013; Rogers *et al.*, 2010). SCC of the skin, a sub-type of NMSC, shows a greater potential for invasion and metastasis (Kim and Armstrong, 2012). Cutaneous SCC is a neoplasm of epidermal keratinocytes with a complex pathogenesis. Mutations in the tumor suppressor, p53 are identified in 58% of SCCs (Brash, 2006; Kim and Armstrong, 2012; Li *et al.*, 1998). p53 plays a key role in the surveillance of keratinocytes excessively damaged by ultraviolet light (Brash, 2006; Li *et al.*, 1998). SCCs have also been shown to have mutations in H*ras*, Notch, MYC, p16 (INK4A), and EGFR (Jacobs *et al.*, 2013; Mortier *et al.*, 2002; Proweller *et al.*, 2006; Yuspa, 1994). Clearly there are multiple genes and pathways important in SCC development. Here we identify a previously unreported tumor suppressor, PKK, in keratinocytes, which may play a crucial role in human SCC development.

PKK is a membrane-associated serine-threonine kinase that interacts with protein kinase C (PKC) and mediates responses to cellular stress (Meylan and Tschopp, 2005; Zhang *et al.*, 2010). PKK has an N-terminal kinase, intermediate region for cleavage by caspases, and C-terminal ankyrin repeats. Previously it has been shown that disruption of PKK in mice leads to severe skin defects (Holland *et al.*, 2002). PKK knockout mice die at birth with fusion of multiple skin folds and orifices. The knockout shows marked acanthosis (epidermal thickening) of the skin (Holland *et al.*, 2002). However there is no increase in BrdU staining observed in the knockout mouse (Holland *et al.*, 2002). Moreover when a plasmid, encoding an siRNA targeting PKK, was transiently transfected with liposomes into HaCaT or Tca-8113 cells, there was no significant alteration in cell proliferation (Adams *et al.*, 2007; Wang *et al.*, 2014).

A recent study showed increased proliferation in cell cultures after inactivation of PKK through insertional mutagenesis studies (Heim *et al.*, 2014). Heim, *et al* also showed that PKK expression was decreased in 80% of hepatocellular carcinomas examined. In addition low levels of PKK expression were associated with more aggressive human SCCs of the tongue and with increased anchorage independent growth of cells in culture, although as noted, not alteration in keratinocyte proliferation (Wang *et al.*, 2014). These studies suggest that in some cell types or model systems, PKK can function as a tumor suppressor.

Interestingly, in multiple, non-keratinocyte cell lines, PKK expression can activate WNT signaling and cause stabilization of beta-catenin, suggesting that overexpression of PKK can promote tumorigenesis. These findings were noted to be cell-type dependent (Huang *et al.*, 2013). Additionally, PKK mediates NF-κB activation in B cells and is required for survival in certain B cell lymphomas (Kim *et al.*, 2008). Therefore the current literature supports that PKK can function as both an oncogene or tumor promoter and a tumor suppressor in a cell-type dependent manner.

In our current study, we demonstrate, in skin keratinocytes, PKK functions as a tumor suppressor rather than as an oncogene. We have utilized multiple models which provide

mutually reinforcing data to support this previously unreported role for PKK in keratinocytes. We have identified multiple mechanistic pathways, which are affected by PKK, in skin keratinocytes, namely the p63 and NF-κB pathways.

TP63 is a master regulator of epidermal cell proliferation and differentiation (Mills *et al.*, 1999; Yang *et al.*, 1999). Humans with mutations in *p63* develop human ectodermal dysplasia syndromes such as ectrodactyly, ectrodermal dysplasia and clefting (EEC) or ankyloblepharon-ectodermal dysplasia-clefting (AEC) among others. (van Bokhoven *et al.*, 2001) Humans with a catalytically inactive PKK kinase have skin and limb defects that are similar to those observed after mutation of *p63* in humans (Kalay *et al.*, 2012; van Bokhoven *et al.*, 2001). This suggests TP63 and PKK are on the same or parallel pathways. Moreover p63 is known to be important in the development of SCC (Li *et al.*, 2011; Westfall *et al.*, 2003). p63 negatively regulates p21 expression in keratinocytes, which controls the cell cycle and proliferation in skin keratinocytes (Truong *et al.*, 2006; Westfall *et al.*, 2003). Therefore p63 is one potential target of PKK in keratinocytes. We provide data that PKK increases p63 pathway activity, thereby altering p21 levels.

NF-κB also plays major roles in epidermal proliferation and differentiation (Wullaert *et al.*, 2011) In normal epidermis, NF-κB proteins are in the cytoplasm of basal cells and in the nuclei of suprabasalilar cells. This indicates that NF-κB activity may be involved in the switch from proliferation to growth arrest and differentiation (Seitz *et al.*, 1998). Inhibition of NF-κB by expressing dominant-negative NF-κB inhibitory proteins in transgenic mice induced hyperplastic epithelium (van Hogerlinden *et al.*, 1999). In addition, application of a pharmacologic inhibitor of NF-κB to intact skin induced epidermal hyperplasia and overexpression of p50 and p65 NF-κB subunits in epithelium and leads to hypoplasia and growth inhibition (Seitz *et al.*, 1998). It is known that PKK mediates NF-κB activity in 293T cells (Meylan *et al.*, 2002; Muto *et al.*, 2002). Moreover a functional analysis of PKK has shown that normal PKK can activate an NF-κB dependent luciferase reporter while mutant forms from patients with Bartsocas-Papas Syndrome cannot (Kalay *et al.*, 2012). In this report we show that PKK exerts an effect on NF-κB activity in keratinocytes.

Results

PKK expression is decreased in human SCCs

To study PKK function in SCC, we developed a tumor bank of skin SCCs excised during Mohs Micrographic Surgery. Tumors from one patient are coupled with adjacent, normal control tissue from the same patient (non-cancerous skin) when available. We examined the mRNA expression level of PKK in skin samples. Quantitative PCR was performed for *PKK* and *GAPDH* as an internal control. When compared to normal skin, *PKK* expression was decreased in 11 of 12 SCC tumors compared to the average, normalized control (blue line) (Fig. 1a). Therefore, this strong association of decreased *PKK* expression in human SCCs of the skin supports that PKK may play an important role in tumorigenesis.

In order to determine if the decreased expression of PKK mRNA was associated with decreased expression of PKK protein, we performed immunohistochemistry on tissue samples from Figure 1a. PKK expression in normal skin was highest in the cytoplasm of

basal keratinocytes but also present throughout all layers of the epidermis (1b and c). Unlike normal skin, SCC tumors showed very low PKK expression in almost all keratinocytes. Figure 1d and e show SCC-17 as a representative sample. Therefore, because both PKK mRNA and protein expression are low in SCC, we further explored the function of PKK in the skin.

Suppression of PKK expression in keratinocytes promotes cell proliferation

In order to evaluate PKK function in SCC, two retroviral vectors carrying two different short hairpin RNAs (shRNAs, named shPKK-1 and shPKK-2) and a control viral construct (shControl) were created to target the *PKK* mRNA. The cells transduced with the retrovirus were selected to establish GFP-positive cell lines (shControl, shPKK-1 or shPKK-2). After establishing cell lines that had stable transfection (i.e. close to 100% GFP positivity) of the shRNA vectors, cells were re-plated and assessed on multiple days. Trypan blue exclusion studies established that knockdown of PKK in keratinocytes leads to an increased number of cells in culture compared to cells transfected with GFP control vector. Multiple keratinocyte cell lines were examined including: HaCaT cells, A431 cells, and two previously described keratinocyte cell lines from SCCs (Mantel, 2014). (Supplemental Fig. S1) Representative cultures from day 3, when cells were at 70–80% confluence and in a logarithmic growth phase, are shown (Fig 2a.). Total living cells are represented. An immunoblot for PKK confirmed the knockdown in SCC cells transduced with both shPKK-1 and shPKK-2 (Fig. 2b). Therefore knockdown of PKK expression is associated with an expansion of cell number in HaCaT, A431, and two human SCC cell cultures.

Suppression of PKK leads to cell cycle progression but does not affect apoptosis in keratinocytes

Since suppression of PKK promotes cell number expansion, we next analyzed the cell cycle distribution of PKK knockdown cells with flow cytometry. Similar results were found with HaCaT, human foreskin and multiple SCC cell cultures (flow cytometry from a representative SCC cell culture is shown). 5-ethynyl-2′-deoxyuridine (EdU) was used to label the cells. As shown in Fig. 2c, there was an increase (48.3% and 39.2%) of EdU positive cells in SCC cells expressing shPKK-1 and shPKK-2, respectively, compared with control cells (27%). Further, PKK knockdown is associated with an increased S phase population (ShPKK-1, 47.7 % and shPKK-2, 36.4%), when compared with SCC cells transduced with control viruses (29.7%) (Fig. 2d).

In light of increased cell numbers, apoptosis was examined. There was no difference in Annexin V staining between control and PKK knockdown cells (Fig. 2e). Therefore suppression of PKK in human keratinocytes leads to increased cell proliferation but not decreased cell survival.

Since PKK knockdown promotes cell cycle progression, we next analyzed cell cycle-related protein expression in keratinocytes from SCCs stably expressing shControl, shPKK-1 or -2. Fig. 2f shows that the cyclin-dependent kinase 4 (Cdk4) and Cyclin E, cell cycle stimulators were markedly increased and p21, a cell cycle inhibitor, was greatly decreased after PKK knockdown.

TP63 nuclear accumulation occurs after PKK knockdown

The cyclin dependent kinase inhibitor, p21 plays a major role in controlling keratinocyte proliferation. The presence of p21 leads to a growth arrest and terminal differentiation (Parker *et al.*, 1995) and inhibits multiple cyclins, inhibiting cells from entering S phase (Xiong *et al.*, 1993). In skin keratinocytes, p21 has been previously shown to be regulated by TP63 (Truong *et al.*, 2006; Westfall *et al.*, 2003) Therefore we next examined TP63 expression in keratinocytes after PKK RNA interference. Nuclear immunofluorescence of TP63 showed a significantly increased nuclear TP63 level in PKK knockdown cells compared to control (Fig. 3a). Multiple mechanisms for TP63 activity have been proposed including binding p53-response elements of targeted genes or activity of its transactivation domain. In each case, nuclear localization of TP63 is associated with its activity (Su *et al.*, 2009; Truong *et al.*, 2006; Westfall *et al.*, 2003). Nuclear preparations of keratinocyte cultures showed that TP63 was increased in nuclei of keratinocytes containing shPKK (Fig. 3b). Whole cell lysates of these keratinocytes showed that SCC cell lines and normal human keratinocytes had an increase in TP63 levels after shPKK transduction whereas HaCaT cells did not (Fig. 3c). Therefore in multiple keratinocyte cell lines, PKK knockdown leads to increased cellular TP63 and decreased p21.

Suppression of PKK decreases NF-κB activation in keratinocytes through inhibition of IKK

Our laboratory has a special interest in the NF-κB pathway and we have previously reviewed the importance of this transcription factor in keratinocyte proliferation (Poligone *et al.*, 2013). Because both increased and decreased NF-κB levels can be associated with keratinocyte tumorigenesis, we next examined whether suppressing PKK has effect on NF-κB signaling in keratinocytes. We analyzed expression levels by Western blot of the NF-κB inhibitor protein IκBα, the hallmark of NF-κB activation, in HaCat and SCC cells expressing shPKK. Consistent with a previous report (Adams and Munz, 2010), knockdown of PKK increases basal IκBα protein levels in the shPKK cells compared to cells with normal PKK expression (Fig. 4a). Keratinocyte cultures were treated for various time points with phorbol 12-myristate 13-acetate (PMA) in order to stimulate the NF-κB pathway. Phosphorylation of IκBα is required for the activation of NF-κB. Phosphorylated IκBα was decreased in cells expressing shPKK, which have been treated with PMA (Fig 4a). We next examined NF-κB transcriptional activity after PMA treatment of SCC keratinocytes. The luciferase activity of untreated cells was normalized to 1 for comparison. Control cells had a greater than 12 fold increase in luciferase activity after PMA treatment, whereas keratinocytes containing shPKK showed much less of an increase (Fig 4b). Lastly a phospho-specific IKK antibody was utilized to examine IKK activity after PMA treatment of keratinocytes. PKK knockdown leads to decreased phosphorylated IKK after PMA treatment, which was not due to an effect on IKKα levels (Fig 4c).

PMA-induced cell cycle arrest is reduced with shPKK

PMA is a PKC and NF-κB activator, which causes cell growth arrest in keratinocytes (Sur *et al.*, 2009). It has previously been shown that NF-κB activity is required for PMA-induced keratinocyte growth arrest (Takao *et al.*, 2003). We hypothesized that suppression of PKK expression can override PMA-mediated growth inhibition in keratinocytes. Therefore

keratinocytes transduced with shControl or shPKK-1 were treated with 100 ng/ml of PMA for 12 hours. As expected, PMA treatment of control keratinocytes (shControl) led to a decrease of keratinocytes in S-phase (3.59%) (Fig 4d) representing a 6.4 fold reduction in S phase (22.9/3.6). PMA treatment of keratinocytes with knockdown of PKK had less reduction (18.0%) or a 1.7 fold reduction in S phase (31.8/18.0) (Fig 4d). Similar results were found in HaCaT cells (data not shown). Therefore NF-κB is a downstream effector of PKK function and the hyperproliferation observed after PKK knockdown is at least partly due to inhibition of NF-κB signaling through IKK.

Inhibition of PKK expression promotes tumorigenesis

With mechanistic evidence that PKK can promote cell proliferation through effects on the cell cycle through p63 and NF-κB, we postulated that suppression of PKK can promote tumor formation in keratinocytes. SCC keratinocytes that were stably transduced with shControl or shPKK viruses were plated in soft agar assays. Soon after plating, a striking difference was noted. Control cells grew fewer, smaller (width of colony), and less dense (GFP intensity) colonies compared to PKK knockdown cells (Fig. 5a). Mean colony numbers are shown in Figure 5b from two different keratinocyte cell lines from SCCs expressing the shPKK retroviral constructs. This shows that knockdown of PKK leads to an increased anchorage-independent growth in human keratinocytes.

To test this result *in vivo*, NOD/SCID mice were utilized to assess tumor growth in a xenograft model. SCC cells that were stably transduced with shControl or shPKK-1 were injected with a subcutaneous bolus on the left and right flank. All experiments were performed in triplicate (i.e. in three mice or six injection sites). SCC cells expressing shPKK produced much larger tumors than SCCs transduced with shControl retroviruses (Fig. 5c). The tumor size of the shPKK keratinocytes was often dramatic and required removal of the animal from the study in 4 weeks (Fig. 5d). Tumor weights were obtained showing a statistically significant increase in tumor weight associated with the shPKK construct (Fig. 5e). The tumors were analyzed histologically and maintained the histology of SCC (Fig. 5f). Ki-67 staining was evaluated by immunofluorescence and there was increased Ki-67 staining in the shPKK tumors compared to shControl tumors (Fig 5h). The percentage of Ki-67 positive cells per high powered (40X) field was increased in shPKK tumors compared to shControl tumors. (Fig. 5h). shPKK tumors harvested at the end of the study continued to have decreased PKK expression compared to the shControl tumors by RT-PCR (Fig. 5g).

Discussion

Using two different small hairpin RNAs to target knockdown of *PKK* in cell cultures, we show that PKK functions as a tumor suppressor in human skin keratinocytes. Knockdown of PKK in cells causes an increase in cell proliferation with an increased S phase. A previous study in the skin of mice with deletion of PKK showed no increase in BrdU staining, although there was increased epidermal thickness (Holland *et al.*, 2002). Our results suggest that despite negative BrdU staining in the epidermis, the increased thickness observed in the knockout may be due to increased proliferation of keratinocytes. Moreover when PKK is overexpressed in keratinocytes (K14-PKK), there was no effect on tumor formation during

chemical carcinogenesis experiments (Rountree *et al.*, 2010). This is also consistent with our finding that PKK is a tumor suppressor in skin keratinocytes and not an oncogene as suggested by a study in ovarian cells (Huang *et al.*, 2013). Although targeting of PKK by siRNA in two previous studies failed to reveal increased proliferation after PKK knockdown (Adams *et al.*, 2007; Wang *et al.*, 2014), we observed significant increases in proliferation after PKK knockdown. It is possible that due to our retroviral transduction and development of stable cultures, in which we observed almost 100% GFP positive cells, we were able to observe proliferation that was not possible with lower transfection efficiencies and transient transfections.

Here we show that PKK activity regulates cell cycle proteins including cyclin E, cdk4 and p21 in human keratinocytes in a manner that promotes cell cycle progression when PKK activity is diminished. Because of the increased nuclear TP63 observed after PKK knockdown and the well-established control of TP63 of p21 in keratinocytes, we were particularly interested to find that PKK knockdown leads to decreased p21. This is likely due to TP63 and must be further explored in other models. In addition to the decreased p21 levels, the increase in cdk4 after PKK knockdown is also noteworthy. cdk4 has been previously shown to enhance malignant progression to SCC of the skin (Miliani de Marval *et al.*, 2004). Moreover knockdown of TP63 has been shown to inhibit the expression of cdk4 in keratinocytes (Antonini *et al.*, 2010), which could explain the increased cdk4 after increased TP63 associated with PKK knockdown. Ultimately the increased TP63 leading to altered cell cycle proteins may at least partly explain why keratinocytes with knockdown of PKK show increased anchorage-independent growth and tumor formation in immunodeficient mice (Fig. 4). One remaining question is why the basal level of TP63 in HaCaT was elevated compared to SCC and foreskin keratinocyte cultures and PKK knockdown did not alter nuclear TP63 levels in HaCaT cells. This may be a cell-line dependent observation.

It was previously reported that IκB was elevated in cells transduced with shRNA against PKK (Adams *et al.*, 2007). In addition to confirming this data, we have now shown that phosphorylation of IκB is inhibited and NF-κB transcriptional activity is decreased (Fig 4a and b) after PKK knockdown. It appears this is at least partly due to decreased IKK activity. Decreased NF-κB activity has been associated in multiple model systems with increased keratinocyte proliferation. Therefore the decrease in NF-κB transcriptional activity associated with decreased PKK levels may explain the observation of increased keratinocyte proliferation after PKK knockdown. Indeed in the setting of various mutations such as with *Hras*, decreased NF-κB is pro-tumorigenic in keratinocytes (Dajee *et al.*, 2003). The increased tumor formation associated with PKK knockdown, which was observed in multiple SCC lines during xenotransplantation experiments, may be due to this effect of PKK on NF-κB. It is possible that increased NF-κB activity in the setting of normal PKK may lead to keratoacanthoma formation but not SCC (Poligone *et al.*, 2013), while decreased NF-κB activity due to PKK disruption causes SCC tumors, suggesting PKK may mediate multiple pathways.

We noted in several xenotransplant mice that the tumor behaved in an aggressive manner with large tumors forming in just weeks (Fig 5d). This is similar to the finding that

decreased PKK was associated with more aggressive tongue SCC (Wang *et al.*, 2014). Although PKK can affect proliferation and pathways important in SCC tumorigenesis, it might also play an important role in the invasiveness of tumors. It has been shown that during normal wound healing, PKK expression is decreased (Adams *et al.*, 2007). It is possible that when this normal reparative pathway is disrupted by mutation, increased invasiveness and tumorigenesis is a consequence. Ultimately the importance of our findings in the development of SCC must be further explored. As one of the most common cancers in the United States, there is great need to identify the causal pathways, and it is exciting to recognize PKK as an important tumor suppressor in human SCC of the skin.

Materials and Methods

Cell Cycle and Apoptosis Analysis

Keratinocytes expressing shControl or shPKK were evaluated with the Click-iT EdU flow Cytometry Assay Kits (BD), following the manufacturer instructions.

The apoptosis assays were performed using the annexin V-PE apoptosis detection kit I (BD Bioscience, San Jose, CA).

RNA isolation and real-time qRT-PCR

Primary human tissue was collected from discarded tissue in order to generate a tissue biorepository which was approved by the investigational review board at the University of Rocheser (RSRB 00042616 and RSRB 00046521). Patient consent was not necessary as all skin was considered discarded. qRT-PCR was performed as described in Supplementary Material.

Soft agar colony formation assay

Soft agar colony formation assays were performed as previously described (Ren *et al.*, 2006) and described in Supplementary Material.

Western Blots and Immunofluorescence

Antibodies and standard protocols are described in Supplementary Material.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry and Immunofluorescence were performed per standard protocols and are described in Supplementary Materials.

Luciferase Reporter Assay

The assay was performed as previously described (Kim *et al.*, 2008) and in Supplementary Materials.

Retrovirus constructs and transduction

Retroviral and lentiviral RNA interference was performed as previously described (Kim *et al.*, 2008) and in Supplementary Materials.

SCC Cells

The SCC cell lines: SCCAM1 (SCC-1 in Figure 2A) and SCCJV (SCC-2 in Figure 2A) have been previously reported (Mantel *et al.*, 2014.).

In vivo xenografts

Mice were housed and animal experiments performed in the University of Rochester School of Medicine Vivarium in compliance with IACUC guidelines (Protocol #101225, UCAR-2008-042R). Xenografting is described in the Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Adams S, Munz B. RIP4 is a target of multiple signal transduction pathways in keratinocytes: implications for epidermal differentiation and cutaneous wound repair. Experimental cell research. 2010; 316:126–37. [PubMed: 19818768]

Adams S, Pankow S, Werner S, et al. Regulation of NF-kappaB activity and keratinocyte differentiation by the RIP4 protein: implications for cutaneous wound repair. J Invest Dermatol. 2007; 127:538–44. [PubMed: 17039240]

Antonini D, Russo MT, De Rosa L, et al. Transcriptional Repression of miR-34 Family Contributes to p63-Mediated Cell Cycle Progression in Epidermal Cells. J Invest Dermatol. 2010; 130:1249–57. [PubMed: 20090763]

Brash DE. Roles of the transcription factor p53 in keratinocyte carcinomas. The British journal of dermatology. 2006; 154(Suppl 1):8–10. [PubMed: 16712710]

Dajee M, Lazarov M, Zhang JY, et al. NF-[kappa]B blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature. 2003; 421:639–43. [PubMed: 12571598]

Heim D, Cornils K, Schulze K, et al. Retroviral insertional mutagenesis in telomerase-immortalized hepatocytes identifies RIPK4 as novel tumor suppressor in human hepatocarcinogenesis. Oncogene. 2014

Holland P, Willis C, Kanaly S, et al. RIP4 is an ankyrin repeat-containing kinase essential for keratinocyte differentiation. Current biology: CB. 2002; 12:1424–8. [PubMed: 12194825]

Huang X, McGann JC, Liu BY, et al. Phosphorylation of Dishevelled by protein kinase RIPK4 regulates Wnt signaling. Science. 2013; 339:1441–5. [PubMed: 23371553]

Jacobs MS, Persons DL, Fraga GR. EGFR and MYC gene copy number aberrations are more common in squamous cell carcinoma than keratoacanthoma: a FISH study. Journal of cutaneous pathology. 2013; 40:447–54. [PubMed: 23521519]

Kalay E, Sezgin O, Chellappa V, et al. Mutations in RIPK4 cause the autosomal-recessive form of popliteal pterygium syndrome. American journal of human genetics. 2012; 90:76–85. [PubMed: 22197489]

Karia PS, Han J, Schmults CD. Cutaneous squamous cell carcinoma: estimated incidence of disease, nodal metastasis, and deaths from disease in the United States, 2012. Journal of the American Academy of Dermatology. 2013; 68:957–66. [PubMed: 23375456]

Kim RH, Armstrong AW. Nonmelanoma skin cancer. Dermatologic clinics. 2012; 30:125–39. ix. [PubMed: 22117874]

Kim SW, Oleksyn DW, Rossi RM, et al. Protein kinase C-associated kinase is required for NF-kappaB signaling and survival in diffuse large B-cell lymphoma cells. Blood. 2008; 111:1644–53. [PubMed: 18025152]

Li G, Tron V, Ho V. Induction of Squamous Cell Carcinoma in p53-Deficient Mice after Ultraviolet Irradiation. J Invest Dermatol. 1998; 110:72–5. [PubMed: 9424091]

Li Q, Sambandam SA, Lu HJ, et al. 14-3-3sigma and p63 play opposing roles in epidermal tumorigenesis. Carcinogenesis. 2011; 32:1782–8. [PubMed: 21926108]

Mantel, A.; Carpenter-Mendini, A.; VanBuskirk, J., et al. [Accepted manuscript online: 10 JUN 2014] Aldo-keto Reductase 1C3 (AKR1C3) is overexpressed in skin squamous cell carcinoma (SCC) and affects SCC growth via prostaglandin metabolism. Experimental Dermatology. 2014. http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1600-0625/accepted

Mantel A, Carpenter-Mendini A, VanBuskirk J, Pentland AP. Aldo-keto Reductase 1C3 (AKR1C3) is overexpressed in skin squamous cell carcinoma (SCC) and affects SCC growth via prostaglandin metabolism. Submitted Journal of Investigative Dermatology. 2014

Meylan E, Martinon F, Thome M, et al. RIP4 (DIK/PKK), a novel member of the RIP kinase family, activates NF-kappa B and is processed during apoptosis. EMBO reports. 2002; 3:1201–8. [PubMed: 12446564]

Meylan E, Tschopp J. The RIP kinases: crucial integrators of cellular stress. Trends in biochemical sciences. 2005; 30:151–9. [PubMed: 15752987]

Miliani de Marval PL, Macias E, Conti CJ, et al. Enhanced malignant tumorigenesis in Cdk4 transgenic mice. Oncogene. 2004; 23:1863–73. [PubMed: 14647432]

Mills AA, Zheng B, Wang XJ, et al. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999; 398:708–13. [PubMed: 10227293]

Mortier L, Marchetti P, Delaporte E, et al. Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the p16(INK4a) tumor suppressor. Cancer letters. 2002; 176:205–14. [PubMed: 11804749]

Muto A, Ruland J, McAllister-Lucas LM, et al. Protein kinase C-associated kinase (PKK) mediates Bcl10-independent NF-kappa B activation induced by phorbol ester. J Biol Chem. 2002; 277:31871–6. [PubMed: 12091384]

Parker SB, Eichele G, Zhang P, et al. p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. Science. 1995; 267:1024–7. [PubMed: 7863329]

Poligone B, Hayden MS, Chen L, et al. A Role for NF-kappaB Activity in Skin Hyperplasia and the Development of Keratoacanthomata in Mice. PLoS One. 2013; 8:e71887. [PubMed: 23977171]

Proweller A, Tu L, Lepore JJ, et al. Impaired notch signaling promotes de novo squamous cell carcinoma formation. Cancer Res. 2006; 66:7438–44. [PubMed: 16885339]

Ren Q, Kari C, Quadros MR, et al. Malignant transformation of immortalized HaCaT keratinocytes through deregulated nuclear factor kappaB signaling. Cancer Res. 2006; 66:5209–15. [PubMed: 16707445]

Rogers HW, Weinstock MA, Harris AR, et al. Incidence estimate of nonmelanoma skin cancer in the United States, 2006. Arch Dermatol. 2010; 146:283–7. [PubMed: 20231499]

Rountree RB, Willis CR, Dinh H, et al. RIP4 regulates epidermal differentiation and cutaneous inflammation. J Invest Dermatol. 2010; 130:102–12. [PubMed: 19626033]

Seitz CS, Lin Q, Deng H, et al. Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. Proc Natl Acad Sci U S A. 1998; 95:2307–12. [PubMed: 9482881]

Su X, Cho MS, Gi YJ, et al. Rescue of key features of the p63-null epithelial phenotype by inactivation of Ink4a and Arf. The EMBO journal. 2009; 28:1904–15. [PubMed: 19494829]

Sur I, Ulvmar M, Jungedal R, et al. Inhibition of NF-kappaB signaling interferes with phorbol ester-induced growth arrest of keratinocytes in a TNFR1-independent manner. Journal of receptor and signal transduction research. 2009; 29:44–51. [PubMed: 19519169]

Takao J, Yudate T, Das A, et al. Expression of NF-kappaB in epidermis and the relationship between NF-kappaB activation and inhibition of keratinocyte growth. The British journal of dermatology. 2003; 148:680–8. [PubMed: 12752124]

Truong AB, Kretz M, Ridky TW, et al. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev. 2006; 20:3185–97. [PubMed: 17114587]

van Bokhoven H, Hamel BC, Bamshad M, et al. p63 Gene mutations in eec syndrome, limb-mammary syndrome, and isolated split hand-split foot malformation suggest a genotype-phenotype correlation. American journal of human genetics. 2001; 69:481–92. [PubMed: 11462173]

van Hogerlinden M, Rozell BL, Ahrlund-Richter L, et al. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res. 1999; 59:3299–303. [PubMed: 10416581]

Wang X, Zhu W, Zhou Y, et al. RIPK4 is downregulated in poorly differentiated tongue cancer and is associated with migration/invasion and cisplatin-induced apoptosis. The International journal of biological markers. 2014:0. [PubMed: 25362935]

Westfall MD, Mays DJ, Sniezek JC, et al. The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol Cell Biol. 2003; 23:2264–76. [PubMed: 12640112]

Wullaert A, Bonnet MC, Pasparakis M. NF-[kappa]B in the regulation of epithelial homeostasis and inflammation. Cell Res. 2011; 21:146–58. [PubMed: 21151201]

Xiong Y, Hannon GJ, Zhang H, et al. p21 is a universal inhibitor of cyclin kinases. Nature. 1993; 366:701–4. [PubMed: 8259214]

Yang A, Schweitzer R, Sun D, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999; 398:714–8. [PubMed: 10227294]

Yuspa SH. The Pathogenesis of Squamous Cell Cancer: Lessons Learned from Studies of Skin Carcinogenesis—Thirty-third G. H. A. Clowes Memorial Award Lecture. Cancer Research. 1994; 54:1178–89. [PubMed: 8118803]

Zhang D, Lin J, Han J. Receptor-interacting protein (RIP) kinase family. Cellular & molecular immunology. 2010; 7:243–9. [PubMed: 20383176]

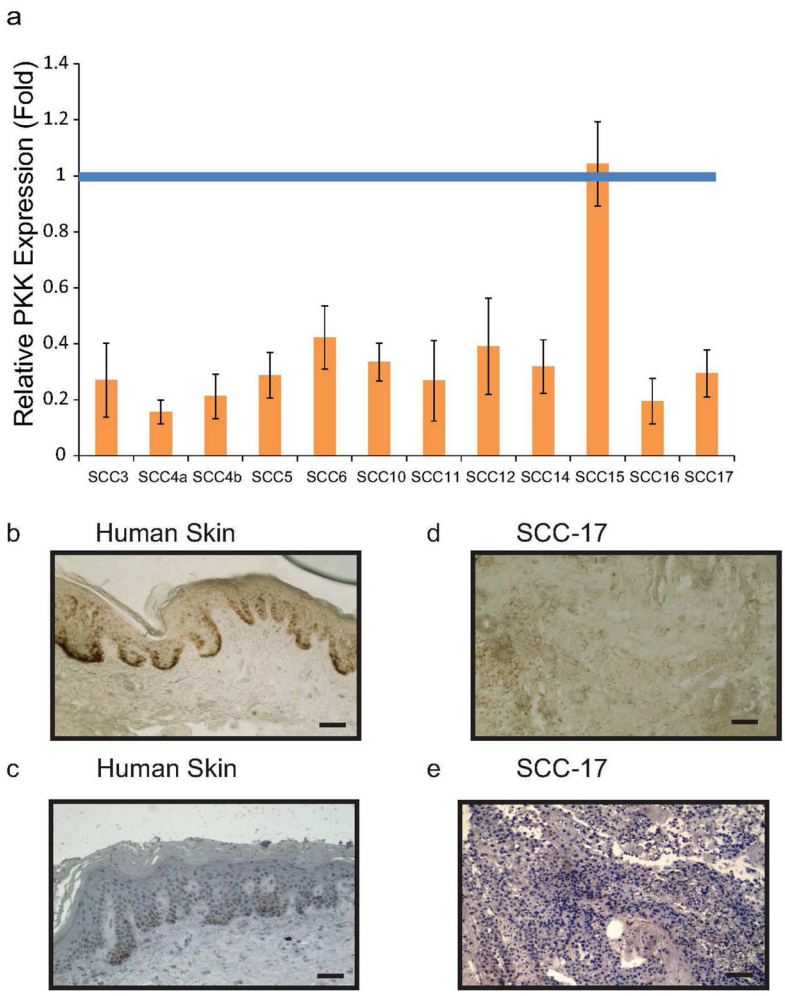

Fig. 1. Relative PKK expression in primary human SCC tumors

(a) Real time PCR analysis of *PKK* gene expression in primary human SCC tumors. The *PKK* expression was represented as a relative fold compared with average level of *PKK* gene expression in normal skin samples, the level of which was indicated with the blue line (normalized as 1). There was a significant difference for all samples except SCC15 compared to the normalized *PKK* expression ($p<0.05$). (b and c) Human skin stained against PKK without a nuclear counterstain (b) and with a nuclear counterstain (c). (d and e) SCC tumor stained with PKK without a nuclear counterstain (d) and with a nuclear counterstain (e). Bar = (a,c = 100μm) (d,e = 50μm).

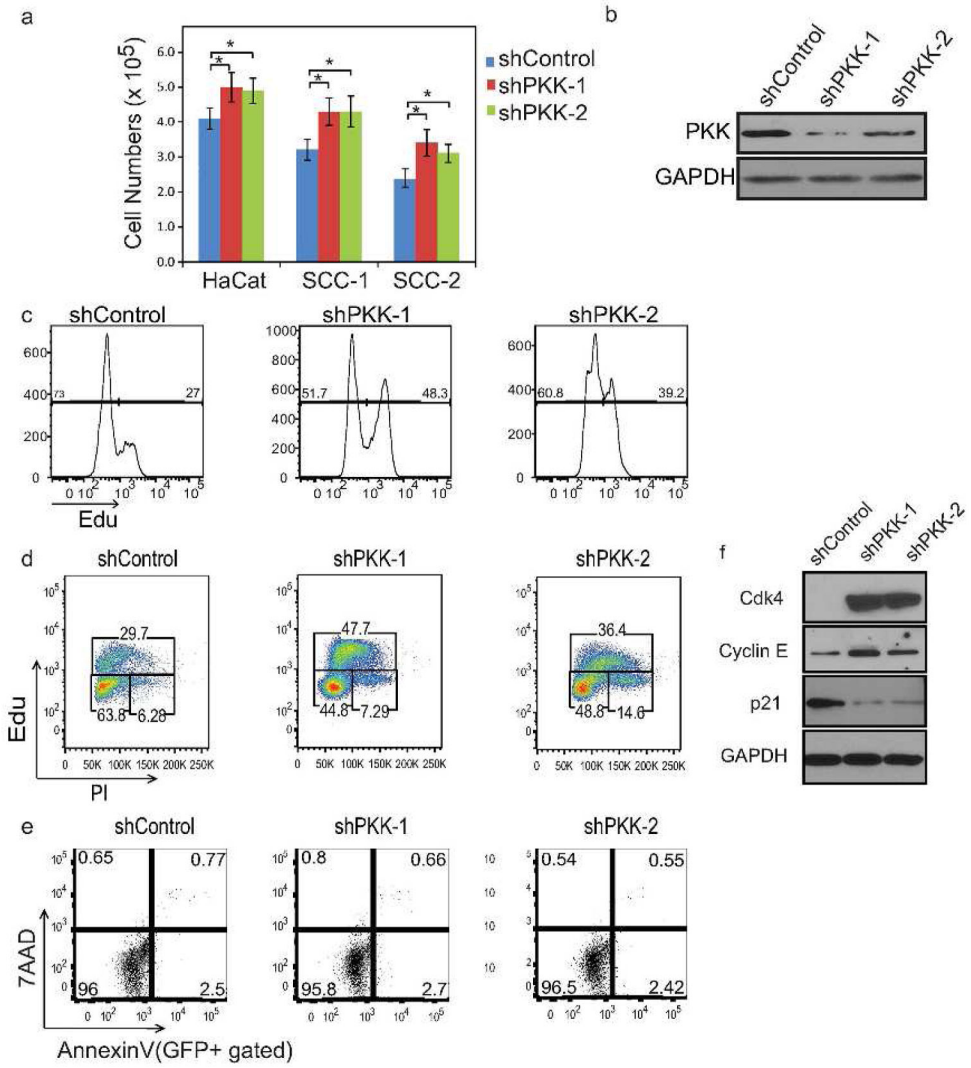

Fig. 2. Effect of PKK knockdown on keratinocyte growth

(a) Keratinocytes expressing shControl, shPKK-1, or shPKK-2 were plated in triplicate. On day 3, cells from each well were counted after staining with Trypan Blue. The total living cell numbers are represented. Error Bars represent the standard error of the mean (SEM) of triplicate measurements. *p<0.05. (b) Immunoblot of PKK knockdown. Anti-GAPDH was loading control. (c) Cell cycle analysis of SCC cells stably expressing shControl or shPKK-1 or shPKK-2. The representative FACS analysis is shown. (d) FACS analysis of GFP-gated keratinocytes labeled with EdU. (e) Keratinocytes gated for GFP, expressing shControl or shPKK-1 or shPKK-2, were stained with Annexin V and 7AAD. (f) SCC keratinocytes expressing shControl, shPKK-1, or shPKK-1 were examined for Cdk4, Cyclin E, p21, and GAPDH by immunoblot analysis.

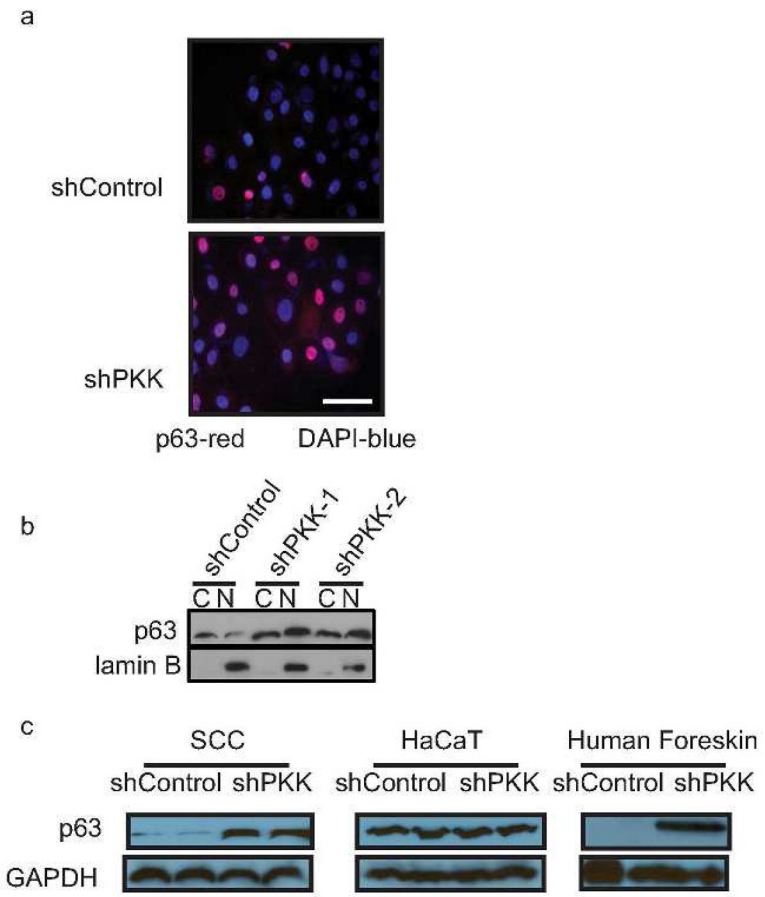

Fig 3. Suppression of PKK expression promotes SCC cell proliferation and increased nuclear TP63

(a) Immunofluorescence microscopy of SCC cells stained with anti-TP63. Cells transduced with shControl or shPKK, which were in log phase growth were analyzed. Bar = 100μm. (b) Nuclear extracts from SCC cells were examined by Western blot against TP63. Lamin B was used as a nuclear loading control. (c) Whole cell lysates were analyzed by Western blot for TP63 levels.

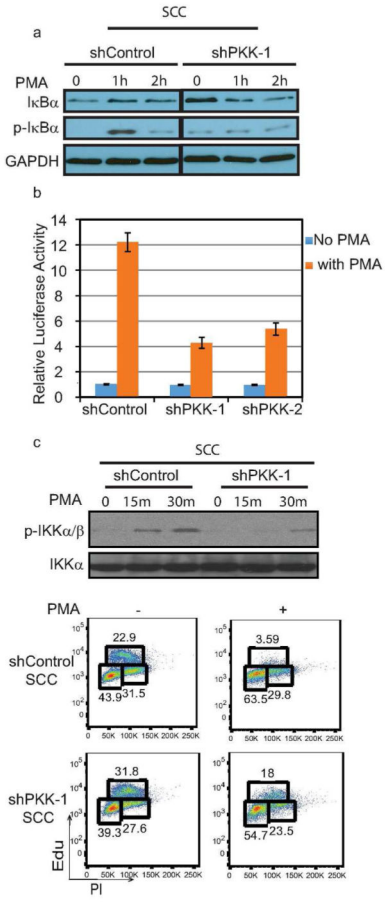

Fig 4. Suppression of PKK expression inhibits IKK leading to decreased NF-κB activity

(a) Western blot analysis of whole cell extracts of SCC cells after PMA treatment. (b) Luciferase Promoter Assay with an NF-κB reporter of SCC cells before and after PMA treatment. Untreated samples were normalized to 1. (c) Western blot analysis for IKK activity with IKKα and a phospho-specific IKK antibodies in SCC cells treated with PMA or control. (d) Cell cycle analysis of SCC cells stably expressing shControl or shPKK-1. PMA treated cells and untreated control cells were labeled with 10 um EdU for two hours. Standard flow cytometry methods were used to determine the percentage of cells in the population with EdU uptake. The representative FACS analysis is shown. Data was confirmed with multiple, independently-run assays.

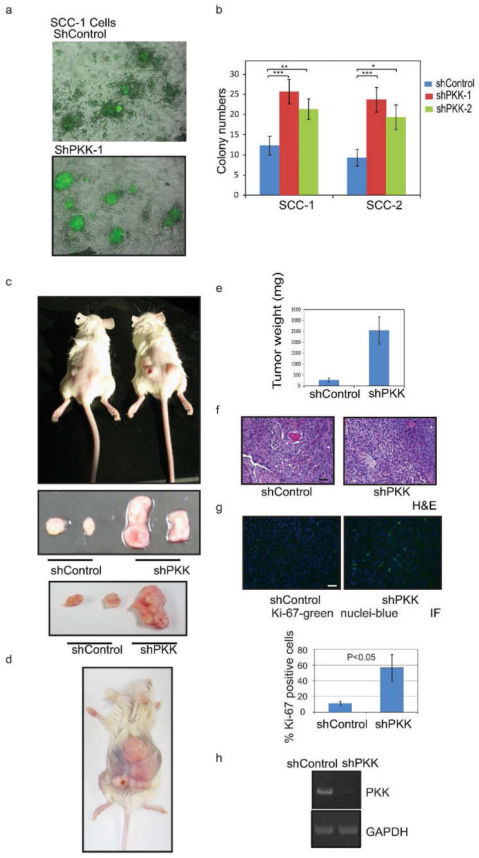

Fig. 5. PKK knockdown promotes SCC growth in soft agar

(a) 5×10^5 keratinocyes stably expressing shControl of shPKK-1 were studied with soft agar colony formation assays. (b) Bar graphs showed increased colony numbers after PKK knockdown. Results are representative of the mean of three independent experiments. *p≤0.05. **p≤0.01. ***p≤0.005. (c) Knockdown of PKK promotes tumor growth of SCC cells injected into NOD/SCID mice. (d) A representative mouse with shPKK-1 tumors. (e) The tumor weight measured at day 28 after implantation. The average tumor weight from 3 injected mice was shown. p≤0.005. (f) A representative H&E stain of tumors isolated from NOD/SCID mice. (h) Immunofluorescence stain with anti-Ki67 of SCC cells (top) Percentage of Ki-67 positive cells within the xenografted tumor (bottom). Bar = 50μm. (g) RT-PCR of xenografted tumors for PKK and GAPDH expression. Bar = 25μm.