Suppressive function of RKTG on chemical carcinogen-induced skin carcinogenesis in mouse

Xiaoduo Xie, Yixuan Zhang, Yuhui Jiang, Weizhong Liu, Hong Ma, Zhenzhen Wang and Yan Chen*

Key Laboratory of Nutrition and Metabolism, Institute for Nutrition and Metabolism Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

*To whom correspondence should be addressed. Tel: +86 21 54920916; Fax: +86 21 5492091; Email: ychen3@sibs.ac.cn

Raf kinase trapping to Golgi (RKTG) is a newly characterized negative regulator of the Ras–Raf–MEK–ERK signaling pathway via sequestering Raf-1 to the Golgi apparatus. However, little is known about the physiological functions of RKTG in mitogenic pathway and carcinogenesis. Here, we describe a suppressive role of RKTG in skin carcinogenesis by analyzing chemical carcinogen-induced tumorigenesis. Epidermis hyperplasia and proliferation are increased in RKTG-deficient mice (RKTG−/−) after acute treatment with 7, 12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). Using a two-stage DMBA/TPA carcinogenesis protocol on mouse skin, the number and size of papillomas are increased in RKTG−/− mice, accompanied by shortened tumor latency and enhanced keratinocyte proliferation. The regression of the carcinogen-induced tumors is also prolonged in RKTG−/− mice. Consistently, the levels of Raf-1 and extracellular signal-regulated kinase phosphorylation in primary keratinocytes as well as skin tumors are elevated when RKTG is disrupted. Collectively, our results indicate that RKTG has a suppressive activity in chemical carcinogen-induced mitogenesis and tumor formation in mouse skin.

Introduction

The Ras–Raf–mitogen-activated and extracellular signal-regulated kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway regulates many fundamental cellular functions including cell proliferation, apoptosis, differentiation, motility, senescence and metabolism. This pathway is also implicated in many human diseases especially in cancers (1, 2). Activating Ras mutations occur in ~30% of all human cancers and various Ras genes are mutated in different malignancies (1). Skin cancer is one of the most common malignancies affecting human beings. Two major cell types are commonly affected in the human skin cancers, including keratinocytes and melanocytes. Skin cancers arise predominantly from keratinocytes, with the most common forms being basal cell carcinoma and squamous cell carcinoma. On the other hand, malignant melanoma originates from melanocytes, the specialized pigment-producing cells interspersed among epithelial cells and also in the eye (3). Malignant melanoma is the rarest form of skin cancer but by far the most deadly due to its high metastatic potential and resistance to treatment. Recently, an array of molecular alterations in skin neoplasia has been identified. These alterations affect conserved regulators of cellular proliferation and viability signaling pathways, including Ras-Raf, Sonic Hedgehog, ARF/p53 and p16INK4A/Rb pathways (4, 5).

Abbreviations: BrdU, bromodeoxyuridine; DMBA, 7, 12-dimethylbenz(a)anthracene; ERK, extracellular signal-regulated kinase; HPF, high-power microscope field; IHC, immunohistochemistry; MEK, mitogen-activated and extracellular signal-regulated kinase kinase; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; RKTG, Raf kinase trapping to Golgi; TPA, 12-O-tetradecanoylphorbol-13-acetate; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

Many proteins are implicated in the regulation of Ras–Raf–MEK–ERK signaling cascade and several components within this pathway have been found to be regulated by subcellular compartmentalization (6, 7). Ras can be shuttled between different endomembrane vesicles and plasma membrane to send subcellular-specific signaling through translational modifications, interacting factors or different lipid anchors (8–11). MEK/ERK can be recruited to the Golgi by Sef and such spatial regulation blocks the Ras signaling to the nucleus but not to the cytosol (12). In addition, many Raf-interacting proteins are able to regulate Ras-mediated signaling such as Raf kinase inhibitor protein, β-arrestin, Sprouty and its related protein Spred. These proteins bind directly to Raf or to its downstream targets, whereby affecting the assembly of the Raf–MEK–ERK complexes (13–16). We recently identified that Raf kinase trapping to Golgi (RKTG) serves as a spatial regulator of Raf-1. RKTG blocks the signaling and function of Raf-1 by sequestering it to the Golgi apparatus (17). However, the physiological outcomes underlying the regulation of Ras–Raf–MEK–ERK signaling pathway by RKTG remain unclear. Here, we demonstrate that RKTG inhibits the proliferation of epidermis cells. RKTG deletion promotes tumor formation induced by chemical carcinogens in mouse skin. These findings, therefore, uncover a tumour-suppressive function of RKTG in skin carcinogenesis in mouse.

Materials and methods

Mouse and materials

RKTG disrupted mice (in C57BL/6J × 129Sv genetic background) were generated and identified as described previously (17). 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), acetone, bromodeoxyuridine (BrdU), 7, 12-dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), MEK-specific inhibitor PD98059 (P-215) and monoclonal anti-BrdU antibody were from Sigma–Aldrich (St Louis, MO). Antibodies against phosphorylated ERK (E-4) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Ki67 antibody was from BD Biosciences Pharmingen (San Diego, CA). Total ERK1/2 antibody and Phospho-c-Raf (Ser338) antibody (56/A6) were from Cell Signaling Technology (Danvers, MA). Rabbit anti-Flaggin polyclonal antibody (ab24584) was from Abcam (Abcam PLC, Cambridge, UK). ApopTag® Peroxidase in Situ Apoptosis Detection Kit was from Chemicon (Temecula, CA). Mouse keratinocytes specific isolation and culture medium Cirl-F07 and Cirl-F02 were from CELLeNT Advanced Cell Systems (Bern, Switzerland).

Skin treatment with chemical carcinogens

For acute treatment, RKTG+/− and RKTG−/− male littermates were treated with five doses of acetone, DMBA (8 nmol/l in 0.2 ml acetone), TPA (0.7 nmol/l in 0.2 ml acetone) or a single dose of DMBA followed by four doses of TPA. The chemicals were applied topically to the shaved backs of the mice every other day. For chronic treatment, RKTG+/−, RKTG−/− or RKTG−/− male mice were treated with a single dose of 200 nmol/l DMBA in 0.2 ml acetone applied topically to the shaved backs of the mice. One week after initiation, 17 nmol/l TPA in 0.2 ml of acetone was applied weekly to the skin for 20 weeks. The occurrence of papillomas was recorded each week starting at 8 weeks after TPA promotion. The treatment was stopped at 20 weeks and the regression of tumor was analyzed afterward until the 38th week.

Histological analysis

The tumor or normal skin was excised promptly after euthanasia and placed in 10% formaldehyde solution, fixed for at least 2 h and then embedded in paraffin. Paraffin sections of 4 μm were used for hematoxylin and eosin staining and immunohistochemistry (IHC). The antibodies were diluted as follows: phosphorylated c-Raf at 1:100; filagrin at 1:1000; phosphorylated ERK at 1:50 and anti-Ki67 at 1:3000. TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed following the manufacturer’s instruction. The IHC results were analyzed by experimenters without knowledge of the coded identity of the samples. To quantify the thickness of skin, five high-power microscope fields (HPFs) for each section were scaled using
Deletion of RKTG promotes proliferation of epidermal keratinocytes upon acute treatment with chemical carcinogens

We recently identified that RKTG functions as a spatial regulator of Raf kinase and negatively modulates the Ras–Raf–MEK–ERK signaling pathway (17). As Ras-mediated signaling pathway plays a central role in cell proliferation and mitogenesis, we hypothesized that RKTG may function as potential tumor suppressor. To address this hypothesis, we analyzed the effect of RKTG deletion on chemical carcinogen-induced skin cell proliferation and tumorigenesis. By reverse transcription–polymerase chain reaction analysis, we found that RKTG was expressed in mouse skin (data not shown). The RKTG-deficient mice were generated by conventional gene targeting technology (17). Under C57BL/6J×129Sv mixed genetic background, the RKTG−/− mice were viable and fertile and displayed no obvious developmental and functional defects. Intercross between RKTG heterozygous mice yielded all three genotypes at a ratios compatible to the expected Mendelian distribution (relative ratio 1:1.6:0.9, n = 302) with equal sexual ratio.

We first examined the skin structure of RKTG−/− mice after acute treatment with carcinogens DMBA and TPA, as both of them have been commonly used to induce Ras activation and oncogenesis in the skin (18). As shown in Figure 1A, no histological lesions were observed in the acetone-treated group. However, upon treatment with DMBA, TPA or DMBA/TPA, the thickness of the epidermis was apparently different between RKTG−/− and RKTG+/+ mice (Figure 1A). The average thickness of the epidermis was significantly increased in RKTG−/− mice than in the wild-type mice (Figure 1B), indicating an increased proliferation rate of skin cells upon DMBA and TPA treatment in the RKTG-deficient animals. This result was supported by IHC staining with a proliferation marker Ki67 (Figure 1C). The number of Ki67-positive cells was significantly increased in RKTG−/− mice in comparison with the wild-type controls (Figure 1D).
together, these results suggest that skin cell proliferation was accelerated in RKTG-deficient mice upon acute treatment with chemical carcinogens.

**RKTG deficiency promotes DMBA/TPA-induced skin carcinogenesis**

To analyze the effect of RKTG deficiency on skin tumorigenesis, we used a two-stage carcinogenesis protocol as illustrated in Figure 2A. The mice were first treated with a single dose of DMBA to initiate skin neoplasia formation and TPA was applied weekly to promote the tumorigenesis for 20 weeks. We first analyzed the timing of tumor occurrence. Papillomas began to appear at 11 weeks in RKTG\(^{-/-}\) mice, at 12 weeks in RKTG\(^{+/+}\) mice and at 15 weeks in RKTG\(^{+/+}\) mice, respectively (Figure 2B, left panel), indicating that tumor latency was shortened in RKTG-deficient animals. After 20 weeks of TPA treatment, 80% RKTG\(^{-/-}\) mice developed papillomas, whereas only 40% wild-type animals had the tumors. It is noteworthy that papillomas also occurred earlier and more frequent in the heterozygous mice than the wild-type controls. This finding suggests that haploinsufficiency of RKTG might be sufficient to accelerate skin tumor formation. Nevertheless, these data indicate that loss of RKTG is associated with increased tumor incidence in the carcinogen-induced skin cancer model.

We next analyzed the number of tumors with these animals. The number of papillomas per mouse was markedly increased in RKTG\(^{+/+}\) and RKTG\(^{-/-}\) mice (Figure 2B, middle panel). In average, >3 papillomas per mouse was observed in RKTG\(^{-/-}\) group and <1 tumor per mouse was found in RKTG\(^{+/+}\) group. Furthermore, the average number of large tumors (>5 mm in diameter) per tumor was also profoundly increased in RKTG-deficient mice (Figure 2B, right panel). These data, therefore, indicated that RKTG deficiency is able to promote the growth of skin tumors induced by DMBA/TPA. We also analyzed the process of tumor regression. At 20th week, TPA treatment was stopped and the changes of the tumors were recorded. In all three groups, the tumors had a tendency to desiccate and regress starting from the 23rd week. It appeared that the tumors in wild-type mice were regressing more rapidly than the tumors in RKTG\(^{-/-}\) and RKTG\(^{+/+}\) mice (Figure 2C).

**RKTG deficiency enhances cell proliferation upon chronic treatment with DMBA and TPA**

We hypothesized that the increased tumor formation in RKTG-deficient mice is associated with an elevated cell proliferation. Both Ki67 staining and BrdU incorporation were used to analyze the proliferative status of epidermis after chronic treatment with DMBA and TPA (Figure 3A and B). In comparison with the wild-type animals, RKTG\(^{-/-}\) mice had a markedly increased Ki67-positive and BrdU-positive cells in the skin, indicating that proliferation of keratinocytes was greatly enhanced in the absence of RKTG.
Fig. 3. Increased skin cell proliferation and reduced tumor apoptosis in RKTG-deficient mice. (A) Hematoxylin and eosin (HE) staining and Ki67 IHC staining of dorsal skin at the 20th week after DMBA initiation. The magnification is given below the images. (B) Summary of Ki67-positive and BrdU-positive cells in dorsal skin at the 20th week after DMBA initiation. Values are mean ± SD for five sections from different mice with the same genotype. *P < 0.05 and **P < 0.01. (C) Hematoxylin and eosin staining of skin tumors at the 20th week after DMBA initiation. The magnification is given below the images. (D) Hematoxylin and eosin staining of skin tumors at the 38th week after DMBA initiation. The magnification is given below the images. (E) Hematoxylin and eosin staining and immunohistochemical analysis of skin tumors at the 38th week after DMBA initiation. Ki67 was used to analyze cell proliferation and the TUNEL assay was used...
To further investigate cell proliferation and tumor progression, we excised the tumors and performed histological analysis. At the 20th week after DMBA/TPA treatment, most papillomas in RKTG+/+ mice continued to grow, whereas papillomas in wild-type mice appeared to be undergoing growth arrest and desiccation (data not shown). Examination by light microscopy revealed polypoid tumors in both wild-type and RKTG-deficient animals at the 20th week following carcinogen treatment. The skin tumors induced by DMBA/TPA in these animals were superficially papillomas consisting of an inner connective tissue with a vascular core, a stratified squamous epithelium with basal cells and an outer surface of keratin. However, some papillomas in RKTG−/− mice progressed to malignant carcinomas manifested as severely dysplastic papillomas with focal invasion. Most tumors occurred in wild-type mice appeared to be benign papillomas with hyperplastic epidermis (Figure 3C). At the 38th week after DMBA/TPA treatment, most tumors in wild-type animals are desiccated. However, the tumors in RKTG−/− mice remained malignant in histological features (Figure 3D). Consistently, Ki67-positive cells were much more in RKTG−/− mice than in the wild-type controls (Figure 3E), indicating a sustained cell proliferation in RKTG-deficient tumors. The increased epidermis thickness and hyperproliferation of the skin tumors upon carcinogen treatment in RKTG-deficient mice indicate an increased proliferation rate of keratinocytes in these animals. It is noteworthy that the observed hyperproliferation of the epidermis and tumors could also be caused by changes in apoptosis and or differentiation. To clarify these possibilities, we evaluated apoptosis. The magnification is given below the images. The inset shows a magnified representative area.

![Figure 4](https://academic.oup.com/carcin/article-abstract/29/8/1632/2476770)

**Fig. 4.** Increased Raf-1 and ERK phosphorylation in RKTG-deficient mouse skin. (A) Western blot analysis of total ERK and phosphorylated ERK (p-ERK) in the dorsal skins of RKTG+/+ or RKTG−/− mice without treatment. (B) Immunohistochemical analyses of phosphorylated ERK and phosphorylated Raf-1 (p-Raf-1) with the dorsal skin of RKTG+/+ and RKTG−/− mice without treatment. The magnification is ×40. (C) Immunohistochemical analysis of phosphorylated Raf-1 and phosphorylated ERK in the skin tumors of RKTG+/+ and RKTG−/− mice at the 38th week after DMBA/TPA treatment. The magnification is ×20.
and/or PD98059 treatment is indicated. C3 proliferation rate in primary keratinocytes as determined by MTT assay. TPA of the cells with TPA and/or MEK inhibitor PD98059 is indicated. (B) Cell proliferation rate in primary keratinocytes as determined by MTT assay. TPA and/or MEK inhibitor PD98059 is indicated. *(P < 0.05 and **P < 0.01 within the same treatment group.

performed IHC staining to elucidate the profiles of apoptosis and terminal differentiation in the skin samples. Intriguingly, cell apoptosis was markedly reduced in RKTG-deficient tumors in comparison with the tumors from wild-type mice (Figure 3E and F). However, the differentiation pattern as analyzed by filaggrin expression was not different between wild-type and RKTG−/− mice. Treatment of the cells with TPA and/or MEK inhibitor PD98059 is indicated. (B) Cell proliferation rate in primary keratinocytes as determined by MTT assay. TPA and/or PD98059 treatment is indicated. *(P < 0.05 and **P < 0.01 within the same treatment group.

RKTG deficiency increases Raf-1 and ERK phosphorylation and proliferation in primary keratinocytes

Over 90% of DMBA/TPA-induced skin tumors contain activated Ha-Ras (19). Thus, our observation that RKTG deficiency promotes DMBA/TPA-induced skin carcinogenesis by stimulating skin cell proliferation raises the possibility that RKTG is essential for tumor suppression in vivo against an activated Ras–Raf–MEK–ERK signaling pathway. To address this issue, we examined the phosphorylation levels of ERK and Raf-1 with the skin samples. The basal phosphorylation levels of ERK and Raf-1 were indeed increased in skin cells (Figure 4A and B). We also found that in DMBA/TPA-induced tumors, the levels of ERK and Raf-1 phosphorylation were much higher in RKTG-deficient tumors than the tumors from wild-type animals (Figure 4C). These observations were consistent with our previous report in which we found that RKTG inhibits ERK phosphorylation by insulating Raf-1 from its upstream target Ras and downstream target MEK (17).

RKTG deficiency enhances Raf-1/ERK phosphorylation and TPA-stimulated cell proliferation in primary keratinocytes

To further evaluate the growth-suppressive activity of RKTG in the skin, we isolated primary keratinocytes from RKTG+/+ and RKTG−/− mice. As shown in Figure 5A, the basal phosphorylation levels of ERK and Raf-1 were elevated in RKTG-deficient keratinocytes. TPA treatment could stimulate ERK phosphorylation and such stimulation was further enhanced by RKTG deletion. Consistent with our observation that RKTG inhibits signaling from Ras to MEK (17), treatment with a MEK-specific inhibitor PD98059 could completely abrogate the basal and TPA-induced ERK phosphorylation in RKTG-deficient keratinocytes (Figure 5A). We further analyzed the cell proliferation rate of the primary keratinocytes by a MTT assay. In the presence of TPA, RKTG−/− keratinocytes grew significantly faster than the wild-type cells and such effect was abrogated by pretreatment of the cells with PD98059 (Figure 5B). Collectively, these data suggest that RKTG deficiency is able to stimulate ERK signaling in vitro and enhance keratinocyte proliferation upon TPA treatment, consistent with our observations from in vivo experiments.

Taken together, our results demonstrate for the first time that RKTG, a newly found Raf kinase regulator, may function as a tumor suppressor in skin carcinogenesis in mouse. The majority of skin cancers are originated from keratinocytes with the most common forms being basal cell carcinoma and squamous cell carcinoma. The two-stage mouse model using DMBA and TPA is mainly applied to analyze the molecular pathogenesis of skin cancers of keratinocyte origin. Treatment of the mouse skin with DMBA and TPA was able to profoundly increase cell proliferation and tumor formation when RKTG was deleted. The tumor-suppressive activity of RKTG appeared to associate with its regulation on ERK signaling pathway. Deletion of RKTG led to increased Raf-1/ERK phosphorylation and keratinocyte proliferation upon TPA stimulation both in vivo and in vitro. These data, therefore, strongly indicate that RKTG has a suppressive function in skin carcinogenesis.

Our results also provided additional evidence that Ras–Ras–MEK–ERK pathway is critical in tumorigenesis as revealed by a majority of in vivo studies. For example, some of key Ras effectors including PLC and RalGDS are implicated in skin tumorigenesis in two-stage skin cancer mouse model. RalGDS-deleted mice showed reduced tumor incidence, size and progression to malignancy in skin carcinogenesis (20). PLC−/− mice exhibit delayed onset and markedly reduced incidence of skin squamous tumors in the skin (21). In addition, knock-out of Sprouty-2, a negative regulator of Raf kinase, promotes lung tumorigenesis in the background of K-rasG12D-transgenic mice (22). In present study, we found that deletion of another negative regulator of Raf kinase promotes DMBA/TPA-induced keratinocyte proliferation and carcinogenesis. Therefore, our study suggests that RKTG may possess a suppressive activity in skin carcinogenesis in the mouse model via negative regulation of the Ras–Raf–MEK–ERK pathway. Considering such important function of RKTG in carcinogenesis, the next challenge will be to uncover its regulation and function in human cancer formation.

Funding
Chinese Academy of Sciences (One Hundred Talents program and the Knowledge Innovation Program KSCX1-YW-02); National Natural Science Foundation of China (30588002 and 30470870); Ministry of Science and Technology of China (2006CB943902 and 2007CB947100) to Y.C.

Acknowledgements

Conflict of Interest Statement: None declared.


Received January 17, 2008; revised April 25, 2008; accepted June 3, 2008