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YAP Signaling is Involved in Keratinocyte Proliferation and Tumorigenesis Mediated by RIPK4 Knockdown

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ABSTRACT

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer of the skin, with approximately 800,000 new cases diagnosed yearly in the US, and its incidence is continuing to increase. Although local cSCC is curable with conventional treatments, cSCC exhibits a significant risk of metastasis, and no effective treatment is currently available for patients with the metastatic disease, leading to a survival rate of less than 20% over 10 years. Thus, a better understanding of the molecular mechanisms of cSCC pathogenesis is urgently needed for developing novel, effective therapies to combat this disease. Recently, new genomic studies identified the protein kinase RIPK4 (also known as PKK) as a recurrently mutated gene in aggressive and metastatic cSCCs. This aligns with our previous studies, which suggested RIPK4 functions as a tumor suppressor. Although we showed that the NFkB and p63 pathways are involved in the RIPK4-mediated cSCC development, the link of RIPK4 with the Hippo pathway has not been reported. In our current study, we investigated whether YAP1, a transcription regulator downstream of the Hippo pathway, is involved in RIPK4-mediated tumor formation. By using small-hairpin RNA (shRNA) targeting the RIPK4 gene and small interfering RNA targeting the YAP1 gene, we demonstrated that suppression of RIPK4 led to tumor development in keratinocytes via upregulating YAP signaling. More strikingly, silencing YAP1 expression led to a dramatic decrease in the size of colonies formed due to RIPK4 depletion. Our results may provide a novel therapeutic strategy by targeting the YAP signaling pathway in RIPK4-deficient cSCC, potentially improving outcomes for patients with this aggressive and metastatic disease.

Keywords: YAP, RIPK4, cutaneous squamous cell carcinoma, human cancer

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer of the skin, with approximately 800,000 new cases diagnosed yearly in the US, and its incidence is continuing to increase.[1,2] cSCC is characterized by abnormal, accelerated growth of squamous cells. Metastatic cutaneous squamous cell carcinoma and aggressive cSCC, which are associated with frequent recurrence or metastasis, have very poor clinical outcomes with the current available treatments.[1-4] Very little is known about the molecular pathways underlying the pathogenesis of aggressive and metastatic cSCC, which has hampered the development of effective targeted therapies. Thus, a better understanding of the molecular mechanisms of these diseases is needed for the identification of therapeutic targets.

Recent genomic studies have identified a number of recurrent putative driver mutations in aggressive and metastatic cSCC, which include RIPK4 mutations in 28% (10 out of 34) and 24% (7 out of 29) aggressive and metastatic cSCCs, respectively.[1,2] RIPK4 is a serine threonine kinase and belongs to the RIP kinase family. RIPK4 was originally identified by us and others as a protein kinase C beta and protein kinase C delta interacting kinase.[3,4] We previously showed that RIPK4 regulates NF-kB activation and proliferation and survival of diffuse large B-cell lymphoma cells.[5,6,7] A role for RIPK4 in Wnt signaling and IRF6 pathway has also been reported.[3,8] Humans with RIPK4 mutations develop Bartsocas-Papas syndrome, a form of limb pterygium syndrome, which is generally lethal at birth.[9,10] Notably, we found that human cSCC samples have significantly lower levels of RIPK4 mRNA than normal human keratinocytes.[11] Moreover, we demonstrated that depletion of RIPK4 expression by small hairpin RNA

(shRNA) greatly increased the proliferation of human cSCC cells in vitro and the tumor growth of the implanted human cSCC, suggesting that RIPK4 may have a tumor-suppressive function in cSCC.[11] Recently, using our keratinocyte-specific conditional knockout mouse model, we demonstrated that RIPK4 deletion promotes tumorigenesis after chemical carcinogenesis, further supporting the note that RIPK4 functions as a tumor suppressor.[12]

RIPK4 plays an important role in normal skin as well as SCC development. We and others have shown that mice with RIPK4 deletion in keratinocytes exhibit thickened epidermis with upregulation of K14 (a basal keratinocyte marker) expression in the granular layer. Additionally, differentiation markers such as Filaggrin, Loricrin, and Involucrin are aberrantly expressed in the spinous and granular layers, indicating delayed keratinocyte differentiation and an expanded basal layer.[13] Interestingly, using a specific keratinocyte RIPK4 knockout mouse, we demonstrated that RIPK4 deficiency promotes SCC formation as a tumor suppressor in skin keratinocytes.[11] However, the molecular mechanism underlying basal layer expansion in the thickened epidermis after RIPK4 loss is not clear, and the molecular signaling pathways involved in RIPK4-mediated cSCC formation have not been completely elucidated.

Skin tumors are characterized by excessive cell proliferation and abnormal differentiation. Yes-associated protein 1 (YAP1, also known as YAP), a member of the Hippo signaling pathway,[14] plays important roles in regulating cell growth, apoptosis, and controlling tissue and organ sizes.[15] YAP has been found to drive cSCC formation and progression.[16] Our previous study using keratinocyte-specific knockout mice and in vitro studies clearly demonstrate that loss of RIPK4 in keratinocytes promotes cell proliferation and cSCC formation. Therefore, we hypothesize that YAP signaling is involved in cSCC formation induced by suppression of RIPK4 expression. To our knowledge, this report is the first study establishing a link between RIPK4 and YAP signaling pathways in cSCC.

METHODS

Cell Lines

The human keratinocyte HaCaT cell line was kindly provided by Dr. Jiyong Zhao (University of Rochester). The cell line was cultured in DMEM (Fisher Scientific #11-960-069), supplemented with 10% FBS. The SCC-1 and SCC-2 cell lines were kindly provided by Dr. Alice Pentland (University of Rochester) and were cultured in a keratinocyte serum-free medium with supplements (Fisher Scientific #17005042).

RIPK4 and YAP1 Knockdown

RIPK4 expression was suppressed by RNA interference using small hairpin RNA (shRNA) specific for human RIPK4 mRNA. One specific effective targeting sequence for RIPK4 (shRIPK4: 5′-GGCCCACCTTCCAAGAAATTA) was selected based on our previous study.11 This shRIPK4 RNA was then cloned into the retroviral pRetro-H1G vector (referred to as shRIPK4). A random hairpin sequence (5′-GTTCTCCGAACGTGTCACG) was also cloned into the same vector (referred to as shControl). To generate HaCat, SCC-1, or SCC-2 cells that stably express shRIPK4 or shControl, the cells were infected with the recombinant retroviruses, and the transduced cells were selected with puromycin for 10 days. After drug selection, bulk infected cells were used in the experiments to avoid clonal variations.

To suppress YAP expression, the SMARTpool siRNA targeting YAP (referred to as siYAP) or scrambled control siRNA (referred to as siScramble as a negative control) was purchased from Thermo Scientific Dharmacon (Pittsburgh, PA, USA). Cells were transfected with 100 nmol/l of the siRNAs against YAP and control siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The resulting cells were referred to as shRIPK4-siYAP-SCC or shRIPK4-siScramble-SCC. After the cells were transfected for 36 hours, 2/3 of the cells were used for a colony formation assay, and 1/3 of the cells were used for Western blot analysis to confirm the suppression of YAP1 expression.

Cell Proliferation Assay

Cell proliferation was measured using Trypan Blue exclusion assays. Live cells (Trypan Blue negative) were counted, and relative total live cell numbers were plotted, with the live control cells set to 1. Triplicates were used for each cell line, and four independent experiments were performed.

Colony Forming Assay

The colony forming assay was carried out based on the method described by Crowley et al.[17] Briefly, 1x104 cells in 2 ml growth medium were plated in 6 well plates. After incubation for 7 days, the medium was removed, and the plates were washed gently with PBS. The cells were then fixed with 100% methanol at room temperature for 20 minutes. 1 ml crystal violet staining solution (0.5% crystal violet in 25% methanol) was added in each well, and the plates were incubated at room temperature for a 5-minute stain. After staining, the crystal violet solution was removed, the plates were washed with H2O, and the colonies were counted. Representative pictures were taken under a microscope. Three independent experiments were conducted. Each cell line had triplicate wells in each experiment.

Western Blot Analysis

The cells were harvested and lysed with a lysis buffer containing 1 M Tris pH 8.0, 5 M NaCl, 1 M NaF, 0.1 M Na3VO4, 1% NP-40, 10% glycerol, and 0.5 M EDTA (pH 8.0). Proteins were separated on 8% SDS-polyacrylamide gel electrophoresis. After transfer to PVDF membranes, the membranes were washed with TBST and incubated with primary antibodies for 1 hour at room temperature. After washing three times with TBST, the membranes were incubated with secondary antibodies for 1 hour. Following another three washes with TBST, the membranes were probed with the ECL system. The antibodies used in this study were anti-RIPK4 antibody (Cell Signaling #12636), anti-YAP1 (Santa Cruz, sc-101199), GAPDH (Santa Cruz, sc-365602). and Anti-cyclin D (Santa Cruz sc-8396). The protein expression level was qualified with Image J.

Statistical Data Analysis

To compare the two groups, data were analyzed by Welch's t test. For the comparison of multiple groups, data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test using the GraphPad Prism (Version 5). All values are expressed as means ± SEM. When p values were <0.05, the differences were considered significant.

RESULTS

Knockdown RIPK4 Expression in Keratinocytes Promotes Cell Proliferation

In order to evaluate whether YAP is involved in RIPK4-mediated keratinocyte proliferation, we aimed to first knock down the RIPK4 gene in SCC cells and then examine the effects of RIPK4 suppression on keratinocyte proliferation. Two human cSCC cell lines, SCC-1 and SCC-2, and HaCaT cells as a "normal" keratinocyte control were utilized.[18] RIPK4 gene expression was suppressed in these cells using a retroviral system that ectopically expresses either a retroviral vector carrying a short hairpin RNA (shRNA, named shRIPK4) or a scrambled shRNA (shControl). The efficiency of RIPK4 suppression was confirmed in these SCC cells.[11] Following viral infection and puromycin selection, stable cell lines carrying shRIPK4 or shControl were established, as previously described. [11] As shown in Fig. 1, a successful RIPK4 knockdown in these cells was confirmed with Western blot analysis (Fig. 1A).

Next, we examined whether the knockdown of RIPK4 has any effect on cell proliferation. After establishing stable transfection with the shRNA retroviral vectors, the same number of cells per cell line were plated in 6-well plates, and cell viability was assessed at day 3 using Trypan blue exclusion assays. As shown in Fig. 1B, knockdown of RIPK4 in keratinocytes led to an increased number of cells in culture compared to the cells with shControl of each cell line.

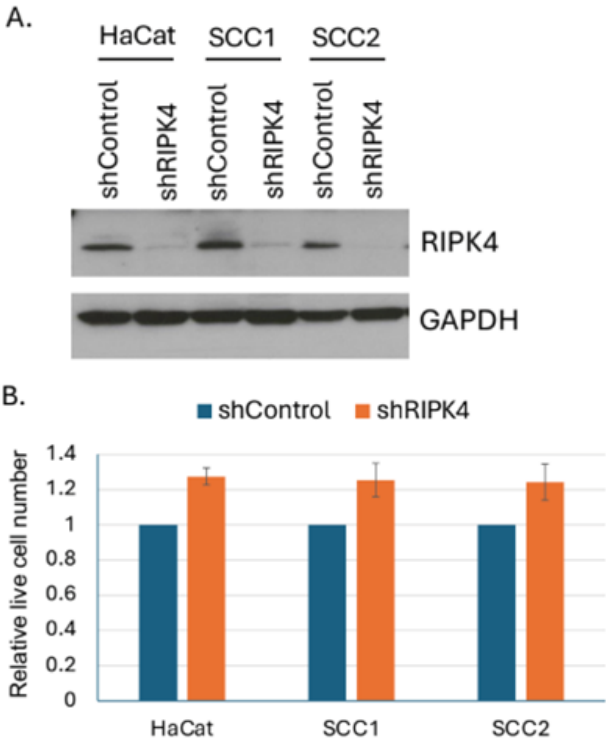

Figure 1. Effect of suppression of RIPK4 on SCC cell growth (A) Western blotting analysis of RIPK4 knockdown in keratinocytes. Total protein extracts isolated from HaCat cells, SCC1and SCC2 transduced with shRIPK4 retroviral construct or shControl. GAPDH was used as a loading control. **(B)** Cell Proliferation Assay. HaCat and SCC cells expressing shRIPK4 and shControl were plated in triplicate. On day 3, the cells from each well were stained with Trypan Blue, and live cells (Trypan Blue negative) were counted. Relative total live cell numbers were presented compared to the live control cells in each cell line, which were set to 1. Triplicates were used for each cell line, and four independent experiments were performed. *P ≤ 0.05.

Knockdown of RIPK4 in Keratinocytes Induced YAP1 Expression

After confirming the RIPK4 knockdown, we examined YAP1 expression in these shRIPK4 cells. As shown in Fig. 2A, YAP1 expression was greatly increased in all shRIPK4 SCC cell lines, as well as in shRPK4 HaCaT cells. We also detected upregulation of Cyclin D, a downstream target of YAP1 that promotes cell proliferation. These results support the notion that YAP1 is involved in the RIPK4-mediated cell proliferation pathway in keratinocytes.

Suppression of YAP1 Expression Inhibited Tumorigenesis Mediated by RIPK4 Gene Knockdown in SCC Cells

Our previous research has shown that RIPK4 functions as a tumor suppressor in SCC development.[11,12] In our current study, we observed that YAP is upregulated in RIPK4 knockdown cells. This led us to postulate that suppression of YAP can decrease tumorigenesis. We tested this hypothesis using the colony formation assay.

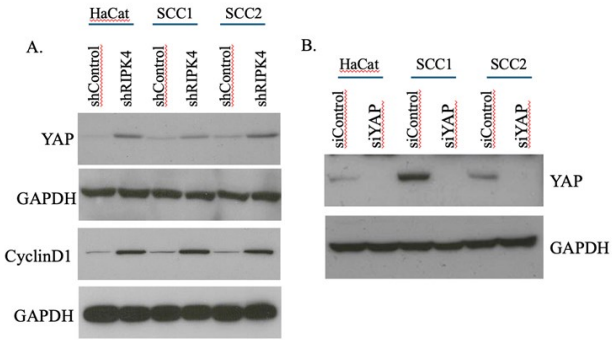

Figure 2. YAP1 expression was upregulated in shRIPK4 keratinocytes. Knockdown of YAP dramatically reduces colony size compared with those formed from shRIPK4 SCC cells. (A) Western Blot analysis of YAP1 expression. YAP target Cyclin D1 expression is also upregulated. Whole cell lysate was used. Anti-GAPDH was a loading control. **(B)** Western Blot analysis of YAP1 knockdown. Whole cell lysate was used. Anti-GAPDH was a loading control.

SCC cells that were stably transduced with shRIPK4 or shControl viruses were used to generate cells with YAP knockdown. The specific small interfering RNA targeting YAP gene was utilized. After confirming both RIPK4 and YAP knockdown in cells (referred to as shRIPK4-siYAP-SCC1, shRIPK4-siScramble-SCC1; SCC2-shRIPK4-siYAP, and SCC2-shRIPK4-siScramble) by Western blot analysis (Fig. 2B), these cells were used for colony formation assays to evaluate tumorigenesis in vitro.

As shown in Fig. 3A and B, a striking difference in colony size was observed between shRIPK4-siYAP-SCC and shRIPK4-siScramble-SCC cells. YAP knockdown greatly reduced the size of the colonies compared to those formed by shRIPK4 SCC cells without YAP1 suppression. Mean colony numbers are shown in Fig. 3C and D for SCCs expressing shRIPK4 and siYAP1. As expected, the area of colonies in each well was greatly decreased for SCCs expressing both shRIPK4 and siYAP1 compared with that of SCC expressing shRIPK4 alone (0.087% vs 0.79% for SCC1 and 0.05% vs 0.18% for SCC2, data not shown). The results indicate that YAP is required for shRIPK4-induced tumor formation in keratinocytes.

CONCLUSION

In the current study, using small-hairpin RNA (shRNA) and small interfering RNA(siRNA) techniques, we investigated the role of the transcription regulator YAP1, a downstream target of the Hippo pathway, in RIPK4-mediated tumorigenesis. We found that YAP1 was upregulated in keratinocytes with RIPK4 expression knockdown. We demonstrated that YAP is required for RIPK4 suppression-induced tumorigenesis by colony formation assay. To our knowledge, this is the first report that linked YAP signaling to RIPK4 mediated tumorigenesis pathway.

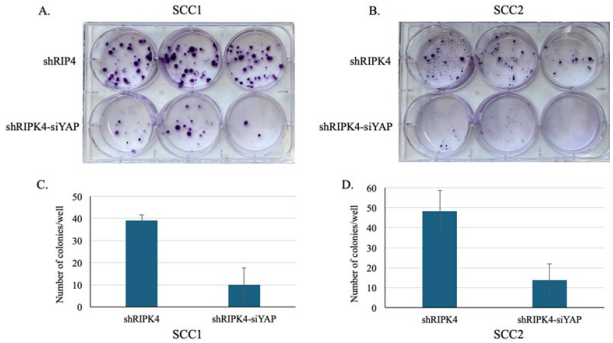

Figure 3. YAP1 knockdown significantly reduces colony sizes of shRIPK4SCC1 and shRIPK4 SCC2. Colony forming assay. $1x10^4$ SCC cells expressing shRIPK4 and siYAP1 were used for colony forming assays. Representative images were shown (3A and 3B). Bar graphs showed reduced colony numbers after YAP1 knockdown (3C and 3D) (n = 2; mean ± SD). P =0.002 for SCC1, P=0.015 for SCC2.

Interestingly, RIPK4 has multiple functions, such as being a tumor suppressor or oncogenic, depending on tissues and cells. In non-keratinocyte cells such as B cells, RIPK4 is required for some lymphoma growth, and in ovarian cells, RIPK4 was reported to be overexpressed, suggesting it is tumorigenic.[7,17] However, in keratinocytes, we and others discovered it functions as a tumor suppressor.[11,12] In our previous study, suppression of RIPK4 expression by small hairpin RNA (shRNA) dramatically increased the proliferation of human cSCC cells in vitro and the tumor growth of the implanted human cSCC, suggesting that RIPK4 may have a tumor-suppressive function in cSCC.[11] Recently, using keratinocyte-specific conditional knockout mice, we demonstrated that RIPK4 deletion promotes tumorigenesis after chemical carcinogenesis.[12] As expected, we found that human cSCC samples displayed a significantly lower level of RIPK4 expression, further demonstrating that RIPK4 functions as a tumor suppressor in keratinocytes.[11] In this study, using the small-hairpin RNA technique, we generated multiple SCC stable cell lines with RIPK4 knockdown. Our results further support the notion that suppression of RIPK4 in keratinocyte promotes cell proliferation.

The Hippo-YAP pathway plays a critical role in organ size, tissue homeostasis, and tumorigenesis in mammals. YAP1 is a transcription regulator at downstream of the Hippo signaling pathway.[20] Abnormal upregulation or nuclear localization of YAP1/TAZ occurs in many human malignancies, including SCCs.[21] However, whether YAP1 plays any role in SCC induced by suppression of RIPK4 has not been reported. In this

study, we observed a marked upregulation of YAP1 as well as cyclin D (downstream target of YAP1) in all keratinocytes with shRIPK4 (suppression of RIPK4 expression). Furthermore, using small interfering RNA of YAP1, YAP1 expression was suppressed in those shRIPK4 SCC stable cell lines. We performed colony assays using these cell lines with both RIPK4 and YAP1 knockdown, as shown in Fig. 2B, and the SCC cells with siYAP1 displayed a dramatic decrease in the colony sizes compared to cells with only knocked down of RIPK4 expression. This result indicated that YAP1 is required for tumorigenesis mediated with RIPK4 suppression in vitro colony formation assay, suggesting an interplay between YAP with RIPK4 signaling.

How do the two proteins YAP1 and RIPK4 interact? We have failed to detect YAP1 being phosphorylated by RIPK4 directly. It has been shown that YAP1 regulates stem cell proliferation and is primarily expressed in the basal layer of the skin.[22] We have shown that knockdown RIPK4 upregulates p63, a master transcription factor in the basal layer of skin.[11] Therefore, we postulated that YAP1 interacts with p63. Interestingly, it was reported that in SCC cells, the accumulation of YAP1 stabilizes p63 to enhance tumor formation.[23] In addition, in the present study, we did not address whether YAP signaling plays a role in SCC metastasis, and RIPK4 downregulation links HPV induced cSCC risk. Currently, investigation of the molecular mechanism by which YAP1 interacts in RIPK4 tumorigenesis is underway. Understanding RIPK4-YAP1 signaling might lead to new therapeutic avenues for cSCC treatment.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: LC

Methodology: LC

Investigation: TZ, LC

Supervision: LC

Writing – original draft: TZ, KM, LC

Writing – review & editing: TZ, KM, LC