

Report

Promoted Papilloma Formation in a Two-Stage Carcinogenesis Model in *Rassf6* Knockout Mice

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Received January 27, 2023; Accepted March 15, 2023

We have previously reported the generation of *Rassf6* knockout mice using CRISPR/Cas technology. Furthermore, we have reported that RASSF6 is implicated in the regulation of the canonical nuclear factor (NF)- κ B signaling pathway and that 7,12-dimethylbenz(a)anthracene induced skin inflammation in *Rassf6* knockout mice more remarkably than in wild-type mice. In this study, we investigated the role of RASSF6 in skin tumorigenesis using a two-stage carcinogenesis model in *Rassf6* knockout mice. *Rassf6* knockout mice initially developed significantly more papillomas than did the control wild-type mice. Additionally, macrophages were detected and the canonical NF- κ B signal was elevated in papillomas of *Rassf6* knockout mice. These results indicated that canonical NF- κ B signaling may be involved in papilloma formation. The suppression of NF- κ B signaling may have implications for preventing cancer initiation and progression. The findings of this study indicate a tumor suppressive role of RASSF6.

Key words *Rassf6* knockout mice, nuclear factor- κ B, two-stage carcinogenic experiments

INTRODUCTION

Ras-association domain family (RASSF) comprises 10 genes in the human genome. Six of them, RASSF1–6, belong to a subgroup called C-RASSF.¹⁾ C-RASSF expression is often suppressed in human cancers, and cancers with low expression of C-RASSF become malignant and have a poor prognosis; therefore, it is considered a tumor suppressor molecule.

In a previous study, we generated *Rassf6* knockout mice using the CRISPR/Cas technology to confirm that RASSF6 deficiency enhances tumor susceptibility.²⁾ Nevertheless, *Rassf6* knockout mice did not develop tumors. Moreover, *Rassf6* knockout mouse embryonic fibroblasts (MEF) were found to be less prone to p53-dependent cell death and cell-cycle arrest and exhibited inhibition of cell senescence and delayed DNA damage repair. Furthermore, the introduction of activated KRAS into *Rassf6*-null MEF promoted colony formation, whereas the introduction of MYC, activated TAZ, and oncogenic p53 did not result in colony formation. RNA sequencing analysis showed that nuclear factor (NF)- κ B signaling was enhanced in *Rassf6*-null MEF. Moreover, *Rassf6* knockout mice exhibited increased NF- κ B signaling and more remarkable skin inflammation than wild-type mice. RASSF6 deficiency might have compromised p53 function and enhanced NF- κ B signaling, causing tumorigenesis.

As mentioned previously, *Rassf6* knockout mice did not exhibit spontaneous tumorigenesis. However, it has been reported that mice knocked out for *Rassf1a*, which encodes another C-RASSF, are susceptible to spontaneous tumorigenesis and chemical-induced tumorigenesis.³⁾ RASSF6 has also

been reported to exert a tumor suppressive effect mediated by p53 and Rb in experiments using human cancer cells.^{4,5)} Therefore, it is presumed that the susceptibility to tumorigenesis would be changed in deficient mice if tumorigenesis was promoted. In this study, we performed a two-stage carcinogenesis experiment to clarify the signaling of RASSF6 during tumorigenesis.

MATERIALS AND METHODS

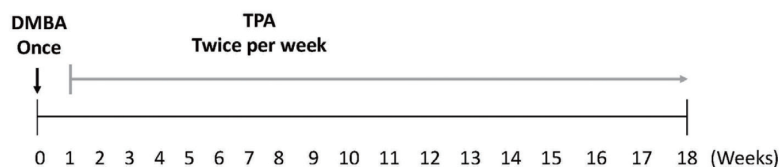
Mice We used 7–9-week-old female wild-type and *Rassf6* knockout mice ($n = 5$ per group) in the animal study.²⁾ All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Approval #A2021-392).

In Vivo Two-Stage Skin Carcinogenesis Experiment After hair shaving, the mice were painted on the dorsal skin with 100 μ g of 7,12-dimethylbenz[a]anthracene (DMBA; Tokyo Chemical Industry, Co. Ltd) in 200 μ L of acetone for tumor initiation. One week later, 30 μ g of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Nacalai Tesque, Japan) in 200 μ L of acetone was applied to the same area twice a week for 18 weeks, to promote papilloma formation.⁶⁾ The number of papillomas was assessed weekly. Developing papillomas were counted when they reached at least 1 mm in diameter.

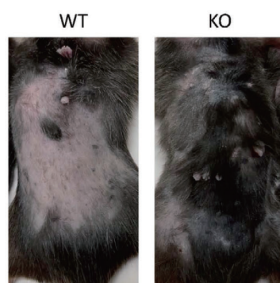
Antibodies and Reagents The antibodies and used here reagents were obtained from the commercial sources mentioned below: rabbit anti- α -tubulin (PM054) (Medical and Biological Laboratories Co. Ltd.); rabbit anti-I κ B α (4812) and rabbit anti-phospho-I κ B α (2859) (Cell Signaling Technology); and rat anti-F4/80 (ab6640) (Abcam) and anti-DMBA

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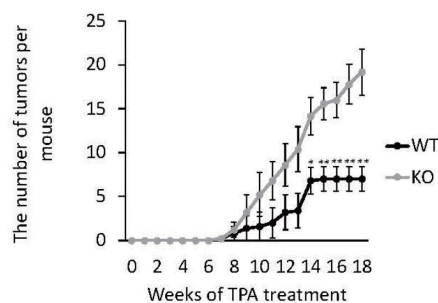
(a)



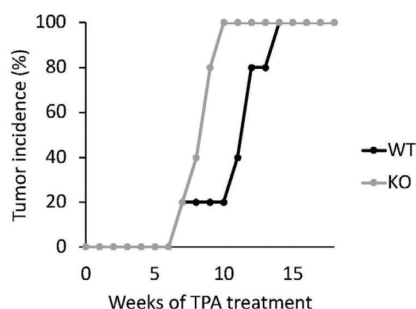
(b)



(d)



(c)



(e)

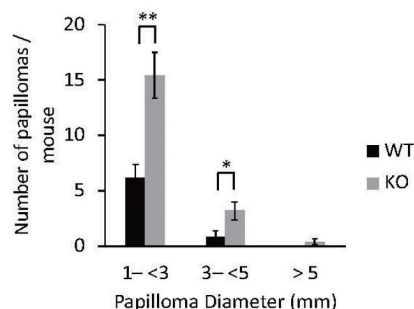


Fig. 1. Promoted Two-Stage Skin Carcinogenesis in the Absence of RASSF6

- (a) Schematic diagram of a two-stage carcinogenic experiment in the mouse skin.
 (b) Formation of papillomas in DMBA/TPA-induced WT and *Rassf6* knockout mice at 18 weeks.
 (c) Incidence of papillomas in the different treatment groups.
 (d) Number of papillomas per mouse in the indicated groups.
 (e) Average numbers of papillomas per mouse in group with different tumor diameters. Data are presented as the mean \pm SD. *, $P < 0.05$; **, $P < 0.01$.

(D0677) (Tokyo Chemical Industry, Co. Ltd.); TPA (27547-14) (Nacalai Tesque, Japan).

Western Blotting Five papillomas less than 5 mm were collected in one mouse as one sample. Three samples were created per group and used for the experiment. Papillomas were treated in RIPA buffer (50 mM Tris \cdot HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 1 mM APMSF). After that, they were homogenized and protein concentration determined. Moreover, they were dissolved in SDS sample buffer (0.5 M Tris/HCl, pH 6.8 62.5 mM, SDS 2%, glycerol 10%, 2-mercaptoethanol 5%, Bromophenol blue 0.02%), and boiled at 96°C for 5 min. Appropriate amounts of proteins were separated on 8%–15% acrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) milk in TBS–Tween buffer (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.1% [v/v] Tween 20, pH 7.4) and incubated overnight at 4°C with the indicated antibodies. The mem-

branes were washed in TBS–Tween buffer, incubated with the appropriate secondary antibodies for 1 h at room temperature with shaking, and washed again in TBS–Tween buffer. Signals were detected using the ECL (GE Healthcare) reagent and X-ray films. The signals were measured with ImageJ.

Statistical Analysis For the comparison between two samples, statistical analyses were performed using Student's *t*-test with the R software, ver. 4.05 GUI 1.74.

RESULTS

Promoted Two-Stage Skin Carcinogenesis in the Absence of RASSF6 Papillomas were initiated with a single dose of DMBA and promoted using TPA twice per week (Fig. 1a, 1b). Compared with WT mice, *Rassf6* knockout mice exhibited a remarkable increase in both the incidence (Fig. 1c) and the number (Fig. 1d) of skin papillomas. The period necessary for all mice to form papillomas was 14 weeks for WT and 9 weeks

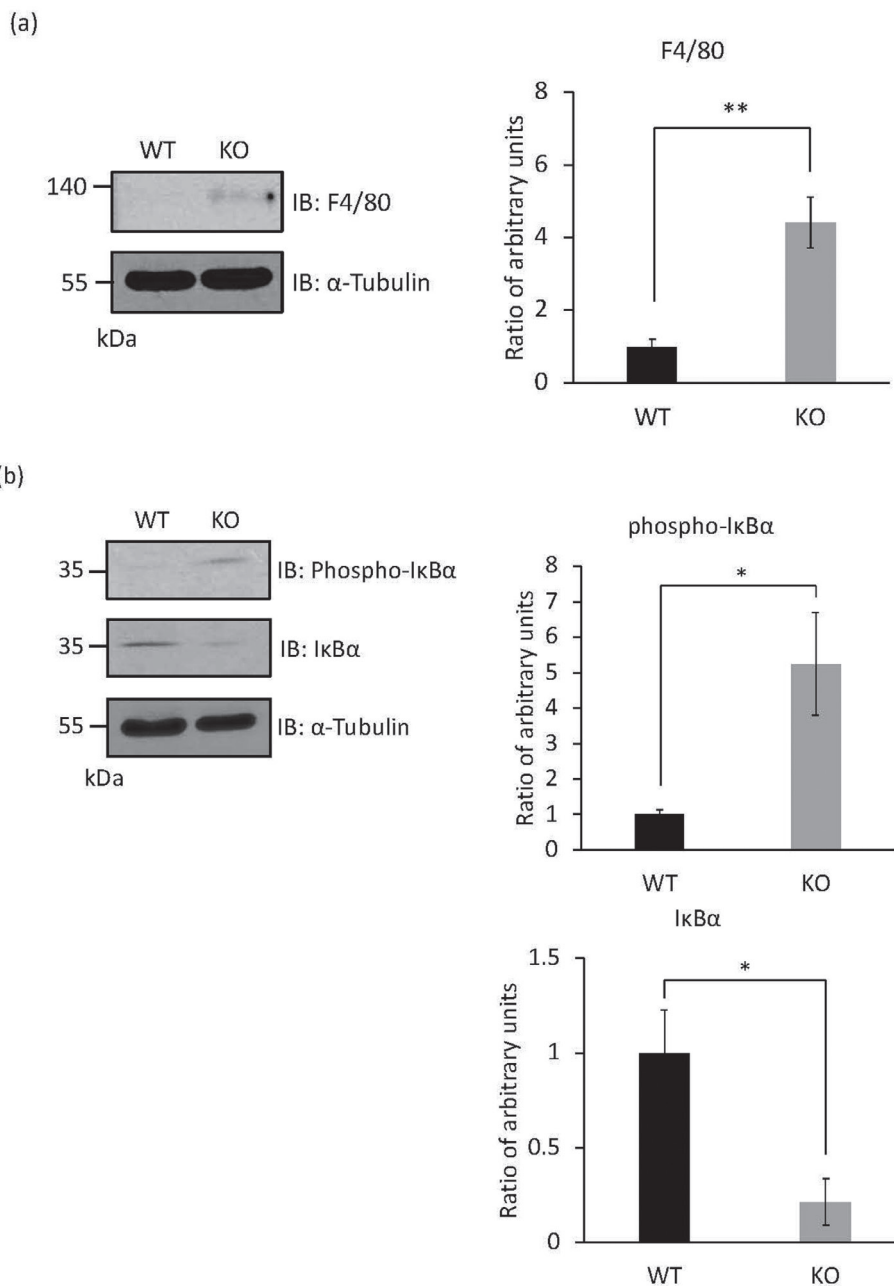


Fig. 2. The Canonical NF- κ B Signaling Pathway Is Enhanced in Papillomas of *Rassf6* Knockout Mice

(a) The tissue lysates from papillomas of wild-type and *Rassf6* knockout mice were immunoblotted with the indicated antibodies. Quantification graphs are shown with α -Tubulin as an endogenous control for protein expression. Protein levels are represented as mean ratio values quantified from protein bands of each marker versus α -Tubulin compared to wild-type or *Rassf6* knockout mice.

(b) The tissue lysates from papillomas of wild-type and *Rassf6* knockout mice were immunoblotted with the indicated antibodies. Quantification graphs are shown with α -Tubulin as an endogenous control for protein expression. Protein levels are represented as mean ratio values quantified from protein bands of each marker versus α -Tubulin compared to wild-type or *Rassf6* knockout mice.

Data are presented as the mean \pm SD. *, $P < 0.05$; **, $P < 0.01$.

for *Rassf6* knockout mice. In *Rassf6* knockout mice, the period until papilloma formation was shortened in all mice compared with WT mice. When examined separately according to tumor diameter at 18 weeks, both 1– < 3 mm and 3– < 5 mm tumors showed increased papilloma formation in *Rassf6* knockout mice compared with WT mice. At a diameter > 5 mm, there was no papilloma formation in WT mice, whereas there was papilloma formation in *Rassf6* knockout mice (Fig. 1e).

Macrophage Infiltration Was Detected in Papillomas of *Rassf6* Knockout Mice Immunoblotting with anti-macrophage F4/80 antibody revealed macrophage infiltration in

papillomas of *Rassf6* knockout mice (Fig. 2a).

The Canonical NF- κ B Signaling Pathway Was Enhanced in Papillomas of *Rassf6* Knockout Mice The immunoblotting experiment revealed the enhancement of I κ B α phosphorylation and the downregulation of I κ B α (Fig. 2b). Therefore, the canonical NF- κ B signaling pathway seems to be enhanced in papillomas of *Rassf6* knockout mice.

DISCUSSION

We reported previously the upregulation of NF- κ B and the

presence of hyperinflammatory responses in the skin of *Rassf6* knockout mice.²⁾ Here, we found that NF- κ B was similarly upregulated in papillomas of *Rassf6* knockout mice. However, no spontaneous tumorigenesis was observed, and it is conceivable that chemical stimulation promotes tumorigenesis.

In this study, we also clarified that macrophage infiltrated in papillomas of *Rassf6* knockout mice. In a previous study, it reported that the *Rassf6* mRNA level is inversely correlated with the number of macrophages in adipose tissues.⁷⁾ The molecular mechanism via which RASSF6 suppresses NF- κ B signaling warrants further investigation. The analogy with RASSF1A and RASSF2 predicts that RASSF6 binds to TBK1 and IKK α/β .^{8,9)}

In addition, two scenarios are hypothesized from the viewpoint of the pathological significance of RASSF6-deficiency-induced NF- κ B signal enhancement. First, inflammatory cytokines are secreted from cancer cells with decreased expression of RASSF6, and inflammatory cells, such as macrophages, are recruited around the cancer tissue, thus creating a favorable microenvironment for cancer growth. The second scenario is one in which decreased expression of RASSF6 is involved in the establishment of chronic inflammation, which is a major cause of tumorigenesis.

In both scenarios, suppression of NF- κ B signaling may have implications for preventing cancer initiation and progression. In a previous research, canonical NF- κ B signaling promotes DMBA/TPA-induced skin carcinogenesis.¹⁰⁾ Therefore, higher canonical NF- κ B signaling is predicted to promote papilloma growth. Moreover, for application in human cancers, it is necessary to investigate whether inflammatory cell infiltration is observed around cancers with low RASSF6 expression using human tissue. Furthermore, it is necessary to analyze whether RASSF6 expression is already reduced during chronic inflammation prior to tumorigenesis.

Acknowledgment This work was supported by JST SPRING, Grant Number JPMJSP2120. We thank Dr. Yutaka Hata (Tokyo Medical and Dental University) for various materials. The authors would like to thank Enago (www.enago.jp) for the English language review.

Conflict of interest The authors declare no conflict of interest.

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