

Inhibition of 7,12-Dimethylbenz(a)anthracene-Induced Skin Tumorigenesis in C57BL/6 Mice by Sulforaphane Is Mediated by Nuclear Factor E2-Related Factor 2

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Abstract

Sulforaphane, a dietary isothiocyanate, possesses potent chemopreventive effects through the induction of cellular detoxifying/antioxidant enzymes via the transcription factor nuclear factor E2-related factor 2 (Nrf2). To investigate carcinogenesis mechanisms related to the regulation of Nrf2, we examined the tumor incidence and tumor numbers per mouse in Nrf2 wild-type (+/+) and Nrf2 knockout (-/-) mice. 7,12-Dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate treatments resulted in an increase in the incidence of skin tumors and tumor numbers per mouse in both genotypes; however, both indices were markedly higher in Nrf2(-/-) mice as compared with Nrf2(+/-) mice. Western blot analysis revealed that Nrf2 as well as heme oxygenase-1, a protein regulated by Nrf2 were not expressed in skin tumors from mice of either genotype, whereas expression of heme oxygenase-1 in Nrf2(+/-) mice was much higher than that in Nrf2(-/-) mice in nontumor skin samples. Next, we examined the chemopreventive efficacy of sulforaphane in mice with both genotypes. Topical application of 100 nmol of sulforaphane once a day for 14 days prior to 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate applications decreased the incidence of skin tumor in the Nrf2(+/-) mice when compared with the vehicle-treated group. Importantly, there was no chemoprotective effect elicited by sulforaphane pretreatment in the Nrf2(-/-) mice group. Taken together, our results show for the first time that Nrf2(-/-) mice are more susceptible to skin tumorigenesis and that the chemopreventive effects of sulforaphane are mediated, at least in part, through Nrf2. (Cancer Res 2006; 66(16): 8293-6)

Introduction

Numerous studies provide strong evidence of an inverse relationship between the intake of crucifers and the risk for certain cancers. This relationship seems to be stronger than that between general fruit/vegetable intake and cancer risk. Epidemiologic studies have shown inverse associations between crucifer intake and the incidence of lung, pancreas, bladder, prostate, ovarian, skin, stomach, and colon cancer (1, 2). The phytochemicals in

crucifers are responsible for the up-regulation of many detoxification and cellular defense enzymes *in vivo*. These phase II detoxifying and antioxidant enzymes include NAD(P)H:quinone reductase, epoxide hydrolase, γ -glutamylcysteine synthetase, and heme oxygenase-1 (HO-1; ref. 3). The regulation of both basal and inducible expression of these phase II detoxifying enzymes is known to be mediated, in part, by the antioxidant response element (ARE), a *cis*-acting sequence found in the 5'-flanking region of many genes encoding phase II enzymes. Nuclear factor E2-related factor 2 (Nrf2), which belongs to the Cap'n'Collar family of basic region-leucine zipper transcription factors, has been shown to be an essential component of the ARE-mediated transcriptional machinery (4). Nrf2 plays an important role in regulating the expression of many detoxifying and antioxidant enzymes in response to oxidative or electrophilic stress. The chemopreventive role of this transcription factor is underscored by the increased susceptibility of Nrf2-deficient mice to carcinogenesis (5, 6).

Sulforaphane, one of the most abundant isothiocyanates in cruciferous vegetables, is highly effective in preventing or reducing tumor formation induced by carcinogens in animal models (7, 8). Studies have shown that sulforaphane and other isothiocyanates inhibit cancer cell growth, induce cancer cell apoptosis, and retard the growth of cancer cell xenografts *in vivo*. The underlying mechanisms involve caspase-mediated apoptosis, regulation of the mitogen-activated protein kinase (MAPK) cascade, induction of phase II enzymes, cell cycle regulation, and inhibition of the I κ B kinase (IKK)-I κ B α -nuclear factor κ B (NF- κ B) signaling pathway (9, 10). Most recently, topical application of sulforaphane-containing broccoli sprout extracts was found to be effective in protection against UV light-induced skin carcinogenesis in SKH-1 high-risk mice (11).

Skin cancer incidence is rising steadily. The average increase in new skin cancer cases has been around 3% to 8% per year since the 1960s, with >1 million new cases per year in the U.S. (12). Previous studies have indicated that antioxidant-supplemental diet such as green tea (13) and curcumin (14) were effective in blocking both chemically induced and UV-induced skin carcinogenesis. Although, the exact mechanisms of how these phenolic antioxidants work are not very clear; however, shifting the balance between intracellular processes that generate reactive intermediates (e.g., electrophiles, reactive oxygen species, and nitrogen species) to that of promoting detoxification and radical scavenging reactions would seem to be a major driving force in the protective mechanisms (15).

The development of skin cancer is a multistep process including initiation, promotion, and progression, and the model mimicking this process can serve as a useful method for the study of carcinogenesis and chemoprevention. Treatment with 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate

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(TPA) in mice is a widely used and reliable model for skin cancer study. In this study, we determined the role of Nrf2 in the development of skin cancer, and then examined the chemoprevention mechanism of sulforaphane in this model. We first examined the incidence of skin tumor induced by DMBA/TPA treatment in Nrf2 wild-type (+/+) and Nrf2 knockout (-/-) mice, and then we examined the chemopreventive efficacy of sulforaphane. To our knowledge, this is the first study showing a significant increase of skin tumorigenesis in Nrf2(-/-) mice when compared with Nrf2(+/+) mice using DMBA/TPA skin cancer model. In addition, using this skin cancer model, we have shown that sulforaphane has cancer-chemopreventive activity in the Nrf2(+/+) mouse but not in the Nrf2(-/-) mouse.

Materials and Methods

Reagents. Sulforaphane was obtained from LKT Laboratories (St. Paul, MN). Antibodies against Nrf2 and HO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal care and treatment. Nrf2(-/-) mice (C57BL/SV129) have been described elsewhere (16). Nrf2(-/-) mice were backcrossed with C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME). To confirm each animal's genotype, DNA was extracted from the tail and analyzed by PCR using the following primers: 3'-primer, 5'-GGAATGGAAAA-TAGCTCTGCC-3'; 5'-primer, 5'-GCCTGAGAGCTGTAGGCC-3'; lacZ primer, 5'-GGGTTTCCAGTCACGAC-3'. Nrf2(-/-) and Nrf2(+/+) mice exhibited one band at ~200 and ~300 bp, respectively, whereas Nrf2(+/-) mice exhibited both bands. The third generation of female Nrf2 knockout mice was used in this study. Female Nrf2(+/+) C57BL/6J mice were purchased from The Jackson Laboratory. Mice 10 to 12 weeks of age were housed at the Rutgers Animal Facility and maintained under 12-hour light/dark cycles. All animals were allowed water and food *ad libitum*. All animal use procedures were in accordance with the NIH Guide for the Care and Use for Laboratory Animals and were approved by the Rutgers Institutional Animal Care and Use Committee. The dorsal region of each mouse was shaved 2 days prior to any application of DMBA or sulforaphane. To induce skin tumorigenesis, animals received one topical application of 200 or 20 nmol of DMBA in 100 μ L of acetone. One week later, 8 or 16 nmol of TPA in 200 μ L of acetone was applied to the same site. The TPA treatment was continued twice weekly for 25 consecutive weeks. To examine the chemopreventive effects of sulforaphane, animals were treated with topical applications of 100 nmol of sulforaphane in 100 μ L acetone once a day for 14 consecutive days. Then pretreatment with sulforaphane was stopped, and mice were treated topically with a single dose of 200 nmol of DMBA on the second day, 1 week later, the mice were promoted topically with 8 nmol TPA twice weekly for 25 weeks as before. The number of skin tumors >1 mm in diameter were counted and recorded every week.

Histopathologic examinations. Skin samples were fixed in 10% formalin before being embedded in paraffin. Sections (5 μ m/L) were stained with H&E as previously described (17).

Western blot analysis. Whole cell protein extracts were obtained by homogenizing skin samples, flash-frozen in the liquid nitrogen, in whole cell lysis buffer (10 mmol/L Tris-HCl, 250 mmol/L sodium chloride, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 \times proteinase inhibitor mixture, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μ mol/L sodium orthovanadate, 2 mmol/L iodoacetic acid, and 5 mmol/L ZnCl₂). Protein concentrations were determined by Bio-Rad (Richmond, CA) protein assay according to the manufacturer's instructions. Twenty micrograms of total protein from each sample were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes using a semidry transfer system from Fisher (Suwanee, GA). The membranes were then blocked with 5% bovine serum albumin or 5% nonfat milk in TBST buffer [2.42 g/L Tris-HCl, 8 g/L NaCl, and 1 mL/L Tween 20 (pH 7.6)] and incubated overnight at 4°C with primary antibodies suspended in TBST buffer. Membranes were then incubated with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnol-

ogy), and visualized with enhanced chemiluminescence Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis. The difference between the average number of skin tumors per mouse was determined by Student's *t* test, whereas the difference between the percentages of mice with skin tumors was determined by Fisher's exact test.

Results

Nrf2(-/-) mice were more susceptible to skin tumorigenesis. A single topical application of 200 nmol DMBA followed by topical application of 8 nmol TPA twice a week for 25 weeks, as described in Materials and Methods, resulted in 65% skin tumor incidence of Nrf2(+/+) mice versus 80% of Nrf2(-/-) mice ($P = 0.24$; Fig. 1A). Interestingly, the topical application of 20 nmol DMBA followed by 16 nmol of TPA twice a week resulted in a tumor incidence of 45% in Nrf2(+/+) mice versus 95% in Nrf2(-/-) mice; the difference was statistically significant ($P < 0.01$; Fig. 1A). The incidence of skin tumors (95%) in Nrf2(-/-) mice treated with a low dose of DMBA (20 nmol) and a high dose of TPA (16 nmol) was slightly higher than the incidence (80%) in Nrf2(-/-) mice treated with a high dose of DMBA (200 nmol) and a low dose of TPA (8 nmol; $P = 0.17$). In contrast, the incidence of skin tumors (45%) in Nrf2(+/+) mice treated with a low dose of DMBA (20 nmol) and a high dose of TPA (16 nmol) was not statistically different from the incidence in Nrf2(+/+) mice (65%) treated with a high dose of DMBA (200 nmol) and a low dose of TPA (8 nmol; $P = 0.17$; Fig. 1A).

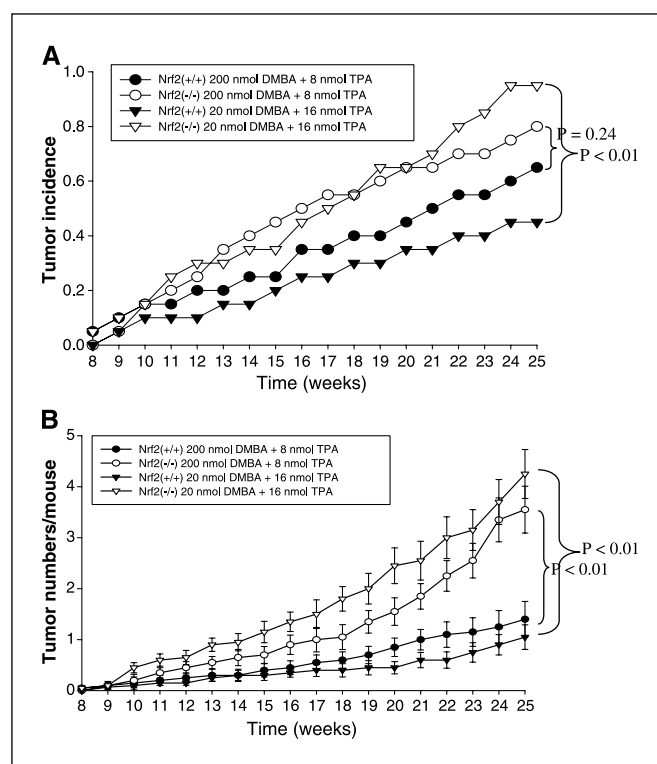


Figure 1. Nrf2(-/-) mice are more susceptible to skin tumorigenesis after treatment with DMBA and TPA. Mice were treated with one application of 200 nmol of DMBA or 20 nmol of DMBA in 100 μ L of acetone. One week later, 8 nmol of TPA or 16 nmol of TPA in 200 μ L of acetone was applied twice a week for 25 consecutive weeks. Skin tumors that were >1 mm in diameter were counted and recorded every week. Points, mean number of skin tumors per animal; bars, \pm SE. A, incidence of skin tumor in Nrf2(+/+) and Nrf2(-/-) mice after treatment with DMBA/TPA. B, number of skin tumors/mouse induced by DMBA/TPA in Nrf2(+/+) and Nrf2(-/-) mice.

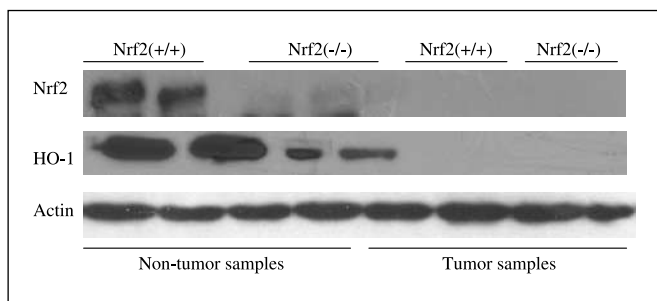


Figure 2. Effect of DMBA/TPA treatment on Nrf2 and HO-1 expression levels in the epidermis of Nrf2(+/+) and Nrf2(-/-) mice. Epidermal cell extracts obtained from Nrf2(+/+) and Nrf2(-/-) animals treated with 200 nmol of DMBA and 8 nmol of TPA were analyzed by Western blot analysis. Each group comprised two animals. Actin was included to show that all lanes were equally loaded (the results in animals treated with 20 nmol of DMBA and 16 nmol of TPA were similar to the data shown above).

A single application of 200 nmol DMBA, followed by 8 nmol of TPA twice a week, to the backs of Nrf2(+/+) and Nrf2(-/-) mice resulted in 1.45 ± 0.35 and 3.55 ± 0.46 tumors/animal, respectively ($P < 0.01$). The application of 20 nmol of DMBA and 16 nmol of TPA to the backs of Nrf2(+/+) and Nrf2(-/-) mice resulted in 1.05 ± 0.24 and 4.25 ± 0.48 tumors/animal, respectively ($P < 0.01$; Fig. 1B).

In terms of skin tumor papillomas, more papillomas were observed in the Nrf2(-/-) mice as compared with Nrf2(+/+) mice after treatment with DMBA and TPA. However, the incidence and the number of squamous cell carcinomas after treatment with DMBA and TPA did not differ between Nrf2(+/+) and Nrf2(-/-) mice (data not shown).

Nrf2 and Nrf2-regulated HO-1 expression is decreased in skin tumors. Nrf2 was not expressed in tumors or nontumor areas of the skin of Nrf2(-/-) mice, as seen by Western blot analysis (Fig. 2). In Nrf2(+/+) mice, Nrf2 was strongly expressed in nontumor skin samples, whereas there was very little or no detectable Nrf2 protein in skin tumors (Fig. 2). The expression level of Nrf2 in normal skin without any vehicle treatment was very high, which was similar to the expression level in nontumor samples after DMBA/TPA treatment (data not shown). HO-1 protein was detected in both Nrf2(+/+) and Nrf2(-/-) mice, and the expression level in Nrf2(+/+) mice was higher than that in Nrf2(-/-) mice. Interestingly, HO-1 was not detected in skin tumors from Nrf2(+/+) or Nrf2(-/-) mice (Fig. 2).

Sulforaphane significantly inhibits skin tumorigenesis in Nrf2(+/+) mice but not in Nrf2(-/-) mice. Next, we examined the anticarcinogenesis effects of sulforaphane on Nrf2 genotypes. Nrf2(+/+) and Nrf2(-/-) mice were treated topically with 100 nmol of sulforaphane once a day for 14 days, and 1 day after the last sulforaphane treatment, the mice were initiated with a single dose of DMBA (200 nmol) and then promoted with TPA (8 nmol) twice a week for 25 weeks. Pretreatment with sulforaphane in the Nrf2(+/+) mice significantly decreased the incidence of skin tumor from 60% to 20% ($P < 0.05$). The number of tumors/mouse was also decreased from 1.35 ± 0.28 to 0.35 ± 0.17 ($P < 0.05$). In contrast, with the Nrf2(-/-) mice, the incidence of tumors and tumor numbers per mouse were not significantly altered as a result of sulforaphane pretreatment (Fig. 3A and B). Furthermore, topical treatment of Nrf2(+/+) mice with 100 nmol of sulforaphane once a day for 14 days was associated with robust increases in Nrf2 protein levels (Fig. 3C). These data show that

sulforaphane pretreatment inhibits skin tumorigenesis in the Nrf2(+/+) mice, but not in the Nrf2(-/-) mice, and that this chemopreventive effect is associated with Nrf2.

Discussion

In this study, a single topical application of DMBA followed by repeated applications of TPA for 25 weeks to the dorsal region of the mice produced skin tumors in both Nrf2(+/+) and Nrf2(-/-) mice. That the incidence of tumorigenesis and number of tumors/mouse were markedly higher in Nrf2(-/-) mice as compared with Nrf2(+/+) mice, at two different doses of DMBA and TPA, indicates that Nrf2 plays important roles both in the initiation and the promotion phases of the skin tumorigenesis.

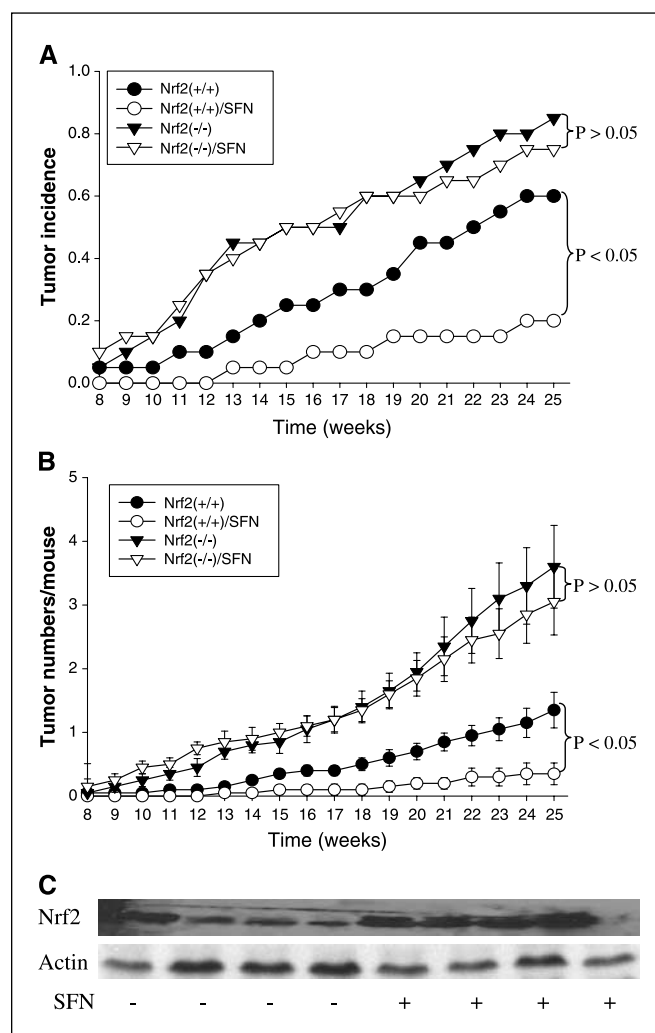


Figure 3. Sulforaphane significantly inhibited skin tumorigenesis in Nrf2(+/+) mice but not in Nrf2(-/-) mice. Animals were pretreated with 100 nmol of sulforaphane once a day for 14 days prior to a single dose of 200 nmol of DMBA applied on the second day. One week later, 8 nmol of TPA was applied topically twice a week for 25 weeks. Skin tumors >1 mm in diameter were counted and recorded every week. Points, mean number of skin tumors per animal; bars, \pm SE. A, effect of sulforaphane pretreatment on the incidence of skin tumor. B, effect of sulforaphane pretreatment on tumor numbers per mouse. C, effect of sulforaphane on Nrf2 expression in Nrf2(+/+) mice. Nrf2(+/+) animals were treated topically with 100 nmol of sulforaphane in 100 μ L of acetone once a day for 14 days, the control group was treated with 100 μ L of acetone. Whole cell extracts from these animals ($n = 4$ in each group) were analyzed for Nrf2 protein levels. Actin was included to show that all lanes were loaded equally.

As has been shown recently, Nrf2 is an important transcriptional factor regulating a number of phase II detoxifying and antioxidant enzymes via the ARE (18). Sulforaphane, one of the isothiocyanates derived from cruciferous vegetables, is highly effective in preventing or reducing the risk of cancer induced by carcinogens in animal models (7, 8). Our results showed that sulforaphane, given prior to DMBA/TPA treatment, effectively inhibits skin tumorigenesis in the Nrf2(+/-) mice, but not in the Nrf2(-/-) mice. The decrease in tumor incidence and tumor numbers/mouse in the Nrf2(+/-) mice suggests that sulforaphane blocked and retarded the initiation and promotion of existing skin lesions as well as tumor growth. The inability of sulforaphane pretreatment to inhibit skin tumorigenesis in Nrf2(-/-) mice, together with the finding that sulforaphane's chemoprotective effects in Nrf2(+/+) animals were coupled with a strong induction of Nrf2 expression prior to DMBA/TPA treatment, indicates that Nrf2 is an important mediator of the sulforaphane's chemoprotective effects in this model. Recent studies have shown that the chemoprotective efficacy of sulforaphane and other isothiocyanates may involve caspase-mediated apoptosis, regulation of the MAPK cascade, cell cycle regulation, and inhibition of the IKK-I κ B α -NF- κ B signaling pathway (9, 10). Other than the regulation of Nrf2, the role of caspase, MAPK, cell cycle, and NF- κ B in the inhibition of skin tumorigenesis elicited by sulforaphane is presently unknown, and needs further investigation.

DMBA treatment produces DNA-carcinogen adducts which may induce G \rightarrow A transitions or A \rightarrow T transversions. Such mutations are frequently observed at exons 5 to 8 of the *p53* gene and at codon 61 of the *Ki-ras* gene in DMBA-induced carcinomas. Furthermore, topical applications of DMBA may induce inflammation and oxidative DNA damage in the skin, which may, in turn, play an important role in the promotion and progression of tumorigenesis and carcinogenesis (19). Nrf2 is an important transcriptional factor regulating phase II detoxifying enzymes and antioxidant response gene expression. As such, Nrf2(-/-) mice would not be expected to mount a coordinated up-regulation of phase II detoxifying enzymes and antioxidant response proteins in response to DMBA, and therefore would not be capable of

eliminating DNA-carcinogen adducts and oxidative DNA damage produced by DMBA. This may account for the high incidence of skin tumorigenesis in the Nrf2(-/-) mice and for sulforaphane's ability to inhibit skin tumorigenesis only in the Nrf2(+/-) mice. This hypothesis will need to be tested in future experiments.

Recent studies show that sulforaphane can induce phase I enzymes including CYP1A1/2, CYP3A1/2, CYP2E1, and phase II enzymes such as epoxide hydrolase in rats. The modulation of xenobiotic-metabolizing enzymes system may shift the balance of carcinogen metabolism toward deactivation (20, 21). In our study, sulforaphane was given only during the initiation phase in both Nrf2(+/-) and Nrf2(-/-) mice, the possible chemopreventive mechanisms of sulforaphane against skin carcinogenesis induced by DMBA/TPA may involve both the phase I and II drug-metabolizing enzymes regulated by Nrf2, leading to the detoxification of DMBA, rapid metabolism of DMBA by P450, epoxide hydrolase, and conjugated to glutathione and/or sulfate. The precise mechanism of the chemopreventive effects by sulforaphane is currently under investigation.

In summary, our results are in agreement with previous studies demonstrating an anticarcinogenesis effect for sulforaphane in other models of tumorigenesis. To our knowledge, this is the first report of sulforaphane-induced inhibition of skin tumorigenesis acting prior to the initiation stage that is dependent on Nrf2. In conclusion, our results show that Nrf2(-/-) mice are more susceptible to skin tumorigenesis and that the chemopreventive effects of sulforaphane on skin tumorigenesis are mediated and require Nrf2.

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