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# Suppression of Skin Tumorigenesis in c-Jun $NH_2$-Terminal Kinase-2-Deficient Mice[1]

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## ABSTRACT

**Previous studies have shown that c-Jun $NH_2$-terminal kinase (JNK) belongs to the mitogen-activated protein kinase (MAPK) family of signal transduction components that are rapidly initiated and activated by many extracellular stimuli. However, the potential role of JNK in mediating tumor promotion and carcinogenesis is unclear. We show here that in JNK2-deficient (*Jnk2*$^{-/-}$) mice, the multiplicity of papillomas induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was lower than that in wild-type mice. Papillomas on wild-type mice grew rapidly and were well vascularized compared with *Jnk2*$^{-/-}$ mice. After the 12th week of TPA treatment, the mean number of tumors per mouse was 4.13–4.86 in wild-type mice but only 1.13–2.5 in *Jnk2*$^{-/-}$ mice. TPA induced phosphorylation of extracellular signal-regulated kinases and activator protein-1 DNA binding activity in wild-type mice, but the phosphorylation of extracellular signal-regulated kinases and activator protein-1 DNA binding were inhibited in *Jnk2*$^{-/-}$ mice. These data suggest that JNK2 is critical in the tumor promotion process.**

## INTRODUCTION

The MAPK[3] family, comprised of Erks, JNKs, and p38 kinases (1), activates distinct groups of substrates and is implicated in the regulation of cell proliferation, tumorigenesis, differentiation, and apoptosis (1, 2). JNKs (*i.e.*, stress-activated protein kinases) are activated in response to cellular stresses including osmotic shock, UV irradiation, arsenic, and tumor necrosis factor-$\alpha$ (2–5). The physiological significance of JNK signaling was documented by genetic analysis in *Drosophila* and mice (6, 7). JNKs were shown to phosphorylate c-Jun and increase AP-1 transcription activity (8). JNKs are implicated in apoptosis (9) and cell proliferation (10). We reported that JNKs are required for tumor necrosis factor-$\alpha$-induced cell transformation (2). Recently, *Jnk2*-knockout (*Jnk2*$^{-/-}$) mice were generated, and they developed normally without apparent phenotype expression except deficiency of differentiation of precursor $CD4^+$ T cells into effector T helper 1 cells (11). Because JNK2 enhances AP-1 transcription activity, we hypothesize that JNK2 may mediate the tumor promotion process. Therefore, we used *Jnk2*$^{-/-}$ mice to study the mechanism of TPA-induced tumor promotion.

## MATERIALS AND METHODS

**Materials.** DMBA, TPA, aprotinin, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). Antibodies used were rabbit polyclonal anti-phosphorylated Erks (New England BioLabs, Inc., Beverly, MA) and monoclonal antibodies for JNK1 and JNK2 (Santa Cruz Biotechnology Inc., Santa Cruz Biotechnology, CA). Primers for PCR were synthesized by Life Technologies, Inc. (Rockville, MD).

**Tumor Induction Experiments.** *Jnk2*$^{-/-}$ mice were originally from C57BL/6(B6)-injected D3 ES cells with the construct pJNK2KO (11). The expression of the endogenous *Jnk2* gene was examined by reverse transcription-PCR using total RNA isolated from the thymus, and *Jnk2* mRNA was not detected in homozygous *Jnk2*$^{-/-}$ mice (11).

Experimental groups consisted of 29–35 mice, 5 mice/cage. Mice were shaved at 7–8 weeks of age and treated once with 100 $\mu$g of DMBA. Two weeks later, tumor growth was promoted by treating with 17 nmol of TPA twice each week for 29 weeks. Visible skin tumors were counted every 2 weeks. The papilloma incidence, expressed as the percentage of animals with one or more papillomas, and the papilloma multiplicity, expressed as the number of papillomas per surviving mouse, were calculated each time tumors were counted.

**Nuclear Protein Analysis.** Gel shift assays were used to detect AP-1 binding activity. Nuclear extracts were prepared as described previously (12). In brief, the skin tissues were lysed with 500 $\mu$l of lysis buffer (50 mM KCl, 0.5% NP40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin, 20 $\mu$g/ml aprotinin, and 100 $\mu$M DTT). After centrifugation at 14,000 rpm for 1 min, the nuclei were washed with 500 $\mu$l of the same buffer but without NP40 and then placed into 200 $\mu$l of extraction buffer (500 mM KCl and 10% glycerol with the same concentration of the other reagents as in the lysis buffer). After centrifugation at 14,000 rpm for 5 min, the supernatant fraction was harvested as the nuclear protein extract and stored at −70°C. An AP-1 binding sequence from the human collagenase promoter region, 5′-AGCATGAGTCAGACACCTCTGGC-3′, was synthesized and labeled with [$^{32}$P]dCTP using the Klenow fragment (Life Science Co., Gaithersburg, MD). Protein concentration was determined using the Modified Lowry Protein Assay (Pierce Chemical), and equal amounts of nuclear protein (3 $\mu$g) were added to the DNA binding buffer, which contained $5 \times 10^4$ cpm $^{32}$P-labeled oligonucleotide probe, 1.5 $\mu$g of poly(deoxyinosinic-deoxycytidylic acid), and 3 $\mu$g of BSA. The reaction mixture was incubated on ice for 10 min, followed by incubation at room temperature for 20 min. The DNA-protein complexes were resolved in a 6% nondenaturing acrylamide gel. The gel was dried and scanned using the Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Western Blotting.** Skin was harvested from mice and placed on dry ice. Each sample was cut into small pieces, placed on ice, and incubated in 500 $\mu$l of SDS lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM DTT] for 60 min. The lysate was sonicated four times, 5 s each, at power 3 and centrifuged at 14,000 rpm in a microcentrifuge at 4°C for 10 min. The supernatant fraction was diluted with three volumes of acetone and left on ice for 10 min. The suspension was centrifuged at 14,000 rpm at 4°C for 10 min, and the pellet was resuspended in 800 $\mu$l of acetone and centrifuged at 14,000 rpm at 4°C for 10 min. The pellet was then dissolved in 200 $\mu$l of SDS lysis buffer. The protein concentration was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA).

Samples containing equal amounts of protein were resolved in an 8% SDS-polyacrylamide gel, and proteins were subsequently transferred and analyzed as described previously (13). Immunoblotting for proteins of Erks and JNKs was carried out using MAPK antibodies against Erks, JNK1, and JNK2 as described previously (13). Antibody-bound proteins were detected by chemifluorescence (ECF; Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed using the Storm 840 PhosphorImager (Molecular Dynamics).

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[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated protein kinase; JNK, c-Jun $NH_2$-terminal kinase; AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[*a*]anthracene.

## RESULTS

**Identification of Phenotype of Genomic DNA and Proteins of *Jnk2*$^{-/-}$ Mice.** The genomic DNA phenotype and protein expression of JNK2 in knockout mice were confirmed by PCR and Western blotting, respectively. Results indicated that *Jnk2*$^{-/-}$ mice did not express JNK2 protein, whereas expression of JNK1 and Erk were unaffected, agreeing with the results of others (Fig. 1; Ref. 11). Thus, the knockout of the *Jnk2* gene was effective and specific, and mice deficient in JNK2 were used in the present experiments.

**JNK2 Deficiency Inhibited TPA-promoted Tumor Growth.** To induce tumor formation in skin of these mice, we used a classical multistage model in which tumors were initiated with DMBA and promoted with TPA. The dorsal epidermis of mice was shaved 2 days before topical application of 100 $\mu$g of DMBA in acetone. Two weeks later, 200 $\mu$l of acetone (control group) or acetone with 17 nmol of TPA was applied to each mouse twice a week for 29 weeks. After 10 weeks of TPA treatment, tumors appeared. Although tumors of *Jnk2*$^{-/-}$ mice displayed an identical external appearance to tumors of wild-type mice, differences in morphology were observed after 2 more weeks. Most papillomas on wild-type mice grew rapidly and were well vascularized, whereas papillomas on *Jnk2*$^{-/-}$ mice appeared growth arrested and desiccated (Fig. 2).

The percentage of mice that developed tumors was similar until week 17 (Fig. 3*A* and Table 1). Nonetheless, at the end of 15 weeks, the total number of tumors/group was 55 for wild-type mice and 13 for *Jnk2*$^{-/-}$ mice ($P < 0.01$). After 17 weeks of TPA treatment, a rapid, consistent increase in the percentage of wild-type mice having papillomas was found, whereas the incidence of papillomas on *Jnk2*$^{-/-}$ mice rose more slowly (Fig. 3*A* and Table 1; $P < 0.02$). By week 29, 86% of wild-type mice had tumors compared with 62% of *Jnk2*$^{-/-}$ mice with tumors (Fig. 3*A*). Significantly, the total number of tumors in the wild-type group was 164 (35 mice) compared with 57 (29 mice) in the *Jnk2*$^{-/-}$ group ($P < 0.0002$).

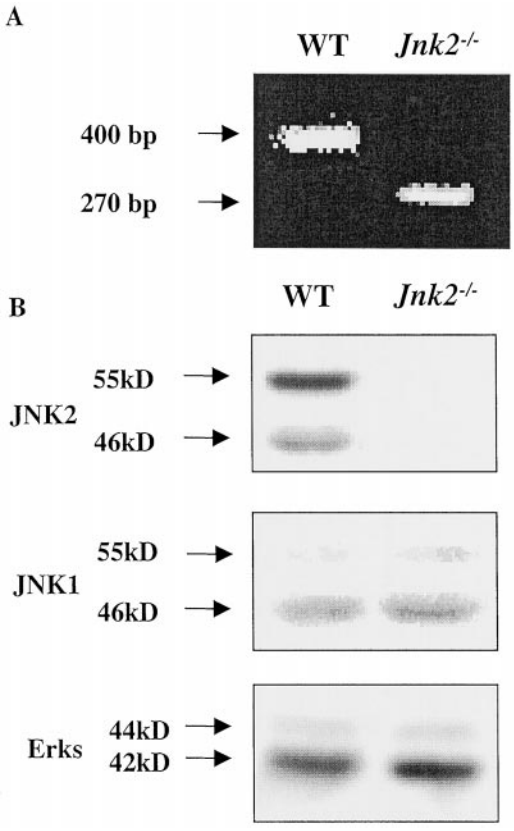


Fig. 1. Identification of the targeted disruption of the murine *Jnk2* gene. *A*, identification of genomic DNA of wild-type (*WT*) and homozygous (−/−) mice by PCR. DNA was isolated from the tail of WT and *JNK2*$^{-/-}$ mice. The top band (*400 bp*) corresponds to the wild-type allele (*WT*), and the bottom band (*270 bp*) corresponds to the mutant allele (*Jnk2*$^{-/-}$). *B*, Western blot analysis of skin lysates from wild-type (*WT*) and homozygous knockout (−/−) mice. The blots were probed with an antibody to JNK1, JNK2, or Erks.

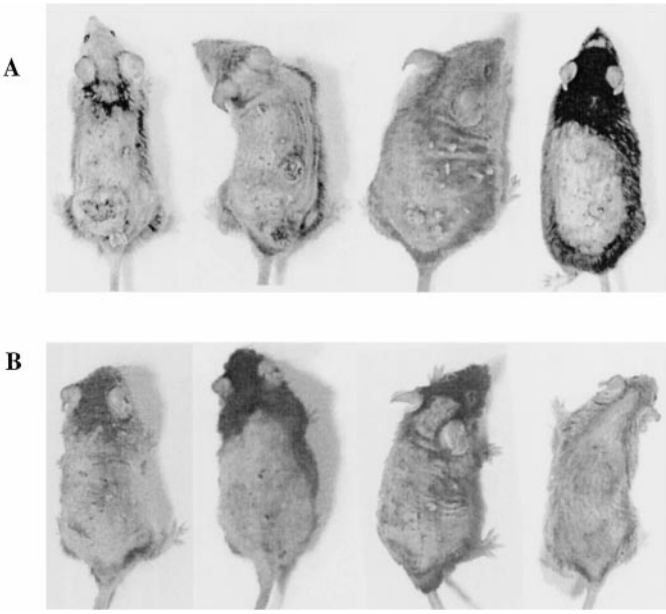


Fig. 2. External appearance of papillomas. Tumors were initiated by treating once with 100 $\mu$g of DMBA, and 2 weeks later, tumors were promoted by treating with 17 nmol of TPA twice per week for 29 weeks. Visible skin tumors were counted once every 1–2 weeks. *A*, four wild-type mice showing the greatest number of tumors. *B*, four *Jnk2*$^{-/-}$ mice showing the greatest number of tumors.

In those mice that developed tumors, the average number was greater in wild-type mice than in *JNK2*$^{-/-}$ mice. Specifically, the average number of tumors/mouse in the wild-type group was 2.7 at week 11 and 5.5 tumors/mouse at week 29. In contrast, the average number in *JNK2*$^{-/-}$ was 1.1 tumors/mouse at week 11 ($P < 0.001$) and 3.2 tumors/mouse at week 29 ($P < 0.0001$; Fig. 3*B*). These results suggest that JNK2 is an important mediator for tumor growth.

In addition to differences in the average number of tumors, we found that tumor size was significantly greater in wild-type mice compared with *Jnk2*$^{-/-}$ mice. Although similar from weeks 11 to 16 (Table 2; Fig. 3*C*), the percentage of tumors >1.5 mm in diameter was greater in wild-type mice than in *Jnk2*$^{-/-}$ mice beginning at week 17 until the end of the study ($P < 0.003$; Table 2 and Fig. 3*C*). At 29 weeks, 62% ($n = 101$) of the total number of tumors ($n = 164$) in the wild-type group were >1.5 mm, whereas only 46% ($n = 26$) of the total number of tumors ($n = 57$) were >1.5 mm in diameter in *Jnk2*$^{-/-}$ mice (Fig. 3*C*; $P < 0.01$). These data strongly suggest that deficiency of the *Jnk2* gene represses formation and growth of DMBA/TPA-induced skin tumors.

**JNK2-deficient Papillomas Appear to Have a Decreased Risk of Undergoing Malignant Conversion.** Malignant lesions grew rapidly and appeared as ulcers. Wild-type mice began to develop malignant skin tumors around week 23 after TPA treatment. The malignant tumors were identified histologically as squamous cell carcinomas (Fig. 4). At week 29, 4 tumors (2.5% in a total of 160 tumors) in wild-type mice became malignant, whereas none became malignant in JNK2-deficient mice, suggesting that JNK2 plays an important role in preventing malignant conversion.

**JNK2 Deficiency Blocked TPA-induced AP-1 DNA Binding Activity.** AP-1 was originally described as a transcription factor that mediates gene expression in response to TPA (14). Evidence indicates

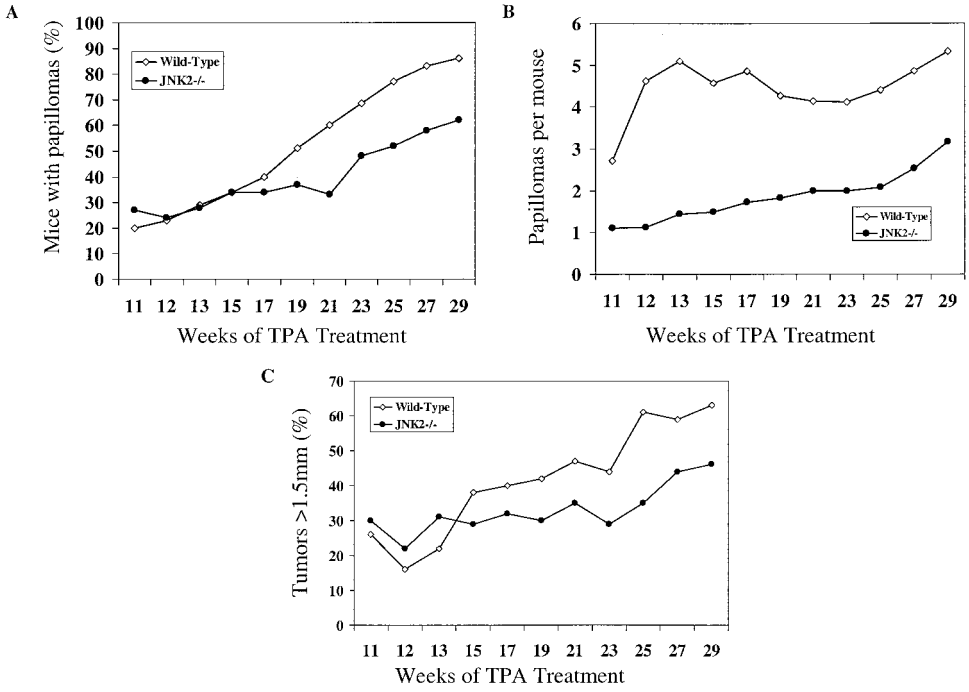


Fig. 3. Tumor formation in DMBA/TPA-treated mice. Tumors were counted and measured once every 1–2 weeks. *A*, papilloma incidence (the percentage of animals having one or more papillomas). *B*, papilloma multiplicity (the number of papillomas/mouse in mice with tumors). *C*, tumor size (the percentage of tumors >1.5 mm in diameter was calculated as the number of tumors with a diameter >1.5 mm divided by the total number of tumors).

Table 1 *Comparison of the percentage of mice with tumors per group*[a]

| | Weeks | |
|---|---|---|
| Group | 11–15 | 17–29 |
| Wild-type | 26.4 ± 3.2[b] | 66.5 ± 6.4 |
| *Jnk2*$^{-/-}$ | 28.5 ± 2.1 | 46.4 ± 4.5 |
| *P* | 0.6 | 0.02 |

[a] Total mice with tumors per group/total mice per group.
[b] Data are expressed as average percentage ± SE.

that acquisition of constitutive AP-1 DNA binding and transactivating ability may be related to carcinogenesis (15). AP-1 binding to the TPA-response element (14) in the promoter of target genes is a key step in TPA-induced tumor promotion. To examine the effect of JNK on TPA-induced AP-1 DNA binding, the dorsal skins of wild-type and *Jnk2*$^{-/-}$ mice were shaved and either treated with acetone (for control) or TPA (17 nmol/mouse). AP-1 DNA binding activity was analyzed by gel-shift assay 24 h later (12). Results show that TPA induced AP-1 DNA binding activity in wild-type mice but not in *Jnk2*$^{-/-}$ mice (Fig. 5), indicating that JNK2 plays a key role in mediating TPA-induced AP-1 DNA binding activity. We also tested whether the fast mobility gel shift bands in samples from *JNK2*$^{-/-}$ mice were also related to AP-1 by using antibodies to identify AP-1 protein components, including c-Jun, JunB, JunD, c-Fos, Fra1, and Fra2, by super gel shift analysis. Compared with TPA-treated wild-type mice, no significant changes were found in *JNK2*$^{-/-}$ mice (data not shown).

**JNK2 Deficiency Blocked TPA-induced Phosphorylation of Erks.** AP-1 activity is modulated by the phosphorylation of c-Jun and c-Fos, whereas MAPKs are the upstream activator kinases responsible for the phosphorylation of the AP-1 family proteins (16–18). Erks, JNKs, and p38 kinases are the three major classes of MAPKs. Because the *Jnk2* gene was absent and p38 kinase is not affected with TPA stimulation, we tested the effect of TPA on Erks phosphorylation. The dorsal skins of wild-type and *Jnk2*$^{-/-}$ mice were shaved and treated with either acetone (control) or TPA (17 nmol/mouse). The skin samples were harvested and lysed in SDS buffer 3 h after treatment. As shown in Fig. 6, TPA-induced phosphorylation of Erks was blocked in JNK2-deficient mice. These results indicate that JNK2 modulation of TPA-induced AP-1 binding activity may be through the inhibition of Erks phosphorylation.

## DISCUSSION

Increasing evidence suggests that JNK signaling mediates oncogenic transformation. However, no direct evidence that JNK is required for tumorigenesis has been reported. We used the well-characterized multistep model of mouse skin carcinogenesis to examine the effect of TPA on tumorigenesis in mice lacking the *Jnk2* gene. Our results demonstrated that JNK2 is involved in enhancing the formation and growth of tumors.

Chemically induced skin cancer in mice has three chronological stages, initiation, promotion, and progression (15, 19). Tumor initiation is a rapid and irreversible process, whereas promotion is a long-term process that requires chronic exposure to a tumor promoter. A tumor promoter increases proliferation of initiated cells, accelerating cancer progression; however, the exact mechanism of promotion is more complicated (19, 20). The role of JNK in mediating carcinogenesis is not clear. Recently, the JNK signaling pathway was found to be constitutively activated in pre-B cells transformed by the leukemogenic oncogene *bcr-abl* (21). The expression of JIP-1, a cytoplasmic inhibitor of JNK, markedly inhibits transformation of pre-B cells by *bcr-abl* (22). These data provide strong support for the hypothesis that the JNK signaling pathway contributes to malignant transformation of pre-B cells. From the current results, after 10 weeks of TPA treatment, tumors appeared in wild-type and *Jnk2* knockout mice at almost the same time, and the incidence of tumors was similar

Table 2 *Comparison of percentage of tumors with a diameter >1.5 mm*[a]

| | Weeks | |
|---|---|---|
| Group | 11–16 | 17–29 |
| Wild-type | 25.6 ± 4.6[b] | 51.7 ± 3.5 |
| *Jnk2*$^{-/-}$ | 28.1 ± 1.9 | 35.8 ± 2.4 |
| *P* | 0.6 | 0.003 |

[a] Total tumors >1.5 mm/total number of tumors.
[b] Data are expressed as average percentage of ± SE.

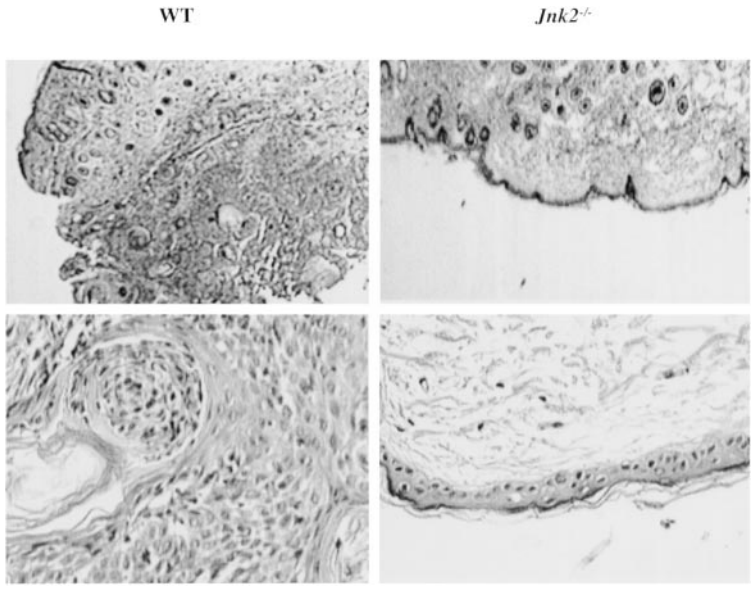


Fig. 4. Pathology of papillomas. H&E-stained sections of tumors. *Top panels*, tumors from a wild-type (*WT*) and a *Jnk2*$^{-/-}$ mouse at the same level of magnification (×25). *Bottom panels*, the same sections at a higher magnification (×200).

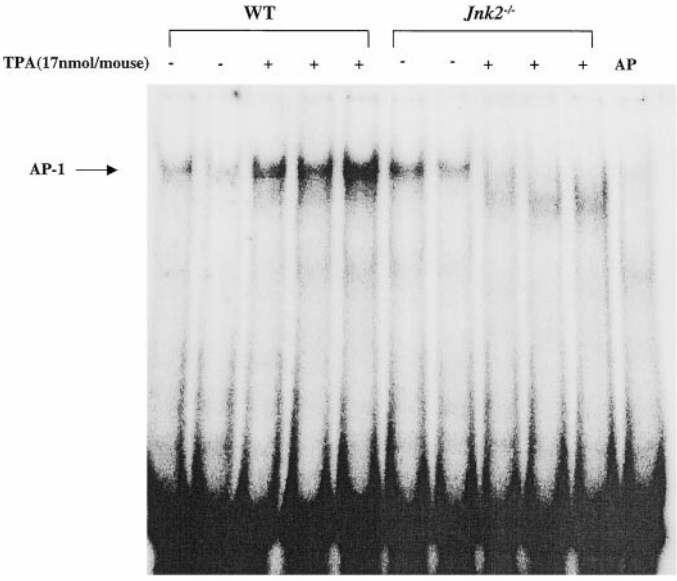


Fig. 5. Inhibition of AP-1 DNA binding activity. The dorsal skins of wild-type (*WT*; $n = 5$) and *Jnk2*$^{-/-}$ ($n = 5$) mice were biopsied, and the sample was incubated in lysis buffer 24 h after treatment with acetone or TPA (17 nmol/mouse). Protein concentration was determined, and equal amounts of protein (3 $\mu$g) were loaded in each lane. Sequence-specific AP-1 DNA binding activity was determined by gel shift analysis using a $^{32}$P-labeled oligonucleotide containing the AP-1 binding site as described in "Materials and Methods." Each *lane* shows a skin sample from a different mouse in each treatment group. A competition experiment (*AP*) using a 10-fold excess of cold AP-1 oligonucleotide indicated that the DNA binding was specific. Representative samples are shown.

between the two groups until week 17. This suggests that JNK2 deficiency does not affect tumor initiation and the beginning of promotion. However, the growth, external appearance and number of tumors were significantly different between the two groups. Moreover, at the end of the experiment (week 29 after TPA treatment), four tumors in wild-type mice were found to be malignant, whereas none were malignant in JNK2-deficient mice. These data indicate that JNK2 may play a more important role in tumor growth and progression than in tumor initiation.

TPA activates protein kinase C and, subsequently, transcription factor AP-1 (23). The activation of signal transduction pathways leading to stimulation of AP-1 is a common mechanism for tumor promotion (24–28). Blocking AP-1 activity prevents TPA-induced cell transformation in JB6 cells and tumor promotion in a mouse skin model (12, 24, 29, 30). The AP-1 family of transcription factors consists of homodimeric or heterodimeric complexes of c-Jun and c-Fos proteins (31). A transgenic mouse model overexpressing c-*fos* developed osteosarcomas and chondrosarcomas (32), and transgenic mice expressing an oncogenic form of *jun* developed fibrosarcomas at sites of wound healing (33). The c-*jun* knockout mutation is embryonically lethal, whereas c-*fos*-deficient tumors fail to undergo malignant conversion (34). Expression of a dominant-negative c-*jun* (Tam67) blocked tumor promoter-induced AP-1 transactivation and showed a dramatic inhibition of papilloma induction in these transgenic animals (35). Topical application of perillyl alcohol inhibited UVB-induced AP-1 transactivation and significantly inhibited tumor incidence and multiplicity (36). All of these data show that components of AP-1 are very important in modulating normal development and carcinogenesis.

AP-1 is one target of MAPK signaling. MAPKs modulate AP-1 activity both by increasing the abundance of AP-1 components and stimulating their activity (37). JNK has been shown to phosphorylate c-Jun at serine 63 and serine 73 residues, resulting in activation of AP-1 (1, 18). The other MAPKs, Erks and p38 kinases, were found to induce c-Fos and c-Jun expression, resulting in increased AP-1 transcriptional activity (38–41). Considering that TPA does not induce AP-1 activity in JNK2-deficient mice (Fig. 5), our present work

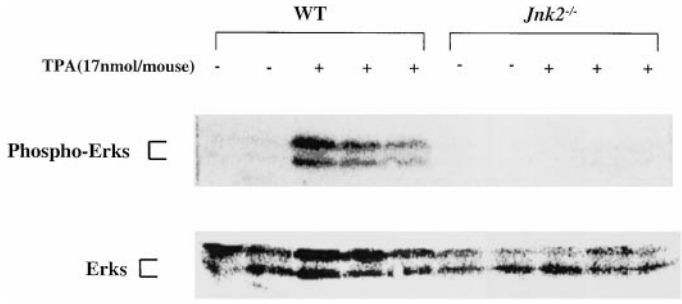


Fig. 6. The effect of TPA on the phosphorylation of Erks. The dorsal skins of wild-type (*WT*; $n = 5$) and *Jnk2*$^{-/-}$ ($n = 5$) mice were biopsied, and the sample was placed in SDS sample buffer 3 h after treatment with acetone or TPA (*17 nmol/mouse*). The tissue lysates were extracted two times with acetone and suspended in SDS sample buffer again. Equal amounts of protein were analyzed by Western blotting using antibodies against phosphorylated Erks (New England BioLabs, Inc.). Each *lane* shows representative skin samples from a different mouse in each treatment group.

suggests that the JNK2 pathway may be very important in mediating TPA-induced AP-1 binding activity. However, we found that TPA-induced c-Jun phosphorylation was not different between wild-type and JNK2-deficient mouse skin (data not shown), suggesting that JNK2 deficiency does not affect TPA-induced c-Jun phosphorylation. Because the phosphorylation of Erks induced by TPA was blocked in JNK2-deficient mice (Fig. 6), the inhibition of Erks phosphorylation may lead to a decrease in AP-1 DNA binding activity. The exact mechanism by which JNK2 mediates Erks phosphorylation is not clear. Because of the lack of direct evidence that JNK2 activates Erks, we suggest that other molecules may mediate the activation of Erks by JNKs. Increasing numbers of additional proteins or protein kinases have been found to be substrates of JNKs. In our laboratory, we reported recently that $p90^{RSK}$ (42), histone 3 (43), and p53[4] are also substrates of JNKs *in vivo* and *in vitro*. Whether these proteins are related to the molecular mechanism of JNK2-mediated carcinogenesis is currently being investigated.

In summary, our studies show that deficiency of the *Jnk2* gene inhibits the incidence, size, and number of TPA-promoted tumors. The fact that TPA treatment does not induce AP-1 DNA binding activity in JNK2-deficient mice may be related to the inhibition of Erks phosphorylation. These results strongly support a critical role for JNK2 in the tumor promotion process. The suppression of TPA-induced tumorigenesis in *Jnk2* gene-deficient mice may be related to the inhibition of AP-1 DNA binding activity.

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[4] Q-B. She and Z. Dong, unpublished data.