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Deficiency of c-Jun-NH_2-terminal Kinase-1 in Mice Enhances Skin Tumor Development by 12-*O*-Tetradecanoylphorbol-13-Acetate[1]

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ABSTRACT

The c-Jun NH_2-terminal kinase (JNK) has been implicated in regulating cell survival, apoptosis, and transformation. However, the distinct role of JNK isoforms in regulating tumor development is not yet clear. We have found previously that skin tumor formation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is suppressed in JNK2-deficient (*Jnk2*$^{-/-}$) mice. Here, we show that JNK1-deficient (*Jnk1*$^{-/-}$) mice are more susceptible to TPA-induced skin tumor development than wild-type mice. The rate of tumor development in *Jnk1*$^{-/-}$ mice was significantly more rapid than that observed in wild-type mice ($P < 0.0001$). At the end of 33 weeks of TPA promotion, the number of skin tumors and tumors >1.5 mm in diameter per mouse in *Jnk1*$^{-/-}$ mice was significantly increased by 71% ($P < 0.03$) and 82% ($P < 0.03$), respectively, relative to the wild-type mice. Furthermore, the carcinoma incidence and the number of carcinomas per mouse were also higher in *Jnk1*$^{-/-}$ mice. Strikingly, *Jnk1*$^{-/-}$ mouse skin was more sensitive to TPA-induced AP-1 DNA binding activity and phosphorylation of extracellular signal-regulated kinases and Akt, which are two important survival signaling components. These results suggest that JNK1 is a crucial suppressor of skin tumor development.

INTRODUCTION

The JNK[3] signal transduction pathway has been known to play an important role in coordinating the cellular stress response, including apoptosis (1). Several lines of evidence also support a role for the JNK pathway in major cellular functions, such as cell proliferation (2–4) and transformation (5–9).

The JNK protein kinases are encoded by three genes, *Jnk1*, *Jnk2*, and *Jnk3*. The *Jnk1* and *Jnk2* genes are expressed ubiquitously, whereas the *Jnk3* gene is largely restricted to brain, heart, and testis. These genes are alternatively spliced to create 10 JNK isoforms (10). Transcripts derived from all three of the genes encode proteins with and without a COOH-terminal extension to create both M_r 46,000 and 55,000 isoforms, but the functional significance of the different isoforms remains unclear. JNKs are identified as members of the mitogen-activated protein kinase family and are known to phosphorylate and activate several transcription factors, including c-Jun (11), ATF-2 (12), Elk-1 (13), and p53 (14). JNK1 and JNK2 demonstrate distinct substrate affinities (10, 15) and may have selective or preferential roles in different biological processes. JNK1 has been shown to be more specifically involved in the regulation of the apoptotic response of small-cell lung cancer cells after UV radiation (16). JNK1 was also shown to be activated preferentially by tumor necrosis factor α in mouse macrophages (17). Inhibition of JNK1 activity using a dominant-negative JNK1 mutant markedly enhanced arsenite-induced JB6 cell transformation (7) and platelet-derived growth factor-mediated anchorage-independent cell growth (9), demonstrating an antagonistic role for JNK1 in cell transformation. On the other hand, JNK2 has a vital role in the survival of inner-medullary collecting duct cells in a hypertonic environment (18). Recent investigations using antisense JNK1 or JNK2 oligonucleotides also suggest that JNK1 and JNK2 may play a differential role in one or more major physiological functions such as cell growth, transformation, or apoptosis (3, 4, 6, 19, 20). However, no direct *in vivo* evidence has shown the distinct role of JNK isoforms in tumor development.

The recent generation of *Jnk1* (21) and *Jnk2* (22) knockout mice provides the opportunity to address the roles of specific JNK isoforms. The multistage model of mouse skin carcinogenesis is a useful system in which biochemical events unique to initiation, promotion, or progression can be studied and related to cancer formation (23, 24). We found previously that two-stage tumor promotion with DMBA and TPA in *Jnk2* knockout (*Jnk2*$^{-/-}$) mice exhibited significant reduction in papilloma burden compared with wild-type controls (25). Here, we provide evidence that *Jnk1* knockout (*Jnk1*$^{-/-}$) mice developed a greater number of papillomas and carcinomas more rapidly than wild-type mice. Our data demonstrated that JNK1 plays an antagonistic role in DMBA/TPA-induced skin tumor development. The molecular mechanism that accounts for the role of JNK1 in regulating skin tumorigenesis is discussed.

MATERIALS AND METHODS

Materials. DMBA, TPA, aprotinin, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO); primers for PCR were synthesized by Life Technologies, Inc. (Rockville, MD); Taq polymerase was from Applied Biosystems (Foster, CA); mouse monoclonal antibody for JNK1/JNK2 was from PharMingen (San Diego, CA); rabbit polyclonal phospho-specific antibody kits for ERKs and p38 kinase, and a rabbit polyclonal antibody to Akt and a phospho-specific antibody for Akt at serine 473 were from Cell Signaling Technology, Inc. (Beverly, MA).

Mice. Jnk1$^{-/-}$ mice were generated as reported previously (21). The number of mice for each experimental group was 30 wild-type and 30 *Jnk1*$^{-/-}$ mice. The mice were housed in groups of five in plastic-bottomed cages in a room with controlled humidity, temperature (22°C), and an automated light cycle in a normal rhythm of 12-h light/12-h dark periods. Food and water were available *ad libitum*. At 7–8 weeks of age, the dorsal skins of the mice were shaved 2–3 days before treatment. The solutions of DMBA and TPA were prepared in acetone and applied to the shaved backs of individual mice in a volume of 0.25 ml each.

Tumor Induction Experiments. Mouse skin tumors were induced by the initiation-promotion regimen. For mouse skin tumor initiation, a single 200-nmol dose of DMBA in 0.25 ml of acetone was applied topically to the shaved backs of the mice. Two weeks after initiation, acetone alone or 17 nmol of TPA in 0.25 ml of acetone was applied twice weekly to the skin for the duration of the experiment (33 weeks). The tumor incidence and burden were observed every 2 weeks starting at 9 weeks of TPA promotion. The papilloma incidence is expressed as the percentage of mice bearing one or more papillomas, and the tumor burden is expressed as the number of tumors per surviving mouse in mice with tumors. Mice were removed from the experiment if they were wounded from fighting. Carcinomas were recorded grossly as downward-

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[3] The abbreviations used are: JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; *Jnk1*$^{-/-}$, JNK1-deficient; *Jnk2*$^{-/-}$, JNK2-deficient; AP-1, activator protein 1; DMBA, 7,12-dimethylbenz(*a*)anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

invading lesions, a subset of which was examined histologically, and malignancy was confirmed as invading the panniculus carnosus.

Histology. The tumor to be examined was excised promptly after euthanasia and immediately placed in 10% formalin. The tumor was fixed for 1 h in formalin and then embedded in paraffin. Sections of 4 μm were cut for H&E staining.

DNA Extraction and PCR. The tail genomic DNAs of wild-type and Jnk1$^{-/-}$ mice were isolated by using the LeMax genomic isolation kit as described previously (21). The primers used to amplify the DNA fragments of *Jnk1*$^{+}$ and *Jnk1*$^{-}$ were: *Jnk1*$^{+}$ forward 5′-GCCATTCTG-GTAGAG-GAAGTTTCTC-3′, *Jnk1*$^{+}$ reverse 5′-CGCCAGTCCAAAATCAAGAATC-3′; *Jnk1*$^{-}$ forward 5′-GCCATTCTGGTAGAGGAAGTTTCTC-3′, and *Jnk1*$^{-}$ reverse 5′-CCAGCTCATTCC-TCCACTCATG-3′. PCR was performed in a 50-μl reaction mixture containing 100 ng of genomic DNA, 0.2 mM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, and 0.25 units of Taq polymerase. The amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized under UV light.

Western Blot Analysis. The dorsal skin of the mice was shaved and depilated 24 h before TPA treatment. The mice were euthanized, and then the dorsal skin was removed and immediately placed on dry ice. The skin was then submerged in 500 μl of lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] and homogenized with a Polytron homogenizer three times (each for 10 s at 4°C at 10-s intervals). The lysate was additionally sonicated four times each for 5 s and placed on ice for 1 h followed by centrifugation at 14,000 rpm for 15 min. The supernatant fractions were saved for Western blot analysis. Equal amounts of protein (50 μg) were separated by an 8% SDS polyacrylamide gel, and proteins were subsequently transferred and analyzed as described previously (26). The levels of phosphorylated ERKs and Akt at serine 473, as well as total ERKs, Akt, JNKs, and p38 kinase, were selectively measured by Western immunoblotting using a specific antibody. The antibody-bound proteins were detected by chemifluorescence and analyzed with the Storm 840 Phospho-Image System (Molecular Dynamics, Sunnyvale, CA).

Gel Mobility Shift Assay. The AP-1 DNA binding assay was performed as described previously (27). Nuclear extracts were prepared from mouse skin treated with TPA or its vehicle acetone. Mice were shaved and depilated before experimentation. The mouse skin was excised, and the skin was ground with a mortar and pestle under liquid nitrogen. The ground skin was homogenized with 500 μl of lysis buffer [25 mM HEPES (pH 7.8), 50 mM KCl, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM DTT]. After centrifugation at 14,000 rpm for 1 min, the pellet was washed once with 500 μl of the same buffer without NP40 and was then resuspended in 200 μl of the extraction buffer (500 mM KCl and 10% glycerol with the same concentration of the other reagents as in the lysis buffer) and incubated at 4°C for 30 min with frequent mixing followed by centrifugation at 14,000 rpm for 5 min. The supernatant fraction was saved as the nuclear protein extract and stored at −70°C until analysis. An AP-1 binding consensus sequence from the human collagenase promoter region 5′-AGCATGAGTCA-GACA-CCTCTGGC-3′ was synthesized and labeled with [^{32}P]dCTP using the Klenow fragment (Life Science Co., Gaithersburg, MD). A DNA-binding reaction mixture of 24 μl, containing 4 mM Tris, 12 mM HEPES (pH 8.0), 12% glycerol, 1 mM EDTA, 1 mM DTT, 1.5 μg of poly(dI·dC), 3 μg of BSA, and 50,000 cpm ^{32}P-labeled oligonucleotide probe, was incubated with 6 μg of nuclear protein on ice for 10 min followed by incubation at room temperature for 20 min. To determine the binding specificity, a 5-fold excess of cold oligonucleotide was incubated in the same reactions. The DNA-protein complexes were resolved in a 5% nondenaturing polyacrylamide gel. The gel was dried and scanned using the Storm 840 Phospho-Image System.

Statistics. Linear regression was used to determine the rate of tumor development between wild-type and Jnk1$^{-/-}$ mice. For comparison between groups, one-way ANOVA was used to determine significant differences. $P < 0.05$ was considered to be significant.

RESULTS

Identification of *Jnk1*$^{-/-}$ Mouse Phenotype. The genomic DNA phenotype of *Jnk1* was examined by PCR. *Jnk1* DNA was not detected in homozygous *Jnk1*$^{-/-}$ mice (Fig. 1*A*). Protein immunoblotting analysis revealed a complete loss of the JNK1 protein in *Jnk1*$^{-/-}$ mice, whereas disruption of the *Jnk1* gene did not alter expression of JNK2, ERKs, or p38 kinase (Fig. 1*B*), which agrees with previous results (21). These data indicate that the knockout of the *Jnk1* gene was effective and specific, and the mice with JNK1 deficiency were used in the present experiments.

JNK1 Deficiency Enhances TPA-promoted Tumor Growth in Mouse Skin. To determine the specific role of JNK1 in tumor development, the *Jnk1*$^{-/-}$ mice were evaluated for their sensitivity to skin tumor promotion by TPA. We used a well-characterized two-stage mouse skin carcinogenesis model in which DMBA was used as an initiator and TPA as a promoter. In these experiments, the shaved backs of *Jnk1*$^{-/-}$ and wild-type mice were initiated by application of a single 200-nmol dose of DMBA and were then treated twice weekly with 17 nmol of TPA in acetone for tumor promotion. The first papillomas appeared after 5 weeks of TPA treatment in both *Jnk1*$^{-/-}$ and wild-type groups (data not shown). After 13 weeks of tumor promotion, the *Jnk1*$^{-/-}$ mice exhibited a rapid and high incidence of papilloma development compared with the wild-type control mice (Fig. 2*A*; Table 1). A papilloma incidence of 95% was achieved by week 25 of promotion in the *Jnk1*$^{-/-}$ group (35 mice). In contrast, the wild-type group (32 mice) did not reach 95% incidence over the entire 33 weeks of tumor promotion (Fig. 2*A*). The rate of tumor development in *Jnk1*$^{-/-}$ mice was significantly more rapid than that observed in wild-type mice during weeks 9–25 (Fig. 2*B*; $P < 0.0001$). The number of papillomas or tumors >1.5 mm in diameter per mouse increased more rapidly in *Jnk1*$^{-/-}$ mice after 27 weeks of tumor promotion (Fig. 2, *C* and *D*; Table 1; $P < 0.03$). At the end of 33 weeks of tumor promotion, compared with wild-type mice (28 mice bearing tumors), *Jnk1*$^{-/-}$ mice (33 mice bearing tumors) had a significantly larger number of papilloma (71%) or tumors >1.5 mm in diameter (82%) per mouse, respectively (Table 2; $P < 0.03$). In addition, the papilloma incidence was also increased in *Jnk1*$^{-/-}$ mice initiated with DMBA but without TPA promotion (data not shown). These data strongly suggest that deficiency of *Jnk1* gene in these mice stimulates formation and growth of skin tumors initiated by DMBA and promoted by TPA.

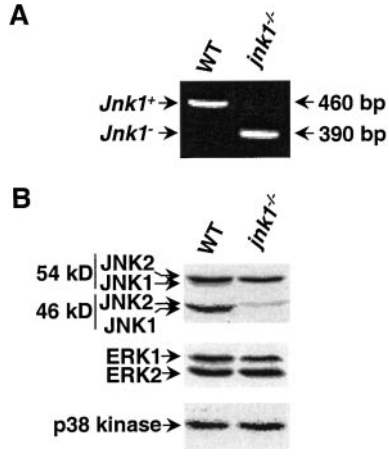

Fig. 1. Identification of the targeted disruption of the murine *Jnk1* gene. *A*, identification of genomic DNA phenotype of *Jnk1* by PCR. DNA was isolated from the tail of wild-type (*WT*) and *Jnk1*$^{-/-}$ mice, and the phenotype of *Jnk1* was confirmed by PCR. The top band (*460 bp*) corresponds to the wild-type allele (*Jnk1*$^{+}$) and the bottom band (*390 bp*) corresponds to the mutant allele (*Jnk1*$^{-}$). *B*, expression of JNKs, ERKs, and p38 kinase. Extracts (50 μg of protein) were prepared from wild-type (*WT*) and *Jnk1*$^{-/-}$ mouse skin. Expression of JNKs, ERKs, and p38 kinase was immunodetected with antibodies for JNK1/JNK2 (PharMingen), ERKs, and p38 kinase (Cell Signaling Technology, Inc.), respectively.

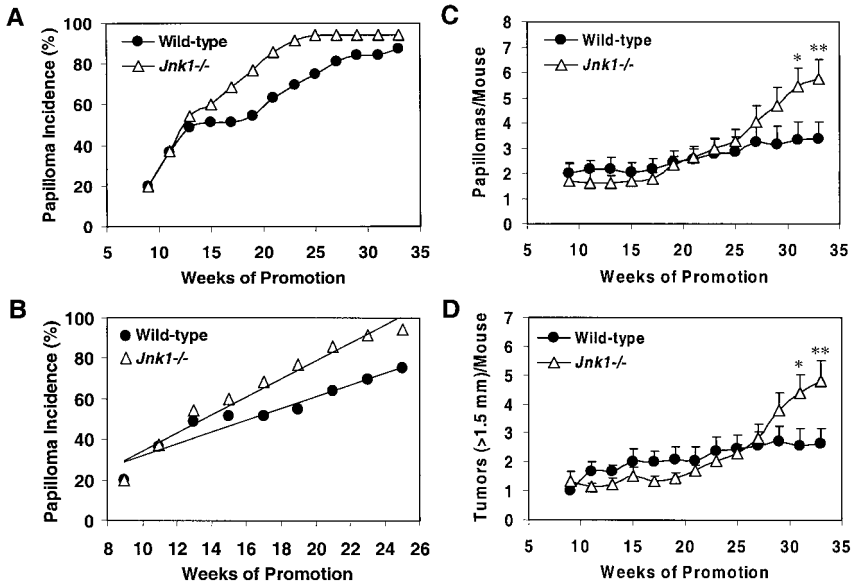

Fig. 2. Susceptibility of *Jnk1*$^{-/-}$ mice to skin tumor promotion by TPA. A single 200 nM dose of DMBA was used to initiate tumor development in wild-type and *Jnk1*$^{-/-}$ mice. Each mouse was then treated twice weekly with 17 nmol of TPA for 33 weeks to promote tumor development. Tumor incidence was observed every 2 weeks. *A*, percentage of mice bearing papillomas at different weeks of promotion. *B*, rate of tumor development during weeks 9–25 of tumor promotion. The rate of tumor development was calculated by regression analysis of papilloma incidence during weeks 9–25 of tumor promotion and was significantly different between wild-type and *Jnk1*$^{-/-}$ mice ($P < 0.0001$). *C*, average number of papillomas per mouse in mice with tumors (*$P < 0.05$; **$P < 0.03$). *D*, average number of tumors >1.5 mm in diameter per mouse (*$P < 0.05$; **$P < 0.03$). *C* and *D*, *bars* ± SE.

JNK1-deficient Papillomas Appear to Have an Increased Risk of Undergoing Malignant Conversion. Malignant lesions grew rapidly, and many carcinomas became ulcerated. Both *Jnk1*$^{-/-}$ and wild-type mice began to develop malignant skin tumors around 23 weeks of TPA promotion. The malignant tumors were identified histologically as squamous cell carcinomas (data not shown). At the end of 33 weeks of tumor promotion, the carcinoma incidence in the *Jnk1*$^{-/-}$ group (35 mice) and the wild-type group (32 mice) was 77% and 53%, respectively (Table 2). Furthermore, the number of carcinomas per mouse in *Jnk1*$^{-/-}$ mice (27 mice bearing carcinomas) tended to be increased compared with the wild-type mice (17 mice bearing carcinomas; Table 2). These observations indicate that JNK1 plays an important role in inhibiting malignant conversion.

JNK1 Deficiency Stimulates TPA-induced Phosphorylation of ERKs and Akt. The ERKs and Akt pathways have been shown to play an essential role in cell survival and tumorigenesis (7, 28–32). Thus, we determined whether disruption of the *Jnk1* or *Jnk2* gene in mice alters the TPA-induced activation of ERKs and Akt to affect the skin tumor formation. Using antibodies to the phosphorylated (activated) ERKs or Akt by probing Western blots of proteins from mouse skin, we found that TPA-induced phosphorylation of ERKs and Akt was significantly enhanced in *Jnk1*$^{-/-}$ mice ($n = 5$) but was repressed in *Jnk2*$^{-/-}$ mice ($n = 5$) for up to 3 weeks of TPA treatment (Fig. 3). These results suggest that JNK1 and JNK2 deficiency modulate different activities of ERKs and Akt, which may account for differences in TPA-induced skin tumor formation.

JNK1 Deficiency Increases TPA-induced AP-1 DNA Binding Activity. AP-1 was originally described as a transcription factor that mediates gene induction by TPA and, thus, its recognition site is known as a TPA response element site (33). Evidence indicates that acquisition of constitutive AP-1 DNA binding and transactivating ability may be related to carcinogenesis. AP-1 binding to the TPA response element in the promoter of target genes is a key step in TPA-induced tumor promotion (34). To examine the effect of JNK isoforms on TPA-induced AP-1 DNA binding, the dorsal skin of wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were shaved and treated with TPA (17 nmol/mouse) in acetone or acetone alone (for control) for 24 h or 3 weeks. Nuclear extracts were prepared from the mouse skin, and AP-1 DNA binding activity was analyzed as described in "Materials and Methods." Results showed that TPA-induced mouse skin AP-1 DNA binding activity was significantly stimulated in *Jnk1*$^{-/-}$ mice ($n = 5$) but was blocked in *Jnk2*$^{-/-}$ mice ($n = 5$) for up to 3 weeks of TPA treatment (Fig. 4), indicating that JNK1 and JNK2 play distinct roles in mediating TPA-induced AP-1 DNA binding activity.

Table 1 *Comparison of papilloma incidence, papilloma burden, and tumors >1.5 mm in diameter per mouse during different periods of TPA treatment for wild-type and Jnk1*$^{-/-}$ *mice*

Experimental details are described in the legend of Fig. 2.

Group	Papilloma incidence weeks 13–33[a]	Papillomas/mouse weeks 21–33	Tumors (>1.5 mm)/mouse weeks 27–33
Wild-type	68 ± 5[b]	3.03 ± 0.11	2.63 ± 0.03
Jnk1$^{-/-}$	83 ± 5	4.11 ± 0.46	3.95 ± 0.43
P value	<0.04	<0.05	<0.03

[a] First significant difference was observed after 13, 21, or 27 weeks of TPA treatment for papilloma incidence, papillomas/mouse, or tumors (>1.5 mm)/mouse, respectively.
[b] Mean ± SE. *P* indicates a statistically significant difference between wild-type and *Jnk1*$^{-/-}$ mice.

Table 2 *Summary of tumor development at 33 weeks of TPA treatment for wild-type and Jnk1*$^{-/-}$ *mice*

Experimental details are described in the legend of Fig. 2.

Group	Papilloma incidence	Papillomas/mouse	Tumors (>1.5 mm)/mouse	Carcinoma incidence	Carcinomas/mouse
Wild-type	87%	3.4 ± 0.7[a]	2.7 ± 0.5	53%	1.8 ± 0.3
Jnk1$^{-/-}$	95%	5.7 ± 0.8	4.8 ± 0.7	77%	2.4 ± 0.3

[a] Mean ± SE. The difference in papillomas/mouse and tumors (>1.5 mm)/mouse between wild-type and *Jnk1*$^{-/-}$ mice was statistically significant at $P < 0.03$.

DISCUSSION

JNKs have been shown to be important components of the signaling pathways involved in the regulation of apoptosis (1), cell growth (2–4), and transformation (5–9). However, the *in vivo* role of individual JNK isoforms in different biological processes, especially in tumor development, has not been demonstrated. Using *Jnk2* knockout mice and a well-characterized two-stage mouse skin carcinogenesis model, we have previously found that JNK2 enhances tumor promotion and progression by TPA (25). In the present study, using *Jnk1* knockout mice, we additionally determined the specific role of JNK1 in tumor development. Our results showed that *Jnk1*$^{-/-}$ mice were hypersensitive to TPA-induced papilloma formation and growth. *Jnk1*$^{-/-}$ mice exhibited a significantly higher papilloma incidence 12 weeks earlier compared with the wild-type mice (21 weeks *versus* 33 weeks, respectively; Fig.

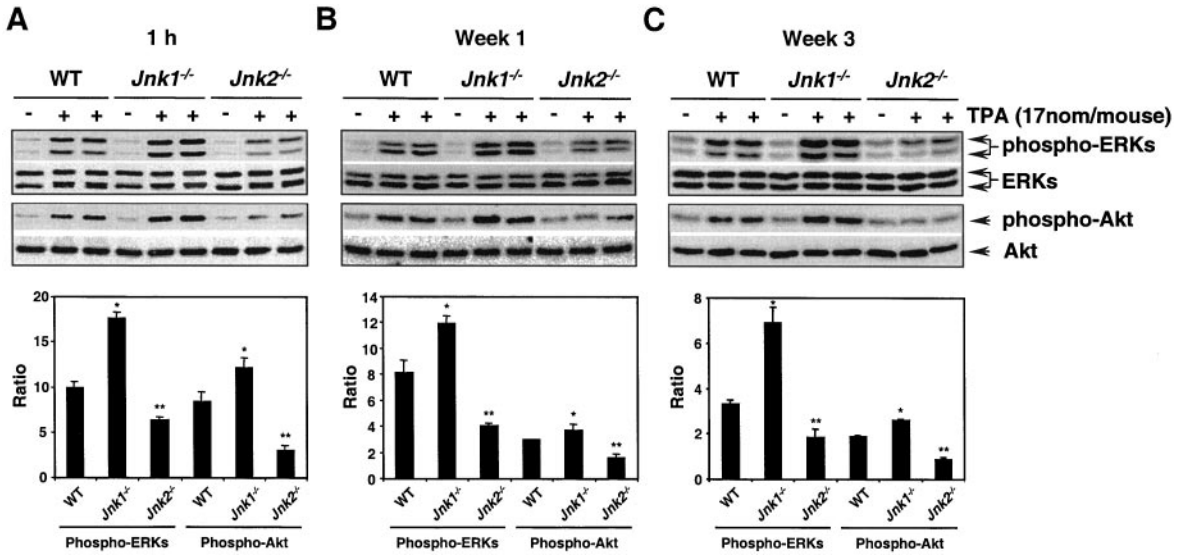

Fig. 3. Disruption of *Jnk1* gene in mice additionally activates TPA-induced phosphorylation of ERKs and Akt. The dorsal skins of wild-type (*WT; n* = 5), *Jnk1*$^{-/-}$ (*n* = 5) and *Jnk2*$^{-/-}$ (*n* = 5) mice were excised at 1 h after last skin painting with TPA (17 nmol/mouse) or its vehicle acetone (Control). Mice were treated with 17 nmol TPA/mouse once (*A*) or twice a week for 1 week (*B*), or twice a week for 3 weeks (*C*). Extracts of the mouse skin were prepared as described in "Materials and Methods." Equal amounts of protein (50 μg) were analyzed by Western blotting using antibodies for phosphorylated or total ERKs and Akt, respectively. Each *lane* shows representative skin samples from a different mouse in each treatment group. The ratio of phosphorylation of ERKs or Akt in TPA-treated sample to control in each experimental group determined by using the TotalLab System (Nonlinear Dynamics, Newcastle, United Kingdom) and represents as mean (*n* = 5) from two independent experiments; *bars* ± SE. $*P < 0.05$, wild-type *versus* *Jnk1*$^{-/-}$ mice; $**$ $P < 0.03$, wild-type *versus* *Jnk2*$^{-/-}$ mice.

2*A*). At the end of 33 weeks of tumor promotion, the number of skin tumors per mouse was 71% higher in *Jnk1*$^{-/-}$ mice, and the number of tumors >1.5 mm in diameter per mouse was 82% higher in *Jnk1*$^{-/-}$ mice compared with the wild-type mice ($P < 0.03$; Fig. 2, *C* and *D*; Table 2). These *in vivo* data clearly demonstrate that JNK1 can suppress skin tumor development. Additionally, the number of skin tumors or tumors >1.5 mm in diameter per mouse was similar between the two groups before 27 weeks of TPA promotion. However, the tumor burden and size per mouse increased more rapidly in *Jnk1*$^{-/-}$ mice compared with the wild-type mice after 27 weeks of tumor promotion (Fig. 2, *C* and *D*). At the end of experiment, the carcinoma incidence and the number of carcinomas per mouse were higher in *Jnk1*$^{-/-}$ mice (Table 2). These results indicate that JNK1 may play a critical role in the suppression of tumor initiation, promotion, and progression.

Jnk1$^{-/-}$ mice have been reported to be defective in T-cell differentiation into Th1 subset, and the T cells preferentially differentiated to Th2 subset (21). However, the *Jnk1*$^{-/-}$ mice were fertile and of normal size. Lymphocyte development appeared normal with typical ratios of T cells:B cells, and naïve to memory T cells in the periphery, indicating that deficiency of JNK1 in mice has no effect on T-cell maturation (21). Recently, JNK1 was shown to not be required for T-cell activation, although it is required for T-cell differentiation (35). Therefore, T-cell differentiation, but not maturation and activation, regulated by JNK1 may be important for the increased skin tumor development in *Jnk1*$^{-/-}$ mice.

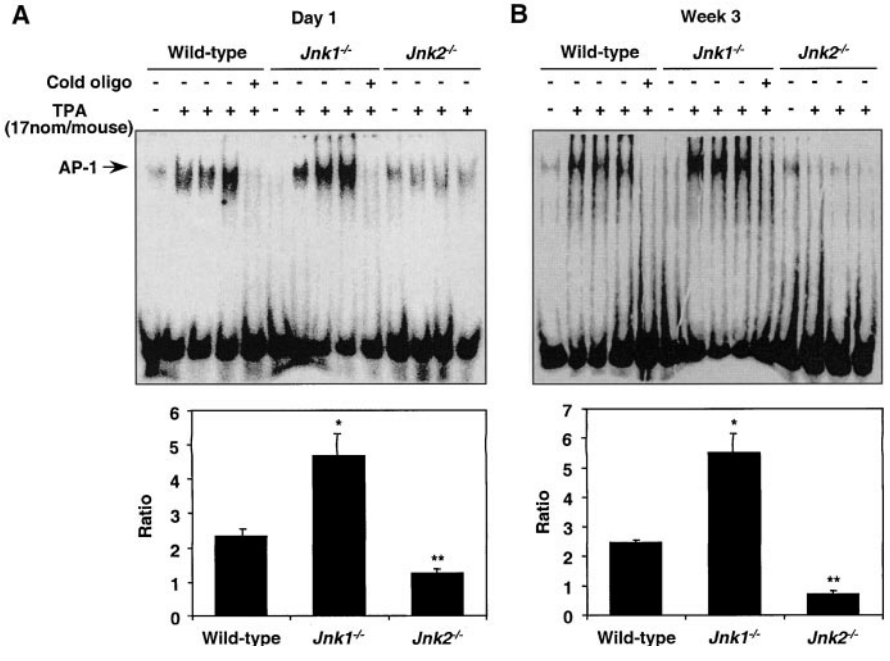

Fig. 4. Disruption of *Jnk1* gene in mice additionally increases TPA-induced AP-1 DNA binding activity. The dorsal skins of wild-type (*n* = 5), *Jnk1*$^{-/-}$ (*n* = 5), and *Jnk2*$^{-/-}$ (*n* = 5) mice were excised at 24 h after last skin painting with TPA (17 nmol/mouse) or its vehicle acetone (Control). Mice were treated with 17 nmol TPA/mouse once (*A*) or twice a week for 3 weeks (*B*). Nuclear extracts (6 μg of protein) were prepared from mouse skin. 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." A competition experiment using a 5-fold excess of cold oligonucleotide indicated that the DNA binding was specific. Representative samples are shown. The ratio of AP-1 DNA binding activity in TPA-treated sample to control in each experimental group determined by using the TotalLab System and represents as mean (*n* = 5) from three independent experiments; *bars* ± SE. $*$ $P < 0.03$, wild-type *versus* *Jnk1*$^{-/-}$ mice; $**$ $P < 0.02$, wild-type *versus* *Jnk2*$^{-/-}$ mice.

Over the past several years, numerous studies have demonstrated that the JNK pathway contributes to apoptosis (1), whereas the ERK and Akt pathways were shown to suppress apoptosis and enhance cell survival or tumorigenesis (7, 28–32). Growing evidence also indicated that cross-regulation between JNK and ERK or Akt signaling may play an important role in determining cell survival or death (28, 36, 37). However, the role of JNK isoforms in cross-regulation of ERK or Akt signaling is not documented. Targeted gene knockout experiments in mice provide an opportunity for the direct biochemical analysis of JNK isoforms in the cross-regulation. Our data demonstrated that disruption of the *Jnk1* gene in mice caused an increase in TPA-induced skin ERKs and Akt activation (Fig. 3). In contrast, disruption of the *Jnk2* gene caused an inhibition of ERKs and Akt activation induced by TPA (Fig. 3). Thus, the increase of skin tumor formation induced by TPA in *Jnk1*$^{-/-}$ mice may be derived from the activation of two important survival signaling components, ERKs and Akt. The exact mechanism by which JNK1 and JNK2 function differently in cross-regulation of ERKs and Akt is not clear. Because of the lack of direct evidence that JNK1 or JNK2 regulate the activation of ERKs and Akt, we suggest that other signaling molecules may be involved. Using cDNA microarray to analyze the gene expression profile, we also confirmed recently that disruption of the *Jnk1*$^{-/-}$ gene significantly up-regulated TPA-induced Akt expression, which was down-regulated by disruption of the *Jnk2* gene.[4] Furthermore, we found that glutathione *S*-transferase P1–1 and Cek5 receptor protein-tyrosine kinase ligand were also markedly up-regulated by deficiency of JNK1 but down-regulated by deficiency of JNK2 in response to TPA treatment (4). Recent studies have shown that glutathione *S*-transferase P1–1 is involved in the regulation of ERK and JNK activation to influence cell proliferation (38, 39). Biochemical and genetic evidence suggest that receptor protein-tyrosine kinases and their membrane-bound ligands play a critical role in transducing signal cascades (40–43). Thus, we propose a hypothesis that the differential regulation of gluthione *S*-transferase P1–1 and Cek5 receptor protein-tyrosine kinase ligand by JNK1 and JNK2 may be related to the modulation of ERKs and Akt activities after TPA treatment. However, additional study will be required to prove this.

The activation of signal transduction pathways leading to increased AP-1 transcriptional activity seems to be a common mechanism for tumor promoter-induced cell transformation (30, 44–47). High AP-1 activity has also been shown to be involved in tumor promotion (48, 49) and progression of various types of cancers, such as lung (50), breast (51), and skin cancer (52–55). TPA has been known to activate protein kinase C and subsequently increase AP-1 activity (56). Using a mouse JB6 epidermal cell line, a well-developed cell culture model for studying tumor promotion (30, 45, 46, 57, 58), and AP-1-luciferase transgenic mice, we and others have reported that blocking AP-1 activity prevents TPA-induced promotion of neoplastic transformation and tumor promotion in a mouse skin carcinogenesis model (30, 45–49, 58, 59). All of these data demonstrate that AP-1 plays an important role in mediating carcinogenesis. AP-1 consists of a homodimeric or heterodimeric complex of c-Jun and c-Fos proteins (44). AP-1 is a target of mitogen-activated protein kinases, including JNKs, ERKs, and p38 kinase (60). JNKs have been shown to phosphorylate c-Jun at serine residues 63 and 73, resulting in activation of AP-1. However, no differences in c-Jun phosphorylation were detected among wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mouse skin after TPA treatment (data not shown), suggesting that JNK1 or JNK2 deficiency does not affect TPA-induced c-Jun phosphorylation. ERKs and p38 kinase were found to induce expression of c-Jun and c-Fos, leading to increased AP-1 transcriptional activity. Our previous work demonstrated that ERKs and phosphatidylinositol-3 kinase, an upstream kinase of Akt, are responsible for TPA-induced cell transformation through increased transactivation of AP-1 activity (30, 46, 47). Considering that TPA also induced an increase in AP-1 binding activity in *Jnk1*$^{-/-}$ mice (Fig. 4), activation of ERKs and Akt in TPA-treated *Jnk1*$^{-/-}$ mice may result in stimulation of AP-1 activity to induce skin tumor development. Because disruption of the *Jnk2* gene in mice inhibited the AP-1 binding activity (Fig. 4), these results suggest that JNK1 and JNK2 play distinct roles in the regulation of ERKs and Akt, and subsequently modulate TPA-induced AP-1 DNA binding activity and tumor formation. However, we may not expect this to be only one mechanism by which JNK1 and JNK2 function differentially in skin tumor development induced by TPA. Recently, by using cDNA microarray analysis, we additionally found that the expression of A20 zinc finger protein, an inhibitor of programmed cell death (61, 62), was markedly induced by TPA in *Jnk1*$^{-/-}$ cells but was blocked in *Jnk2*$^{-/-}$ cells. Whether the differential expression of A20 is also involved in the differential regulation of TPA-induced skin tumor formation by JNK1 and JNK2 is currently being investigated.

In conclusion, we demonstrated for the first time that JNK1 and JNK2 are important but differential regulators of skin tumor development. In contrast to the role of JNK2 as a survival factor for tumor growth (25), JNK1 appears to inhibit papilloma formation. Our results suggest that JNK1 may be a crucial *in vivo* suppressor of tumorigenic transformation. The mechanism by which JNK1 and JNK2 function differentially in TPA-induced skin tumor development may be complex. Our data here offer a possible explanation that the differential regulation of ERKs, Akt, and subsequent AP-1 DNA binding activities in *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice may in part account for the distinct roles of JNK1 and JNK2 in skin tumor development. In practical terms, understanding the differential specificity of JNK1 and JNK2 in the regulation of signaling molecules and tumor development may provide potential targets for pharmaceutical intervention.

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Deficiency of c-Jun-NH_2-terminal Kinase-1 in Mice Enhances Skin Tumor Development by 12- *O* -Tetradecanoylphorbol-13-Acetate

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