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Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines

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There is evidence that reactive oxygen species (ROS) are important mediators of tumor promotion and progression. The molecular mechanisms involved in ROS-mediated signaling, however, are unclear at present. Using ionizing radiation and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as model physical and chemical carcinogens, we have malignantly progressed 308 cells, a papilloma-producing mouse keratinocyte cell line, and investigated the molecular alterations in the progressed phenotypes. In this study, we demonstrate that both MNNG and radiation-progressed malignant variants showed elevated ROS levels that contributed to their proliferative capacity *in vitro* as well as *in vivo*. We found increased Erk-1/2 and p38 MAP kinase activities to be important components of ROS-mediated signaling. The pro-oxidant state also contributed to constitutive elevation of AP-1, NFκB and cAMP response element transactivation in the malignant phenotype. Our data provide evidence for a functional role of elevated ROS levels in tumor progression and implicate Erk-1/2 and p38 MAP kinase activation in the malignant progression of mouse keratinocytes.

Introduction

An accepted paradigm for neoplastic transformation is the multistep model of carcinogenesis in mouse skin, which involves initiation, promotion and progression (1,2). Initiation involves mutational activation of a cellular proto-oncogene. Tumor promotion involves the selective clonal expansion of the initiated cell population (3), whereas the final stage (progression) involves irreversible malignant transformation from pre-neoplastic lesions (4).

Investigations into the molecular mechanisms of tumor progression have revealed that this operational step involves modulation of transcription factors. A causal relationship has been established between neoplastic transformation and transcription factor AP-1 transactivation (5–8). Over-expression of this heterodimeric complex (9) leads to increased neoplastic transformation (10). Constitutive elevation of AP-1 activity has been correlated with malignant conversion of papillomas to carcinomas (11). Tumor promoters like TPA and okadaic acid efficiently induce AP-1 activity (12,13). Inhibition of AP-1 activity by a dominant-negative c-Jun mutant (TAM-67) or by AP-1 transrepressing retinoids blocks cell transformation and attenuates the malignant phenotype (5,7,8,11). Recently, evidence for a functional role of increased NFκB (14) transactivation during tumor progression has been obtained. NFκB transactivation is also elevated by agents that increase AP-1 activity during progression, and inhibitors of NFκB also attenuate AP-1 activity (15,16). These data suggest that common upstream signaling cascades may be involved that mediate elevated AP-1 as well as NFκB transactivation during tumor progression. While some common effectors of NFκB and AP-1 activity are known, their role during malignant progression of skin tumors and the molecular mechanisms involved therein remain poorly understood.

Reactive oxygen species (ROS) are an important class of these effector molecules. ROS such as superoxide ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) are normal biproducts of intracellular metabolic processes. These and other oxidant species are highly reactive and can produce macromolecular damage (17). There is evidence that ROS can act as activators of transcription factors and modulate their activity either directly or indirectly by activating other signaling cascades. NFκB transactivation has been shown to be redox-sensitive at two levels: (i) phosphorylation of the inhibitor I-κB is mediated by oxidation; and (ii) a Ref/thioredoxin-dependent binding of the p50 subunit to the DNA is governed by the reduction of an essential cysteine group. NFκB activation can be triggered by oxidants in the absence of any physiological stimulus and is inhibited by a broad range of chemically unrelated antioxidants (18). AP-1 DNA binding activity has also been shown to be modulated by Ref/thioredoxin-dependent reduction of cysteines in c-Jun and c-Fos (19). In addition, active oxygen has been shown to induce c-*fos* expression (20).

Mitogen-activated protein kinases (MAPKs), a group of serine/threonine-specific, proline-directed protein kinases (21), are also known to modulate transcription factor activities. They are a part of kinase cascades that serve as information relays, connecting extracellular stimuli to specific transcription factors thereby allowing these signals to regulate specific gene expression. There are three subtypes of MAPKs: the extracellular signal regulated kinases (Erks), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs. Current evidence indicates that while Erks are predominantly stimulated by mitogens, JNKs and p38 MAPK are a part of the stress response pathways activated by cellular stress induced by agents like heat, UV and ionizing radiation and inflammatory cytokines mediating inhibition of cell proliferation or cell death (22,23). AP-1 activity has been shown to be modulated by MAPKs in response to various stimuli (24). NFκB activation has been reported to be modulated by MEKK1, a kinase upstream of JNKs (25) as well as p38 MAPK (26). There is evidence that antioxidants can attenuate MAPK activation (23,27,28), thereby suggesting that MAPK signaling cascades are additional targets affected by ROS levels in cells.

Several lines of evidence suggest that pertubations in ROS levels may play a functional role in the pathogenesis of cancer

Abbreviations: CRE, cAMP response element; Erks, extracellular signal related kinases; JNKs, c-Jun N-terminal kinases; MAPKs, mitogen-activated protein; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; PDTC, pyrollidine dithiocarbamate; ROS, reactive oxygen species.

(29,30). Tumor cells have high and persistent oxidative stress (31). Free radicals are involved in initiation (32) as well as promotion/progression stages of tumorigenesis (33,34). Small molecule inhibitors and scavengers of ROS inhibit these stages of tumor development (35,36). There is evidence that TPA, one of the most studied tumor promoters in mouse skin, acts through an oxygen mediated mechanism and that oxygen radicals are critical components of the tumor promotion process (37). Despite this evidence, molecular mechanisms of oxidant mediated cell growth and its targets during tumor progression remain unclear and need further study.

Recent evidence has implicated ROS as a mediator of mitogenic signaling in activated *ras* transformed fibroblasts (38). Since mutational activation of *ras* gene in the mouse skin is a critical event (4,39,40), we modeled tumor progression in a papilloma-producing mouse keratinocyte cell line that carries an activated *ras* gene. Using ionizing radiation and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) as model physical and chemical carcinogens, respectively, we have malignantly progressed 308 cells and investigated the role of ROS in the progressed phenotype. In this study, we demonstrate that the malignant variants showed elevated ROS levels that contributed to their proliferative capacity. We identified constitutive Erk-1/2 and p38 MAPK activities as important components of ROS-mediated mitogenic signaling. These radiation- and MNNG-progressed tumor cell lines also showed constitutive elevations of AP-1, NFκB and cAMP response element (CRE) transactivation potential. These data provide a functional role for a pro-oxidant state, identify its potential targets during tumor progression and, for the first time, implicate Erk-1/2 and p38 MAPK activation in the malignant progression of mouse keratinocytes.

Materials and methods

Cell culture and generation of progressed variants

The mouse keratinocyte cell line 308 was kindly provided by S.H.Yuspa (41). The cells were maintained in minimal essential medium (MEM) supplemented with 7.5% fetal calf serum (FCS), 2.5% calf serum (CS) and 100 U/ml penicillin/streptomycin (Gibco BRL, Grand Island, NY) at 37°C in a humidified atmosphere containing 7.5% CO_2. For *in vitro* malignant transformation, $>10^7$ 308 cells in late log-phase culture were treated with LD_{90} doses of either MNNG (2.5 μM; Sigma, St Louis, MO) or γ rays (750 cGy) from a ^{60}Co source. The cultures were trypsinized immediately following treatment and plated at clonal density. The survivors were pooled, grown to $>10^7$ as before and re-treated. A total of six such generations were created. Parallel flasks of 308 cells were mock treated and passaged under similar conditions as the treated cells.

The sixth generation radiation- or MNNG-progressed as well as mock-treated 308 cells were injected s.c. into athymic nude mice (Charles River Labs, Wilmington, MA) at 10^6 cells per site and followed for tumor growth. One randomly selected tumor from each of the MNNG and radiation groups was put into culture to establish cell lines according to previously established protocols (42). These MNNG (6M90)- and radiation (6R90)-progressed tumor cell lines were maintained in culture under identical conditions as mock-passaged sixth generation 308 cells. All experiments were done using cultures with passage numbers between passage five and 15. The 308 cells described in the experiments are the mock-treated sixth generation 308 cells.

Transactivation analysis

A total of 1.5×10^5 308 cells as well as 6M90 and 6R90 variants were plated in 6-well dishes and transiently transfected in triplicate using DOTAP liposomal transfection reagent (Boehringer Mannheim, Indianapolis, IN). Luciferase reporter construct, driven by either a human collagenase TPA response element (TRE), NFκB sites from the HIV genome (NFκB) or cAMP response element (CRE) from the Intracisternal A-type Particle gene, was used at 5 μg/well. These constructs were a kind gift from Dr Zigang Dong and have been described previously (8,43). Parallel plates were transfected with the empty luciferase vector as controls. The cells were recovered after 24 h post-transfection with or without serum as described in Results. Cell lysates were prepared and analyzed by a luciferase assay as described previously (44). Luciferase activity from a given construct for the progressed variants was compared with the activity seen in 308 cells and expressed as fold activity over 308 cells. In indicated experiments, the cells were treated with antioxidants during the 24 h recovery period. Results from three independent experiments were averaged and 95% confidence intervals (CI) were determined ($P < 0.05$). Transfection efficiencies were determined independently by parallel transfections with CMV-βgal under identical conditions and calculating percentage of cells staining positive for β-galactosidase (β-gal).

DNA synthesis assay and treatment with antioxidants

A total of 2×10^4 cells/cm^2 were plated in 24-well dishes and allowed to grow for 24 h. The cultures were then synchronized by serum starvation for the next 24 h, following which the cells were re-stimulated with serum-containing media. At this point the wells were treated in triplicate with antioxidants N-acetyl-L-cysteine (NAC; Sigma), Trolox (Aldrich, Milwaukee, WI) or other agents in indicated experiments for 16 h. For Mek-1 inhibitor (PD 98059; New England Biolabs, Beverly, MA) and p38 kinase inhibitor (SB 203580; Calbiochem, La Jolla, CA) the cultures were pretreated for 1 h with the agents before shifting to serum-containing media. [^{3}H]thymidine was added to the cultures at 1 μCi/ml during the last 4 h of incubation. The cultures were then harvested and processed for [^{3}H]thymidine uptake according to published protocols (45). Average [^{3}H]thymidine uptake values were calculated as percent of untreated control samples for a cell line. Values from at least three independent experiments were averaged and 95% CI calculated. Toxicity of various agents used in the studies was determined by doing clonogenic survival assays on the cell lines under identical treatment conditions.

Inhibition of in vivo tumor growth

Cells (5×10^6) from the progressed variants were injected s.c. at a single site into athymic nude mice (BALB/c, nu/nu, Harlan Sprague–Dawley). When the tumors reached a mean size between 50 and 100 mm^2, the animals received i.p. injections of either pyrollidine dithiocarbamate (PDTC, 100 mg/kg) or saline three times per week and were observed for the rate of tumor growth. PDTC has previously been shown to be non-toxic at this dose as an antioxidant in these mice (46). Tumor area was estimated weekly as described (47).

Measurement of intracellular ROS levels

Luminol or lucigenin enhanced chemiluminescence assays were used to measure intracellular ROS levels (39,48). Cells were plated at confluent density (1×10^5/cm^2) on cover slips or 12 mm tissue culture inserts (Falcon) and allowed to grow for 24 h. The lucigenin assay was done by placing the cells in 0.25 mM lucigenin in HEPES-buffered PBS (HPBS) and the luminescence recorded immediately for 1 min with a Monolight 2010 luminometer (38). The luminol assay was done by incubating cells in 1 mg/ml luminol in HPBS and measuring luminescence using a Beckman Liquid Scintillation Counter set in a single photon emission mode (48). Background luminescence from inserts alone was subtracted in both assays. For the NAC attenuation studies, the cultures were treated with indicated concentrations of the agent for 16 h prior to the assay. Results from a representative of three experiments are shown.

Growth curves and clonogenic survival assays

Cells (5×10^5) were plated in 100 mm dishes and grown for 3 days as described. These dishes were trypsinized every 12 h in triplicate and the cells counted on a Coulter Counter. Mean cell numbers were plotted against time on a log-linear scale to generate growth curves for all three cell lines. Data from a representative of three experiments are shown. For clonogenic survival assays, 500 cells were plated in 60 mm dishes and treated in triplicate under identical conditions as DNA synthesis assays. The cultures were then grown for another 7–10 days, stained with Coomassie and the number of colonies counted (data not shown).

Isolation of nuclear protein and western analysis

Nuclear protein was isolated from subconfluent cultures using published protocols (49). Briefly, the cells were rinsed with PBS and buffer A, pelleted and then lysed with 0.1% NP-40 in buffer A. Nuclei were pelleted by centrifugation and the proteins extracted in buffer C. The extracts were stored at –80°C diluted 1:6 with buffer D until used. The protein concentrations of the extracts were determined with Bio-Rad reagent (Bio-Rad, Hercules, CA). Nuclear extract (20 μg) was resolved on a 10% SDS–polyacrylamide gel and analyzed by western blot using antibodies against p65 NFκB (1:250 dilution; Santa Cruz Biotechnology, La Jolla, CA) and Threonine–71 phosphorylated ATF-2 (1:100 dilution; New England Biolabs). Individual band intensities were quantified by digitizing the radiographs on EagleEye (Stratagene, Santa Cruz, CA) and then analyzing the image using ONEDSCAN software.

In vitro protein kinase assays

Cells from subconfluent cultures were lysed in 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Protein (100 μg) was incubated with phospho-specific ERK-1/2 antibody, phospho-specific JNK antibody or phospho-specific p38 MAPK antibody (New England Biolabs) with gentle rocking overnight at 4°C. Protein A sepharose beads were added and the mixture rotated for another 3 h, washed twice in lysis buffer and twice in kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate and 10 mM $MgCl_2$). Beads were suspended in 50 μl kinase buffer with 100 μM ATP and 0.5 μg substrate (Erk-1/2, Elk-1; JNK, c-Jun; p38 MAPK, ATF-2; Santa Cruz Biotechnology) and incubated for 30 min at 30°C. Samples were then boiled in SDS sample buffer and resolved on 12.5% SDS–polyacrylamide gels. Phosphorylated substrates were visualized by western blot using phospho-specific antibodies for the different substrates (New England Biolabs). Individual band intensities were quantified by densitometric analysis of the radiographs as before.

Results

In vitro tumor progression model

The benign papilloma-producing mouse keratinocyte cell line 308 has been shown to acquire a transformed phenotype following treatment with tumor promoters (8) and physical carcinogens (6). We used subconfluent cultures of 308 cells to establish an *in vitro* model of malignant progression using ionizing radiation and MNNG as model physical and chemical carcinogens, respectively. After six consecutive LD_{90} treatments, the survivors as well as mock-treated 308 cells were injected s.c. into athymic nude mice and followed for tumor development. The first tumors were seen 8 weeks post-injection in the MNNG group and at 10 weeks in the radiation group. By 12 weeks post-injection, more than two-thirds of the sites injected with either MNNG- or radiation-progressed survivors showed progressively growing tumors. In contrast, mock-treated 308 cells remained non-tumorigenic up to 20 weeks post-injection. Histologically, the radiation and MNNG tumors showed features consistent with moderately differentiated squamous cell carcinoma (data not shown). Randomly selected tumors from the MNNG and radiation groups were put in culture to establish cell lines. When re-injected into athymic nude mice, these MNNG (6M90) as well as radiation (6R90) tumor cell lines produced aggressively growing tumors 2–3 weeks post-injection.

Figure 1 shows the growth characteristics of these cell lines in culture. After an initial lag phase, the 6M90 and 6R90 cultures had higher growth rates as compared with 308 cells and by the end of 5 days had 35–50% more cells in growth assays ($P < 0.05$; Figure 1A). To determine if these differences in growth characteristics were statistically significant, the cell numbers were converted into their log values and plotted against time on a linear scale. The slopes of the curves were calculated by a linear regression analysis (data not shown). A Student's *t*-test was performed to determine the level of significance for the differences observed in the slopes. Based on these analyses the slopes of the curves for the malignant 6M90 and 6R90 cell lines were found to be significantly higher as compared with the benign 308 cells ($P < 0.05$). The plating efficiencies were estimated to be between 75 and 80% for all the cell lines. The cloning efficiencies for 308 cells as well as their malignant variants were also found to be very similar and did not account for the increased proliferation seen in 6M90 and 6R90 cultures (data not shown). DNA synthesis assays (Figure 1B) showed that the malignant cell lines had 30–70% elevation in [^{3}H]thymidine uptake as compared with

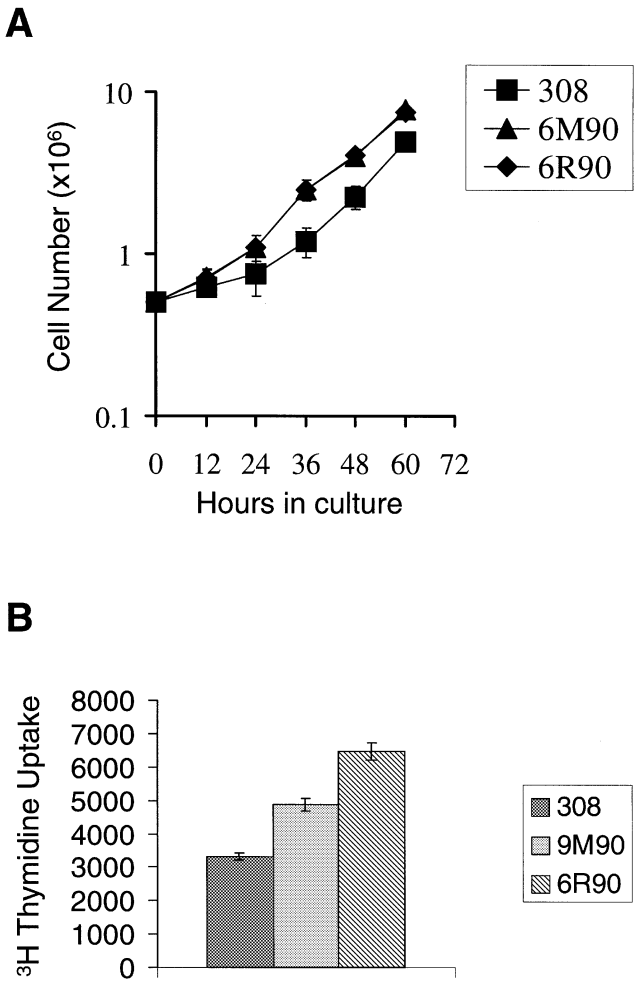


Fig. 1. The 6M90 and 6R90 malignant cell lines have a higher growth rate compared with 308 cells in culture. Cells (5×10^5) from 308 as well the malignant variant cell lines were plated and grown for 3 days. Triplicate dishes were trypsinized every 12 h and the cells counted on a Coulter Counter. (**A**) Representative growth curve from one of three independent experiments. The error bars represent standard deviation from the mean cell number. DNA synthesis in log-phase cultures (**B**) was measured using a tritiated thymidine uptake assay (Materials and methods).

308 cells ($P < 0.05$). These data suggest that the 308 malignant variants have an increase in growth fraction, which accounts for their higher growth rate in culture.

Both 6M90 and 6R90 cells have elevated NFκB and CRE transactivation as well as increased Erk-1/2 and p38 MAPK activity

To determine the transactivation potential via different *cis*-elements, cell lines were transiently transfected with luciferase constructs driven by AP-1, NFκB or CRE dependent promoters. To determine if the transactivation potential through these *cis*-elements was dependent on growth factors, luciferase activities were measured in cultures recovered either in the absence or presence of serum. Figure 2 shows the results from the luciferase assays. In the absence of serum (Figure 2A) the 6M90 showed elevated AP-1 (3.3–9.4-fold), NFκB (5.0–10.5-fold) and CRE (2.3–3.7-fold) transactivation as compared with 308 cells. The radiation-progressed 6R90 did not show any increase in AP-1 activity over 308 cells. However, statistically significant elevations in NFκB (1.7–2.8-fold) and CRE (1.8–2.9-fold) transactivation were seen ($P < 0.05$) in these cells.

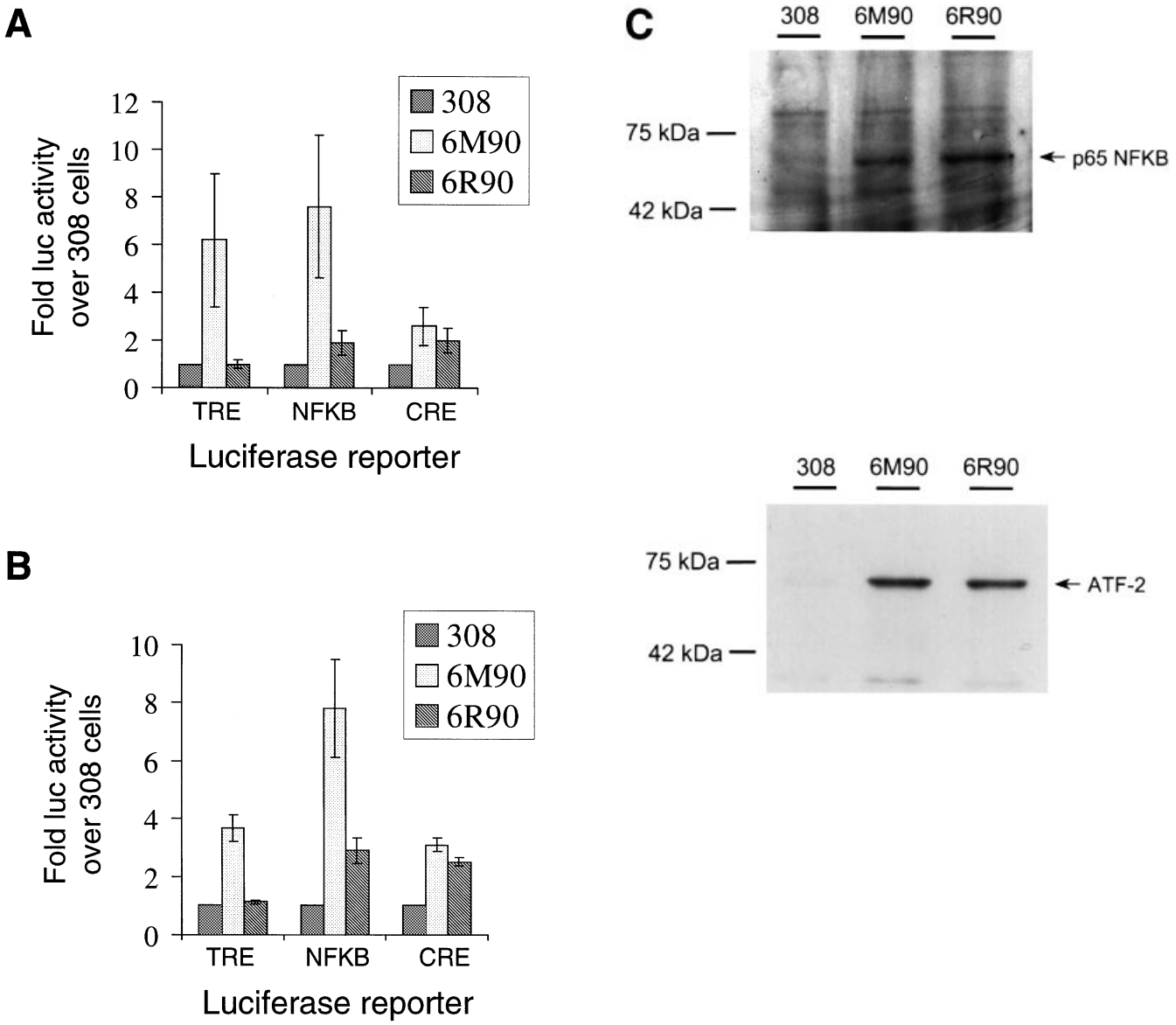


Fig. 2. Malignant variants have constitutively elevated transcription factor activities compared with benign 308 cells. Cells were transiently transfected with luciferase constructs containing a TRE, NFκB or a CRE *cis*-element. The cultures were grown either in the absence (**A**) or in the presence (**B**) of serum for 24 h after transfection and then lyzed. Total protein (30 μg) was analyzed for luciferase activity. The results are means of three independent experiments done in triplicate. Shown are average fold luciferase activity in the cell lines compared with 308 cells. The error bars represent 95% CI for the data.
(**C**) Representative western blots for p65 NFκB and phosphorylated ATF-2 in the nuclear extracts from cells 24 h following serum starvation.

In the presence of serum (Figure 2B), the progressed cell lines maintained significantly elevated transcription factor activities compared with 308 cells, suggesting that elevated transactivation potential from NFκB and CRE *cis*-elements was constitutive in 6M90 and 6R90 malignant cells. In addition, the 6M90 cell line also had a constitutive elevation of AP-1 activity. In order to determine the specificity of this elevation in transactivation potential, transfections with parent luciferase vector lacking these *cis*-elements were done. No significant differences in luciferase activities were found amongst the cell lines (data not shown). The transcription factor specificity of the luciferase constructs is also indicated by the fact that whereas significant elevation in AP-1 transactivation was detected in 6M90 cells, AP-1 activity in 6R90 variants was unaltered as compared with 308 cells. Transfection efficiencies in the three cell lines were determined to be very similar (between 7–9%) by transfecting a CMV-driven β-gal reporter in parallel experiments. Rates of overall protein synthesis, as measured by [^{35}S]methionine uptake were also found to be similar amongst the 308 and the malignant variants (data not shown).

To determine the potential mechanisms for constitutively elevated transactivation via NFκB and CRE *cis*-elements, nuclear extracts from all cell lines were analyzed for NFκB p65 and phosphorylated ATF-2 levels (Figure 2C). Significant elevations in nuclear levels of p65 NFκB were seen in both 6M90 as well as 6R90 cells (2.5–3.8-fold over 308 levels). ATF-2 phosphorylation at Thr-69 and Thr-71 within its N-terminal activation domain has been shown to stimulate its transcriptional activity (50). The 308 nuclear extracts showed barely detectable levels of transcriptionally activated ATF-2, while substantially increased levels of Thr-71 phosphorylated ATF-2 were detectable in 6M90 as well as 6R90 cells. While these findings suggested a potential mechanism of elevated

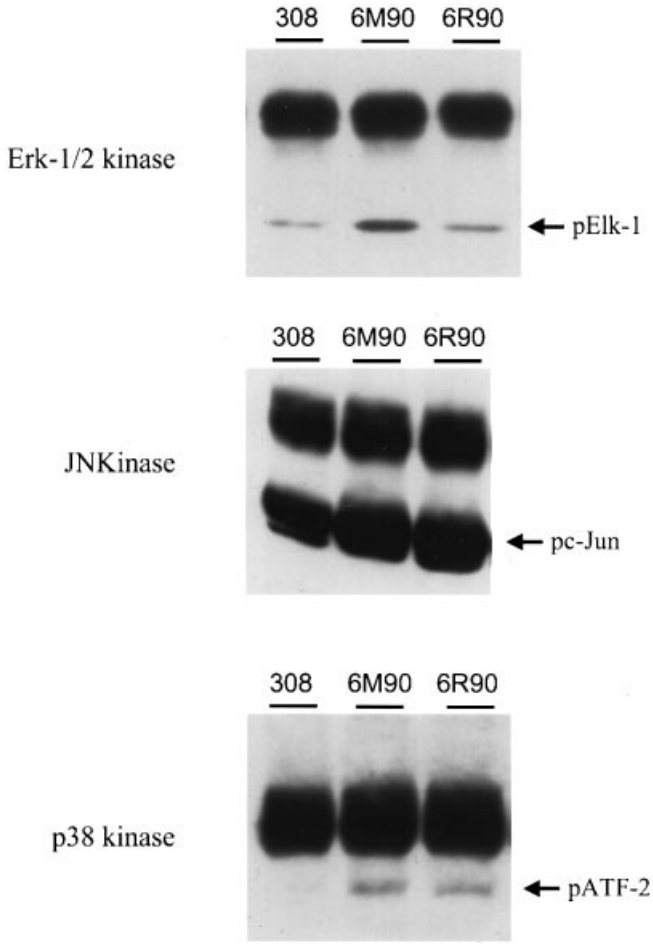


Fig. 3. MAPK activities are elevated in both 6M90 and 6R90 cell lines. Exponentially growing cultures were serum starved for 24 h. The cells were lysed and 100 μg total cell extract were immunoprecipitated with phospho-specific antibodies for Erk-1/2, JNK or p38 MAPK. *In vitro* kinase assays were performed and phosphorylated substrates were detected by western blot analysis using phospho-specific antibodies. Representative blots from three experiments are shown.

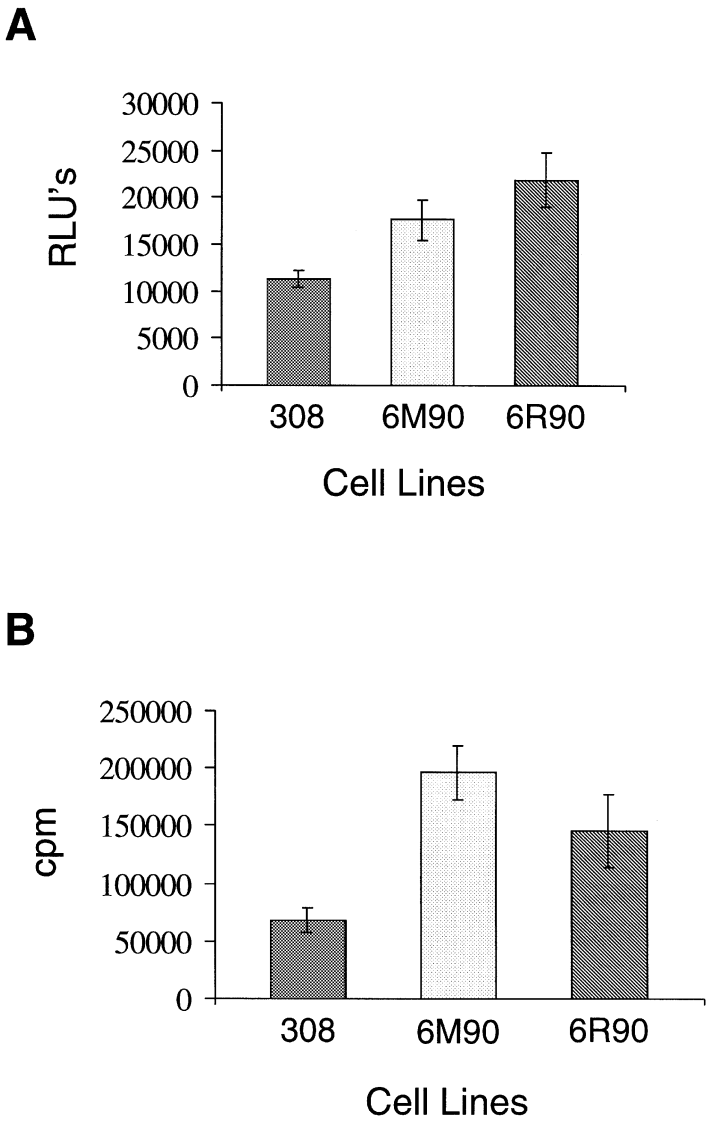


Fig. 4. Malignant variants have increased intracellular ROS levels. Confluent cultures were analyzed for intracellular ROS levels using lucigenin and luminol enhanced chemiluminescence assays. Cells (1×10^6) were grown to confluency for 24 h and placed in 0.25 mM lucigenin. Luminescence was recorded immediately for 1 min in a Monolight 2010 luminometer (**A**). For the luminol assay (**B**), cells were incubated in 1 mg/ml luminol in HPBS and the luminescence measured on a Beckman Liquid Scintillation Counter set in a single photon emmision mode. Results from a representative experiment out of three are shown. The error bars represent standard deviation of triplicates in the same experiment.

NFκB and CRE transactivation potential, the fold elevations in protein and transactivation did not appear to correlate. This suggests additional post-translational differences in NFκB and CRE complexes in these cells.

Having documented a constitutive elevation of transcription factor activities in the malignant phenotype, we then studied MAPK activities in 308 cells and the malignant variants. Total cell extracts from subconfluent cultures grown without serum were analyzed for individual MAPK activities by an *in vitro* kinase assay (Figure 3). In these assays, the 308 cells showed low levels of Erk-1/2 activity. In contrast, Erk-1/2 activity was found to be significantly elevated in 6M90 cells (2.5–4.0-fold). p38 MAPK activity was barely detectable in 308 cells under these conditions whereas both 6M90 as well as 6R90 lysates showed elevated p38 activity. JNK activity did not appear to be significantly different amongst the three cell lines. Taken together, these data show that, in this model, increased transcription factor and MAPK activities correlate with the malignantly progressed phenotype.

Malignant variants have increased intracellular ROS levels that contribute to their proliferative capacity

308 cells have an activated *ras* gene (41). Recently, ROS have been shown to be involved in *ras* signaling (38). We therefore analyzed the ROS status of 308 cells and the malignant variants. Levels of intracellular ROS were measured using lucigenin and luminol enhanced chemiluminescence assays (Figure 4A and B respectively). Lucigenin enhanced chemiluminescence has specificity for $O_2^{-}\cdot$, whereas luminol detects hydrogen peroxide and singlet oxygen generation (38,48). With both assays, the 6M90- as well as 6R90-progressed cells showed a near 2-fold elevation in steady state ROS levels (range 1.6–2.4-fold between experiments; $P = 0.05$). In general, there was a trend towards a higher chemiluminescence with lucigenin in 6R90 cells (Figure 4A), whereas the 6M90 cells showed elevated signals with luminol (Figure 4B), suggesting that there might be differences in ROS species between these cell lines.

Since acute exposure to active oxygen has been shown to

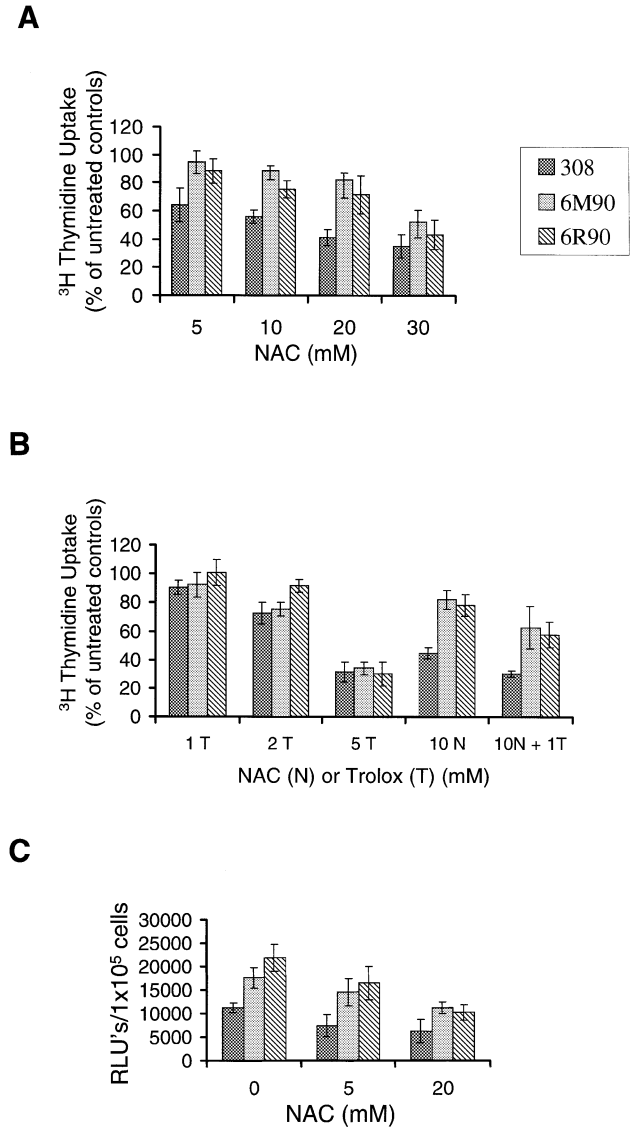


Fig. 5. Increased ROS levels contribute to the proliferative capacity of 6M90 and 6R90 cells. (**A**) Cells (40 000/well) were plated in 24-well dishes. After synchronization with serum starvation the cells were shifted in serum-containing media and grown for 16 h in the presence of increasing doses of different agents as indicated. [^{3}H]thymidine (1 μCi/ml) was added to the wells for the last 4 h of incubation. The cells were washed with PBS and processed for [^{3}H]thymidine incorporation. (**B**) Effects of combination treatments with NAC and Trolox on DNA synthesis in these cell lines. Bars represent means from three independent experiments. Error bars show 95% CI of the means. To determine the specificity of antioxidant treatment, intracellular ROS levels in the presence of increasing doses of NAC were measured by a lucigenin-enhanced chemiluminescence assay (**C**). Error bars represent standard deviation amongst triplicates.

be mitogenic (51), we hypothesized that elevated steady state ROS levels could mediate the observed increases in cell proliferation in the malignant variants. To test this hypothesis, we analyzed the effects of antioxidant treatments on DNA synthesis in the 308 cells as well as the malignant variants. NAC treatment attenuated DNA synthesis in all cell lines in a dose-dependent manner (Figure 5A). 308 cells were found to be significantly more sensitive to inhibition at equimolar doses of NAC than the progressed cell lines. Treatment with as low as 5 mM NAC resulted in a 38% (range 25–45%) inhibition in DNA synthesis in 308 cells whereas the progressed cell lines remained largely unaffected. With 20 mM NAC a 20–30% attenuation in DNA synthesis was seen in the malignant 6M90 and 6R90 cells, whereas a >50% attenuation was seen in 308 cells. Treatment with 30 mM NAC appeared to be necessary to attenuate DNA synthesis in malignant cell lines. Trolox, an analog of Vitamin E and a chemically unrelated antioxidant, was much less effective in attenuating cell proliferation as compared with NAC (Figure 5B). 308 cells were more sensitive to inhibition with 2 mM Trolox as compared with 6R90 cells. Furthermore, an ineffective (1 mM) dose of Trolox potentiated the inhibitory effects of 10 mM NAC, suggesting that these agents predominantly exerted an antioxidant effect in our assays. All the treatment doses were evaluated for toxicity by clonogenic survival assays. No significant differences in cloning efficiencies were observed between treated cells versus control cells (data not shown). We further confirmed the specificity of NAC effects by measuring ROS levels in the presence of 5 and 20 mM NAC. A dose-dependent attenuation in lucigenin enhanced chemiluminescence was seen (Figure 5C). More significantly, higher residual luminescence was observed in both 6M90 and 6R90 cell lines compared with 308 cells at both the doses tested. These data demonstrate that the malignant variants have a higher ROS level as compared with the benign 308 cells. Treatment with equimolar doses of antioxidant NAC attenuates DNA synthesis more effectively in 308 cells. At doses of up to 20 mM NAC, significantly higher residual levels of ROS persist in both 6M90 as well as 6R90 variants that maintain elevated DNA synthesis in these cells. A higher (30 mM) dose of NAC is necessary to attenuate DNA synthesis in these malignant variants to a level similar to low-dose treatment of 308 cells. Taken together, these data functionally implicate elevated ROS levels in the malignant progression of 308 cells.

To test if the tumorigenic phenotype of the malignant variants was dependent on their pro-oxidant state, we studied the effects of another antioxidant, PDTC, *in vivo* by treating athymic nude mice bearing 6M90 or 6R90 tumor xenografts. The choice of this antioxidant was based on the fact that *in vitro* treatment with 100 μM PDTC resulted in >50% attenuation of DNA synthesis in these cell lines as was seen with 30 mM NAC treatment (data not shown) and that the efficacy and safety of the PDTC dose regimen has already been established in the athymic nude mice (46). After the establishment of palpable tumors (mean tumor area 50–100 mm^2), animals received either PDTC or saline three times/week, i.p. As shown in Figure 6, PDTC treatment significantly slowed tumor growth in both 6M90 (panel A) as well as 6R90 (panel B) xenografts over 5 weeks of treatment in comparison with saline treated controls. However, complete cessation of growth or tumor regression was not seen with this treatment. Gross analysis of the cut sections of the tumors at the end of the experiment showed evidence of residual solid, potentially viable, tumor mass in PDTC-treated animals. While these results demonstrate that elevated ROS levels in these malignant variants are important for tumor growth *in vivo* as well as *in vitro*, a lack of complete abrogation of the tumorigenic phenotype points towards additional, ROS-independent mechanisms being involved in the maintenance of the phenotype.

Pro-oxidant state of 6M90 and 6R90 cell lines mediates elevated Erk-1/2 and p38 MAPK activities and, in part, elevated transcription factor activities

Having shown that the elevated ROS levels functionally contribute to the proliferative capacity of the progressed

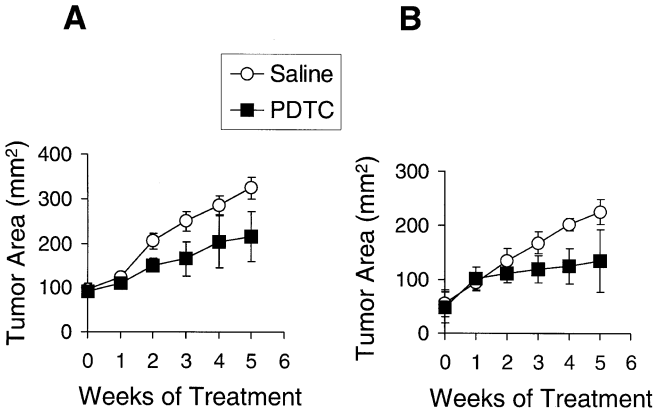


Fig. 6. Antioxidant PDTC attenuates *in vivo* tumor growth from 6M90 and 6R90 cell lines. Cells (5×10^6) from late log-phase cultures were injected s.c. into athymic nude mice. When the tumors from 6M90 (**A**) and 6R90 (**B**) cells reached a mean size of 50–100 mm^2, the mice were given thrice weekly injections of either PDTC (100 mg/kg) or saline and followed for tumor growth. Error bars represent standard deviation of the means. The data represent means from eight mice in each treatment group.

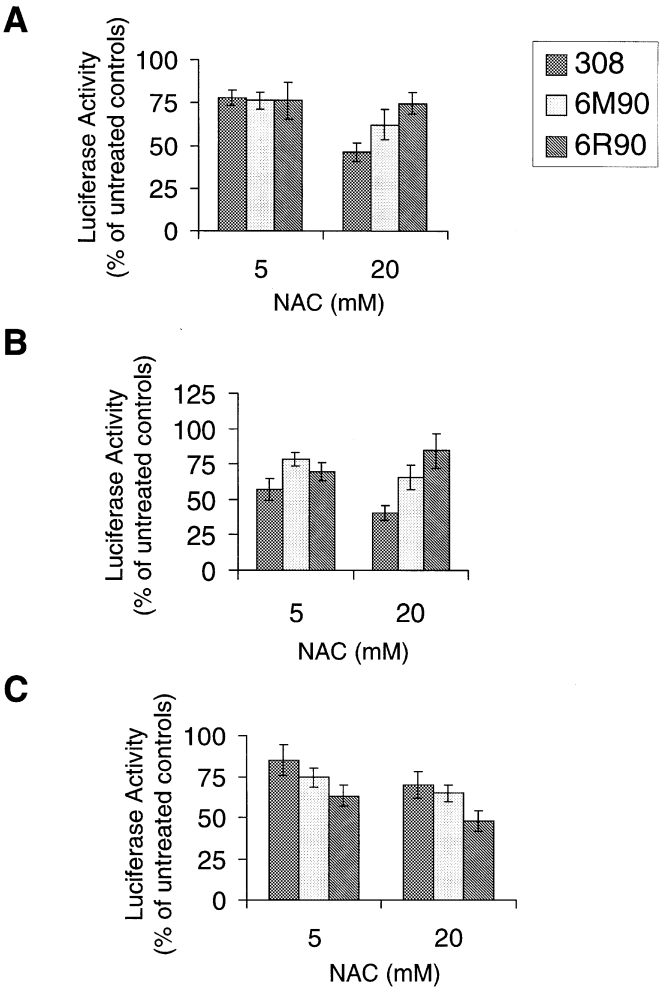


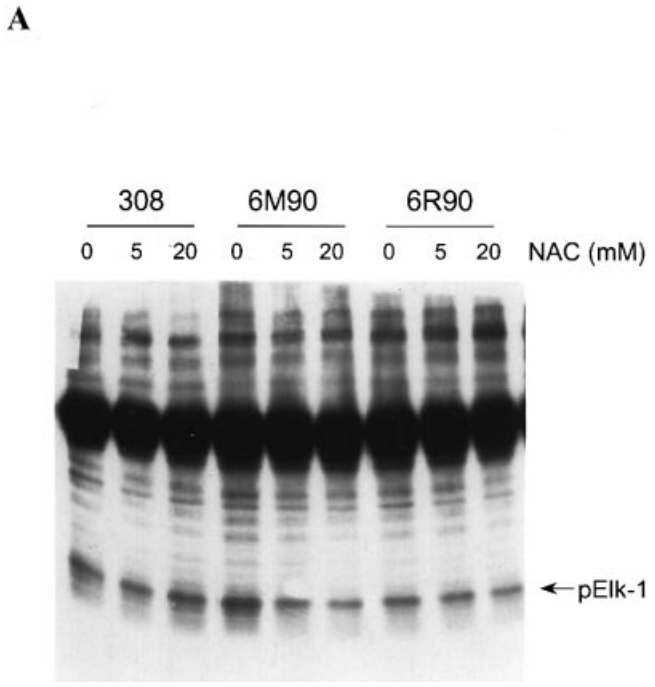
Fig. 7. NAC treatment partially attenuates transcription factor activities in all cells. Log-phase cultures were transfected with luciferase reporter constructs containing either a TRE (**A**), NFκB (**B**) or a CRE (**C**) *cis*-element as before. During recovery from transfection, they were treated with indicated doses of NAC. Luciferase activity was analyzed in the cell lysates and was expressed as a percent of untreated controls in a given cell line. Data shown represent means from three independent experiments.

variants, we proceeded to investigate the potential signaling cascades involved in this process. It has been shown previously that ROS can act as signaling molecules and can modulate transcription factor and MAPK activities (17–20,27,28). In order to determine whether elevated ROS levels signaled via these effector targets in our model, we performed attenuation studies with NAC. Figure 7 shows the effects of NAC treatment on AP-1 (graph A), NFκB (graph B) and CRE (graph C) luciferase reporter activities. NAC treatments (20 mM) resulted in a 50% inhibition of NFκB and AP-1 transactivation in 308 cells (Figure 7A and B), which correlates with the extent of inhibition of DNA synthesis with NAC in these cells. However, a majority (nearly 75%) of the AP-1 as well as NFκB transactivation in 6M90 and 6R90 cells was still detectable at this dose. This could be a reflection of higher residual ROS levels at this dose of NAC treatment. Alternately, it is possible that AP-1 and NFκB activities in the progressed phenotype are, in part, independent of the pro-oxidant state of these cells, and their constitutive elevation involves other mechanisms. The modulation of CRE-dependent transactivation with NAC was interesting in that the transactivation in 6R90 cells appeared to be attenuated to a greater extent than the 308 cells (Figure 7C), suggesting that ROS levels may significantly modulate CRE-dependent transactivation in 6R90 cells. These studies suggested that both ROS-dependent as well as ROS-independent mechanisms influenced signaling via these transcription factors. This prompted us to study the effects of NAC treatment on MAPK activities

Figure 8 shows the effects of increasing doses of NAC on Erk-1/2 and p38 MAPK activities. As was seen in serum-starved cells (Figure 3), Erk-1/2 activity in 6M90 cells was found to be elevated (1.8–2.5-fold) in cycling cells, indicating a constitutively elevated Erk-1/2 signaling cascade in these cells (Figure 8A). Furthermore, a dose-dependent attenuation of Erk-1/2 activity was seen with NAC in this MNNG-progressed malignant variant. Treatment with a 5 mM dose appeared to bring the activity level down to that seen in untreated 308 cells. p38 MAPK activity showed a dose-dependent attenuation in all three cell lines (Figure 8B). 6R90 cells showed a significant elevation of p38 activity (2.3–3.5-fold) as compared with 308 cells. Taken together, the MAPK studies show that elevation of Erk-1/2 and p38 MAPK correlate with the malignant progression of 308 cells. Studies with the antioxidant NAC suggest that elevation of steady state ROS levels in these cells may be responsible for the constitutive activation of these MAPK in the 6M90 and 6R90 malignant variants.

Both Erk-1/2 as well as p38 MAPK signaling pathways are important in maintaining cell proliferation

To investigate the role for ROS-mediated elevated Erk-1/2 and p38 MAPK in proliferative capacity of the malignant phenotype, we used pharmacological inhibitors to attenuate signaling via these kinases. We inhibited Erk-1/2 activity with PD 98059, which is a specific inhibitor of Erk-1/2 activating kinases MEK-1/2 (52). We have shown previously that 50 μM PD 98059 completely abrogates Erk-1/2 activity in 308 cells (44). We also found this to be true for both 6M90 as well as 6R90 cells (data not shown). Inhibition of Erk-1/2 activity in all cell lines resulted in a significant (35–45%) attenuation of DNA synthesis (Figure 9) suggesting that in this model system, the Erk-1/2 cascade transduces a mitogenic signal. SB 203580, a pyridinyl-imidazole compound that inhibits p38 MAPK (53)



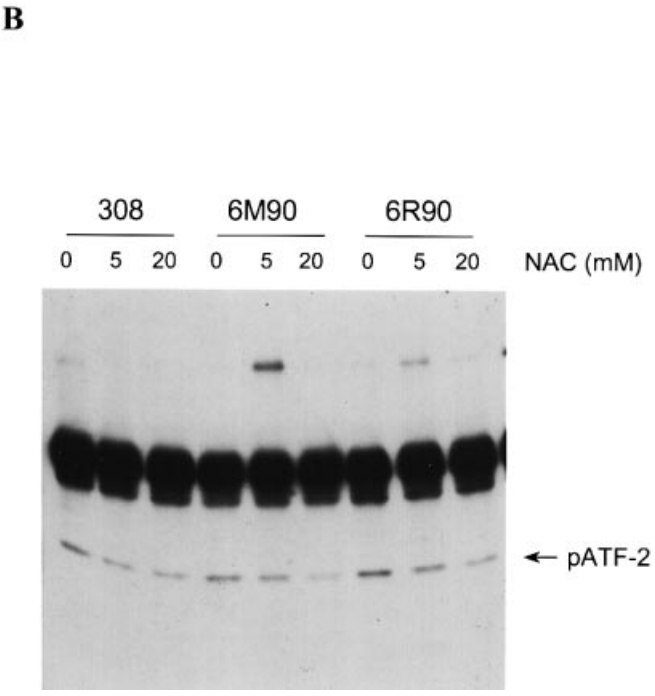


Fig. 8. NAC attenuates elevated Erk-1/2 and p38 MAPK activities in the malignant cell lines. Exponentially growing cultures were treated with increasing doses of NAC. After 16 h of treatment, cells were lysed and 100 μg of total protein were immunoprecipitated with phospho-specific antibodies for Erk-1/2 (**A**) or p38 MAPK (**B**). *In vitro* kinase assays were performed and phosphorylated substrates were detected by western blot analysis using phospho-specific antibodies. Representative blots are shown. The experiment was repeated three times.

was used to study the effects of p38 MAPK inhibition on cell proliferation. SB 203580 (20 μM) inhibited 85–90% MAPK-2 phosphorylation and did not decrease Erk-1/2 or JNK phosphorylation (data not shown) in these cells substantiating its specificity as a p38 MAPK inhibitor. The 6R90 cells were found to be extremely sensitive to p83 MAPK inhibition,

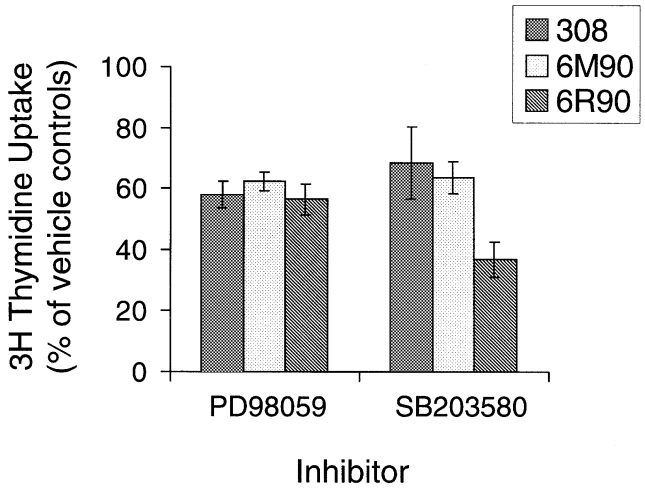


Fig. 9. Treatment with MEK-1 or p38 MAPK inhibitor reduces DNA synthesis in all cell lines. DNA synthesis was measured as before in the presence of 50 μM MEK-1 inhibitor (PD 98059) or 20 μM p38 MAPK inhibitor (SB 203580). The cells were treated with either the inhibitor or DMSO for 1 h prior to serum stimulation. [^{3}H]thymidine incorporation was measured after 16 h of treatment (Materials and methods) and expressed as percent of DMSO controls. Average [^{3}H]thymidine uptake from three independent experiments is shown. The error bars represent 95% CI of the means.

which resulted in a >50% attenuation of DNA synthesis in these cells. The MNNG-progressed 6M90 cells as well as 308 cells were also inhibited but to a lesser extent.

Discussion

Deregulated gene expression due to perturbations in signal transduction pathways can lead to abnormal cellular responses including malignant conversion. Our finding that AP-1 activity is constitutively elevated in 6M90 cells agrees with the evidence for a causal relationship between elevated AP-1 transactivation and malignant progression of murine skin tumors and human keratinocytes (6,11,16). We did not see a similar increase in 6R90 cells, suggesting the involvement of AP-1 independent mechanisms in the progression of this variant. Recently, elevated NFκB activity has also been implicated in this process (8,16). Our analysis of the 6M90 and 6R90 malignant variants extends these observations to include elevated nuclear levels of p65 subunit as a potential mediator of increased NFκB transactivation. This subunit has been shown to be necessary for optimal transactivation and induction of NFκB-dependent genes (54–56). In addition, 'cross-talk' between AP-1 and NFκB transcription factors can also occur via p65 NFκB subunit interaction with AP-1 proteins (57). These findings indicate the essential role of p65 subunit in NFκB activity and provide support for its elevated nuclear levels as a potential mechanism for increased NFκB transactivation observed in the 6M90 and 6R90 malignant variants. This increased nuclear localization could be a result of either a transcriptional induction of p65 or decreased levels of its cytoplasmic inhibitor IKBα (58). Additional post-translational modifications of p65, such as phosphorylation, have been shown to increase its DNA binding (59) and transactivation (60) potential. The contributions of these mechanisms to elevated NFκB transactivation in this model system is an area of further study.

We have made a novel observation of elevated CRE transactivation in the malignant phenotype. CRE is found in the

promoters of genes such as those responsive to protein kinase A activation (61). This octameric site preferentially binds c-Jun/ATF-2 heterodimers (62) and appears to be constitutively occupied in non-stimulated cells (63). Phosphorylation of threonine residues in the transactivation domain of ATF-2 by stress-activated protein kinases, such as p38 MAPKs, are required for transcriptional activation of this prebound complex (50). The CRE *cis*-element has been shown to play an important role in the transcriptional activation of c-Jun (63) and proteases such as uPA (64), genes that may play a functional role during tumor progression. We hypothesize that alterations in CRE activity plays a functional role in malignant progression by effecting expression of such genes.

In our transient transfection studies, we used a CMV-driven β-gal expression vector to normalize for transfection efficiencies. Initially, we cotransfected this expression plasmid with the luciferase reporter constructs and attempted to normalize the data based on the β-gal activity measured in each transfection. However, we found that the β-gal activity, as measured by an enzymatic assay, was always 2–4-fold higher in the malignant variants as compared with 308 cells (data not shown). We suspected that this could be due to differences in CMV promoter activity in the cell lines, rather than a difference in transfection efficiencies. Therefore, we employed an alternative approach and stained the cells under identical transfection conditions for β-gal expression. In these experiments between 7–9% cells stained positive for β-gal. These results suggest that the transfection efficiencies between the cell lines were indeed similar and that the difference in β-gal activity was perhaps due to CMV promoter related differences. Our contention is supported by a recent observation that activation of MAPK up-regulates expression of CMV driven transgene expression in cells (65).

MAPKs are important upstream regulators of transcription factor activities. In this study we demonstrate that the malignant variants have elevated Erk-1/2 and p38 MAPK activities that are important in maintaining optimal cell proliferation. Increased AP-1 and/or NFκB transactivation, as seen in the 6M90 cells, could be among some of the downstream effects of elevated Erk-1/2 activity. Erk-1/2 signaling has been shown to play an functional role in malignant transformation in other cell types (66,67). De-regulation of this signaling cascade may, therefore, be a critical determinant of malignant progression. AP-1 and/or NFκB transcription factors are potential downstream mediators of Erk-1/2 activation.

In our model system, we demonstrate that the 6R90 cells have a constitutively elevated p38 MAP kinase activity that is critical for its proliferative capacity. The role of p38 signaling in cellular responses to various stimuli remains poorly understood and is controversial at present. Both apoptotic (22,68,69) as well as proliferative (70) responses have been correlated with activation of p38 MAPK in various cell types. Our data indicate that p38 MAPK signaling transduces a cell survival/proliferation signal in the malignant variants. A potential explanation for these contrasting results may lie in the p38 MAPK subtypes. The p38 subfamily of MAPKs consists of p38α, p38β, p38γ and p38δ (53,71). Activation of p38 MAPKs stimulates the activity of several transcription factors including ATF-2, CHOP and MEF-2C (72). It has recently been shown that expression of p38β attenuated Fas and UV induced apoptosis. In contrast, p38α expression augmented apoptosis by these agents (73). Therefore, depending on which p38 subtype is induced or is dominant in a cell type, p38 MAPK-mediated responses may be quite different. The p38 MAPK inhibitor SB 23580, which we used in our studies, inhibits only the α and β isoforms of p38 MAPK (53,71). It is possible that both 6M90 as well as 6R90 cells have elevated p38β activity as a dominant component of p38 MAPK activity that mediates a cell survival response. Further studies are needed to characterize the p38 subtypes in modulating cellular responses.

In this study we show that both MNNG-progressed 6M90 as well as ionizing radiation-progressed 6R90 cells show elevated ROS levels that contribute to their increased proliferative capacity. Attenuation studies with the antioxidant NAC have identified Erk-1/2 and p38 MAPK as well as potential targets mediating the mitogenic response of elevated ROS levels. These results are consistent with the observations that acute exposure to X-rays or MNNG can induce MAPKs in a redox-sensitive manner (74,75). We speculate that elevated ROS levels can increase MAPK signaling during malignant progression that provides the necessary growth advantage for the emergence of the transformed phenotype. Our results suggest that elevation of transcription factor activities via MAPK activation and/or redox modulation is also a part of ROS-mediated mitogenic signaling. The mechanism by which ROS modulate the activity of MAPK is unknown at present. Recently, Irani *et al.* (38) have shown that oncogenic *ras* increases the production of superoxide anion that contributes to increased cell proliferation. However, our data are significantly different from the results of their study. ROS-induced mitogenic signaling was found to be independent of MAPKs in the NIH 3T3 fibroblast clone A6 analyzed by Irani *et al.* (38), whereas our results show that elevated Erk-1/2 and p38 MAPK activities are important components of ROS-mediated mitogenic signaling in the malignantly progressed variants. Whereas the *ras* mutation in 308 keratinocytes involves codon 61, the A6 clone caries a valine-12 mutant (38,41). We speculate that different *ras* mutants may activate different signaling cascades. Our contention is supported by studies with another *ras*-transformed 3T3 clone that carries a V12-S35 mutant and has a constitutively activated MAPK pathway (76). Our preliminary analysis of *ras* involvement in our model suggests that cell proliferation in the malignant variants is *ras* dependent (data not shown).

In summary, our results provide evidence that emergence of a pro-oxidant state plays a functional role in the malignant progression of mouse keratinocytes. We have made a novel observation of increased CRE transactivation in the tumorigenic phenotype. Our data suggest that transcription factors and MAPKs are potential targets involved in ROS-mediated mitogenic signaling and, for the first time, implicate elevated Erk-1/2 and p38 MAPK signaling in tumor progression.

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References

1. Hennings,H., Shores,R., Weick,M.L., Spangler,E.F., Tarone,R. and Yuspa, S.H. (1983) Malignant conversion of mouse skin tumors is increased by tumor initiators and unaffected by tumor promoters. *Nature*, **304**, 67–69.
2. Boutwell,R.K. (1974) The function and mechanism of promoters of carcinogenesis. *C. R. Crit. Rev. Toxicol.*, **2**, 419–443.

3. Finch,J.S. and Bowden,G.T. (1996) PCR/RFLP assay for copy number of mutant and wild-type alleles. *Biotechniques*, **21**, 1055–1060.
4. DiGiovanni,J. (1992) Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.*, **54**, 63–128.
5. Dong,Z., Birrer,M.J., Watts,R.G., Matrisian,L.M. and Colburn,N.H. (1994) Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc. Natl Acad. Sci. USA*, **91**, 609–613.
6. Domann,F.E.Jr, Levy,J.P., Finch,J.S. and Bowden,G.T. (1994) Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. *Mol. Carcinog.*, **9**, 61–66.
7. Li,J.J., Dong,Z., Dawson,M.I. and Colburn,N.H. (1996) Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. *Cancer Res.*, **56**, 483–489.
8. Dong,Z., Crawford,H.C., Lavrovsky,V., Taub,D., Watts,R., Matrisian,L.M. and Colburn,N.H. (1997) A dominant negative mutant of jun blocking 12-*O*-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. *Mol. Carcinog.*, **19**, 204–212.
9. Karin,M., Liu,Z.G. and Zandi,E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.*, **9**, 240–246.
10. Watts,R.G., Ben-Ari,E.T., Bernstein,L.R., Birrer,M.J., Winterstein,D., Wendel,E. and Colburn,N.H. (1995) c-*jun* and multistage carcinogenesis, association of overexpression of introduced c-*jun* with progression toward a neoplastic endpoint in mouse JB6 cells sensitive to tumor promoter-induced transformation. *Mol. Carcinog.*, **13**, 27–36.
11. Domann,F.E., Levy,J.P., Birrer,M.J. and Bowden,G.T. (1994) Stable expression of a c-Jun deletion mutant in two malignant mouse epidermal cell lines blocks tumor formation in nude mice. *Cell Growth Differ.*, **5**, 9–16.
12. Bernstein,L.R. and Colburn,N.H. (1989) AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science*, **244**, 566–569.
13. Rosenberger,S.F. and Bowden,G.T. (1996) Okadaic acid stimulated TRE binding activity in a papilloma producing mouse keratinocyte cell line involves increased AP-1 expression. *Oncogene*, **12**, 2301–2308.
14. Baeuerle,P.A. and Baltimore,D. (1996) NF-kappaB: ten years after. *Cell*, **87**, 13–20.
15. Li,J.J., Westergaard,C., Ghosh,P. and Colburn,N.H. (1997) Inhibitors of both nuclear factor-kappaB and activator protein-1 activation block the neoplastic transformation response. *Cancer Res.*, **57**, 3569–3576.
16. Li,J.J., Rhim,J.S., Schlegel,R., Vousden,K.H. and Colburn,N.H. (1998) Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene*, **16**, 2711–2721.
17. Saran,M., Michel,C. and Bors,W. (1998) Radical functions *in vivo*. A critical review of current concepts and hypotheses. *Z Naturforch.*, **53c**, 210–227.
18. Flohe,L., Brigelius-Flohe,R., Saliou,C., Traber,M.G. and Packer,L. (1997) Redox regulation of NF-kappa B activation. *Free Radic. Biol. Med.*, **22**, 1115–1126.
19. Abate,C., Patel,L., Rauscher,F.J.III and Curran,T. (1990) Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science*, **249**, 1157–1161.
20. Amstad,P.A., Krupitza,G. and Cerutti,P.A. (1992) Mechanism of c-*fos* induction by active oxygen. *Cancer Res.*, **52**, 3952–3960.
21. Su,B. and Karin,M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.*, **8**, 402–411.
22. Xia,Z., Dickens,M., Raingeaud,J., Davis,R.J. and Greenberg,M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–1331.
23. Wang,X., Martindale,J.L., Liu,Y. and Holbrook,N.J. (1998) The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem. J.*, **333**, 291–300.
24. Karin,M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.*, **270**, 16483–16486.
25. Lee,F.S., Hagler,J., Chen,Z.J. and Maniatis,T. (1997) Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**, 213–222.
26. Schwenger,P., Alpert,D., Skolnik,E.Y. and Vilcek,J. (1998) Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IkappaB alpha phosphorylation and degradation. *Mol. Cell. Biol.*, **18**, 78–84.
27. Guyton,K.Z., Liu,Y., Gorospe,M., Xu,Q. and Holbrook,N.J. (1996) Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. *J. Biol. Chem.*, **271**, 4138–4142.
28. Wilmer,W.A., Tan,L.C., Dickerson,J.A., Danne,M. and Rovin,B.H. (1997) Interleukin-1beta induction of mitogen-activated protein kinases in human mesangial cells. Role of oxidation. *J. Biol. Chem.*, **272**, 10877–10881.
29. Cerutti,P. (1988) Oxidant tumor promoters. In Colburn,N.H., Moses,H. and Stanbridge,E.J. (eds) *Growth Factors, Tumor Promotors and Cancer Genes*. Alan R.Liss, New York, NY, pp. 239–247.
30. Oberley,L. and Oberley,T. (1986) Free radicals cancer and aging. In Johnson,J.E., Walford,R.Jr, Harman,D. and Miguel,J. (eds) *Free radicals, Aging and Degenerative Diseases*. Alan R.Liss, New York, NY, pp. 325–371.
31. Palozza,P., Agostara,G., Piccioni,E. and Bartoli,G.M. (1994) Different role of lipid peroxidation in oxidative stress-induced lethal injury in normal and tumor thymocytes. *Arch. Biochem. Biophys.*, **312**, 88–94.
32. Loeb,L., James,E., Waltersdorph,A., Stokes,K. and Klebanoff,S. (1988) Mutagenisis by oxygen free radicals *in vitro*. *Proc. Am. Assoc. Cancer Res.*, **28**, 162–168.
33. Troll,W. and Wiesner,R. (1985) The role of oxygen radicals as a possible mechanism of tumor promotion. *Annu. Rev. Pharmacol. Toxicol.*, **25**, 509–528.
34. Cerutti,P. (1986) The role of active oxygen in tumor promotion. In Harris,C. (ed.) *Biochemical and Molecular Epidemiology of Cancer*. Alan R.Liss, New York, NY, pp. 167–176.
35. Kennedy,A., Troll,W. and Little,J. (1984) Role of free radicals in the initiation and promotion of radiation transformation *in vitro*. *Carcinogenesis*, **5**, 1213–1218.
36. Amstad,P., Liu,H., Ichimiya,M., Berezesky,I.K. and Trump,B.F. (1997) Manganese superoxide dismutase inhibits soft agar growth in JB6 clone 41 mouse epidermal cells. *Carcinogenesis*, **18**, 479–484.
37. Troll,W., Frenkel,K. and Teebor,G. (1983) Free oxygen radicals. Necessary contributions to tumor promotion and cocarcinogenesis. In Fujiki,H. (ed) *Cellular Interactions by Environmental Tumor Promoters*. VNU Science Press, Utrecht, The Netherlands, pp. 207–210.
38. Irani,K., Xia,Y., Zweier,J.L., Sollott,S.J., Der,C.J., Fearson,E.R., Sundaresan,M., Finkel,T. and Goldschmidt-Clermont,P.J. (1997) Mitogenic signaling mediated by oxidants in *ras*-transformed fibroblasts. *Science*, **275**, 1649–1652.
39. Yuspa,S.H. (1998) The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. *J. Dermatol. Sci.*, **17**, 1–7.
40. Ananthaswamy,H.N. and Pierceall,W.E. (1990) Molecular mechanisms of ultraviolet radiation carcinogenesis. *Photochem. Photobiol.*, **52**, 1119–1136.
41. Strickland,J.E., Greenhalgh,D.A., Koceva-Chyla,A., Hennings,H., Restrepo,C., Balaschak,M. and Yuspa,S.H. (1988) Development of murine epidermal cell lines which contain an activated *ras*Ha oncogene and form papillomas in skin grafts on athymic nude mouse hosts. *Cancer Res.*, **48**, 165–169.
42. Gupta,A., Andrews,K.L., McDaniel,K.M., Nagle,R.B. and Bowden,G.T. (1999) Experimental induction of rhabdomyosarcoma in mice with fractionated doses of β-irradiation. *J. Cancer Res. Clin. Oncol.*, **125**, 257–267.
43. Galien R., Emanoil-Ravier, R. and Mercier, G. (1994) Differential effects of c-*jun* and CREB on c-AMP response activation by Ha-*ras*. *Oncogene*, **9**, 1101–1108.
44. Rosenberger,S.F., Finch,J.S., Gupta,A. and Bowden,G.T. (1999) Extracellular signal regulated kinase (ERK) 1/2 mediated phosphorylation of JunD and FosB is required for okadaic acid induced AP-1 activation. *J. Biol. Chem.*, **274**, 1124–1130.
45. Game,S.M., Huelsen,A., Patel,V., Donnelly,M., Yeudall,W.A., Stone,A., Fusenig,N.E. and Prime,S.S. (1992) Progressive abrogation of TGF-beta 1 and EGF growth control is associated with tumour progression in *ras*-transfected human keratinocytes. *Int. J. Cancer*, **52**, 461–470.
46. Chinery,R., Beauchamp,R.D., Shyr,Yu., Kirkland,S.C., Coffey,R.J. and Morrow,J.D. (1998) Antioxidants reduce cyclooxygenase-2 expression, prostaglandin production and proliferation in colorectal cancer cells. *Cancer Res.*, **58**, 2323–2327.
47. Barthelman,M., Chen,W., Gensler,H.L., Huang,C., Dong,Z. and Bowden, G.T. (1998) Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation. *Cancer Res.*, **58**, 711–716.
48. Rathakrishnan,C. and Tiku,M.L. (1993) Lucigenin-dependent chemiluminescence in articular chondrocytes. *Free Radic. Biol. Med.*, **15**, 143–149.
49. Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Accurate transcriptional initiation by RNA polymerase II in a soluble extract from isolated nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
50. Livingstone,C., Patel,G. and Jones,N. (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.*, **14**, 1785–1797.
51. Muehlematter,D., Larsson,R. and Cerutti,P. (1988) Active oxygen induced DNA strand breakage and poly ADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells. *Carcinogenesis*, **9**, 239–245.

52. Pang,L., Sawada,T., Decker,S.J. and Saltiel,A.R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.*, **270**, 13585–13588.
53. Lee,J.C., Laydon,J.T., McDonnell,P.C., Gallagher,T.F., Kumar,S., Green,D., McNulty,D., Blumenthal,M.J., Heys,J.R., Landvatter,S.W., Strickler,J.E., McLaughlin,M.M., Siemens,I.R., Fisher,S.M., Livi,G.P., White,J.R., Adams,J.L. and Young,P.R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.
54. Schmitz,M.L. and Baeuerle,P.A. (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J.*, **10**, 3805–3817.
55. Collins,T., Read,M.A., Neish,A.S., Whitley,M.Z., Thanos,D. and Maniatis,T. (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J.*, **9**, 899–909.
56. Beg,A.A., Sha,W.C., Bronson,R.T., Ghosh,S. and Baltimore,D. (1995) Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature*, **376**, 167–170.
57. Stein,B., Baldwin,A.S.Jr, Ballard,D.W., Greene,W.C., Angel,P. and Herrlich,P. (1993) Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. *EMBO J.*, **12**, 3879–3891.
58. Baldwin,A.S.Jr (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Ann. Rev. Immunol.*, **14**, 649–683.
59. Naumann,M. and Scheidereit,C. (1994) Activation of NF-kappa B *in vivo* is regulated by multiple phosphorylations. *EMBO J.*, **13**, 4597–4607.
60. Vanden Berghe,W., Plaisance,S., Boone,E., De Bosscher,K., Schmitz,M.L., Fiers,W. and Haegeman,G. (1998) p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.*, **273**, 3285–3290.
61. Karin,M. and Smeal,T. (1992) Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends Biochem. Sci*., **17**, 418–422.
62. Price,M.A., Rogers,A.E. and Treisman,R. (1995) Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *EMBO J.*, **14**, 2589–2601.
63. Herr,I., van Dam,H. and Angel,P. (1994) Binding of promoter-associated AP-1 is not altered during induction and subsequent repression of the c-*jun* promoter by TPA and UV irradiation. *Carcinogenesis*, **15**, 1105–1113.
64. De Cesare,D., Vallone,D., Caracciolo,A., Sassone-Corsi,P., Nerlov,C. and Verde,P. (1995) Heterodimerization of c-Jun with ATF-2 and c-Fos is required for positive and negative regulation of the human urokinase enhancer. *Oncogene*, **11**, 365–376.
65. Bruening,W., Giasson,B., Mushynski,W. and Durham,H.D. (1998) Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter. *Nucleic Acids Res*., **26**, 486–489.
66. Huang,C., Ma,W.Y., Young,M.R., Colburn,N. and Dong,Z. (1998) Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. *Proc. Natl Acad. Sci. USA*, **95**, 156–161.
67. Cowley,S., Paterson,H., Kemp,P. and Marshall,C.J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, **77**, 841–852.
68. Cobb,M.H. and Goldsmith,E.J. (1995) How MAP kinases are regulated. *J. Biol. Chem*., **270**, 14843–14846.
69. Schwenger,P., Bellosta,P., Vietor,I., Basilico,C., Skolnik,E.Y. and Vilcek,J. (1997) Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. *Proc. Natl Acad. Sci. USA*, **94**, 2869–2873.
70. Ushio-Fukai,M., Alexander,R.W., Akers,M. and Griendling,K.K. (1998) p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. *J. Biol. Chem.*, **273**, 15022–15029.
71. Cuenda,A., Cohen,P., Buee-Scherrer,V. and Goedert,M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.*, **16**, 295–305.
72. Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, **78**, 1027–1037.
73. Nemoto,S., Xiang,J., Huang,S. and Lin,A. (1998) Induction of apoptosis by SB202190 through inhibition of p38 beta mitogen-activated protein kinase. *J. Biol. Chem.*, **273**, 16415–16420.
74. Wilhelm,D., Bender,K., Knebel,A. and Angel,P. (1997) The level of intracellular glutathione is a key regulator for the induction of stress activated signal transduction pathways including Jun N-terminal kinases and p38 kinase by alkylating agents. *Mol. Cell Biol*., **17**, 4792–4800.
75. Stevenson,M.A., Pollock,S.S., Coleman,N. and Calderwood,S.K. (1994) X-irradiation, phorbol esters, and H_2O_2 stimulate mitogen-activated protein kinase activity in NIH 3T3 cells through the formation of reactive oxygen intermediates. *Cancer Res.*, **54**, 12–15.
76. White,M.A., Nicolette,C., Minden,A., Polverino,A., Van Aelst,L., Karin,M. and Wigler,M.H. (1995) Multiple Ras functions can contribute to mammalian cell transformation. *Cell*, **80**, 533–541.

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