Mitogen- and Stress-Activated Kinase 1–Mediated Histone H3 Phosphorylation Is Crucial for Cell Transformation

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Abstract

Mitogen- and stress-activated kinase 1 (MSK1) belongs to a family of dual protein kinases that are activated by either extracellular signal-regulated kinase or p38 mitogen-activated protein kinases in response to stress or mitogenic extracellular stimuli. The physiologic role of MSK1 in malignant transformation and cancer development is not well understood. Here, we report that MSK1 is involved in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced or epidermal growth factor (EGF)–induced neoplastic transformation of JB6 Cl41 cells. H89, a potent inhibitor of MSK1, strongly suppressed TPA-induced or EGF-induced cell transformation. When cells overexpressing wild-type MSK1 were treated with TPA or EGF, colony formation increased substantially compared with untreated cells or cells that did not overexpress MSK1. In contrast, MSK1 COOH terminal or NH_2 terminal dead dominant negative mutants dramatically suppressed cell transformation. Introduction of small interfering RNA-MSK1 into JB6 Cl41 cells resulted in suppressed TPA-induced or EGF-induced cell transformation. In addition, cell proliferation was inhibited in MSK1 knockdown cells compared with MSK1 wild-type cells. In wild-type MSK1-overexpressing cells, activator protein (AP-1) activation increased after TPA or EGF stimulation, whereas AP-1 activation decreased in both MSK1 dominant-negative mutants and in MSK1 knockdown cells. Moreover, TPA-induced or EGF-induced phosphorylation of histone H3 at Ser^{10} was increased in wild-type cells but the induced phosphorylation was abolished in MSK1 dominant-negative mutant or MSK1 knockdown cells. Thus, MSK1 is required for tumor promoter-induced cell transformation through its phosphorylation of histone H3 at Ser^{10} and AP-1 activation. [Cancer Res 2008;68(7):2538–47]

Introduction

The mitogen-activated protein kinase (MAPK) cascades are required for many cellular functions, including growth, proliferation, differentiation, inflammation, apoptosis, and malignant transformation (1–3). The extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 MAPK signaling pathways are among the best characterized of these intracellular protein kinase cascades (4). Mitogen-activated and stress-activated kinase 1 (MSK1) is a serine/threonine protein kinase that is activated by either ERKs or p38 MAPKs in response to stress or mitogenic extracellular stimuli. MSK1 is involved in the transcriptional activation of genes, including the inflammatory gene *interleukin-6* and immediate early response genes, such as *c-fos, c-jun, MAPK phosphatase-1* (*mkp-1*), and *nurr1* (5, 6). MSK1 has also been reported to phosphorylate chromatin proteins histone H3 and high mobility group 14 (HMG-14; ref. 7). Further, MSK1 phosphorylates various transcription factors including cyclic AMP (cAMP)-response element-binding protein (cAMP-responsive element binding protein (CREB)), activating transcription factor 1 (ATF1) and the p65 subunit of nuclear factor-κB (NF-κB; refs. 8–10). Recently, MSK1 was shown to play a positive role in the control of cell proliferation of HaCaT keratinocytes (11) and might play a role in pathogenesis of psoriasis by regulating the production of proinflammatory cytokines in psoriatic epidermis (12). MSK1 was suggested to be activated in response to tumor promoters, such as epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or UVB (7, 13, 14). However, the role of MSK1 in tumorigenesis or cell transformation is not completely clear.

Here, we used MSK1 dominant-negative or small interfering RNA (siRNA)–MSK1 knockdown cells to show that MSK1 is involved in TPA-induced or EGF-induced cell transformation of JB6 Cl41 cells. Results indicated that blocking MSK1 suppressed anchorage-independent transformation of JB6 cells through the inhibition of both phosphorylation of histone H3 and AP-1 activation. Thus, we showed that MSK1 is required for tumor promoter-induced cell transformation.

Materials and Methods

Reagents and antibodies. Chemical reagents, including Eagle's MEM, L-glutamine, and gentamicin, were purchased from Invitrogen. TPA and EGF were purchased from Sigma-Aldrich and EMD Biosciences, Inc., respectively. Fetal bovine serum (FBS) was from Atlanta Biologicals and the polyvinylidene difluoride (PVDF) membrane was from Millipore. The DNA ligation kit (version 2.0) was purchased from Takara Bio, Inc. Antibodies against phosphorylated or total MSK1 were purchased from Cell Signaling Technology, Inc. and Santa Cruz Biotechnology, Inc., respectively. Antibodies to detect histone H3 or phosphorylated histone H3 were purchased from Upstate Biotechnology.

Cell lines and cell cultures. JB6 Cl41 mouse epidermal skin cells and stably transfected JB6 cells were grown in 5% FBS/MEM supplemented with penicillin/streptomycin (100 units/mL; Invitrogen) at 37°C in a humidified 5% CO_2 incubator. The pCMV5-Flag vector, pCMV5-Flag-wild-type MSK1, pCMV-Flag-MSK1-A195/NH_2 terminal kinase dead, and pCMV-Flag-MSK1-A565/COOH terminal kinase dead (generous gifts from Dr. D.R. Alessi, Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee; ref. 8) were each transfected into JB6 Cl41 cells. Stable cell lines were generated and characterized according to previous methods (13).

Construction of siRNA MSK1 and establishing stable cell lines. To construct the siRNA-MSK1 (si-MSK1), the pU6pro vector (a gift kindly

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provided by Dr. David L. Turner, Mental Health Research Institute, University of Michigan; si-MOCK) was digested with *Xba*I and *Bbs*I. The annealed synthetic primers (primer 1, 5′-TTTGAGACCTACTTCAGCGTCTCTTCAAGAGAGAGACGCTGAAGTAGGTCTTTTTT-3′; primer 2, 5′-CTAGAAAAAAGACCTACTTCAGCGTCTCTCTCTTGAAGAGACGCTGAAGTAGGTCT-3′) were then introduced following the recommended protocols found online.[3] The recombinant plasmid of psi-MSK1 was confirmed by agarose gel electrophoresis and DNA sequencing (GENEWIZ, Inc.). Stable JB6 Cl41 cells expressing pU6pro (si-MOCK) or si-MSK1 were established with pcDNA3.1 as the selection marker. Transfected cells were selected in medium containing 400 μg/mL G418, and the expression level of the MSK1 protein was confirmed by Western blot analysis.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium assay. To estimate cell proliferation, stable psi-MSK1 JB6 Cl41 cells (1×10^3) were seeded into 96-well plates in 100 μL of 5% FBS/MEM and incubated at 37°C in a 5% CO_2 incubator. After culturing for various periods of time, 20 μL of the CellTiter 96 Aqueous One Solution (Promega) were added to each well and cells were then incubated for 1 h at 37°C in a 5% CO_2 atmosphere. To stop the reaction, 25 μL of a 10% SDS solution were added, and absorbance was measured at 492 and 690 nm.

Anchorage-independent cell transformation assay. TPA-induced or EGF-induced cell transformation was investigated in JB6 Cl41 and MSK1 dominant-negative cells and psi-MSK1 stably transfected cells. In brief, cells (8×10^3) were exposed to TPA (20 ng/mL) or EGF (10 ng/mL) in 1 mL of 0.3% Basal Medium Eagle (BME) agar containing 10% FBS. The cultures were maintained at 37°C in 5% CO_2 incubator for 10 d (EGF) or 12 d (TPA), and cell colonies were scored using a microscope and the Image-Pro PLUS (v.4) computer software program (Media Cybernetics).

Cell cycle analysis. psi-MOCK or psi-MSK1 stably transfected JB6 Cl41 cells (2×10^5) were seeded into 60-mm dishes and cultured for 24 h at 37°C in a 5% CO_2 incubator. The cells were harvested with trypsin, fixed with ice-cold methanol, stained with propidium iodide (PI), and then analyzed for cell cycle phase by flow cytometry.

Reporter gene assays. For reporter gene assays, transient transfections were carried out using jetPEI (Qbiogene) and cells were cotransfected in triplicate with 250 ng of the *AP-1 reporter* gene and 50 ng of a β-galactosidase (β-Gal)–expressing plasmid. The β-Gal–expressing plasmid was cotransfected for normalizing the transfection efficiency. Routinely, 36 h after transfection, cells were washed and then starved 24 h in 0.1% FBS/MEM, and cell lysates were then prepared 12 h after stimulation with TPA (20 ng/mL) or EGF (10 ng/mL). Luciferase and β-Gal activities were measured using the Luminoskan Ascent (Thermo Electron) and Multiskan MS (Labsystems), respectively.

Isolation of histone proteins. For the isolation of histone proteins, cells (6×10^6) were homogenized in 0.5 mL nuclear preparation buffer [10 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.65% NP40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] in the presence of protein phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 25 mmol/L β-glycerophosphate). Nuclei were recovered by centrifugation at $1,500 \times g$ for 15 min. All centrifugations were carried out at 4°C. Nuclei were resuspended in 0.3 mL resuspension buffer [10 mmol/L Tris-HCl (pH 7.6), 3 mmol/L $MgCl_2$, 10 mmol/L NaCl, 1 mmol/L PMSF, protein phosphatase inhibitors]. Nuclei were extracted with 0.4 N H_2SO_4 to isolate total histone proteins. The samples were precipitated with trichloroacetic acid and then resuspended in double-distilled H_2O.

***In vitro* kinase assay.** The histone H3 protein was used for an *in vitro* kinase assay with active MSK1 (Upstate Biotechnology, Inc). Reactions were carried out at 30°C for 30 min in a mixture containing 50 μmol/L unlabeled ATP and then stopped by adding 6× SDS sample buffer. Samples were boiled and then separated by 15% SDS-PAGE and visualized by Western blotting.

Western blot analysis. Samples containing equal amounts of protein were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated in blocking buffer and hybridized with the appropriate primary antibodies to detect phosphorylated MSK1, total MSK1, phosphorylated histone H3 (Ser^{10} or Ser^{28}), total histone H3, or β-actin. The Western blots were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences Corp.) after hybridization with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody.

[3] http://www.ambion.com/techlib/misc/siRNA_finder.html

Results

TPA or EGF induces MSK1 activation and cell transformation. TPA and EGF are well-known tumor promotion agents used to study malignant transformation in cells and carcinogenesis in animal models (15). By using the JB6 mouse epidermal cell system, we found that TPA or EGF was very effective in dose-dependently promoting the transformation of JB6 Cl41 cells (Fig. 1*A* and *B*). Results also indicated that TPA or EGF caused phosphorylation of MSK1 (Fig. 1*C* and *D*) at the same dose, as was effective for inducing cell transformation. These results suggested that MSK1 phosphorylation might be involved in TPA-induced or EGF-induced cell transformation.

H89, a potent inhibitor of MSK1, suppresses TPA-induced or EGF-induced AP-1 activation and cell transformation. H89 {*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonaime} has been shown to have a potent and selective inhibitory action against cyclic AMP-dependent protein kinase (protein kinase A; ref. 16). H89 also was reported to strongly inhibit MSK1 activity *in vitro* or *in vivo* (17, 18). To test whether H89 could affect cell transformation, JB6 Cl41 cells were incubated with TPA (20 ng/mL) or EGF (10 ng/mL) and various concentrations of H89. Our results showed that H89 dramatically suppressed TPA-induced or EGF-induced cell transformation in JB6 Cl41 cells (Fig. 2*A* and *B*), which supports a role for MSK1.

The AP-1 transcription factor is a dimeric complex that comprises members of the Jun, Fos, ATF1 (activating transcription factor 1), and musculoaponeurotic fibrosarcoma protein families (19). The regulation of cell proliferation by AP-1 is believed to be of crucial importance for the multistage development of tumors (20, 21). To test whether the inhibition of MSK1 by H89 might contribute to the AP-1 activation response to TPA or EGF, we cotransfected the *AP-1 luciferase reporter* plasmid and the *pCMV-β-Gal* gene into JB6 Cl41 cells. Our data showed that H89 effectively suppressed TPA-promoted (Fig. 2*C, left*) or EGF-promoted (Fig. 2*C, right*) AP-1 activation in JB6 cells. We also found that TPA-induced (Fig. 2*D, left*) or EGF-induced (Fig. 2*D, right*) *c-fos* promoter activity in JB6 Cl41 cells was decreased by treatment with H89. These data indicated that H89, a potent inhibitor of MSK1, is very effective in preventing TPA-induced or EGF-induced cell transformation and AP-1 transactivation activity.

A dominant-negative mutant of MSK1 suppresses TPA-induced or EGF-induced cell transformation and AP-1 activation. The COOH terminal kinase domain of MSK1 is essential for activation of its NH_2 terminal domain and an inactivation mutation either in the NH_2 terminal or COOH terminal kinase domain completely abolishes MSK1 activation (8). To further investigate whether MSK1 is directly responsible for TPA-induced or EGF-induced cell transformation, we used JB6 cells transfected with plasmids containing a pCMV5-FLAG vector (pCMV-neo), pCMV5-FLAG-wild-type MSK1 (pCMV-MSK1 WT), pCMV5-FLAG-MSK1-A565 COOH terminal kinase-dead (pCMV-MSK1 CD), or pCMV-FLAG-MSK1-A195 NH_2 terminal kinase-dead (pCMV-MSK1 ND; ref. 13). These cells were treated with TPA (20 ng/mL) or EGF (10 ng/mL) in soft agar and incubated at 37°C in a 5% CO_2 incubator for 10 to 12 days, at which time colony

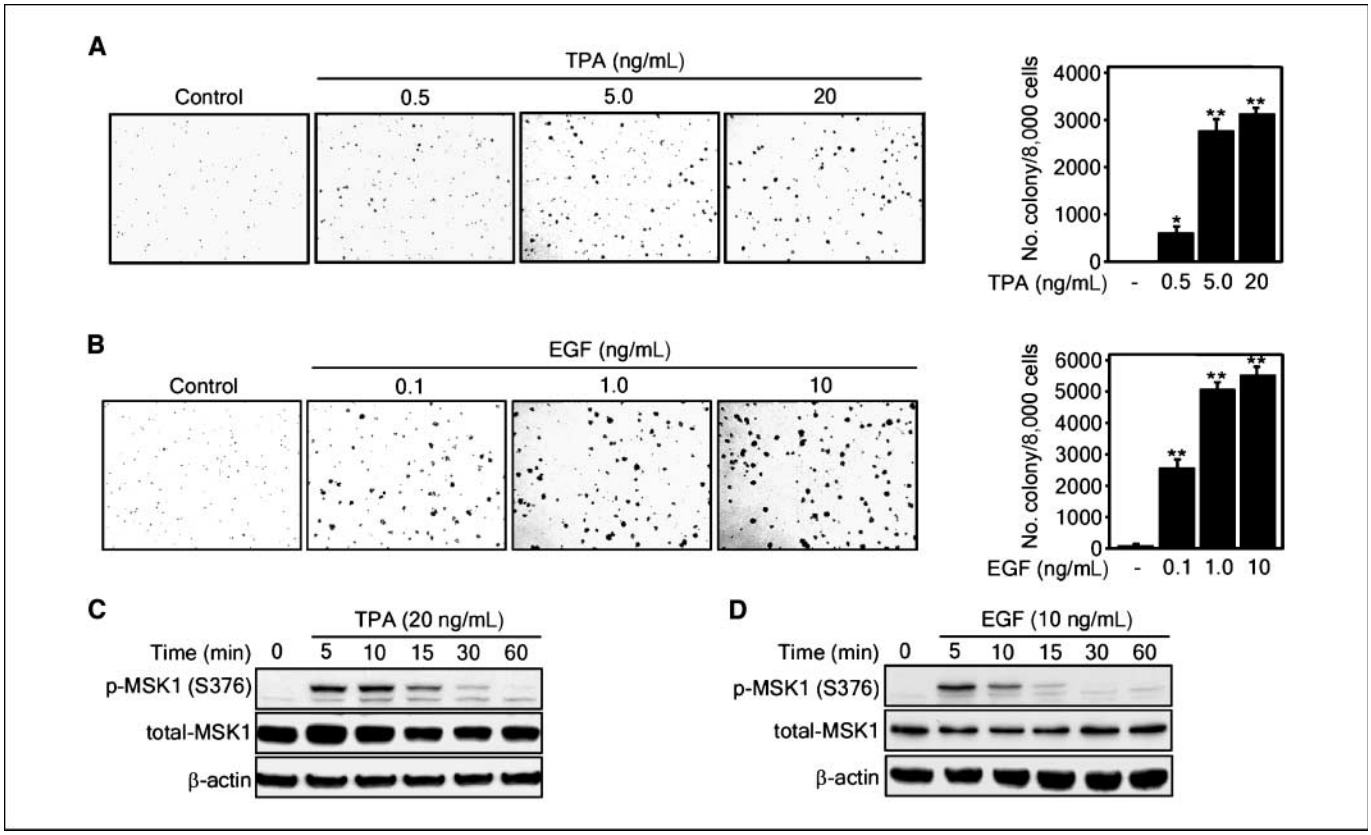

Figure 1. TPA or EGF induces cell transformation and MSK1 phosphorylation in JB6 Cl41 mouse epidermal skin cells. *A* and *B,* JB6 Cl41 cells were assessed for cell transformation in an anchorage-independent soft agar assay in the presence of TPA (*A*) or EGF (*B*). Cells (8×10^3) were exposed to TPA (0, 0.5, 5, or 20 ng/mL) or EGF (0, 0.1, 1, or 10 ng/mL) in 1 mL of 0.3% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 atmosphere for 10 to 12 d, and then colonies were counted using a microscope and the Image-Pro PLUS (v.4) computer software program. The average colony number was calculated (*right*); plates were photographed (*left*) from three separate experiments (*A* and *B*) and representative plates are shown. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant increase in TPA-induced or EGF-induced cell transformation (*, $P < 0.001$; **, $P < 0.0001$) compared with untreated control. *C* and *D,* JB6 Cl41 cells were used to determine the protein levels of endogenous phosphorylated MSK1 in TPA-induced or EGF-induced cell transformation. Cells (2×10^5) were starved in 0.1% FBS-MEM for 24 h at 37°C in a 5% CO_2 atmosphere and then stimulated with 20 ng/mL of TPA (*C*) or 10 ng/mL of EGF (*D*) and finally harvested at various time periods. Cells were disrupted and proteins extracted with NP40 cell lysis buffer and Western blotting was conducted as described in the Materials and Methods using specific antibodies as indicated. β-Actin was used to confirm equal protein loading.

numbers were determined. Our results showed a marked increase in colony numbers, as well as colony sizes in cells overexpressing wild-type MSK1 (pCMV-MSK1 WT) promoted by TPA or EGF compared with control cells (pCMV-neo; Fig. 3*A* and *B*). On the other hand, the overexpression of COOH terminal dead MSK1 caused a dramatic suppression of colony formation induced by TPA or EGF (Fig. 3*A* and *B*). Overexpression of the MSK1 NH_2 terminal dead dominant-negative mutant resulted in a decrease in the number of TPA-induced colonies (Fig. 3*A*), but seemed to exert a lesser effect on EGF-induced colony numbers (Fig. 3*B*). These results strongly suggested that MSK1 mediates cell transformation stimulated by tumor promotion agents, such as TPA or EGF, and the COOH terminal kinase domain of MSK1 plays an important role.

To determine whether TPA- or EGF-induced cell transformation elicited by the induction of AP-1 promoter activity mediated by MSK1, we cotransfected the *AP-1 luciferase reporter* plasmid and the *pCMV-β-Gal* gene into each of the MSK1 WT, COOH terminal or NH_2 terminal dead dominant-negative mutants, or control JB6 Cl41 cell lines. The TPA-promoted or EGF-promoted AP-1 transcriptional activity response in JB6 Cl41 cells was much more pronounced in MSK1 WT overexpressing cells (pCMV-MSK1 WT) compared with mock vector control cells (Fig. 3*C* and *D*). In contrast, a significant inhibition of AP-1 transactivation was observed in COOH terminal and NH_2 terminal kinase dead cells after treatment with TPA (Fig. 3*C*) or EGF (Fig. 3*D*). These data supported our idea that the induction of AP-1 promoter activity by TPA or EGF mediated by MSK1 might be one of the mechanisms explaining the increased anchorage-independent growth of JB6 Cl41 cells.

MSK1-mediated phosphorylation of histone H3 is important in cell transformation. Different covalent modifications, such as acetylation, phosphorylation, and methylation at different sites of the NH_2 terminal tail of histone H3 seem to have various effects on cell function. Mitotic and stimulus-inducible phosphorylation of histone H3 at Ser^{10} and Ser^{28} has been characterized (22–25). Furthermore, MAPK-mediated phosphorylation of Ser^{10} and Ser^{28} has also been shown to occur after stimulation with EGF (25), UVB (13, 26), TPA, or anisomycin (7). To investigate whether histone H3 phosphorylation at Ser^{10} or Ser^{28} specifically induces cell transformation promoted by TPA or EGF, we constructed stable transfectant histone H3 WT and various histone H3 mutants (S10A, S28A and S10/28A). The various cell lines were treated with TPA (20 ng/mL) or EGF (10 ng/mL) in a soft agar matrix and incubated at 37°C in a 5% CO_2 incubator for 10 to 12 d. The results indicated that the number of colonies promoted by either TPA or

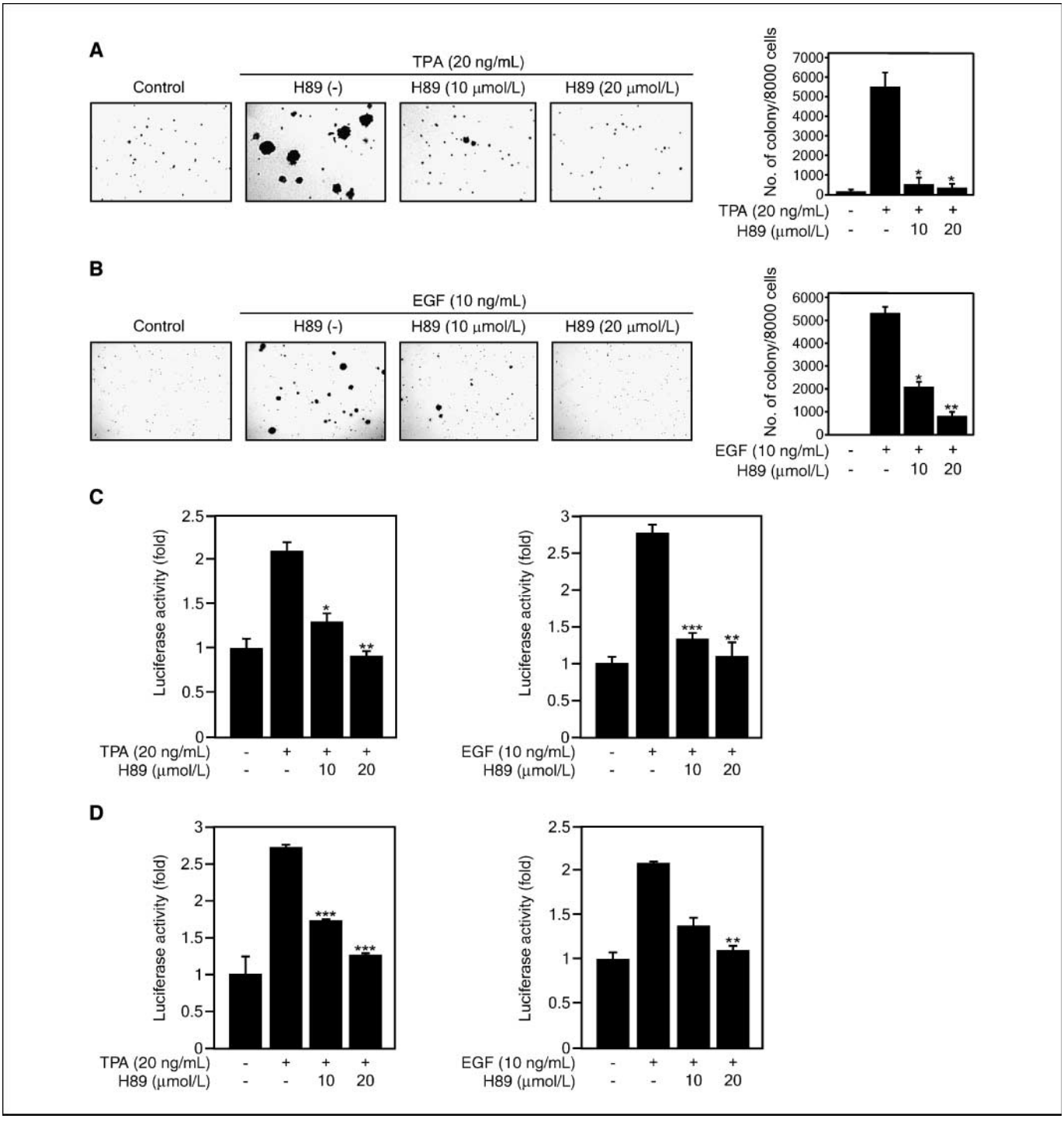

Figure 2. H89, a potent inhibitor of MSK1, strongly inhibits colony formation and AP-1 activation induced by TPA or EGF. *A* and *B,* H89 suppresses TPA-induced or EGF-induced anchorage-independent colony formation in soft agar. Cells (8×10^3) were exposed to TPA (20 ng/mL, *A*) or EGF (10 ng/mL, *B*) and different doses of H89 in 1 mL of 0.3% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 atmosphere for 10 to 12 d, and then colonies were counted using a microscope and the Image-Pro PLUS (v.4) computer software program. The average colony number was calculated (*right*) and plates were photographed (*left*) from three separate experiments (*A* and *B*) and representative plates are shown. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced cell transformation by H89 compared with cells treated with only TPA or EGF (*, $P < 0.005$; **, $P < 0.0001$). *C,* JB6 Cl41 cells were cotransfected with a plasmid mixture containing the *AP-1-luciferase reporter* gene (0.25 μg) and with the *pCMV-β-Gal* gene (0.05 μg) for normalization. At 36 h after transfection, cells were starved for 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere and then pretreated for 30 min with different doses of H89, followed by stimulation with 20 ng/mL of TPA (*left*) or 10 ng/mL of EGF (*right*) for an additional 12 h and then harvested. *D,* JB6 Cl41 cells were cotransfected with a plasmid mixture containing the *c-fos-luciferase reporter* gene (0.1 μg) and the *pCMV-β-Gal* gene (0.05 μg) for normalization purposes. At 36 h after transfection, cells were starved for 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere and then pretreated for 30 min with different doses of H89 followed by stimulation with 20 ng/mL of TPA (*left*) or 10 ng/mL of EGF (*right*) followed by harvesting after 12 h. The firefly luciferase activity was determined in cell lysates and normalized against β-gal activity. Significant differences were evaluated using the Student's *t* test and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced AP-1 activation by H89 compared with cells treated with only TPA or EGF (*, $P < 0.01$; **, $P < 0.005$; ***, $P < 0.0001$).

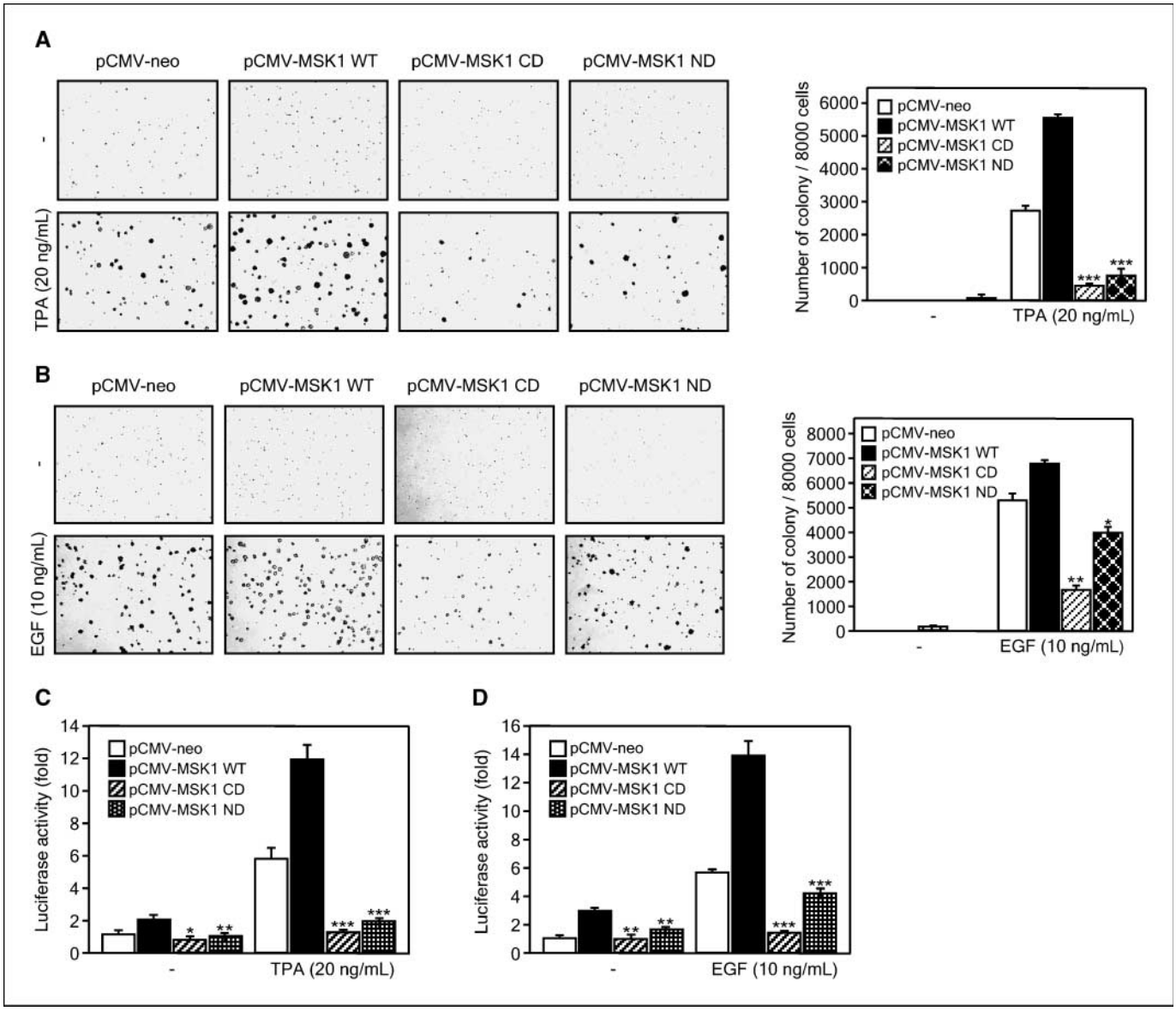

Figure 3. MSK1 dominant-negative mutants suppress cell transformation and AP-1 activation promoted by TPA or EGF. *A* and *B,* cells (8×10^3) transfected with pCMV-neo, pCMV-MSK1 WT, pCMV-MSK1 CD, or pCMV-MSK1 ND were subjected to a soft agar assay in the presence of TPA (20 ng/mL, *A*) or EGF (10 ng/mL, *B*) in 1 mL of 0.3% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 atmosphere for 10 to 12 d, and then colonies were counted using a microscope and the Image-Pro PLUS (v.4) computer software program. The average colony number was calculated (*right*) and photographed (*left*) from three separate experiments (*A* and *B, right*). Representative plates are shown. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced cell transformation compared with cells treated with only TPA or EGF (*, $P < 0.005$; **, $P < 0.0005$; ***, $P < 0.0001$). *C* and *D,* JB6 Cl41 cells were transfected with a plasmid mixture containing the *AP-1-luciferase reporter* gene (0.5 μg) or with the *pCMV-β-Gal* gene (0.05 μg) for normalization. At 36 h after transfection, cells were starved for 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere and then incubated with 20 ng/mL of TPA (*C*) or 10 ng/mL of EGF (*D*) for 12 h. The firefly luciferase activity was determined in cell lysates and normalized against β-gal activity. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced AP-1 activation compared with cells treated with only TPA or EGF (*, $P < 0.005$; **, $P < 0.0005$; ***, $P < 0.0001$).

EGF was decreased in S10A mutant cells compared with the overexpressing histone H3 WT cells (Fig. 4*A* and *B*). The cell transforming activity was also inhibited in H3 S28A mutant cells compared with overexpressing H3 WT cells but to a lesser degree than H3 S10A mutants (Fig. 4*A* and *B*). These finding indicated that the phosphorylation of histone H3 at Ser^{10} is very likely a critical site for mediating cell transformation induced by tumor promoters, such as TPA or EGF.

MSK1 is a well known mitogen-responsive and stress-responsive histone H3 kinase that is activated by the Ras-MAPK pathway (7). To test whether MSK1 directly phosphorylates histone H3, active MSK1 was incubated with human histone H3 and ATP. Immunoblot results showed that MSK1 phosphorylated histone H3 at Ser^{10} and Ser^{28} (Fig. 4*C*). Furthermore, to investigate whether MSK1 is the histone H3 kinase responsible for TPA-induced or EGF-induced phosphorylation of histone H3, we assessed phosphorylation of histone H3 in control cells (pCMV-neo), MSK1 COOH terminal (pCMV-MSK1 CD) or NH_2 terminal dead (pCMV-MSK1 ND) dominant-negative mutants after TPA or EGF stimulation. Our results indicated that TPA-induced or EGF-induced phosphorylation of histone H3 at Ser^{10} and Ser^{28} was decreased in both mutants compared with control mock cells (Fig. 4*D*). These results

show that MSK1 directly phosphorylates histone H3 at Ser^{10} and Ser^{28} in TPA-induced or EGF-induced cell transformation.

Knockdown of MSK1 suppresses cell proliferation by causing accumulation of cells at G_1. To determine whether siRNA-mediated knockdown of MSK1 in JB6 Cl41 could inhibit cell transforming activity, we designed siRNA against MSK1 (si-MSK1) and a general scrambled mock control (si-MOCK; Fig. 5*A*) for transfection into JB6 Cl41 cells. Immunoblot analysis

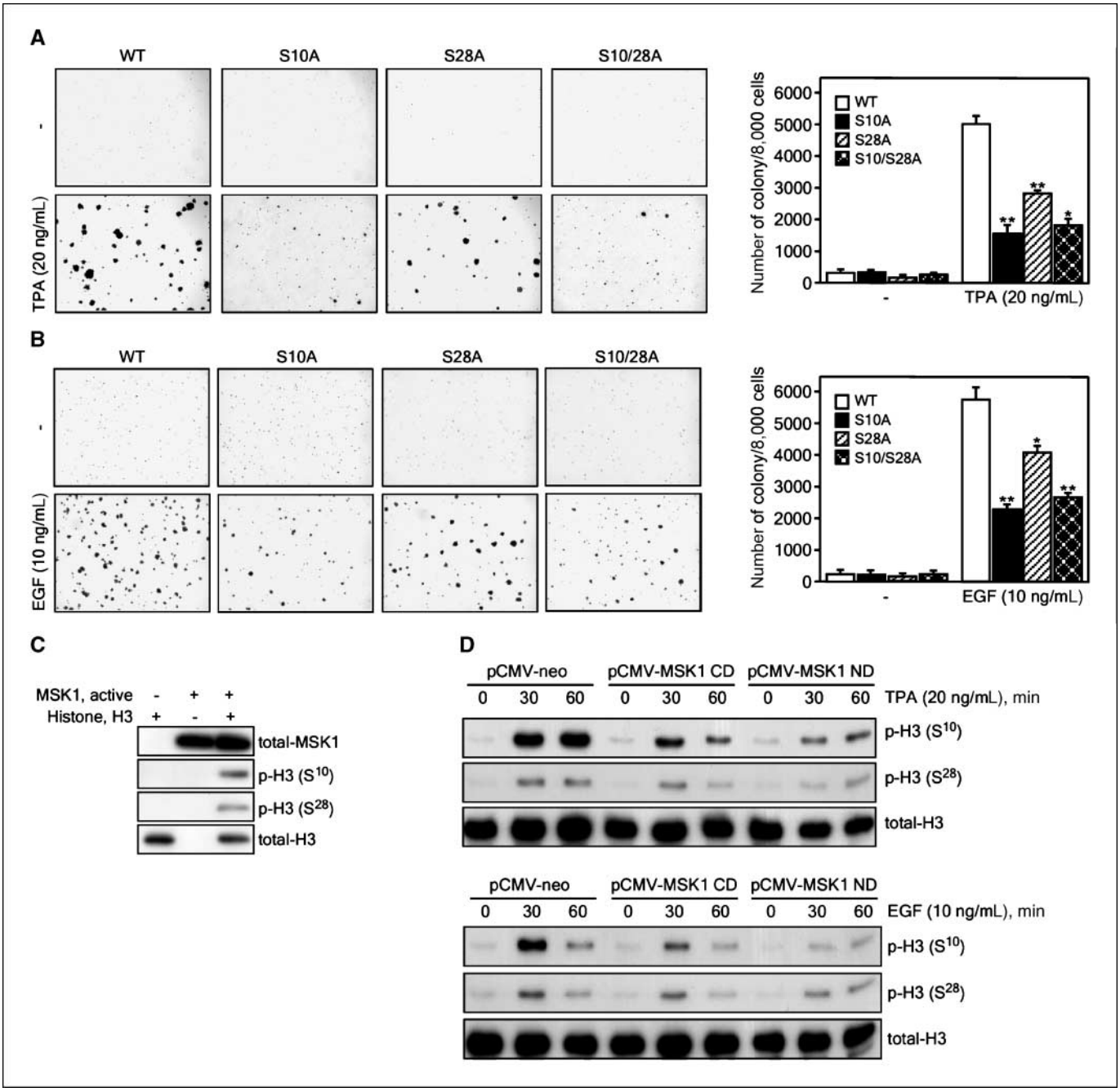

Figure 4. MSK1-mediated phosphorylation of histone H3 at Ser^{10} regulates TPA-induced or EGF-induced cell transformation. *A* and *B,* cells (8×10^3) transfected with histone H3 WT or mutants, S10A, S28A, or S10/28A, were subjected to a soft agar assay in presence of TPA (20 ng/mL, *A*) or EGF (10 ng/mL, *B*) in 1 mL of 0.3% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 atmosphere for 10 to 12 d and then colonies were counted using a microscope and the Image-Pro PLUS (v.4) computer software program. The average colony number was calculated (*right*) and photographed (*left*) from three separate experiments (*A* and *B*) and representative plates are shown. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced cell transformation compared with overexpressed histone H3 WT cells (*, $P < 0.005$; **, $P < 0.0001$). *C,* MSK1 phosphorylates histone H3 at Ser^{10} or Ser^{28}. Active MSK1 (50 ng) was combined with human histone H3 (1 μg) protein and 50 μmol/L ATP. The reaction was carried out at 30°C for 30 min and then stopped by adding 6× SDS sample buffer. The samples were resolved by 15% SDS-PAGE, and the protein bands were visualized by Western blotting with specific antibodies to detect total MSK1 or phosphorylation of histone H3 at Ser^{10} or Ser^{28}. *D,* cells transfected with pCMV-neo, pCMV-MSK1 CD, or pCMV-MSK1 ND were analyzed to detect endogenous phosphorylated histone H3 levels induced by TPA or EGF. Cells (6×10^5/mL) were starved in 0.1% FBS-MEM for 24 h at 37°C in a 5% CO_2 atmosphere and then stimulated with 20 ng/mL of TPA (*top*) or 10 ng/mL of EGF (*bottom*) for various periods of time. Histone proteins (1 μg) were resolved by SDS-15% PAGE and then transferred to membranes for Western blot analysis. The phosphorylation of histone H3 at Ser^{10} or Ser^{28} was detected with specific antibodies. Detection of total histone H3 was used for confirming equal loading of protein.

of si-MSK1–transfected and si-MOCK–transfected JB6 Cl41 cells revealed a suppression of endogenous MSK1 protein expression of up to 80% compared with cells expressing the mock vector (pU6pro; Fig. 5*B*).

MSK1 has been reported to play a positive role in the control of cell proliferation of both HaCaT keratinocytes and the A431 human epidermoid carcinoma line (11). To examine whether the knockdown of MSK1 could affect cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium assay was used to examine proliferation of cells stably transfected with si-MOCK or si-MSK1. Results indicated that si-MSK1 stably transfected cells showed a marked decrease in the rate of proliferation compared with si-MOCK stably transfected cells (Fig. 5*C*). In contrast, JB6 Cl41 cells overexpressing MSK1 exhibited an increased rate of proliferation (data not shown), confirming that MSK1 is involved in cell proliferation. Using these cell lines, we studied differences in cell cycle phases induced by TPA or EGF. Our data indicated that knockdown of MSK1 resulted in less cells occupying the S phase and a greater accumulation of cells in the G_1-G_0 phase of the cell cycle compared with si-MOCK control cell (Fig. 5*D*). These results clearly showed that the knockdown of MSK1 suppressed cell proliferation because of an impaired G_1-S cell cycle transition.

TPA-induced or EGF-induced cell transformation is suppressed by knockdown of MSK1. To examine the effect of suppressing MSK1 expression on TPA-induced or EGF-induced cell transformation, we analyzed colony-forming activity in si-MSK1 and si-MOCK cells. These cell lines were stimulated with TPA (20 ng/mL) or EGF (10 ng/mL) in soft agar and incubated at 37°C in a 5% CO_2 incubator for 10 to 12 days. Our results showed that, as expected, the knockdown of MSK1 suppressed colony formation induced by TPA (Fig. 6*A, top*) or EGF (Fig. 6*A, bottom*). Overall, these data further showed that MSK1 plays a key role in cell transformation induced by tumor promoting agents.

To determine whether the knockdown of MSK1 in JB6 Cl41 cells might affect the AP-1 activation response to TPA or EGF, we cotransfected the *AP-1 luciferase reporter* plasmid and the *si-MOCK* or *si-MSK1* gene into JB6 Cl41 cells. Results showed that TPA-induced or EGF-induced AP-1 activation was suppressed in si-MSK1 stably expressing cells compared with mock control cells (Fig. 6*B*). Similar results were obtained for EGF-induced or TPA-induced *c-fos* promoter activity (Fig. 6*C*), confirming that MSK1 has a role in *c-fos* promoter and AP-1 activation induced by TPA or EGF.

We showed that the TPA-induced or EGF-induced phosphorylation of histone H3 at Ser^{10} and Ser^{28} was reduced in two different MSK1 dominant-negative mutant cell lines (Fig. 4*D*). We therefore next examined the effect of MSK1 knockdown on histone H3 phosphorylation (Ser^{10} or Ser^{28}). Results confirmed that TPA-induced or EGF-induced histone H3 phosphorylation is effectively suppressed in MSK1 knockdown cells compared with control mock cells (Fig. 6*D*). This finding showed that MSK1 is essential for

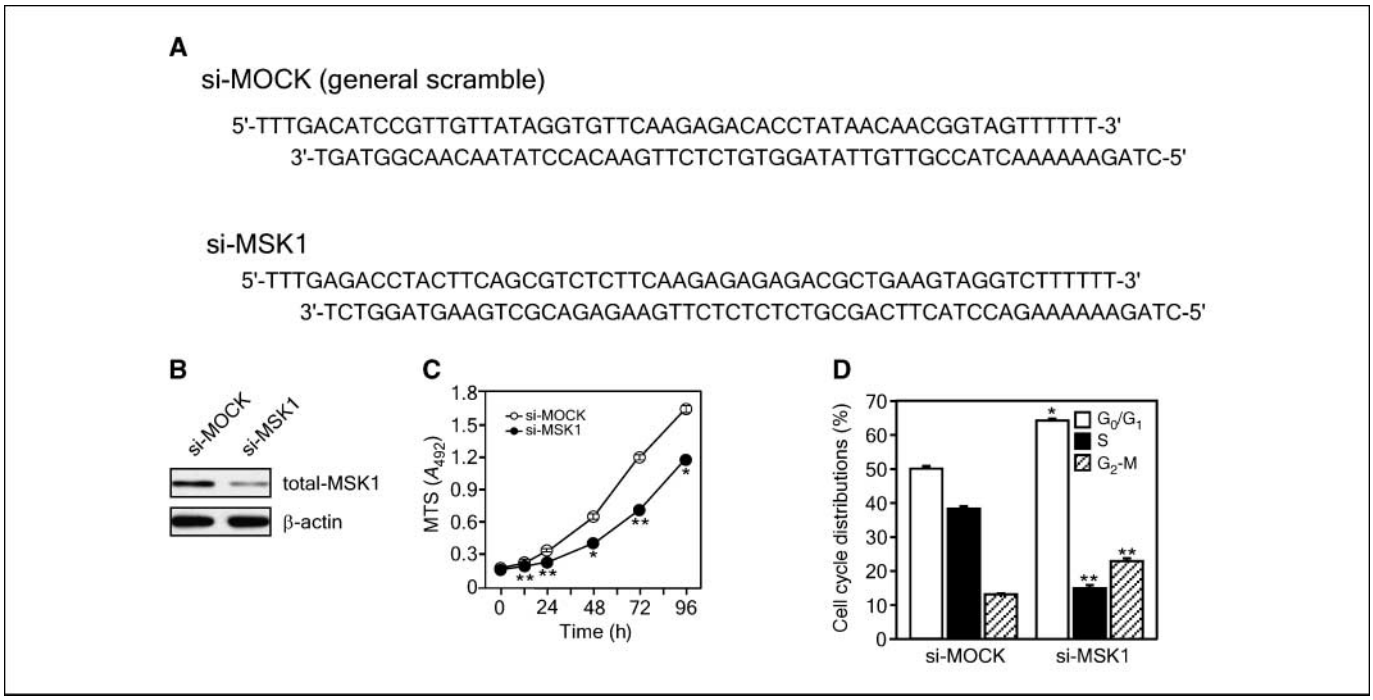

Figure 5. Knockdown of MSK1 suppresses cell proliferation. *A,* DNA sequence of the si-MOCK and si-MSK1 primers. *B,* si-MSK1 efficiently suppresses the endogenous MSK1 protein level. JB6 Cl41 cells were cotransfected with pcDNA3.1-neo and pU6pro control or the *si-MSK1* gene, selected with 400 μg/mL G418 for 12 d, and then pooled. The expression of MSK1 was analyzed in the stable transfectant JB6 Cl41 cells. The lysates were resolved by 10% SDS-PAGE, and the protein bands were visualized by Western blotting with a specific MSK1 primary antibody and an HRP-conjugated secondary antibody. Detection of total β-actin was used to verify equal loading of protein. *C,* cells stably transfected with the si-MOCK or si-MSK1 plasmid were seeded (1×10^3 per well) in 96-well plates in 100 μL of 5% FBS-MEM, and cell proliferation was estimated using the CellTiter 96 Aqueous One Solution detection kit (Promega). Cell proliferation was estimated by absorbance at 492 nm at 24-hour intervals up to 96 h. *Points,* means of triplicate experiments; *bars,* SD. Statistical differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant change in si-MSK1 cells compared with si-MOCK control cells (*, $P < 0.001$; **, $P < 0.0001$). *D,* si-MOCK or si-MSK1 stably expressing (2×10^5) cells were seeded into 60-mm dishes and cultured 24 h. The cells were harvested, fixed with methanol, stained with PI, and then analyzed for cell cycle phase. Data are expressed as the percentage of cells in G_1-G_0, S, or G_2-M phase; *columns,* mean of values obtained from triplicate experiments; *bars,* SD. Statistical differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant change in cell cycle phase distribution in si-MSK1 cells compared with si-MOCK control cells (*, $P < 0.01$; **, $P < 0.0001$).

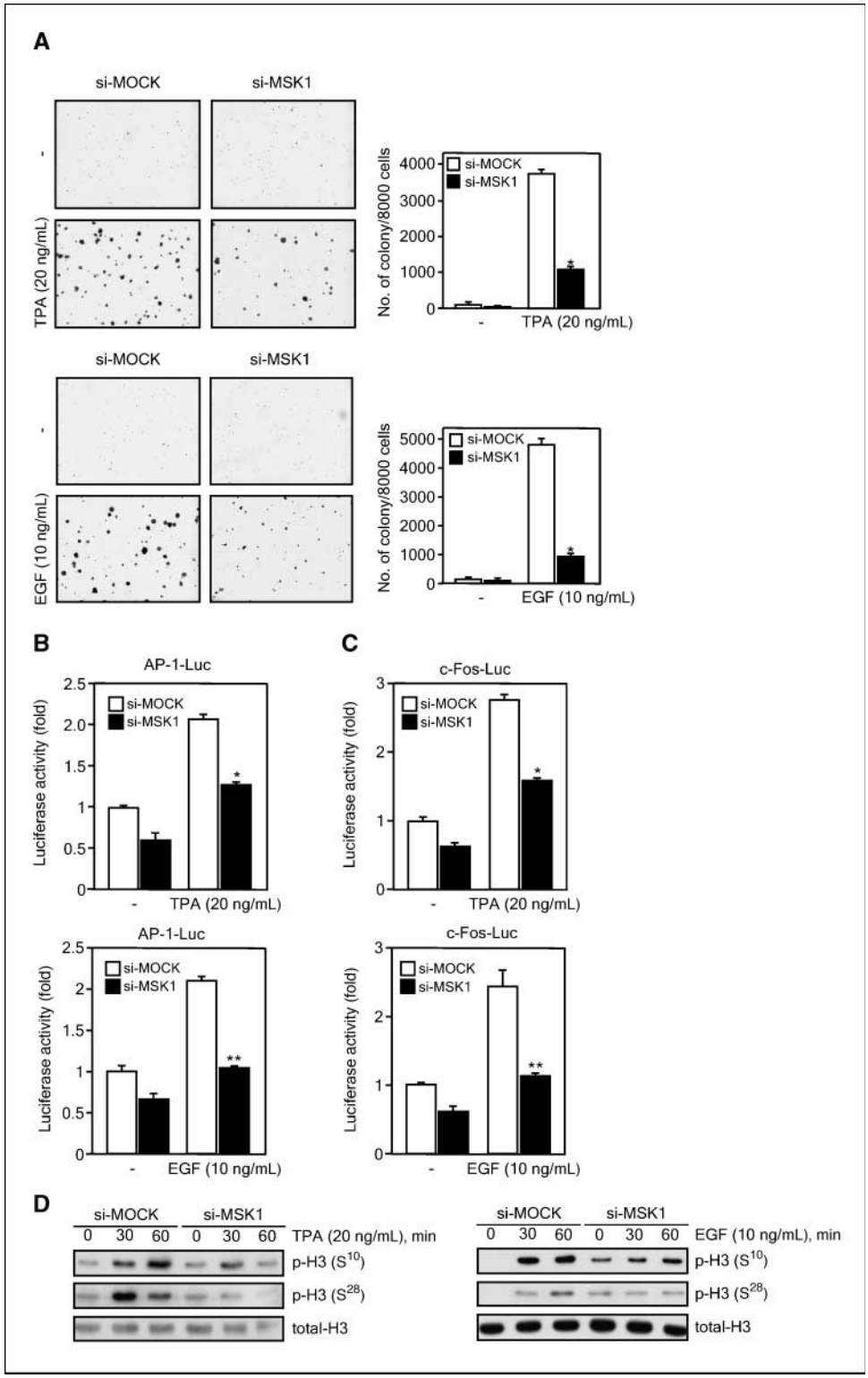

Figure 6. Knockdown of MSK1 suppresses TPA-induced or EGF-induced cell transformation and phosphorylation of histone H3. *A,* si-MOCK and si-MSK1 stably transfected cells (8×10^3) were exposed to TPA (20 ng/mL, *top*) or EGF (10 ng/mL, *bottom*) in 1 mL of 0.3% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 atmosphere for 10 to 12 d, and then colonies were counted using a microscope and the Image-Pro PLUS (v.4) computer software program. The average colony number was calculated and photographed from three separate experiments (*top right,* TPA; *bottom right,* EGF) and representative plates are shown. Significant differences were evaluated using the Student's *t* test and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced cell transformation compared with si-MOCK cells (*, $P < 0.0001$). *B,* JB6 Cl41 cells were transiently transfected with si-MOCK or si-MSK and a plasmid mixture containing the *AP-1-luciferase reporter* gene (0.25 μg) or the *pCMV-β-Gal* gene (0.05 μg). At 36 h after transfection, cells were starved for 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere and then incubated with 20 ng/mL of TPA (*top panel*) or 10 ng/mL of EGF (*bottom panel*) for 12 h. *C,* JB6 Cl41 cells stably expressing si-MOCK or si-MSK1 were transiently transfected with a plasmid mixture containing the *c-fos-luciferase reporter* gene (0.1 μg) or the *pCMV-β-Gal* gene (0.05 μg) for normalizing. At 36 h after transfection, cells were starved for 24 h by incubating in serum-deprived MEM at 37°C in a 5% CO_2 atmosphere and then incubated with 20 ng/mL of TPA (*top*) or 10 ng/mL of EGF (*bottom*) for 12 h. Significant differences were evaluated using the Student's *t* test, and the respective asterisks indicate a significant decrease in TPA-induced or EGF-induced AP-1 activation compared with si-MOCK cells (*, $P < 0.01$; **, $P < 0.005$). *D,* si-MOCK or si-MSK1 transfected cells (6×10^5/mL) were starved in 0.1% FBS-MEM for 24 h at 37°C in a 5% CO_2 atmosphere and then stimulated with 20 ng/mL of TPA (*left*) or 10 ng/mL of EGF (*right*) for various periods of time. Histone proteins (1 μg) were prepared for Western blot analysis and the phosphorylation of histone H3 at Ser^{10} or Ser^{28} expression was detected with specific antibodies. Total histone H3 was used to verify equal protein loading.

TPA-induced or EGF-induced phosphorylation of histone H3 at Ser^{10} and Ser^{28}. Taken together, these results provided strong evidence showing that MKS1 is required for TPA-induced or EGF-induced cell transformation, which might be regulated through MSK1-mediated histone H3 phosphorylation at Ser^{10} and Ser^{28} and AP-1 activation.

Discussion

MSK1 is a nuclear kinase that acts downstream of both the ERK and p38 MAPKs and has been suggested to be activated in response to EGF, TPA, UVB, and anisomycin (7, 8, 13, 14). MSK1 phosphorylates various transcription factors, including CREB,

ATF1, ATF2, signal transducers and activators of transcription 3, and NF-κB (8–10, 27–29). MSK1 plays a critical role in the survival of lipopolysaccharide-stimulated RAW 264.7 cells through the phosphorylation of CREB (30). MSK1 also activates the transcription factor ER81, which is involved in oncogenesis and breast tumor formation (31). MSK1 phosphorylation of these transcription factors may alter their ability to bind to their target DNA and/or recruitment of required coactivators. MSK1 has also been shown to be required for the full induction of several immediate early genes, such as *c-fos, junB,* and *mkp-1*, in response to various stimuli (5, 6). Moreover, MSK1 regulates the transcription of the *Nur77, Nurr1*, and *Nor1* nuclear orphan receptor genes of the NR4A subfamily (32), the up-regulation of which has been implicated in cell transformation (33). MSK1 is also required for chromatin protein histone H3 and HMG-14 phosphorylation (7, 34). The activation of the Ras-MAPK pathway and MSK1 resulting in the elevation of phosphorylated H3 levels may contribute to the aberrant gene expression observed in the oncogene-transformed cells (35). However, the intriguing question is whether the activation of MSK1 directly affects tumor promoter-induced cell transformation.

Tumor promotion agents, such as TPA or EGF, can induce AP-1 activation and promote JB6 Cl41 cell transformation (20, 36). On the other hand, the inhibition of AP-1 transactivation activity suppresses TPA-induced or EGF-induced cell transformation (37). Our results showed the relationship of MSK1 activity and AP-1 transactivation activity in TPA-induced or EGF-induced cell transformation. Expression of c-Fos and c-Jun has been shown to be increased TPA-induced and UVB-induced cell transformation in JB6 cell and in mouse epidermis (38). Bcl-2–overexpressing JB6 cells enhanced TPA-induced cell transformation and also, *bcl-2*–transfected cells expressed significantly more c-Fos, but not c-Jun, after TPA treatment (39). Although overexpression of the Fos and Jun proteins was found to correlate with a positive effect on cell transformation (40), c-Fos might have a more important role than c-Jun during late stage tumorigenesis (41). The importance of c-Fos in tumor invasion has been supported *in vivo*, as the progression of chemically induced papillomas to invasive squamous cell carcinomas was shown to be impaired in c-Fos–deficient mice (42). Activated genes including *c-fos* and *c-jun* coupled with histone H3 phosphorylation at specific loci may contribute to a mechanism that allows them to be rapidly activated in response to external stimuli (25). The stimulation of the activity of the *c-fos* SRE by phosphorylation of histone H3 at Ser^{10} (25) might promote c-Fos expression and c-Fos/c-Jun heterodimer formation leading to expression of AP-1 regulated genes (43). In histone H3 mutant S10A cells, *c-fos* promoter activity and AP-1 activation was significantly decreased compared with control cells (44). Therefore, histone H3 phosphorylation by MSK1 seems to be closely linked with AP-1 transactivation potential. Another implication of the increased AP-1 transactivation activity in cell transformation induced by TPA or EGF is that MSK1 can regulate AP-1 activation through the phosphorylation of transcription factors, such as CREB and ATF1. MSK-mediated phosphorylation of CREB and ATF1 (6, 9) has been implicated in the induction of *c-fos* and *junB* transcription. The function of MSK1 is critical for interleukin-1–induced and CREB-mediated *c-fos* gene expression in keratinocytes and promotes the growth of both keratinocytes and human epidermoid carcinoma cell lines (11). Therefore, increased AP-1 activity induced by MSK1 is very much associated with TPA-induced or EGF-induced cell transformation.

In the present study, we found that MSK1 closely regulated cell transformation and cell proliferation of JB6 Cl41 cells through the phosphorylation of histone H3. Increased phosphorylation of histone H3 at Ser^{10} was found in mitogen-stimulated and oncogene-transformed mouse fibroblasts (45). The mutation of Ser^{10} of histone H3 significantly suppressed cell transformation promoted by EGF (44). Furthermore, we suggest that MSK1 can directly regulate cell transformation through the phosphorylation of histone H3 at Ser^{10}. Histone H3 is a well-known substrate of MSK1 when induced by mitogenic or stress stimuli (35, 45). Our results indicated that MSK1 can directly phosphorylate histone H3 at Ser^{10} *in vivo* and *in vitro*. Moreover, inhibition of MSK1 activation in dominant-negative mutants or knockdown of MSK1 expression using siRNA suppressed the phosphorylation of histone H3 at Ser^{10} and cell transformation induced by TPA or EGF. Other studies using MSK1 and MSK2 knockout murine embryonic fibroblasts revealed that MSK1 regulates phosphorylation of histone H3 at Ser^{10} and Ser^{28}, as well as phosphorylation of HMG-14 (7, 46). Increased mitotic histone H3 Ser^{10} phosphorylation was also observed in various colorectal tumor cells with high AIM-1, a mammalian Ipl1/aurora kinase involved in histone H3 phosphorylation (47). These previous studies strongly support our finding that MSK1 might have an important role in carcinogenesis through its phosphorylation of histone H3 at Ser^{10}.

Although many factors have been shown to interact with and promote cell transformation, we found that the MSK1 plays an important role in cell transformation induced by tumor promoting agents, such as TPA or EGF. MSK1 can induce cell transformation leading to phosphorylation of histone H3 and induction of AP-1 activity, which may include its transactivating potential and DNA binding capacity and the stability of AP-1 components. By linking MSK1 to histone H3 phosphorylation and AP-1 transactivation activity, these findings provide an attractive explanation of how MSK1 might be related to cell transformation induced by tumor promotion agents. Taken together, these results strongly suggested that MSK1 is required for cell transformation and the relationship of MSK1 and histone H3 might be a crucial target for cancer chemotherapy or gene therapy in the future.

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