Microtubule Disruption and Tumor Suppression by Mitogen-Activated Protein Kinase Phosphatase 4

Yuangang Liu, James Lagowski, Aaron Sundholm, Alexandra Sundberg, and Molly Kulesz-Martin

Department of Dermatology, Oregon Health and Science University, Portland, Oregon

Abstract

The extracellular signal-regulated kinase (Erk) is one of the downstream effectors of the Ras pathway whose activation is essential for the proliferation and survival of cancer cells. Erk activation is negatively regulated by mitogen-activated protein kinase (MAPK) phosphatases (MKP), which are generally up-regulated by Erk activation, thus forming a feedback loop for regulation of Erk activity. In searching for early alterations in the Ras pathway in epidermal carcinogenesis, we identified MKP4, a cytosolic MKP with specificity to not only Erk, but also, to a lesser extent, c-jun-NH_2-kinase and p38. MKP4 is down-regulated at initiation and lost at malignant conversion in a clonal model of epidermal carcinogenesis that lacks Ras mutation. The loss of MKP4 was associated with squamous cell carcinoma (SCC) but not benign papilloma clonal lineages and with independently induced SCC relative to benign tumors in mouse skin. Reconstitution of MKP4 expression in malignant tumor cells leads to cell death and tumor suppression. Unlike Erk inhibition that blocks cell cycle entry, MKP4 reconstitution resulted in G_2-M associated cell death and microtubule disruption. Thus, microtubule disruption by MKP4 provides a novel mechanism for tumor suppression by a cytosolic MKP and implies a novel therapeutic strategy through combined MAPK inhibitions that mimic the function of MKP4. [Cancer Res 2007;67(22):10711–9]

Introduction

Ras activation is one of the most common alterations in human carcinogenesis (1). Ras mutation activates a signaling cascade that leads to the activation of a number of downstream effectors essential for carcinogenesis. One of these is the extracellular signal-regulated kinase (Erk), which is activated by mitogen-activated protein (MAP)/Erk kinase (MEK), a dual-specificity kinase that phosphorylates threonine and tyrosine residues in the TXY motif of Erk. The phosphorylated Erk is translocated from the cytosol to the nucleus, where it phosphorylates numerous substrates needed for cell cycle entry. Erk activation is essential for carcinogenesis (2), and constitutively activated Erk is found in a variety of human cancers (3). Constitutive activation of MEK is sufficient to transform mammalian cells (4). Furthermore, Erk inhibition by small-molecule inhibitors of MEK has been shown to result in tumor regression *in vivo* (5).

Requests for reprints: Yuangang Liu, Department of Dermatology, L468R, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239. Phone: 503-418-4268; Fax: 503-418-4266; E-mail: liuy@ohsu.edu and Molly Kulesz-Martin, Department of Dermatology, L468R, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239. Phone: 503-494-9933; Fax: 503-418-4266; E-mail: kuleszma@ohsu.edu.

doi:10.1158/0008-5472.CAN-07-1968

Normally, Erk activity is tightly regulated by MAP kinase (MAPK) phosphatases (MKP). MKPs are dual-specificity phosphatases that dephosphorylate the corresponding TXY residues phosphorylated by MEK (6). There are at least nine distinct mammalian MKPs, and these share a highly conserved COOH-terminal catalytic domain. The specificity of MKPs seems to be achieved through MAPK interaction with the less-conserved NH_2-terminal domains (7). The substrate specificity of MKPs may not be simply a biochemical issue. Other properties of MKPs, such as cell type–specific expression, cellular localization, and gene regulation, may contribute to the substrate specificity and function of MKPs (8). Many MKPs are induced by activated MAPKs, thus forming a negative feedback loop. MKP1 is the prototype of MKPs, first identified as an immediate early gene induced by mitogen and oxidative stress (9, 10). The Erk inhibition by MKP1 is sufficient to block DNA synthesis and cell cycle entry activated by oncogenic Ras (11). In addition to transcriptional induction, MKP1, MKP2, and MKP3 proteins are stabilized by Erk-mediated phosphorylation. Furthermore, the enzymatic activity of MKP1 and MKP3 is allosterically activated by Erk binding to their NH_2-terminal noncatalytic domain. MKP1 and MKP2 are localized in the nucleus, whereas MKP3 and MKP4 are mainly cytosolic, suggesting distinct roles to inactivate Erk in different cellular compartments. Multiple regulations of Erk by the different MKPs provide a feedback mechanism to fine-tune Erk activity for its biological action.

With the identification of MKPs as negative regulators of Erk, it was originally predicted that MKPs may function as tumor suppressors in carcinogenesis (11). However, analysis of MKP1 expression in human cancer suggests that MKP1 does not behave as a tumor suppressor. Overexpression of MKP1 is found in a variety of human cancers, including breast cancer (12), non–small cell lung cancer (13), and prostate cancer (14). Furthermore, down-regulation of MKP1 suppresses tumorigenicity of pancreatic cancer cells *in vivo* (15). Our studies of mouse epidermal carcinogenesis suggest that MKPs may have different roles at different stages of carcinogenesis. We found elevated MKP1 expression in the initiated keratinocyte (03C), followed by normalized MKP1 expression in their paired malignant cell derivative (03R; ref. 16). Cytosolic MKP3 protein, on the other hand, is overexpressed in mild dysplasia as well as in severe dysplasia/carcinoma *in situ* in pancreatic ducts, but MKP3 is underexpressed in poorly differentiated pancreatic carcinoma (17). The tumor suppressor activity of MKP3 has been shown by MKP3-mediated growth arrest and apoptosis in pancreatic cancer cells (17), as well as suppression of Ras-dependent tumorigenesis by a green fluorescent protein (GFP)–fused MKP3 protein (18). The identification of cytosolic MKP3 but not nuclear MKP1 as a tumor suppressor raises questions about the role of cytosolic Erk in carcinogenesis and about the effect of Erk inhibition by cytosolic MKPs.

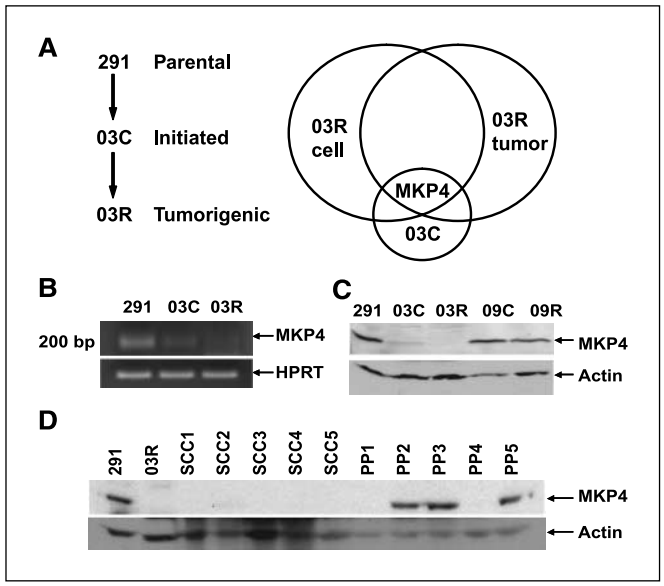

Figure 1. Identification of MKP4 loss as an oncogenic alteration in an epidermal model of carcinogenesis. *A,* the strategy to identify MKP4 from the multistage epidermal cell model of carcinogenesis by microarray analysis. *B,* analysis of MKP4 mRNA level in the epidermal model of carcinogenesis by RT-PCR with HPRT as a loading control. *C,* analysis of MKP4 protein in cell lysates from malignant SCC lineages (initiated 03C and tumorigenic 03R), benign papilloma lineages (initiated 09C and tumorigenic 09R), and their parental cell (291) by immunoblotting with anti-MKP4 antibody. *D,* analysis of MKP4 protein in lysates from UV-induced SCC tissue and DMBA-TPA–induced papillomas by immunoblotting with anti-MKP4 antibody.

MKP4 is another cytosolic MKP with MAPK selectivity Erk > p38 ≥ c-jun-NH_2-kinase (JNK) (19, 20). It shows closest sequence similarity to MKP3 (57% identity) and MKPx (also a cytosolic MKP, 61% identity) in the MKP family. Deletion of the MKP4 gene results in embryonic lethality due to placental insufficiency (21). In the current study, we identified MKP4 as a potential tumor suppressor in epidermal carcinogenesis. The loss of MKP4 expression is associated with malignant epidermal tumor lineages but not benign papilloma lineages from a common non-transformed keratinocyte progenitor clone. Reconstitution of MKP4 in tumor cells leads to microtubule disruption, G_2-M associated cell death, and tumor suppression *in vivo*. This suggests that the microtubule is the target of cytosolic MAPKs regulated by MKP4, having implications for the use of MAPK inhibitors that mimic the activities of MKP4 in human cancer therapy.

Materials and Methods

Cell culture and Affymetrix GeneChip analysis. Non-transformed epidermal cells (291) were grown in fibroblast-conditioned low calcium medium as described (22). The initiated cell lines (03C and 09C) as well as the tumorigenic cell lines (03R and 09R) were maintained in high-calcium EMEM. H1299 cells were grown in DMEM supplemented with 10% FCS. Total RNAs were isolated from each cell type performed in biological triplicate using TRIzol reagent according to the instructions of the manufacturer (Invitrogen). The Affymetrix GeneChip analysis was performed by the Gene Microarray Shared Resource at Oregon Health and Science University. The expression level of MKP4 was confirmed by reverse transcription-PCR (RT-PCR) with a set of MKP4-specific primers: ACCCACCTTCCTTCTTTACTACCC (forward) and TTCTACTCTGTTCCTGCCTTGCTC (reverse), which amplified a 220-bp cDNA fragment of mouse MKP4. A set of primers specific to hypoxanthine-guanine phosphoribosyltransferase (HPRT), CCTGCTGGATTACATTAAAGCACTG (forward) and GTCAAGGGCATATCCAACAACAAAC (reverse), was used in each RNA sample to amplify HPRT cDNA as a reference.

Antibody production and immunoblotting. The anti-MKP4 antibody was produced by immunizing rabbits with the synthetic peptide corresponding to the last 20 amino acids of murine MKP4, NH_2-Asp-Pro-Pro-Ser-Phe-Phe-Thr-Thr-Pro-Thr-Ser-Asp-Gly-Val-Phe-Glu-Leu-Asp-Pro-Thr-COOH. The specificity of the antisera was determined by immunoblotting analysis of MKP4 protein in 03R cells infected with MKP4 recombinant lentivirus. Antibodies for dual-phosphorylated Erk (T202/Y204), JNK (T183/Y185), and p38 (T180/Y182) were from Cell Signaling Technology. Antibody for MKP3 was from Santa Cruz Biotechnology.

Recombinant lentivirus and transduction. The mouse MKP4 cDNA digested with *Bam*HI (blunted) and *Sma*I from MKP4 cDNA vector (ATCC no. MGC-6681) was subcloned into *Xho*I- (blunted) and *Sma*I-digested pSL35 lentiviral vector (obtained from Luigi Naldini, HSR-TIGET, Milan, Italy; ref. 23), which contains an internal ribosome entry site (IRES) sequence for coexpression of GFP. The mouse MKP3 cDNA was amplified from MKP3 cDNA vector (ATCC no. MGC-6625) and cloned into *Xho*I and *Eco*RI sites of pSL35 lentiviral vector. Recombinant lentiviruses were generated using the four-plasmid system by cotransfection of 293T cells with pSL3, which expresses the vesicular stomatitis virus G envelope protein; pSL4, which expresses the HIV-1 *gag/pol* genes; pSL5, which expresses the *rev* gene; and pSL35 containing MKP4 or MKP3. Lentivruses were harvested at 48 and 72 h after the transfection and concentrated by ultracentrifugation at 500,000 × *g* for 90 min. The recombinant virus titer was determined for use of minimal viral particles to achieve ≥90% infection of 03R cells.

Tetracycline-inducible MKP4 expression. Tet-responsive cell line (H1299-Tet) was generated by transfection of H1299 cell with pIRES-TetR vector constructed by cloning *Nhe*I/*Sma*I TetR fragment from pcDNA6/TR (Invitrogen) into pIRES2-enhanced green fluorescent protein (EGFP) vector (Clontech). The H1299-Tet clones were maintained in G418 medium and were selected by measuring luciferase activity induced by tetracycline (5 μg/mL) after transient transfection of pcDNA4/TO-Luciferase reporter. The treatment of tetracycline at 5 μg/mL had no effect on the growth of H1299-Tet clones (data not shown). Murine wild-type MKP4 cDNA was cloned into pcDNA4/TO vector (Invitrogen) and transfected into H1299-Tet cells with zeocin selection. The tetracycline-responsive clones (H1299/MKP4-Tet) were selected by Western blot analysis of MKP4 protein levels in response to tetracycline treatment.

Tubulin immunostaining. Cells were plated on collagen-coated coverslips. Before fixation, cells were permeabilized with 0.2% Triton X-100 for 10s to release free tubulin. The cells on the coverslips were fixed with 4% paraformaldehyde and stained with anti–α-tubulin (Sigma). The image was captured and processed with a Nikon confocal imaging system and a Leica imaging system.

Flow cytometry. Cells were harvested at 2, 4, and 6 days after MKP4 induction by tetracycline (1 μg/mL). The cells were fixed with 100% cold ethanol, treated with RNase, and stained with propidium iodide (50 μg/mL). The DNA content was analyzed by a Becton Dickinson FACSCalibur analyzer. All analyses were performed in duplicate.

***In vivo* tumorigenesis.** Animal studies were performed according to institutional guidelines. Two million lentivirus-infected 03R cells were s.c. inoculated into the back of BALB/c neonates. Tumor sizes were compared between groups infected with GFP lentivirus versus MKP4 lentivirus (six mice each group). For tetracycline-inducible MKP4, 5 million H1299-Tet/MKP4 cells were s.c. injected into the flanks of 8-week-old female nude mice (Taconic). MKP4 expression was induced by administering doxycycline water (1 mg/mL) when visible tumor appeared ($\sim$50 mm^3). The tumor size was measured and calculated by the formula: 0.4 × (tumor length) × $(\text{tumor width})^2$.

Results

MKP4 loss is associated with malignancy. In an attempt to identify alterations that activate the Ras pathway in a non-ras mutation model of epidermal carcinogenesis (22), we profiled the gene expression of epidermal cells representing different

carcinogenesis stages by means of Affymetrix GeneChip analysis (Fig. 1*A*). We selected candidate gene defects based on the criteria that it must be altered at the initiation stage (03C) and sustained or further altered in the malignant stage (03R). The selection was further narrowed down by comparing tissue-cultured 03R cells with 03R tumor tissue. The selection with these criteria led to the identification of MKP4, a negative regulator of MAPK. At the mRNA level, MKP4 was down-regulated in the initiated cells and was further lost in malignant tumor cells (Fig. 1*B*). To evaluate MKP4 expression at the protein level, we developed a MKP4 polyclonal antibody that recognizes the COOH terminus of MKP4. MKP4 loss was specific to squamous cell carcinoma (SCC)–producing

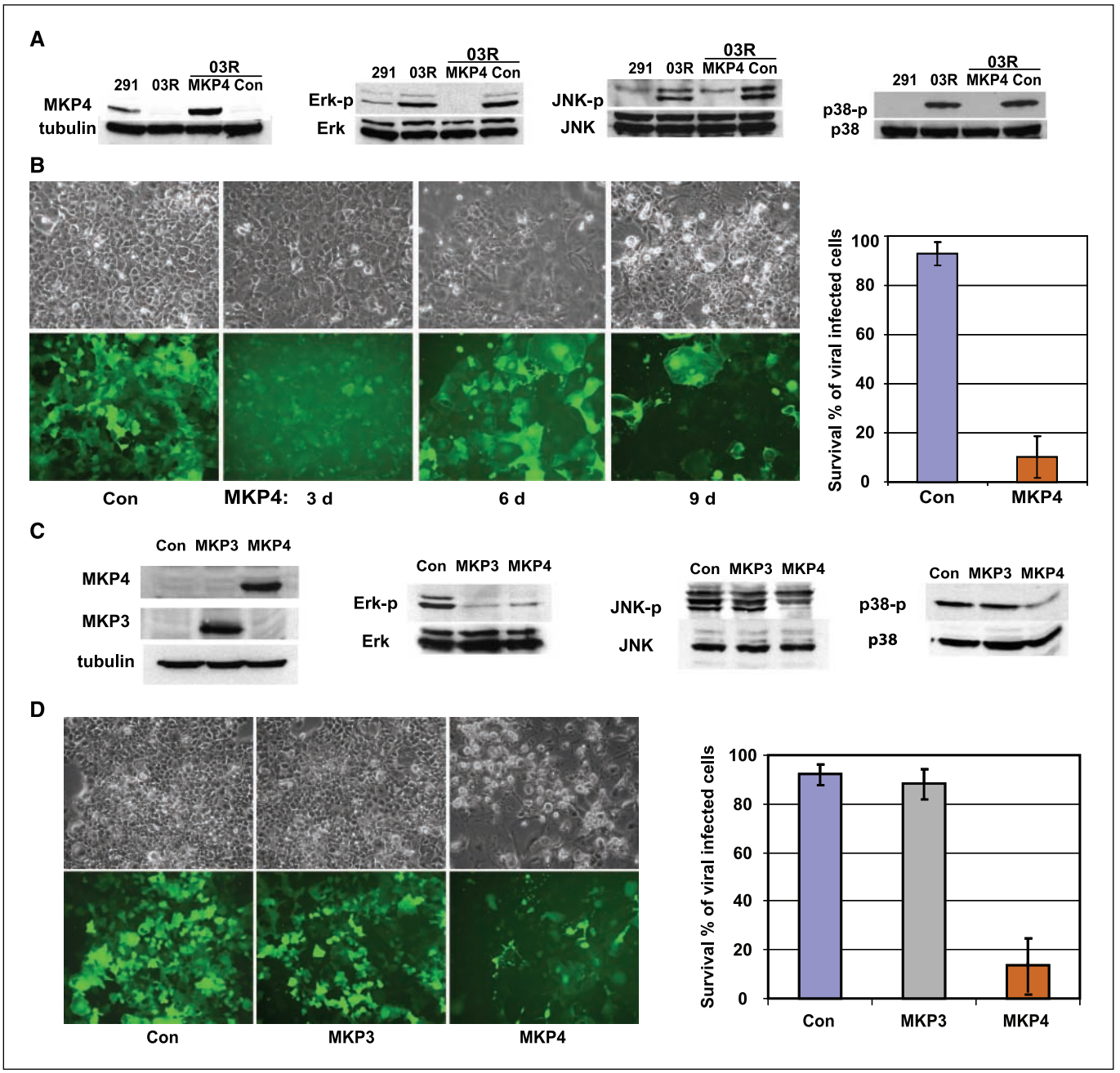

Figure 2. Tumor cell death by MKP4 reconstitution. *A,* analysis of Erk, JNK, and p38 MAPK activities in 03R tumor cells reconstituted with MKP4 by MKP4 recombinant lentivirus. 03R cells were infected with MKP4 lentivirus (MKP4) or GFP lentivirus as control (*Con*). The phosphorylation status of Erk, JNK, and p38 was measured by direct immunoblotting with antibodies specific to dual-phosphorylated Erk, JNK, and p38, respectively. The parental 291 cell was included as negative control. *B,* 03R tumor cells were infected with recombinant lentivirus in which MKP4 wild-type cDNA and GFP are coexpressed from an IRE expression cassette (*MKP4*), with lentivirus containing GFP only as a control. GFP expression was monitored by immunofluorescence to track both groups of lentivirus-infected cells. *Left,* morphology of 03R cells infected with MKP4 virus at days 3, 6, and 9 and control virus at day 9. The percent survival of MKP4 lentivirus-infected 03R cells was calculated by relative ratio of the number of GFP-positive cells 9 d after the infection to the initial infection efficiency (*right*). *C,* comparison of phosphatase activities of MKP3 and MKP4 in 03R cells (as in *A*). *D,* evaluation of the effect of MKP3 expression in 03R tumor cells by infection with MKP3 lentivirus. *Left,* morphology of 03R cells infected with control virus, MKP3 virus, and MKP4 virus at 9 d after the infection. *Right,* survival percentage of MKP4 lentivirus-infected 03R cell was calculated as in *B* above.

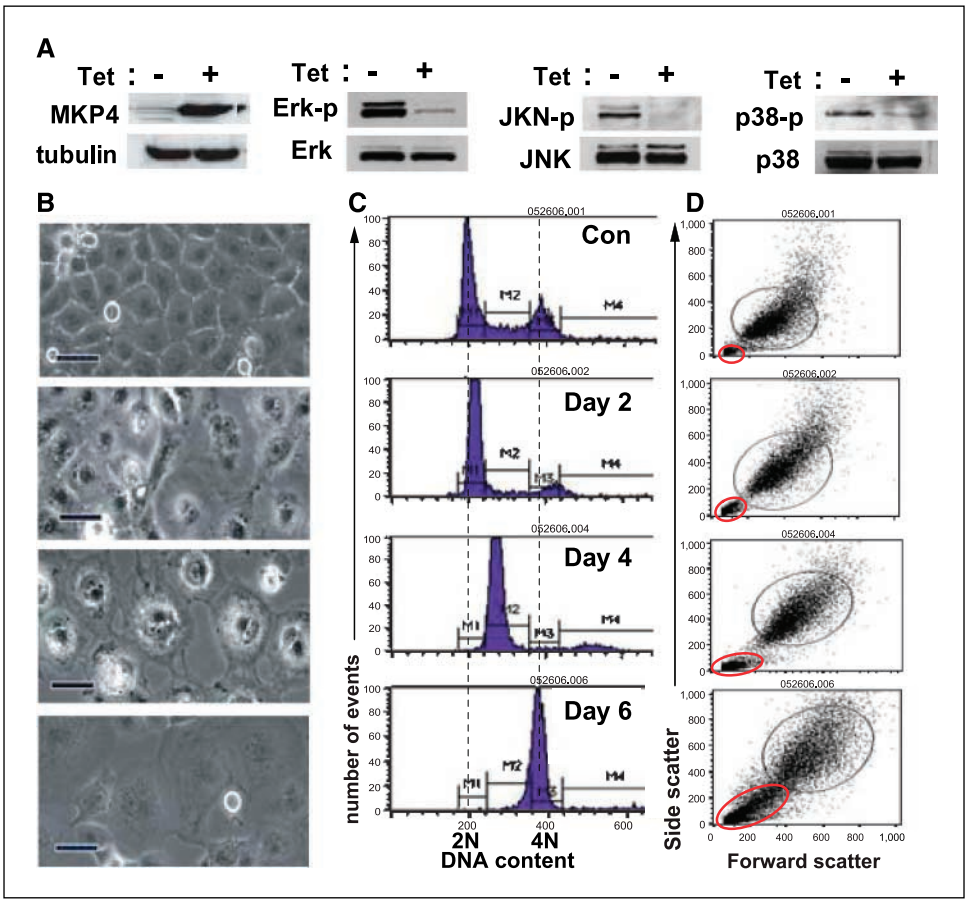

Figure 3. G_2-M associated cell death by MKP4. *A,* a tetracycline-controlled MKP4 expression system was established in H1299 cells. The tetracycline-dependent MKP4 expression and its phosphatase activity were measured by immunoblotting with MKP4 antibody and phospho-specific antibodies to Erk, JNK, and p38, respectively. *B,* the morphologic changes of H1299/MKP4-Tet cells at 2, 4, and 6 d after tetracycline induction. *C,* DNA content changes following MKP4 induction. *D,* cell death profiles of H1299/MKP4-Tet cells after tetracycline induction. *Red circles,* dead cell population.

(03C progressing to 03R) but not papillomagenic lineages (09C progressing to 09R; Fig. 1*C*). To address the broader significance of MKP4 loss as a potential initiation event and to test the hypothesis that its loss or inactivation is associated with malignancy, we examined tumors independently generated by the application of chemical or physical carcinogens in mice (Fig. 1*D*). MKP4 loss occurred in all (100%, $n = 5$) rapidly invasive SCC tested, induced by UVB light, but fewer than half (40%, $n = 20$) of 7,12-dimethylbenz(a)anthracene (DMBA)–12-*O*-tetradecanoylphorbol-13-acetate (TPA) chemically induced papillomas. The finding of MKP4 loss in a subset of the DMBA-TPA–induced tumors corresponds to the ~50% malignant conversion rate of these papillomas in the clonal carcinogenesis model (24). MKP4 loss in SCC-producing lineages and UVB-induced SCC suggests that MKP4 loss is an initiation event that is associated with the progression of carcinogenesis to a more invasive and metastatic phenotype.

Tumor cell death by MKP4 reconstitution. We first determined whether the malignant 03R cells, as a result of their sporadic MKP4 expression loss, showed elevated Erk, JNK, and p38 activity relative to 291 cells, measured by immunoblotting with their corresponding dual phospho-specific antibodies (Fig. 2*A, first two lanes of each panel*). The phosphorylated Erk, JNK, and p38 MAPKs were significantly reduced in 03R cells infected with MKP4 lentivirus (Fig. 2*A, third lane* relative to *fourth lane* of each panel), which is consistent with previous reports that MKP4 is a phosphatase with MAPK specificity for Erk > p38 ≥ JNK (19, 20).

To test whether MKP4 reconstitution could counteract tumor cell characteristics, we constructed a recombinant MKP4 lentivirus with a bicistronic cassette in which MKP4 and GFP are coexpressed through an IRES, allowing us to monitor phenotypes of infected cells. We adjusted viral titers to the minimum that achieved ≥90% infection of 03R cells. To test whether the MKP4 expressed from the lentivirus is functional, the phosphatase activity of MKP4 was evaluated by examining the phosphorylation status of endogenous MAPKs. Both MKP4 and control viruses infected more than 90% of 03R cells (comparing GFP expression throughout the cells in MKP4 (3 days) and Con; Fig. 2*B*). The MKP4 virus–infected cells progressively enlarged over time after the infection. The MKP4 virus–infected cells were selected again, gradually being replaced by the small subset of uninfected cells (GFP negative) as a result of cell death by MKP4 (Fig. 2*B*), such that by 9 days after the infection, nearly 90% of the infected cells were eliminated.

To test whether 03R tumor cell death can be induced by other MKP members of more restricted MAPK substrate specificity, we compared the activity of MKP4 with MKP3, a cytosolic MKP with MAPK selectivity predominantly to Erk. We generated a recombinant MKP3 lentivirus with MKP3 and GFP coexpression in the bicistronic cassette. MKP3 showed a similar Erk dephosphorylation, but had no effect on JNK and p38, as compared with MKP4 (Fig. 2*C*). Although MKP3 inactivated Erk as effectively as MKP4, MKP3 lentivirus-infected 03R cells showed no signs of enlargement or cell death, whereas nearly 90% of MKP4 lentivirus-infected 03R cells died by 9 days after the infection (Fig. 2*D*). Although both MKP3 and MKP4 are cytosolic MKPs with phosphatase activity to Erk, failure to induce 03R tumor cell death by MKP3 suggests that the inactivation of other MAPKs associated

with MKP4 is required to cooperate with Erk inactivation to mediate the death of these highly invasive, metastatic tumor cells.

MKP4 induces G_2-M associated cell death but not G_1 growth arrest. Cellular proliferation is normally determined by extracellular signal(s)–mediated Erk activation that engages the expression of cyclin D1 and the assembly of the cyclin D–cyclin-dependent kinase 4 complex needed for cell cycle entry (25, 26), with small-molecule inhibitors of Erk such as CL-1401 blocking cell cycle progression at the G_1-S boundary (5). Therefore, we evaluated whether MKP4 reconstitution similarly blocked cell cycle progression in G_1. Because 03R cells exhibit strong homophilic cell adhesion, we expanded these studies to the human small cell lung carcinoma cell line H1299 cells, also characterized by undetectable MKP4 protein expression (data not shown), to evaluate the effect of MKP4 on cell cycle progression and tumor suppression. Induction of MKP4 expression was highly responsive to tetracycline in the H1299 stable lines (Fig. 3*A*). MKP4 induction resulted in dephosphorylated Erk, JNK, and p38, consistent with the phosphatase activity of MKP4. Upon MKP4 induction, H1299/MKP4-Tet cells became enlarged with decondensed chromatin, followed by cell death (Fig. 3*B*). In contrast to G_1 growth arrest, as expected from Erk inhibition (5), cells with MKP4 induction accumulated in the G_2-M phase of the cell cycle (Fig. 3*C*), corresponding to cell death (Fig. 3*D*). In conjunction with cell enlargement and decondensed chromatin in MKP4-expressing cells, the G_2-M associated cell death by MKP4 suggests that these cells are undergoing mitotic catastrophe, a general term applied to cell death occurring during mitosis as a result of DNA damage or defective spindle formation in tumor cells (27).

Microtubule disruption by MKP4. Because death occurred upon MKP4 induction without added DNA-damaging agents, we examined the microtubule structure in response to MKP4 induction to see if defective spindle formation is the cause of G_2-M associated cell death. Indirect immunofluorescence staining of polymerized microtubules with anti-tubulin antibody revealed microtubule disruption, as indicated by the collapsed microtubule structure in malignant 03R cells infected with MKP4 lentivirus (Fig. 4*A, top right*), as compared with a typical network of polymerized mictotubules in cells infected with control lentivirus (Fig. 4*A, top left*). Although the staining pattern of microtubule structure in H1299 cells was different from that in 03R tumor cells, the changes in microtubule structure was also observed in H1299 cells with MKP4 expression induced by tetracycline (Fig. 4*B bottom right* compared with *bottom left*).

Tumor suppression by MKP4. We then explored MKP4 activity as a tumor suppressor by two approaches, one in a tumorigenicity assay and the other by the reconstitution of MKP4 by tetracycline induction of established tumors. To evaluate the effect of MKP4 on tumorigenesis *in vivo*, the 03R cells were infected with MKP4-containing virus and immediately inoculated s.c. to BALB/c neonates (Fig. 5*A*). Tumors reached an average of 72 mm^3 in mice injected with 03R cells infected with GFP virus by day 22 after the inoculation compared with no tumors in six mice injected with cells infected with MKP4 virus. By 30 days, tumors in 6/6 GFP alone, control cell–injected mice averaged 319 mm^3 compared with only 43 mm^3 for the tumors in mice injected with the MKP4 virus. Immunostaining of the tumor sections revealed that GFP was negative in tumors from mice injected with 03R cells infected with virus coexpressing MKP4 and GFP (Fig. 5*B, right*), indicating that these tumors were likely derived from the small fraction of uninfected 03R cells in the injected cell population. In contrast, tumors generated from cells infected with control lentivirus were

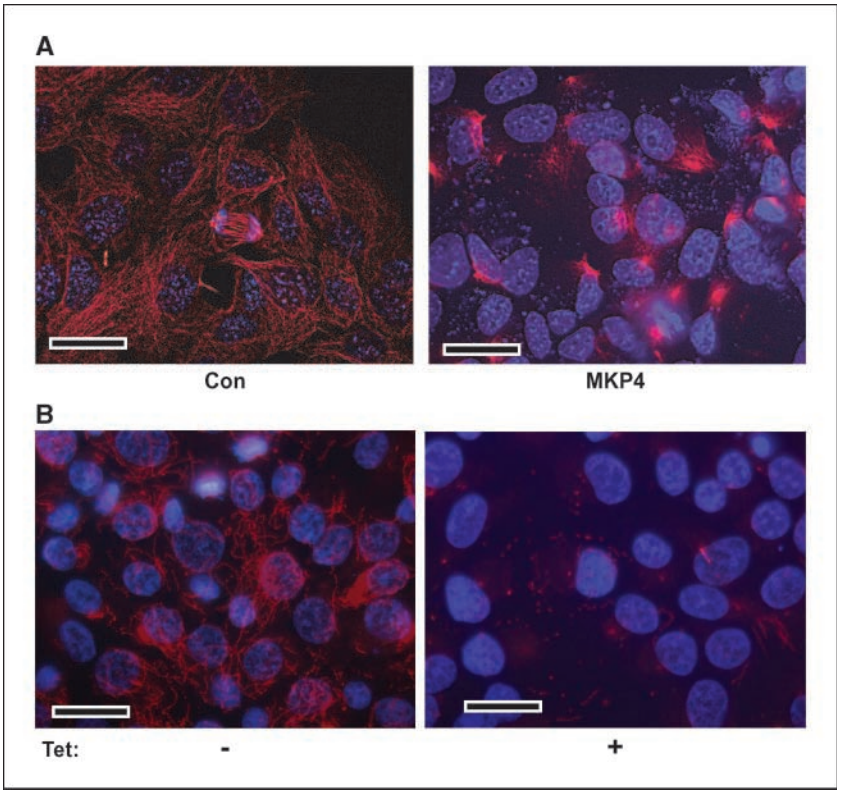

Figure 4. Microtubule disruption by MKP4. *A,* 03R cells were infected with control lentivirus (*Con*) and MKP4 lentivirus (*MKP4*), respectively. Four days after the infection, cells were permeablized to release free tubulin before fixation. Microtubules were stained with anti–tubulin-α antibody (*red*). The nuclei were stained with Hoechst 33342. The images were captured and processed with a Nikon confocal imager system. *B,* analysis of microtubule structure of H1299/MKP4-Tet cells 4 d after MKP4 induction by tetracycline. The images were captured with Leica imager system. *Bars,* 50 μm.

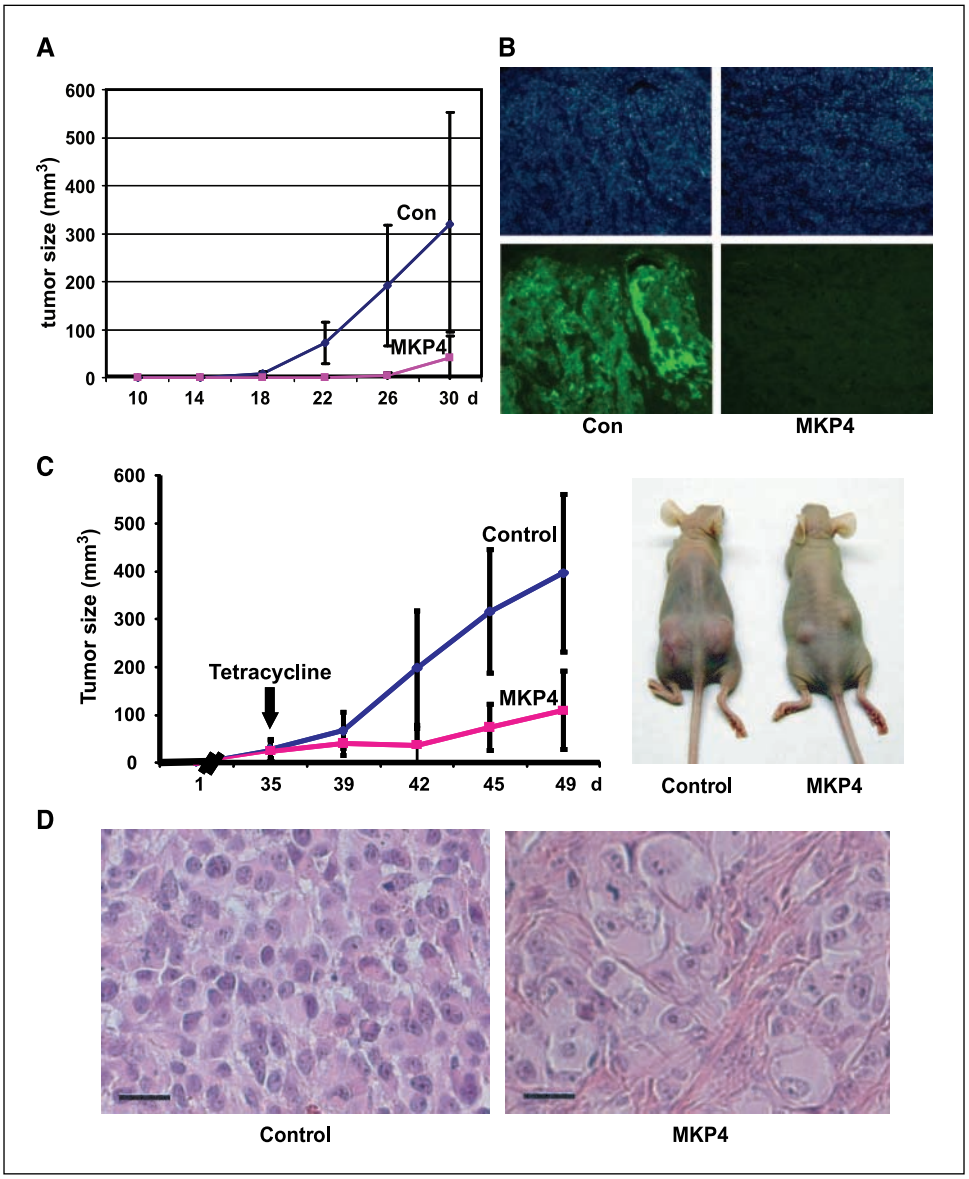

Figure 5. Tumor suppression by MKP4. *A,* suppression of 03R tumor formation by MKP4 lentivirus. Two million each of 03R cells infected with MKP4 lentivirus (*MKP4*) or control GFP lentivirus (*Con*) were inoculated s.c. into BALB/c neonates. The tumor size was monitored beginning at 10 d after the inoculation. Tumor size was calculated by using the equation: tumor size (in mm^3) = 0.4 × length × $width^2$. *Bars,* SD from the mean. *B,* GFP expression in tumor sections was measured by immunostaining with anti-GFP antibody to track lentiviral infected 03R cells. The nuclei in tumor sections were stained with Hoechst. *C,* suppression of preexisting tumor xenographs by tetracycline-induced MKP4 expression. Two million H1299-MKP4-Tet cells were s.c. inoculated into each flank of nude mice. When visible tumor appeared (~50 mm^3) at 35 d after the inoculation, MKP4 expression was induced by the administration of tetracycline in the drinking water. Tumor sizes were measured over time after MKP4 induction by tetracycline. Tumor size was calculated as above. *Right,* example of tumors in the nude mice on day 10 after MKP4 induction by tetracycline. *D,* histology of H1299 tumor specimens from mice on day 8 after MKP4 induction by tetracycline. *Bar,* 50 μm.

GFP positive (Fig. 5*B, left*). The evidence from both the *in vitro* assays and *in vivo* tumorigenesis studies suggests that MKP4 has tumor-suppressive activities.

We next evaluated the ability of MKP4 to suppress growth in well-established tumors, by s.c. inoculating H1299/MKP4-Tet cells into the flanks of nude mice. MKP4 was induced when visible tumors appeared, by administering tetracycline to the mice in their drinking water 35 days after the inoculation. The tumors with MKP4 induction had significantly reduced tumor size compared with control tumors (Fig. 5*C*). Histology of tumor specimens from MKP4-induced mice showed distinct morphologic changes as compared with tumors from control mice (Fig. 5*D*). The tumor cells were evidently enlarged with decondensed nuclei, consistent with the morphology observed in cultured cells shown in Fig. 3*B*. Although the tumor growth curve suggests tumor stasis, the enlarged volume of the tumor cells after MKP4 induction suggests that MKP4 has a tumoricidal effect that contributes to tumor suppression. Taken together, the evidence from these *in vitro* assays and *in vivo* tumorigenesis studies suggests that MKP4 can act as a tumor suppressor, and that its activities could be exploited in developing novel approaches to molecular-targeted cancer therapy.

Growth arrest but not cell death by MKP4 in nontumorigenic keratinocytes. Ideally, therapies should be selective for tumor cells while leaving normal cells unaffected. To test whether normal cells are spared from the action of MKP4, we compared the effect of MKP4 on nontumorigenic keratinocyte precursor cells (291) versus their tumorigenic derivative cells (03R). 291 and 03R cells were infected with MKP4 lentivirus in which MKP4 and GFP are cotranslated from a bicistronic transcript, providing GFP as an indicator for MKP4 expression. The 291 and 03R cells showed similar infection efficiency and MKP4 expression as indicated from the level of GFP and MKP4 in both cell lines (Fig. 6*A*). As expected, the MKP4-positive 03R cells showed significant cell enlargement followed by cell death. At 9 d after the infection, MKP4-positive 03R cells were decreased more than 6-fold in number, and those that remained were significantly enlarged. The enlarged 03R cells

gradually disappeared and were replaced with noninfected, GFP-negative cells. However, over the same time period after MKP4 expression, the nontumorigenic 291 cells did not show significant morphologic changes, and there was no significant reduction in number of MKP4-positive 291 cells (Fig. 6*B* and *C*). Thus, MKP4 expression caused cell death in the 03R malignant cells but not in phenotypically normal 291 keratinocytes. As growth arrest in normal cells can be reversible or consistent with adult tissue function, the differential effect of MKP4 expression in nontumorigenic cells versus tumorigenic cells is promising for selective cancer therapy designed to mimic the effect of MKP4.

Discussion

The concept of the function of MKP as a tumor suppressor has been a matter of speculation ever since MKP1 was identified as a negative regulator of Erk (9, 28). Because MKPs are downstream in the Ras/Erk pathway, their inactivation should be essential for sustained Erk activation in carcinogenesis. However, the involvement of MKPs in carcinogenesis remains controversial. In this study, we show several features of MKP4 as a tumor suppressor. MKP4 was down-regulated in initiated keratinocytes and was lost in their malignant derivatives. Reconstitution of MKP4 expression in tumor cells triggered cellular enlargement, microtubule disruption, and G_2-M associated cell death, features of mitotic catastrophe. Furthermore, MKP4 reconstitution suppressed tumorigenicity *in vivo* in established human non–small cell lung carcinoma H1299 xenografts. The above lines of evidence strongly suggest that MKP4 is a tumor suppressor. Further confirmation of MKP4 tumor suppression will require prospective tumorigenesis studies using conditional MKP4 knock out mice, given that MKP4 knock-out is embryonically lethal (21).

These results are the first evidence for MKP4 as a tumor suppressor, including MKP4 loss in sporadic multistage carcinogenesis and tumor suppression *in vivo*, and support the original concepts of MKPs as negative regulators of oncogenic Erk activation and putative tumor suppressors. Furthermore, our studies provide evidence for a mechanism of action of tumor suppression by cytosolic MKP4 that involves the disruption of microtubules and cell death. The current data point toward MKP localization as a determinant for its contribution in carcinogenesis. Because both MKP1 and MKP2 are nuclear MKPs induced by activated Erk, they seem to be good candidates for forming negative feedback loops to inactivate Erk in the nucleus and opposing the role of nuclear Erk in activation of cell cycle entry. However, the evidence from MKP1 and MKP2 overexpression in human cancer argues against their role in tumor suppression. Although MKP3 showed robust anti-Erk activity without inducing cell death of the 03R tumor cells, the tumor suppression activity of MKP3 has been suggested from its ability to induce pancreatic carcinoma cell death (17) and fibroblast growth inhibition (18). Because MKP3 is an Erk-specific phosphatase, the different effects of MKP3 on different tumor cell types are likely determined by their dependence on Erk. Such a difference may explain why some tumor cells are resistant to Erk inhibition, whereas others are sensitive (29). Nevertheless, cytosolic MKP3 and MKP4 are the only MKPs reported to have tumor suppressor types of activities, indicating the importance of the activation of cytosolic MAPK(s) in carcinogenesis. Although activated MAPKs are generally localized in the nucleus for transcriptional regulation, tumor suppression by cytosolic MKP3 and MKP4 suggests that the inhibition of cytosolic MAPK activity is critical for tumor suppression. However, the contributions of cytosolic MAPKs in carcinogenesis have been largely unknown.

In this study, we found that the reconstitution of MKP4 leads to microtubule disruption, implicating the regulation of microtubule formation by MKP4 and its MAPK substrates. In fact, both Erk and JNK were originally identified as microtubule-associated protein kinases long before being recognized as mitogen-activated protein kinases (30, 31). Despite the fact that about half of activated Erk activity is associated with microtubules (32), the role of Erk activation in microtubule regulation is largely overlooked. Not only are microtubules structural proteins contributing to cell shape maintenance and cell polarity, but they also act as molecular motors for intracellular transport and mitosis. Microtubules undergo constant polymerization and depolymerization, termed as microtubule dynamic instability. This process is regulated through stabilizing factors such as microtubule-associated protein 2 (MAP2) and destabilizing factors such as stathmin or oncoprotein 18 (33). Microtubule poisons disrupt microtubules by

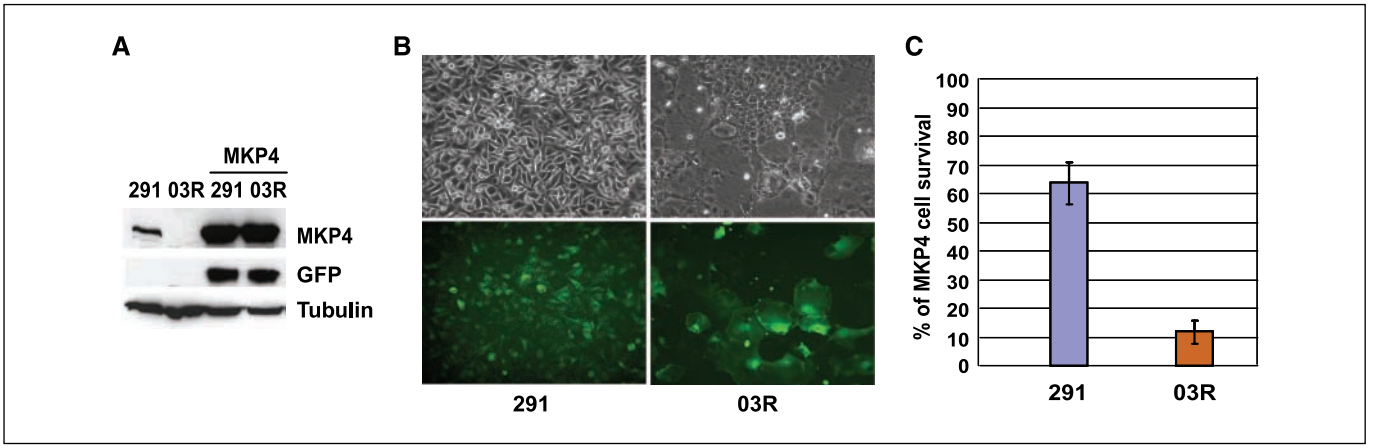

Figure 6. MKP4 expression results in growth arrest in nontumorigenic cells and cell death in tumorigenic cells. Nontumorigenic cells 291 and tumorigenic cells 03R were infected with MKP4 lentivirus. *A,* the infection efficiency was evaluated by immunoblotting of MKP4 and GFP. *B,* the morphology of MKP4 lentivirus-infected 291 and 03R cells at 9 d after the infection. *C,* the percentage of MKP4-positive cells that survived was calculated by dividing the number of MKP4-positive cells at 9 d by the number of MKP4-positive cells at 3 d.

perturbing either polymerization (colchicines) or depolymerization (taxol). In addition to Erk, other MAPKs, JNK, and p38 are also implicated in microtubule regulation. MAP2 is also a substrate of JNK1. Compromised microtubule integrity was observed in neuronal cells from JNK1-deficient mice (34). Stathmin, a microtubule-destabilizing factor, is phosphorylated by p38 (35). Stathmin phosphorylation inhibits its function as a destabilizing factor; thus, p38 also potentially contributes to microtubule polymerization. Microtubule disruption by MKP4, a phosphatase with specificity to Erk, JNK, and p38, suggests that the regulation of microtubule polymerization/depolymerization is a combined effect from different MAPKs.

Erk activation is generally considered to contribute to cell cycle entry by activating the expression of cyclins needed for the cell cycle, such as cyclin D1. However, cyclin D1 expression and cell cycle entry are independent of Erk activation in many malignant tumor cells (29). This is probably due to the inactivation of the Rb pathway in those tumor cells, for example, the loss of p16 in 03R cells (data not shown). Because both Rb inactivation and Ras activation are required for malignant transformation as suggested from the transformation of primary human mammary epithelial cells (36), sustained Erk activation by oncogenic Ras should contribute to activities other than the activation of cell cycle entry in tumor cells. Although the mechanisms of microtubule regulation by Erk remain largely unclear, perturbation of microtubule dynamic instability by MKP4 suggests the involvement of Erk in coordination of microtubule activity in cell cycle progression. We speculate that the regulation of Erk and other MAPK activities by MKP4 is a mechanism to coordinate DNA synthesis and microtubule formation to prevent premature cell division. MKP4 induction is particularly critical when JNK and p38 are activated under stress.

In this study, novel approaches to cancer therapy are suggested by our evidence for the mechanism of MKP4 action in tumor suppression through its ability to induce tumor cells to undergo mitotic catastrophe. The actively cycling tumor cells, being defective in the G_1-S and G_2-M DNA structure or spindle checkpoints, undergo mitotic catastrophe instead of growth arrest upon MKP4 reconstitution, providing a basis for cancer therapy strategies using combinations of MAPK inhibitors. Erk inhibition by small pharmacologic molecules has shown promising therapeutic value in animal models (5). However, Erk inhibition alone has been found to be ineffective in many tumor cells (29) and has failed in clinical trials for treatment of advanced human tumors (37). Overexpression of Erk-specific MKP3 in tumor cells results in growth arrest but not mitotic catastrophe (18) and, in this study, had no effect on 03R tumor cell growth and survival (Fig. 2*D*). In addition, Erk inhibition by UO126 has no effect on the growth and survival of 03R and H1299 cells (data not shown). These lines of evidence suggest that Erk inactivation is not sufficient to induce mitotic catastrophe in cancer, and that other activities associated with MKP4 are required. In addition to Erk, at least, JNK and p38 are inactivated by MKP4. Therefore, combinations of MAPK inhibitors and, possibly, specificity for MAPK isoforms warrant exploration aimed toward mimicking the activities of MKP4 as a more effective strategy in cancer therapy.

Acknowledgments

Received 5/30/2007; revised 7/25/2007; accepted 8/27/2007.

Grant support: NIH CA98893 and Oregon Health and Science University Cancer Institute CA69533.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank G. Tim Bowden and Henry Hennings for providing mouse tumor specimens, Luigi Naldini for providing lentiviral packaging plasmids, Mandy Boyd for flow cytometry assistance, Carl Pelz and Rosalie Sears for Affymetrix GeneChip analysis, and Robin Dickinson and Stephen Keyse for providing MKP4 antibody, MKP3 cDNA, and helpful discussion. Y. Liu is the recipient of a Career Development Award from the Dermatology Foundation.

References

1. Katz ME, McCormick F. Signal transduction from multiple Ras effectors. Curr Opin Genet Dev 1997;7:75–9.
2. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res 1998;74:49–139.
3. Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 1999;18:813–22.
4. Mansour SJ, Matten WT, Hermann AS, et al. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 1994;265:966–70.
5. Sebolt-Leopold JS, Dudley DT, Herrera R, et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat Med 1999;5:810–6.
6. Keyse SM. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr Opin Cell Biol 2000;12:186–92.
7. Fjeld CC, Rice AE, Kim Y, Gee KR, Denu JM. Mechanistic basis for catalytic activation of mitogen-activated protein kinase phosphatase 3 by extracellular signal-regulated kinase. J Biol Chem 2000;275:6749–57.
8. Dickinson RJ, Keyse SM. Diverse physiological functions for dual-specificity MAP kinase phosphatases. J Cell Sci 2006;119:4607–15.
9. Sun H, Charles CH, Lau LF, Tonks NK. MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. Cell 1993;75:487–93.
10. Keyse SM, Emslie EA. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature 1992;359:644–7.
11. Sun H, Tonks NK, Bar-Sagi D. Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. Science 1994;266:285–8.
12. Loda M, Capodieci P, Mishra R, et al. Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. Am J Pathol 1996;149:1553–64.
13. Vicent S, Garayoa M, Lopez-Picazo JM, et al. Mitogen-activated protein kinase phosphatase-1 is overexpressed in non-small cell lung cancer and is an independent predictor of outcome in patients. Clin Cancer Res 2004;10:3639–49.
14. Magi-Galluzzi C, Mishra R, Fiorentino M, et al. Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. Lab Invest 1997;76:37–51.
15. Liao Q, Guo J, Kleeff J, et al. Down-regulation of the dual-specificity phosphatase MKP-1 suppresses tumorigenicity of pancreatic cancer cells. Gastroenterology 2003;124:1830–45.
16. Wang Z, Liu Y, Mori M, Kulesz-Martin M. Gene expression profiling of initiated epidermal cells with benign or malignant tumor fates. Carcinogenesis 2002;23:635–43.
17. Furukawa T, Sunamura M, Motoi F, Matsuno S, Horii A. Potential tumor suppressive pathway involving DUSP6/MKP-3 in pancreatic cancer. Am J Pathol 2003;162:1807–15.
18. Marchetti S, Gimond C, Roux D, Gothie E, Pouyssegur J, Pages G. Inducible expression of a MAP kinase phosphatase-3–GFP chimera specifically blunts fibroblast growth and ras-dependent tumor formation in nude mice. J Cell Physiol 2004;199:441–50.
19. Muda M, Boschert U, Smith A, et al. Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. J Biol Chem 1997;272:5141–51.
20. Dickinson RJ, Williams DJ, Slack DN, Williamson J, Seternes OM, Keyse SM. Characterization of a murine gene encoding a developmentally regulated cytoplasmic dual-specificity mitogen-activated protein kinase phosphatase. Biochem J 2002;364:145–55.
21. Christie GR, Williams DJ, Macisaac F, Dickinson RJ, Rosewell I, Keyse SM. The dual-specificity protein phosphatase DUSP9/MKP-4 is essential for placental function but is not required for normal embryonic development. Mol Cell Biol 2005;25:8323–33.
22. Kulesz-Martin MF, Penetrante R, East CJ. Benign and malignant tumor stages in a mouse keratinocyte line treated with 7,12-dimethylbenz[*a*]anthracene *in vitro*. Carcinogenesis 1988;9:171–4.
23. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463–71.
24. Kulesz-Martin MF, Blumenson LE, Manly KF, Siracky J, East CJ. Tumor progression of murine epidermal cells after treatment *in vitro* with 12-*O*-tetradecanoylphorbol-13-acetate or retinoic acid. Cancer Res 1991;51:4701–6.
25. Cheng M, Sexl V, Sherr CJ, Roussel MF. Assembly of cyclin D–dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc Natl Acad Sci U S A 1998;95:1091–6.

26. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002;2:103–12.
27. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. Oncogene 2004;23:2825–37.
28. Brondello JM, Brunet A, Pouyssegur J, McKenzie FR. The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. J Biol Chem 1997;272:1368–76.
29. Solit DB, Garraway LA, Pratilas CA, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006;439:358–62.
30. Ray LB, Sturgill TW. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine *in vivo*. Proc Natl Acad Sci U S A 1988;85:3753–7.
31. Kyriakis JM, Avruch J. pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. J Biol Chem 1990;265:17355–63.
32. Reszka AA, Seger R, Diltz CD, Krebs EG, Fischer EH. Association of mitogen-activated protein kinase with the microtubule cytoskeleton. Proc Natl Acad Sci U S A 1995;92:8881–5.
33. Desai A, Mitchison TJ. Microtubule polymerization dynamics. Annu Rev Cell Dev Biol 1997;13:83–117.
34. Chang L, Jones Y, Ellisman MH, Goldstein LS, Karin M. JNK1 is required for maintenance of neuronal microtubules and controls phosphorylation of microtubule-associated proteins. Dev Cell 2003;4:521–33.
35. Mizumura K, Takeda K, Hashimoto S, Horie T, Ichijo H. Identification of Op18/stathmin as a potential target of ASK1–38 MAP kinase cascade. J Cell Physiol 2006;206:363–70.
36. Elenbaas B, Spirio L, Koerner F, et al. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 2001;15:50–65.
37. Rinehart J, Adjei AA, Lorusso PM, et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non–small-cell lung, breast, colon, and pancreatic cancer. J Clin Oncol 2004;22:4456–62.

Microtubule Disruption and Tumor Suppression by Mitogen-Activated Protein Kinase Phosphatase 4

Yuangang Liu, James Lagowski, Aaron Sundholm, et al.

Cancer Res 2007;67:10711-10719.