Regulation of Arsenic Trioxide-induced Cellular Responses by Mnk1 and Mnk2*

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Arsenic trioxide (As_2O_3) is a potent inducer of apoptosis of malignant cells *in vitro* and *in vivo*, but the precise mechanisms by which it mediates such effects are not well defined. We provide evidence that As_2O_3 induces phosphorylation/activation of the MAPK signal-integrating kinases (Mnks) 1 and 2 in leukemia cell lines. Such activation is defective in cells with targeted disruption of the *p38α* MAPK gene, indicating that it requires upstream engagement of the p38 MAPK pathway. Studies using Mnk1$^{-/-}$ or Mnk2$^{-/-}$, or double Mnk1$^{-/-}$Mnk2$^{-/-}$ knock-out cells, establish that activation of Mnk1 and Mnk2 by arsenic trioxide regulates downstream phosphorylation of the eukaryotic initiation factor 4E at Ser-209. Importantly, arsenic-induced apoptosis is enhanced in cells with targeted disruption of the *Mnk1* and/or *Mnk2* genes, suggesting that these kinases are activated in a negative-feedback regulatory manner, to control generation of arsenic trioxide responses. Consistent with this, pharmacological inhibition of Mnk activity enhances the suppressive effects of arsenic trioxide on primary leukemic progenitors from patients with acute leukemias. Taken together, these findings indicate an important role for Mnk kinases, acting as negative regulators for signals that control generation of arsenic trioxide-dependent apoptosis and antileukemic responses.

The use of arsenic-containing compounds in the treatment of leukemias and other malignancies dates several decades back in time (1). Despite the known existence of arsenic compounds for hundreds of years, only recently has a derivative of this heavy metal, arsenic trioxide (As_2O_3), found an established role in the treatment of a human disease. Extensive work has now established that As_2O_3 exhibits potent pro-apoptotic effects against malignant cells and has important antineoplastic activities *in vitro* and *in vivo* (2–6). Importantly, As_2O_3 has been approved for the treatment of acute promyelocytic leukemia

(APL)[2] in humans, and its introduction in the therapy of this form of acute leukemia has had a major impact in medical oncology (1, 7–10). Notably, APL is a form of leukemia with unusual sensitivity to the effects of arsenic trioxide. It is well established that induction of differentiation of APL cells occurs at low concentrations (0.5 μM), whereas higher ($\geq$2 μM) concentrations are required for the generation of its pro-apoptotic effects in other cell types (2–6). As_2O_3 is also currently under investigation for the treatment of other hematological malignancies and clonal disorders, including chronic myelogenous leukemia, multiple myeloma, and myelodysplastic syndromes (2, 4, 11–13). A remaining challenge in introducing arsenic trioxide in the treatment of other malignancies is the development of means to enhance arsenic-dependent apoptosis at lower final concentrations. Thus, identification of cellular pathways that could be targeted to enhance the antineoplastic properties of As_2O_3 are of high translational potential and interest.

Previous work has suggested that the pro-apoptotic effects of As_2O_3 on APL cells correlate with targeting and degradation of the abnormal PML-RARα fusion protein (5, 14, 15), although independent mechanisms also exist (16). Among the genes regulated by the PML-RARα fusion protein is the mitogen-activated protein kinase (MAPK)-interacting kinase 1 (Mnk1) (17, 18), a kinase that was recently shown to be post-translationally stabilized by PML-RARα fusion protein and participate in the control of differentiation of myeloid cells (19). Mnk1 and the related Mnk2 are known to be activated downstream of MAPKs, via phosphorylation at Thr-197 and Thr-202 located in their activation loop (20–23), and after their activation, they in turn phosphorylate the cap binding eukaryotic initiation factor 4E (eIF4E) at Ser-209 in response to mitogens and stress signals (22, 23).

In previous work, we had demonstrated that the p38 MAPK is activated in response to treatment of leukemic cells with As_2O_3 (24) and shown that such activation occurs in a negative feedback regulatory manner, to control and limit arsenic-dependent apoptosis. This was established by studies demonstrating that pharmacological inhibitors of p38 promote generation of As_2O_3-dependent apoptosis (24), whereas pro-apoptotic

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[2] The abbreviations used are: APL, acute promyelocytic leukemia; MAPK, mitogen-activated protein kinase; Mnk1, MAPK-interacting kinase 1; eIF4E, eukaryotic initiation factor 4E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CFU-L, leukemic CFU-blast; siRNA, small interference RNA; MEF, mouse embryonic fibroblast; Mek, MAPK/Erk kinase; Erk, extracellular signal-regulated kinase.

responses are enhanced in p38α knock-out cells (25). We have been interested in identifying downstream effectors of the p38 MAPK that may be engaged during As$_2$O$_3$ treatment of leukemic cells to counteract its antileukemic effects, because such proteins could be conceivably targeted for the treatment of hematological malignancies and other tumors. In the present study we sought to identify downstream effectors of p38 that may account for the negative regulatory properties of the p38 pathway in the induction of arsenic trioxide responses. Our data demonstrate that the kinases Mnk1 and Mnk2 are activated in an As$_2$O$_3$-inducible manner and regulate downstream phosphorylation of eIF4E. Such engagement of Mnk1 by As$_2$O$_3$ requires upstream activation of the p38 MAPK pathway, as evidenced in studies using p38α knock-out cells. The induction of arsenic trioxide-induced apoptosis is enhanced in cells with targeted disruption of the *Mnk1* and/or *Mnk2* genes, indicating that activation of Mnk kinases negatively regulates As$_2$O$_3$-dependent apoptosis. Consistent with this, inhibition of Mnk kinase activity or siRNA-mediated knockdown of Mnk1/Mnk2 expression enhances the antileukemic properties of As$_2$O$_3$ on primitive hematopoietic progenitors from patients with acute leukemia *in vitro*, raising the possibility that targeting Mnk kinases may be an effective approach to enhance the antileukemic properties of As$_2$O$_3$ *in vivo*.

MATERIALS AND METHODS

Cells and Reagents—The CML-derived K562 cell line, the NB-4 human APL cell line and the U937 human acute myelomonocytic leukemia cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Immortalized mouse embryonic fibroblasts from p38α knock-out mice (26) were kindly provided from Dr. Angel Nebreda (CNIO (Spanish National Cancer Center), Madrid, Spain). Immortalized fibroblasts from Mnk1/Mnk2 double knock-out and Mnk1 and Mnk2 single knock-out mice (27) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. Arsenic trioxide (As$_2$O$_3$) was purchased from Sigma. Antibodies against the phosphorylated forms of Mnk1 (Thr-197/202), p42/p44 MAPK (Thr-202/Tyr204), p38 MAPK (Thr-180/Tyr-182), and eIF4E (Ser-209) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against eIF4E were also obtained from Cell Signaling Technology, Inc. Antibodies against p38α MAPK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from Chemicon International (Temecula, CA), respectively. The Mnk1 inhibitor, CGP57380, the MEK1/2 inhibitor, U0126, and anisomycin were purchased from Calbiochem.

Cell Lysis and Immunoblotting—Cells were treated with the indicated doses of As$_2$O$_3$ for the indicated times and subsequently lysed in the phosphorylation lysis buffer as previously described (28–30). In the experiments in which pharmacological inhibitors were used, the cells were pretreated for 60 min at the indicated final concentrations of inhibitors and subsequently treated for the indicated times with As$_2$O$_3$, in the continuous presence of the inhibitors, prior to cell lysis in phosphorylation lysis buffer. Immunoblotting using an enhanced
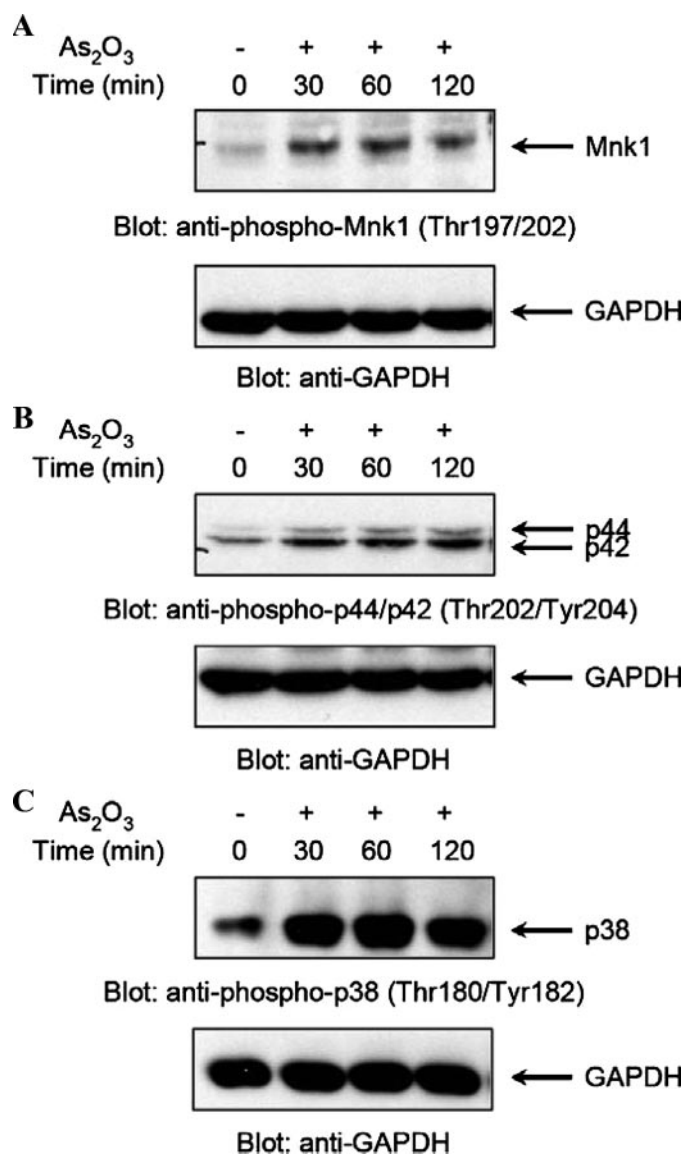

FIGURE 1. **As$_2$O$_3$-dependent phosphorylation of the Mnk1 in NB4 acute promyelocytic leukemia cells.** *A*, NB-4 cells were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-Mnk1 (Thr-197/202) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*). *B*, NB-4 cells were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-p44/p42 (Thr-202/Tyr-204) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for loading (*lower panel*). *C*, NB-4 cells were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-p38 (Thr-180/Tyr-182) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for loading (*lower panel*).

chemiluminescence (ECL) method was done as previously described (28–30).

Cell Proliferation Assays—Cells were treated with the indicated doses of As$_2$O$_3$, in the presence or absence of CGP57380 (2 μM), for 7 days. Cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide method were performed as in our previous studies (31, 32).

Evaluation of Apoptosis—Cells were exposed to the indicated doses of As$_2$O$_3$ for the indicated time period. Flow cytometric

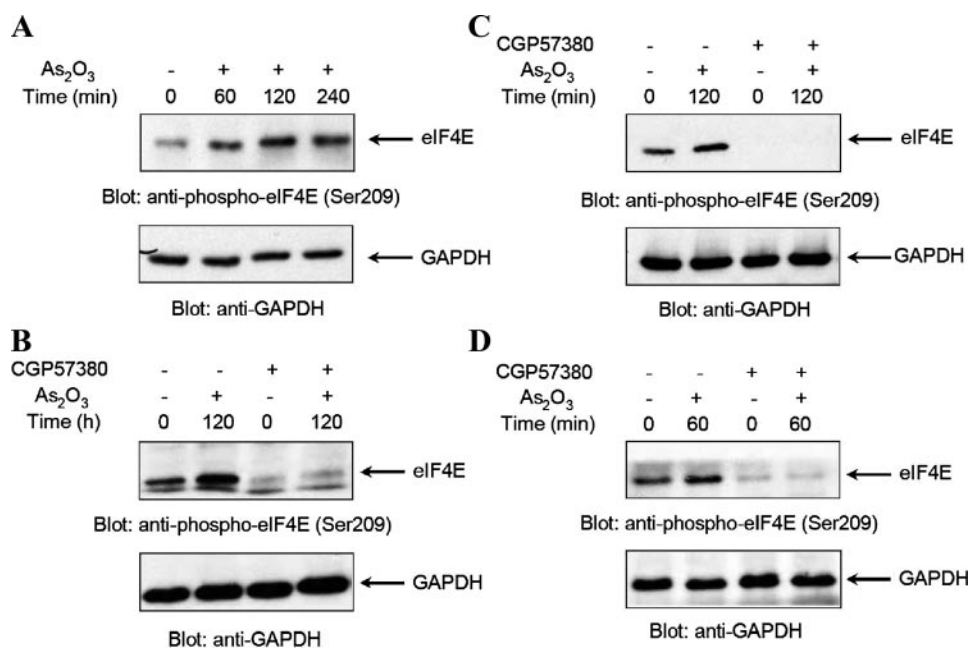FIGURE 2. **As$_2$O$_3$ induces phosphorylation of eIF4E in an Mnk1-dependent manner in leukemia cell lines.** *A*, NB-4 cells were incubated in the absence or presence of As$_2$O$_3$ (2 µM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*). *B*, NB-4 cells were pre-treated for 60 min with CGP57380 (20 µM), before treatment with As$_2$O$_3$ (2 µM) for 120 min, as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *C*, K562 cells were pre-treated for 60 min with CGP57380 (20 µM), before treatment with As$_2$O$_3$ (2 µM) for 120 min, as indicated. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *D*, U937 cells were pre-treated for 60 min with CGP57380 (20 µM), before treatment with As$_2$O$_3$ (5 µM) for 60 min, as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for protein loading (*lower panel*).

assays to evaluate apoptosis by Annexin and propidium iodide staining were done essentially as previously described (33).

Human Hematopoietic Progenitor Cell Assays—Bone marrow or peripheral blood from patients with acute leukemia were collected after obtaining consent approved by the Institutional Review Board of Northwestern University. The effects of arsenic trioxide on the growth of leukemic progenitors were assessed by clonogenic assays in methylcellulose, as in previous studies (24, 31, 33, 34). The cells were cultured in the presence or absence of As$_2$O$_3$ (0.5 µM) in the presence or absence of the indicated concentration of CGP57380 (10 µM). Leukemic CFU-blast (CFU-L) colonies were scored on day 14 of culture.

siRNA-mediated Knockdown of Mnk1/2 in Human Leukemic Cells—For knockdown of Mnk1 and Mnk2 simultaneously, U937 cells were transfected with a mixed pool of pre-designed Mnk1- and Mnk2-specific siRNAs from Dharmacon (Lafayette, CO), using Amaxa Biosystems Nucleofector Kit V (Gaithersburg, MD), whereas the control set of U937 cells was transfected with a non-targeted siRNA pool. Cells were subsequently cultured for 48 h prior following quantification real-time reverse transcription-PCR method using Mnk1/2-specific primers. These studies were performed as previously described (61). The suppressive effects of arsenic trioxide on leukemic progenitor (CFU-L) colony formation from U937 leukemic

cells transfected with Mnk1/2-specific siRNAs were assessed by clonogenic assays in methylcellulose (24, 31, 33, 34). The cells were cultured in the presence or absence of As$_2$O$_3$ (0.5 µM) and leukemic CFU-blast (CFU-L) colonies were scored on day 5 of culture.

RESULTS

We initially determined whether As$_2$O$_3$ treatment of leukemic cells induces phosphorylation/activation of Mnk1. NB4 cells were treated with arsenic trioxide for different times, ranging from 30 to 120 min, and after cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of Mnk1 on Thr-197 and Thr-202. As$_2$O$_3$ treatment triggered phosphorylation of Mnk1 that occurred rapidly, within 30 min, and was still detectable after 120 min of treatment of the cells (Fig. 1*A*). Such a time pattern of Mnk1 phosphorylation was similar to the patterns seen for arsenic-inducible phosphorylation of MAPKs, including Erk1/Erk2 (Fig. 1*B*) and p38 (Fig. 1*C*). Thus, Mnk1 is phosphorylated/activated during treatment of leukemia cells with As$_2$O$_3$, suggesting that it may play a role in the generation of arsenic trioxide responses.

In response to mitogen and diverse cellular stresses, phosphorylated/activated Mnk1, and the related kinase, Mnk2, regulate the phosphorylation of the cap-binding eIF4E at the physiologically relevant site, Ser-209 (22, 23, 35). Consistent with the observed phosphorylation/activation of Mnk1, As$_2$O$_3$ treatment of NB4 cells was found to induce strong phosphorylation of eIF4E (Fig. 2*A*). In experiments in which cells were pretreated with the specific Mnk kinase inhibitor (36, 37), CGP57380, we found that the As$_2$O$_3$-dependent phosphorylation of eIF4E was blocked (Fig. 2*B*), indicating that Mnk activity is essential for such phosphorylation. As$_2$O$_3$-dependent inducible phosphorylation of eIF4E was also detected in other hematopoietic cell lines of diverse origin, including the CML-derived K562 cell line (Fig. 2*C*), and the acute myelomonocytic leukemia-derived U937 cell line (Fig. 2*D*). As in the case of NB4 cells, cotreatment of either cell line with the Mnk kinase inhibitor, CGP57380, resulted in abrogation of eIF4E phosphorylation (Fig. 2, *C* and *D*), establishing a requirement for Mnk activity in As$_2$O$_3$-dependent engagement of eIF4E.

It is well established that phosphorylation of Mnk1 in response to stress signals requires upstream activation of MAPKs (21). As in previous work we had demonstrated that As$_2$O$_3$ induces activation of p38 MAPK (24, 25), we sought to determine if p38 activity is required for activation of Mnk1 in
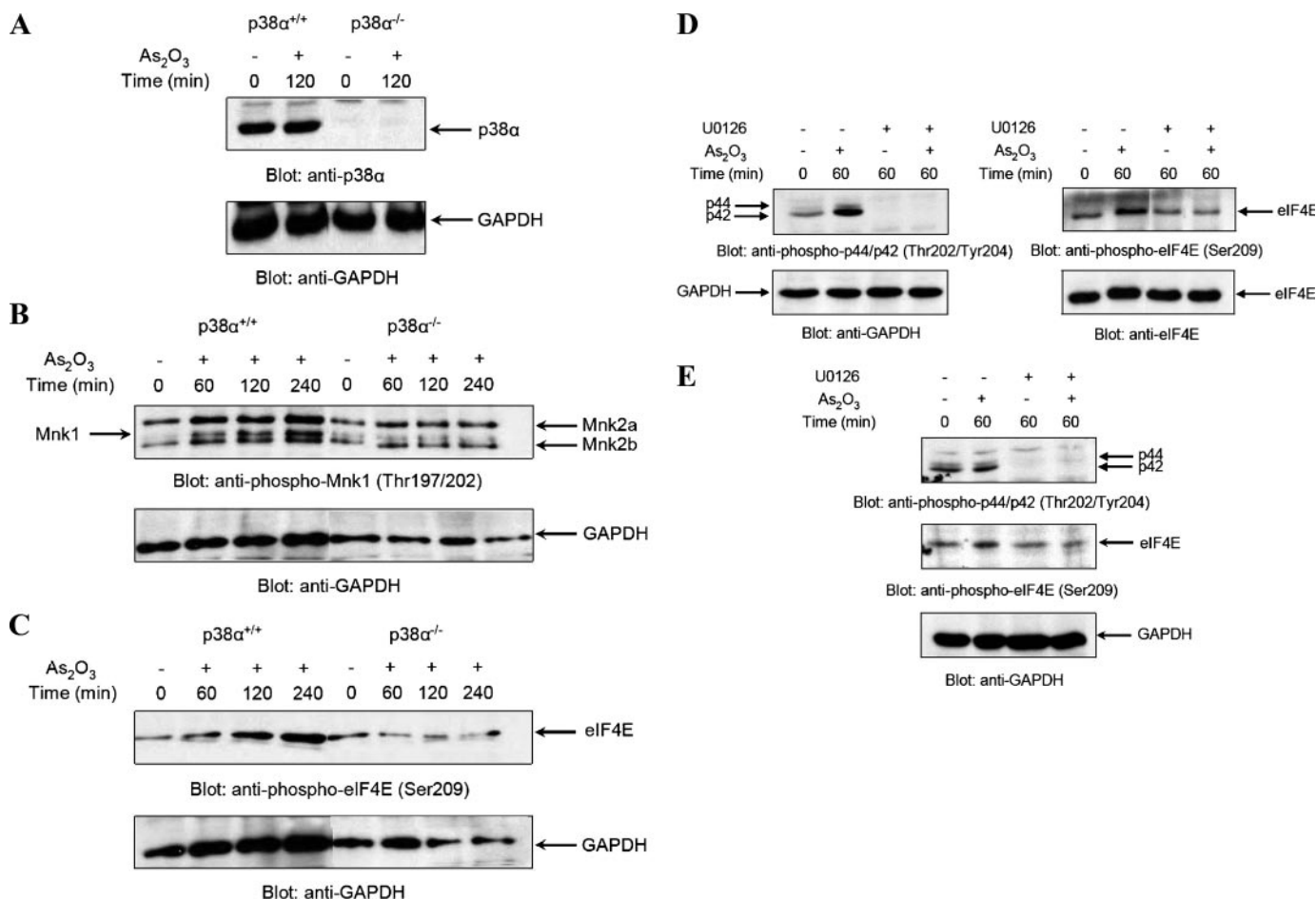
FIGURE 3. **As$_2$O$_3$-dependent phosphorylation/activation of Mnk1 and Mnk2 and phosphorylation of the eIF4E is MAPK-dependent.** *A*, total cell lysates from As$_2$O$_3$ (2 μM)-treated p38$\alpha^{+/+}$ or p38$\alpha^{-/-}$ MEFs were resolved by SDS-PAGE and immunoblotted with an anti-p38α antibody (*upper panel*). The same blot was re-probed with an anti-GAPDH antibody to control for loading (*lower panel*). *B*, p38$\alpha^{+/+}$ and p38$\alpha^{-/-}$ MEFs were treated with As$_2$O$_3$ (2 μM) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-Mnk1 (Thr197/202) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *C*, p38$\alpha^{+/+}$ and p38$\alpha^{-/-}$ MEFs were treated with As$_2$O$_3$ (2 μM) for the indicated times or with anisomycin (1 μg/ml) as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *D*, NB4 cells were pre-treated for 60 min with MEK1/2 inhibitor, U0126 (10 μM), before treatment with As$_2$O$_3$ (1 μM) for indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immunoblotted either with an anti-phospho-Erk (Thr-202/Tyr-204) antibody (*left side*; *upper panel*) or with an anti-phospho-eIF4E (Ser-209) antibody (*right side*; *upper panel*). Blots were then stripped and reprobed with either an anti-GAPDH antibody (*left side*; *lower panel*) or with an anti-eIF4E antibody (*right side*; *lower panel*) to control for loading. *E*, U937 cells were pre-treated for 60 min with MEK1/2 inhibitor, U0126 (10 μM), prior to treatment with As$_2$O$_3$ (1 μM) for the indicated times, in the continuous presence or absence of the MEK inhibitor. Equal amounts of total cell lysates were resolved by SDS-PAGE, and the upper part of the blot was immunoblotted with an anti-phospho-Erk (Thr-202/Tyr-204) antibody (*upper panel*), while the lower part of blot was immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*central panel*). The upper part of blot was then stripped and reprobed with an anti-GAPDH antibody to control for loading (*lower panel*).

response to arsenic trioxide. To address this, we used immortalized mouse embryonic fibroblasts (MEFs), obtained from mice with targeted deletion of the *p38α* gene (26). As expected, there was no detection of the predominant p38 isoform (38), p38α, in such knock-out MEFs (Fig. 3*A*). Subsequently, experiments were performed in which p38$\alpha^{+/+}$ and p38$\alpha^{-/-}$ MEFs were incubated either in the presence or absence of As$_2$O$_3$ for different times, and after cell lysis, lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form the protein on Thr-197/202. As shown in Fig. 3*B*, Mnk1 was rapidly phosphorylated in response to arsenic treatment of the parental, p38$\alpha^{+/+}$, cells (Fig. 3*B*). In addition, As$_2$O$_3$ treatment induced phosphorylation of the two forms of Mnk2, Mnk2a and Mnk2b, which were expressed in such MEFs (Fig. 3*B*). On the other hand, As$_2$O$_3$-inducible phosphorylation of both Mnk1 and Mnk2 was defective in the p38$\alpha^{-/-}$ cells,

indicating a requirement for p38α MAPK in As$_2$O$_3$-dependent Mnk phosphorylation. Consistent with the suppression of Mnk1/2 phosphorylation/activation in p38 knock-out cells, phosphorylation of eIF4E by As$_2$O$_3$ was completely abrogated in the p38α knock-out MEFs (Fig. 3*C*). Taken altogether, these studies established that As$_2$O$_3$ phosphorylates/activates both Mnk1 and Mnk2, and that such activation requires upstream engagement of the p38 MAPK pathway.

Mnk1 and Mnk2 have been previously identified as Erk kinase substrates in other systems (20, 21) and are known to be phosphorylated/activated *in vitro* by both Erk and p38 in response various stimuli such as growth factors, cellular stresses and inflammatory cytokines (20, 21, 22, 35). Because Erk1/2 are phosphorylated/activated by As$_2$O$_3$, we determined whether Erk kinase activity is required for As$_2$O$_3$-dependent Mnk1/2 activation. To address this, experiments were per-
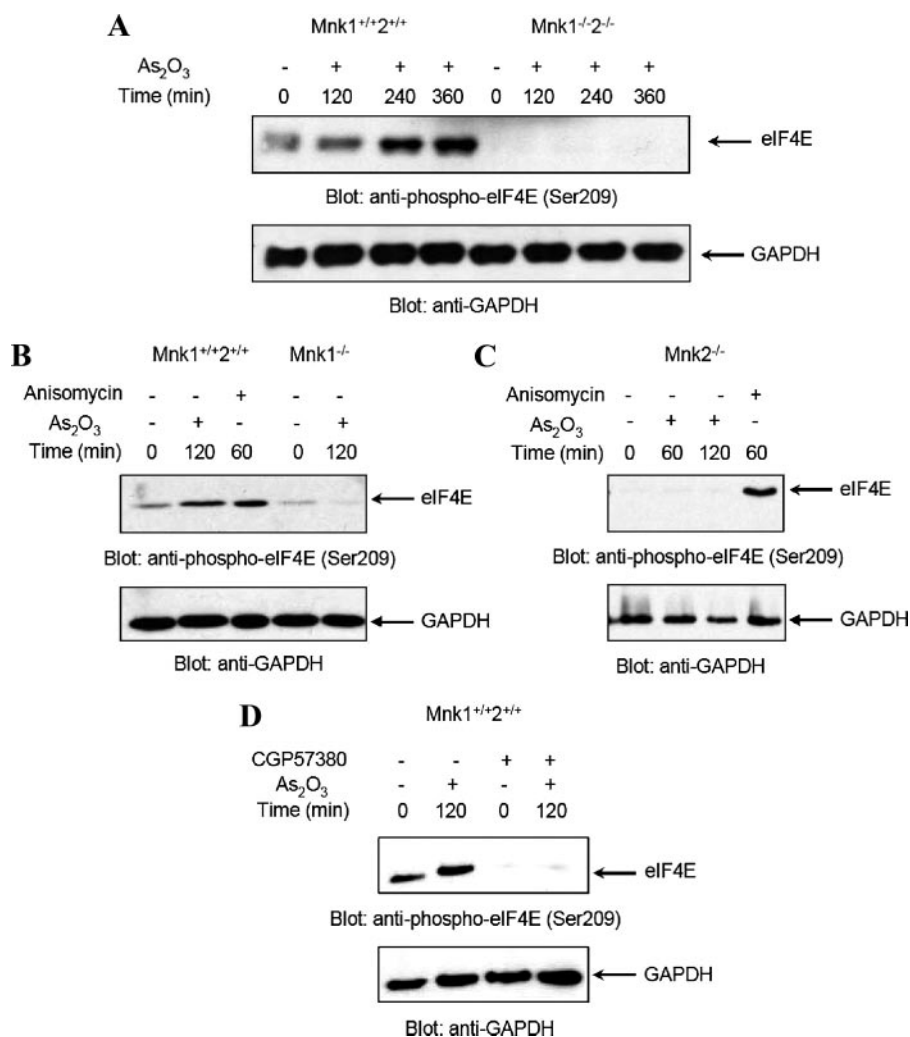
FIGURE 4. **As$_2$O$_3$-dependent phosphorylation of the eIF4E in Mnk1$^{+/+}$2$^{+/+}$, Mnk1$^{-/-}$2$^{-/-}$, Mnk1$^{-/-}$, and Mnk2$^{-/-}$ MEFs.** *A*, Mnk1$^{+/+}$2$^{+/+}$ and Mnk1$^{-/-}$2$^{-/-}$ MEFs were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for the indicated times. Equal amounts of total cell lysates were resolved by SDS-PAGE and immuno-blotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The blot was then stripped and re-probed with an anti-GAPDH antibody to control for loading (*lower panel*). *B*, Mnk1$^{+/+}$2$^{+/+}$ and Mnk1$^{-/-}$ MEFs were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for indicated times or in the presence of anisomycin (1 μg/ml) for 60 min. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immu-noblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *C*, Mnk2$^{-/-}$ MEFs were incubated in the absence or presence of As$_2$O$_3$ (2 μM) for indicated times or in the presence of anisomycin (1 μg/ml) for 60 min. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was then stripped and reprobed with an anti-GAPDH antibody to control for loading (*lower panel*). *D*, Mnk1$^{+/+}$2$^{+/+}$ MEFs were pre-treated for 60 min with CGP57380 (20 μM), before treatment with As$_2$O$_3$ (2 μM) for 120 min as indicated. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-phospho-eIF4E (Ser-209) antibody (*upper panel*). The same blot was reprobed with an anti-GAPDH antibody to control for loading (*lower panel*).

with As$_2$O$_3$, and the phosphoryla-tion of eIF4E was assessed. Arsenic trioxide treatment induced strong phosphorylation o eIF4E (Fig. 4*A*) in parental MEFs, but not in Mnk1$^{-/-}$ Mnk2$^{-/-}$ MEFs. Interestingly, such As$_2$O$_3$-inducible phosphoryla-tion of eIF4E was also blocked in either single Mnk1 (Fig. 4*B*) or Mnk2 (Fig. 4*C*) knock-out cells, indicating that the functions of both Mnk1 and Mnk2 are essential for eIF4E phosphorylation in response to arsenic trioxide. It should be also noted that pretreatment of Mnk1$^{+/+}$ Mnk2$^{+/+}$ MEFs with CGP57380, also resulted in suppression of eIF4E phosphorylation (Fig. 4*D*).

In subsequent studies, we sought to determine the functional role of Mnk1 and Mnk2 in the generation of As$_2$O$_3$-mediated apoptosis. When the induction of apoptosis by arsenic trioxide was determined in immortalized MEFs with targeted disruption of the *Mnk1* and/or *Mnk2* genes, we found enhanced As$_2$O$_3$-induced apoptosis in cells lacking either Mnk1, or Mnk2 and in double knock-out MEFs for both Mnk1 and Mnk2, as compared with the parental cells (Fig. 5). Interest-ingly, there was slightly more apo-ptosis in the single Mnk1 knock-out MEFs as compared with the single Mnk2 knockouts, or the double Mnk1/Mnk2 knockouts, but the generation of arsenic-dependent apoptosis was enhanced in all of them when compared with wild-type MEFs. Paired *t* test analysis of Mnk1$^{-/-}$2$^{-/-}$, Mnk1$^{-/-}$, and Mnk2$^{-/-}$ cells compared with Mnk1$^{+/+}$2$^{+/+}$ cells displayed *p* val-ues of 0.0192, 0.0078, and 0.0037, respectively (Fig. 5), firmly estab-lishing that both Mnk1 and Mnk2 play essential roles in the generation of anti-apoptotic signals in response to As$_2$O$_3$.

To further explore the role of Mnk kinases in the genera-tion of the effects of As$_2$O$_3$ in a more physiologically relevant system, we examined the effects of pharmacological inhibi-tion of Mnk1 and Mnk2 in the induction of the suppressive effects of As$_2$O$_3$ on primary leukemic progenitors from patients with AML. Bone marrow or peripheral blood from different AML patients were exposed to As$_2$O$_3$ in the absence or presence of the pharmacological Mnk inhibitor, CGP57380, and leukemic progenitor colony formation (CFU-L) was assessed by clonogenic assays in methylcellu-

formed using the specific MEK inhibitor U0126. Pretreatment of cells with U0126 inhibited phosphorylation of both Erk1/2 and eIF4E in both NB4 (Fig. 3*D*) and U937 cells (Fig. 3*E*), strongly suggesting that, beyond p38, Erk activity is required for As$_2$O$_3$-dependent engagement of the Mnk/eIF4E pathway.

In subsequent studies, we sought to determine the functional relevance of Mnk1 and Mnk2 in the generation of arsenic tri-oxide responses. For that purpose, we used cells with targeted deletion of either the *Mnk1* or the *Mnk2* genes alone, or double knock-out cells for both *Mnk1* and *Mnk2* (27). In initial exper-iments, wild-type Mnk1$^{+/+}$Mnk2$^{+/+}$ MEFs and double knock-out Mnk1$^{-/-}$Mnk2$^{-/-}$ MEFs were treated for different times
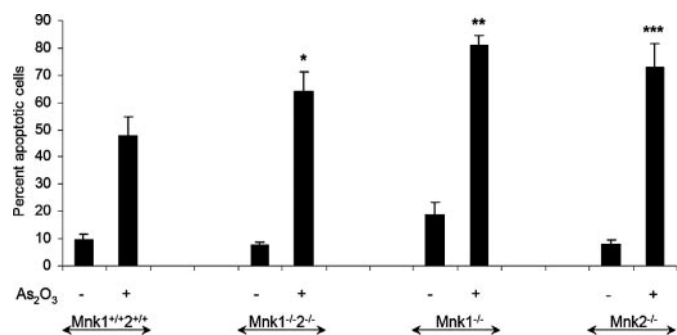
FIGURE 5. **Targeted disruption of the Mnk1 and Mnk2 genes potentiates As₂O₃-induced apoptosis.** Mnk1$^{+/+}$2$^{+/+}$, Mnk1$^{-/-}$2$^{-/-}$, Mnk1$^{-/-}$, and Mnk2$^{-/-}$ MEFs were incubated in the absence or presence of As₂O₃ (10 μM) for 48 h. The percentage of apoptotic cells was determined by flow cytometric analysis for annexin V/propidium iodide staining. Data are expressed as means ± S.E. of six independent experiments. Paired t test analysis of Mnk1$^{-/-}$2$^{-/-}$, Mnk1$^{-/-}$, and Mnk2$^{-/-}$ MEFs compared with Mnk1$^{+/+}$2$^{+/+}$ MEFs (*columns* marked with *asterisks*), showed $p = 0.0192$, $p = 0.0078$, and $p = 0.0037$, respectively.
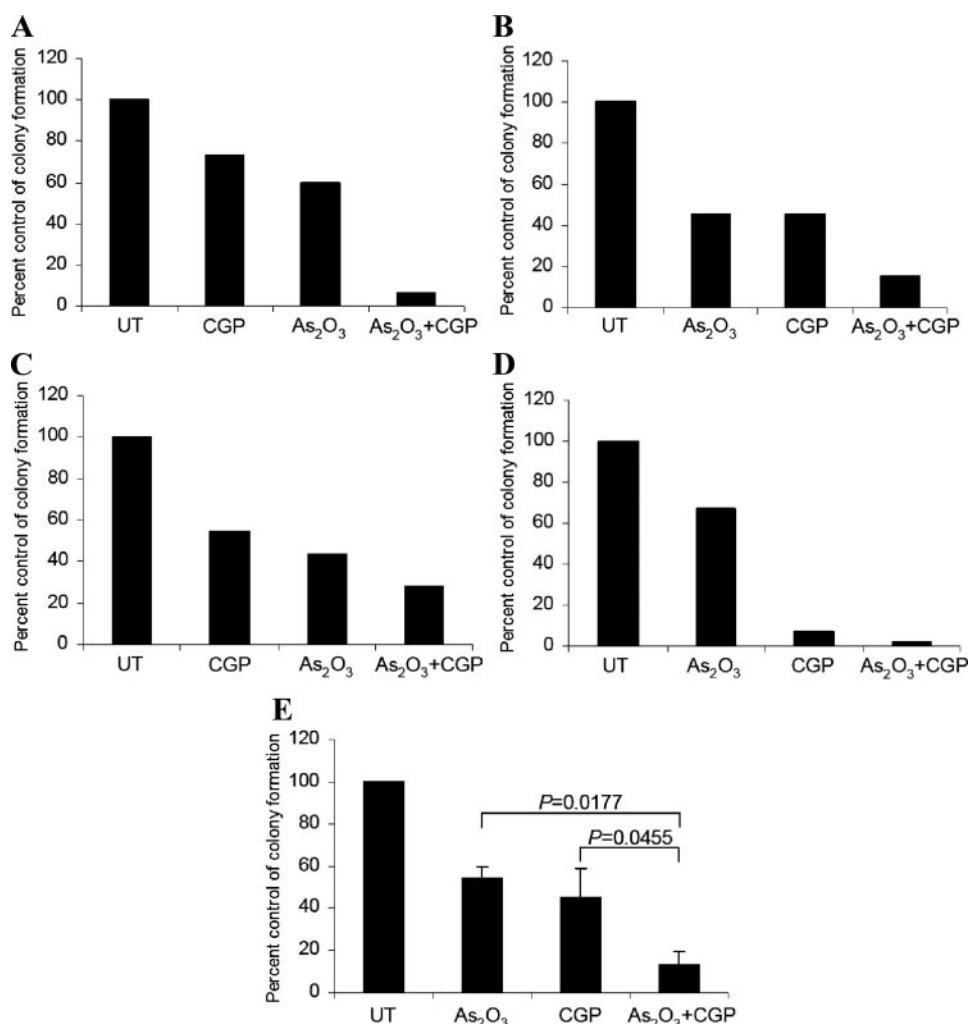

FIGURE 6. **Pharmacological inhibition of Mnk1 promotes As₂O₃-induced growth suppression of CFU-L colony formation from AML patients and promotes sensitivity of leukemic blasts at very low concentrations of As₂O₃.** *A–D*, bone marrow or peripheral blood mononuclear cells from four AML patients were plated in a methylcellulose assay system with As₂O₃ (0.5 μM), in the presence or absence of CGP57380 (10 μM). The data are expressed as percent control of CFU-L colony numbers for untreated cells. *E*, means ± S.E. of the values from the experiments using different patient samples (*A–D*) are shown. Paired t test analysis showed $p = 0.0177$ for the combination of CGP57380 and As₂O₃ *versus* As₂O₃ alone and $p = 0.0455$ for the combination of CGP57380 and As₂O₃ *versus* CGP57380 alone.

lose. Addition of As₂O₃ to the cultures suppressed leukemic progenitor colony formation in all different cases (Fig. 6, *A–D*). Interestingly, the Mnk inhibitor CGP58370 also suppressed leukemic colony formation to a similar degree (Fig. 6). However, concomitant addition of CGP58370 and As₂O₃ resulted in strong synergistic effects, as shown by paired t test analysis ($p = 0.0177$ for the combination of As₂O₃ plus CGP57380 *versus* As₂O₃ alone; and $p = 0.0455$ *versus* CGP57380 alone) (Fig. 6*E*).

To better understand the mechanisms of enhanced generation of the antileukemic effects by the combination of As₂O₃ with CGP58370, the effects of such combination on the generation of As₂O₃-dependent apoptosis in different leukemic cell lines were assessed. As expected, treatment of U937 (Fig. 7*A*) or K562 (Fig. 7*B*) cells with arsenic trioxide resulted in induction of apoptosis, but concomitant addition of the Mnk inhibitor, CGP58370, led to enhanced apoptosis that was significant (paired p value = 0.000271 for U937 and paired p value = 0.0384 for K562 cells) (Fig. 7, *A* and *B*). We also performed dose-response experiments, to determine whether pharmacological inhibition of the kinases Mnk1 and Mnk2 promotes generation of antileukemic effects in response to As₂O₃ on AML cells, at low concentrations that normally do not result in such effects. Due to limitations in availability of primary leukemic progenitors, such studies were also performed using U937 acute myelomonocytic leukemia cells. As shown in Fig. 7*C* arsenic trioxide treatment did not result in any significant growth inhibitory effects on such cells when used at concentrations <2 μM. However, concomitant addition of CGP57380 dramatically enhanced arsenic-dependent growth suppression, resulting in substantial growth inhibition at the very low concentration of 0.25 μM (Fig. 7*C*). Thus, pharmacological inhibition of Mnk kinases strongly enhances the antileukemic properties of As₂O₃ and results in the generation of antileukemic responses at very low concentrations of As₂O₃.

To directly assess the functional relevance of Mnk1 and Mnk2 in the generation of antileukemic effects of As₂O₃, we determined whether siRNA-mediated knockdown of Mnk1 and Mnk2 enhances the suppressive effects of As₂O₃ on leukemic progenitor (CFU-L) colony formation. U937 cells were transfected with either
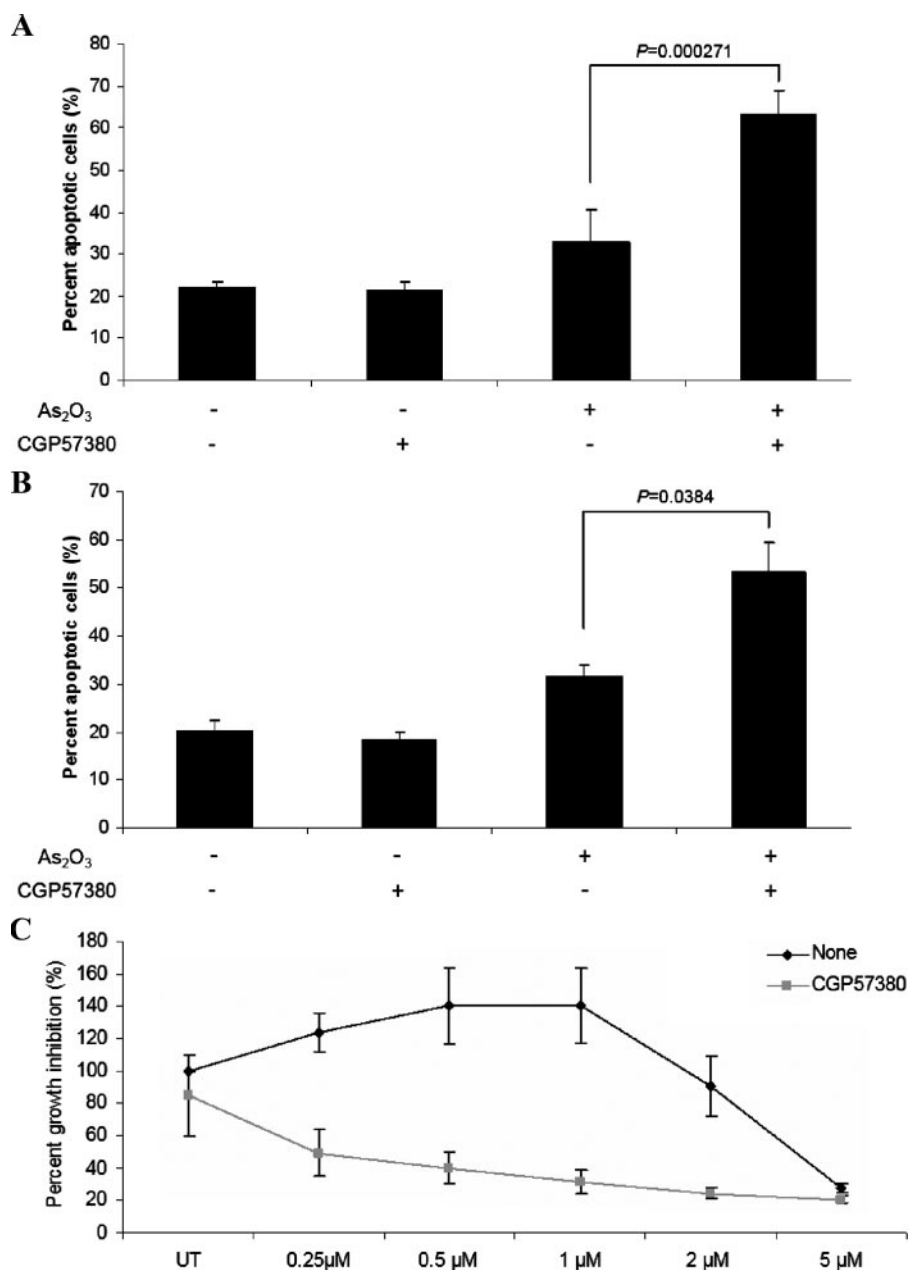
FIGURE 7. **Pharmacological inhibition of Mnk1 promotes As$_2$O$_3$-induced apoptosis and growth suppression of human leukemic cell lines.** *A*, U937 cells were pre-treated for 60 min with CGP57380 (20 μM) and then incubated in the absence or presence of As$_2$O$_3$ (2 μM) for 96 h. The percentage of apoptotic cells was determined by flow cytometric analysis for annexin V/propidium iodide staining. Data are expressed as means $\pm$ S.E. of five independent experiments. Paired *t* test analysis showed $p = 0.000271$ for the combination of CGP57380 and As$_2$O$_3$ *versus* As$_2$O$_3$ alone. *B*, K562 cells were pre-treated for 60 min with CGP57380 (20 μM) and then incubated in the absence or presence of As$_2$O$_3$ (2.5 μM) for 96 h. The percentage of apoptotic cells was determined by flow cytometric analysis for annexin V/propidium iodide staining. Data are expressed as means $\pm$ S.E. of three independent experiments. Paired *t* test analysis showed $p = 0.0384$ for the combination of CGP57380 and As$_2$O$_3$ *versus* As$_2$O$_3$ alone. *C*, U937 cells were incubated for 7 days in the presence or absence of the indicated doses of As$_2$O$_3$, in the presence or absence of CGP57380 (2 μM). Cell proliferation was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as means $\pm$ S.E. of five independent experiments.

nonspecific siRNA or siRNAs specific targeting Mnk1 and Mnk2 (Fig. 8*A*). The cells were exposed to As$_2$O$_3$, and CFU-L colony formation was assessed in clonogenic assays in methylcellulose. As$_2$O$_3$-dependent suppression of CFU-L colony formation was strongly enhanced in cells transfected with Mnk1/Mnk2 siRNAs over controls (paired $p = 0.017$) (Fig.

8*B*), firmly establishing that the Mnk pathway plays a critical regulatory role in the generation of the effects of As$_2$O$_3$ on leukemic progenitors.

DISCUSSION

It is well established that arsenic trioxide induces apoptosis and suppresses the growth of different types of neoplastic cells *in vitro* and *in vivo* (1–6, 39). Despite that, the precise up-stream signals that control induction of programmed cell death by As$_2$O$_3$ remain to be elucidated. Previous work has demonstrated that decreasing the mitochondrial membrane potential may be a key mechanism by which arsenic trioxide induces its pro-apoptotic effects (11–13). Such an As$_2$O$_3$-dependent decrease of mitochondrial membrane potential results in the release of cytochrome *c* and activation of caspases, which ultimately lead to apoptotic cell death (1–6, 11–13, 40–42). Other work has established that activation of the JNK MAPK pathway is essential for As$_2$O$_3$-dependent apoptosis (43), whereas inhibition of transcriptional NF-κB activity is also involved in the generation of arsenic trioxide responses (44–46). It should be also noted that the generation of reactive oxygen species depends on cellular glutathione stores (47) and that ascorbic acid (48) or buthionine sulfoximine (49) enhance the pro-apoptotic effects of As$_2$O$_3$.

Despite the potent pro-apoptotic and antitumor effects of arsenic trioxide, it is well documented that malignant cells develop resistance to its effects *in vitro* and *in vivo* (1–6), but the precise cellular mechanisms responsible for such resistance remain unknown. In previous work, we demonstrated that the p38 MAPK (24) and its upstream effectors Mkk3 and Mkk6 (25) are activated during treatment of different cell types with As$_2$O$_3$, whereas pharmacological or molecular targeting of theses kinase was found to enhance As$_2$O$_3$-mediated apoptosis and antiproliferative responses (24, 25). These findings have strongly suggested that this MAPK cascade may be negatively regulating generation of As$_2$O$_3$ responses, likely as a component of a negative feedback regula-
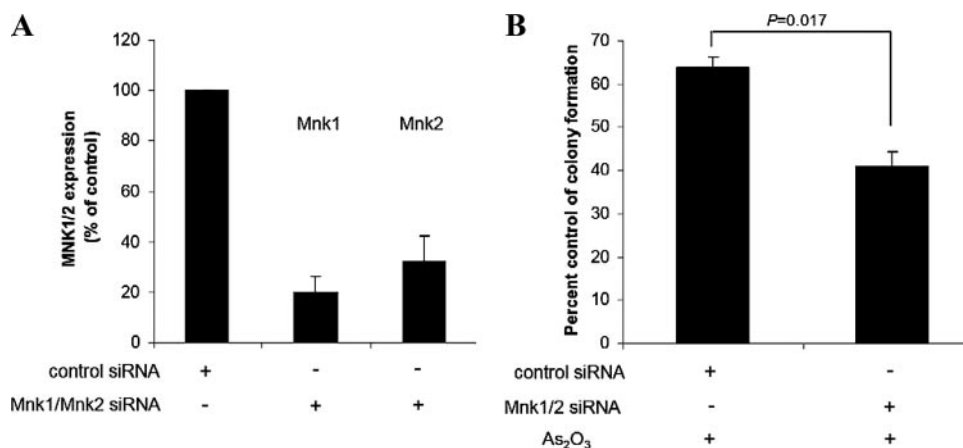
A

B

FIGURE 8. **Knockdown of Mnk kinases enhances the suppressive effects of As$_2$O$_3$ on leukemic progenitor (CFU-L) growth.** *A*, U937 cells were transfected with control siRNA or a mixture of Mnk1- and Mnk2-specific siRNAs. 48 h after transfection, expression of mRNAs for *Mnk1* and *Mnk2* genes was evaluated by quantitative real-time reverse transcription-PCR using *GAPDH* gene for normalization. Data are expressed as -fold increase over control samples and represent means ± S.E. of two independent experiments. *B*, U937 cells were transfected with control siRNA or a mixture of Mnk1- and Mnk2-specific siRNAs and were subsequently incubated in methylcellulose in the presence or absence of As$_2$O$_3$ (0.5 μM), and leukemic CFU-L colony formation was assessed. Data are expressed as percent control colony formation of untreated samples and represent means ± S.E. of three independent experiments are shown. Paired *t* test analysis comparing the effects of As$_2$O$_3$ in the presence or absence of Mnk1/Mnk2 siRNAs showed a paired *p* value = 0.017.

tory loop. The identification of the downstream effector signaling elements that mediate induction of anti-apoptotic responses is therefore of considerable interest, as it may lead to more potent and specific approaches to target anti-apoptotic mechanisms and promote generation of As$_2$O$_3$-dependent antitumor activities.

In the present study, we provide the first evidence that Mnk1 and Mnk2 are activated during treatment of cells with arsenic trioxide. Our data establish that such activation is rapid, occurring within 30 min of treatment of cells, and results in downstream phosphorylation of eIF4E on serine 209. We also demonstrate that activation of Mnk kinases/downstream eIF4E phosphorylation in response to arsenic trioxide is defective in cells with targeted disruption of the p38α MAPK gene and is blocked by pharmacological inhibition of the MEK/Erk pathway, establishing that it requires upstream engagement of the p38 and Erk MAPK pathways. In studies using knock-out cells for Mnk1, Mnk2, or both, we found that the absence of these kinases results in increased generation of As$_2$O$_3$-dependent pro-apoptotic signals and programmed cell death. Consistent with this, our data demonstrate that inhibition of Mnk1/Mnk2 activity in primitive progenitors from patients with AML results in enhanced suppression of leukemic growth and arsenic-dependent apoptosis. Similarly, siRNA targeting of Mnk kinases was also found to enhance the suppressive effects of arsenic trioxide on U937-derived leukemic progenitor colony formation. These findings indicate that Mnk1/Mnk2 are critical regulators of the biological effects of arsenic trioxide and, along the kinase Msk1 (33), may be the key signaling elements downstream of MAPKs that block induction of antileukemic responses.

Mnk1 and Mnk2 are serine threonine kinases that regulate phosphorylation of eIF4E on serine 209 (27), a site important for the activation of the protein (50). Previous work has established that engagement of these kinases in growth factor signal-

ing is MAPK-dependent and regulated by the p38 and/or the Mek/Erk kinase cascades (20, 21). The ability of Mnks to phosphorylate eIF4E may be of importance in the regulation of mRNA translation, as it has been previously demonstrated that eIF4E phosphorylation is relevant to cap-dependent translation and protein synthesis in different cellular systems (51–55). Consistent with this, recent studies have established that Mnk1-mediated phosphorylation of eIF4E occurs in response to oxidants (56). In addition, there is evidence that such phosphorylation is associated with enhanced herpes simplex virus-1 mRNA translation and replication (56) and tumor necrosis factor-α biosynthesis (57). There is also evidence that Mnks phosphorylate AU-rich element-binding proteins such as hnRNP A1,

resulting in decreased binding to tumor necrosis factor-α AU-rich element *in vitro* and tumor necrosis factor-α mRNA *in vivo* (57). On the other hand, there have been studies demonstrating that, under certain circumstances, Mnks may negatively regulate protein translation (37) or that Mnk-mediated phosphorylation in response to oxidant stress negatively regulates global protein synthesis (58). Notably, the functions of Mnk1 and Mnk2 may be regulated by a novel Mnk-specific regulatory mechanism, involving autoinhibition by a reprogrammed activation segment (59).

Despite the significant advances on the mechanisms of activation and function of Mnks, the precise roles of members of this family of kinases in various physiological and pathophysiological processes remains to be established. Our studies suggest a novel function for Mnk1 and Mnk2, acting as negative regulators of arsenic trioxide-dependent apoptosis. Such a function of Mnks appears to be also important for the generation of the antileukemic effects of arsenic trioxide, as shown by our studies with primitive leukemic precursors enriched from the bone marrows or peripheral blood of patients with acute leukemia. Interestingly, a recent study demonstrated that loss of Mnk function sensitizes fibroblasts to serum withdrawal-induced apoptosis (60). The results of this study, taken together with our findings, suggest that Mnk1 and Mnk2 may be important elements in the induction of anti-apoptotic responses, possibly via regulation of translation of anti-apoptotic genes. Although the identities of anti-apoptotic proteins whose expression may be regulated by Mnk kinases remain to be defined in future studies, our data raise the possibility that targeting of Mnks may be a way to enhance antileukemic responses and reverse the resistance that malignant cells develop to the effects of arsenic trioxide, and possibly other antitumor agents.

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