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Role of extracellular signal-regulated kinase (ERK) signaling in nucleotide excision repair and genotoxicity in response to As(III) and Pb(II)*

Ju-Pi Li, Chun-Yu Wang, Yen-An Tang, Yun-Wei Lin, and Jia-Ling Yang‡

Molecular Carcinogenesis Laboratory, Institute of Biotechnology and Department of Life Sciences, National Tsing Hua University, Hsinchu 30013, Taiwan

Abstract: Arsenic and lead can induce genetic injuries and epigenetic signaling pathways in cultured mammalian cells. To test whether signaling pathways affect the extent of genetic injuries, we explored the impacts of extracellular signal-regulated kinase 1 and 2 (ERK) on nucleotide excision repair (NER), cytotoxicity, and genotoxicity following sodium arsenite [As(III)] and lead acetate [Pb(II)]. Sustained ERK activation was observed in human cells exposed to As(III) and Pb(II). As(III) inhibited the cellular NER synthesis capability; conversely, Pb(II) stimulated it. ERK activation contributed to the As(III)-induced NER inhibition and micronucleus formation. In contrast, this signal was required for inducing cellular NER activity and preventing mutagenesis following Pb(II). ERK activation by Pb(II) was dependent on protein kinase C (PKCα) that also exhibited anti-mutagenicity. Enforced expression of ERK signaling markedly elevated the cellular NER activity, which was suppressed by As(III). Nonetheless, ERK activation could counteract the cytotoxicity caused by these two metals. Together, the results indicate that pro-survival ERK signaling exhibits dual and opposing impacts on NER process following As(III) and Pb(II) exposures. The findings also suggest that ERK is an important epigenetic signaling in the determination of metal genotoxicity.

Keywords: ERK; NER; micronucleus; *HPRT* mutagenesis; carcinogenic metal.

INTRODUCTION

Arsenic and inorganic lead compounds are widely distributed environmental toxicants and were classified, respectively, as group 1 (carcinogenic to humans) and group 2A (probably carcinogenic to humans) chemicals by the International Agency for Research on Cancer [1–3]. Human exposure to arsenic is highly associated with increased incidences of cancers in lung, skin, bladder, and other internal organs [1,2], whereas lead exhibits a low yet suggestive correlation to cancer incidences in humans [3]. On the other hand, lead causes kidney, brain, and lung cancers in experimental rodents but evidence for the carcinogenicity of arsenite in animals is limited [1,2]. Arsenite and lead have been considered as tumor promoters because they induce anchorage-independent phenotype and morphologi-

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‡Corresponding author: E-mail: jlyang@life.nthu.edu.tw

cal transformation in mammalian cells [4–6]. The two metals also cause genomic damage including DNA breaks, chromosome aberrations, sister-chromatid exchanges, micronuclei, and gene mutations in mammalian cell culture systems [7,8]. However, some divergent results have been reported. For example, lead shows mutagenic potential in rodent cells [9–11], though it does not induce mutations in diploid human fibroblasts using the hypoxanthine guanine phosphoribosyltransferase (*HPRT*) mutation assay [4]. Whereas, arsenite does not increase gene mutations in mammalian *HPRT* locus but displays mutagenicity in the *S1* locus of A_L human-hamster hybrid cells, probably due to that it stimulates mainly multilocus deletions [12,13]. Arsenite also induces deletions and rearrangements in the *supF* gene of a shuttle vector system [14] and elicits a delayed mutagenesis in human osteosarcoma TE85 cells [15].

The genotoxic effects of arsenite and lead have been linked to the inhibition of DNA repair systems [16–23]. The two metals, however, exhibit distinct cellular and in vitro biochemical effects on DNA repair activity. Treatment of mammalian cells with arsenite markedly inhibits cellular DNA repair processes, specifically, the DNA ligation and incision steps [17–19,22], while the nucleotide excision repair (NER) activity is elevated in cultured human cells after lead exposure [24]. The NER-proficient human fibroblasts are more sensitive to arsenite cytotoxicity than the NER-deficient fibroblasts derived from xeroderma pigmentosum group C [17]. Conversely, lead induces significant higher cytotoxicity and mutagenicity in the NER-deficient than in the NER-proficient rodent cells [24]. On the other hand, lead inhibits the NER process in a cell-free assay [25], while arsenite can activate enzymatic activities of several DNA repair proteins during in vitro reactions [26]. Similarly, lead can inhibit the apurinic/apyrimidinic endonuclease activity of cell lysate in vitro, but arsenite cannot [27]. The opposing cellular effects of arsenite and lead on DNA repair activity is therefore most unlikely mediated via a direct action of metals toward repair proteins. It is conceivable that arsenite and lead may elicit distinct cellular alterations, such as signaling transduction pathways, to indirectly affect DNA repair systems and subsequent metal genotoxicity.

ERK, also termed p44/p42 mitogen-activated protein kinases (MAPKs), are vital intracellular signaling components that become phosphorylated and activated in response to a wide diversity of stimuli including growth factors, cytokines, and environmental stresses [28,29]. Activation of the ERK signaling usually involves the Raf-1–mitogen-activated protein kinase kinase 1 and 2 (MKK1/2)–ERK three-kinase module and the recruitment of Raf-1 to cell membrane via Ras-GTP to connect the activated receptor tyrosine kinases [28,29]. The Raf-1–MKK1/2–ERK module is also regulated by protein kinase C (PKC) partly through increasing Ras-Raf-1 membrane recruitment and Raf-1 phosphorylation [28]. ERK activation requires a dual-phosphorylation by MKK1/2 on the Thr and Tyr residues of the motif Thr-Glu-Tyr within the activation loop [28,29]. Following activation, ERK phosphorylates numerous substrates including transcription factors, kinases, phosphatases, and cytoskeletal proteins for regulation of cell proliferation, differentiation, transformation, survival, and death. The particular function regulated by ERK is likely to depend on the cell type, the stimulus, and the duration and strength of kinase activities.

The ERK signaling is extremely sensitive to arsenite and lead [24,30–34]. Attempting to explore whether signaling transduction pathways affect metal genotoxicity, we have reported that sustained ERK activation by lead acetate [Pb(II)] is required for the enhanced NER synthesis activity in CL3 human non-small-cell lung carcinoma cells; the Pb(II)-elicited ERK also participates in the prevention of cytotoxicity and mutagenicity in HFW diploid human fibroblasts as well as CL3 cells [24]. We further found that activation of PKCα in Pb(II)-exposed CL3 cells functions as an ERK upstream for the prevention of cytotoxicity and mutagenicity [35]. In contrast, the activated-ERK following sodium arsenite [As(III)] is involved in the inhibition of NER synthesis activity and the induction of micronuclei in G1-enriched CL3 cells [34]. To validate the distinct effects of the two metals, we have continued the study of ERK signaling in NER activity affected by As(III) in asynchronous CL3, HFW, and H293 human embryonic kidney cells. Here we compare and discuss the findings that ERK signaling has dis-

tinct impacts on DNA repair and genomic stability in human cells following As(III) and Pb(II) exposures.

METHODS

Cell culture

The CL3 cell line established from a non-small-cell lung carcinoma tumor was cultured in RPMI-1640 complete media. The human diploid fibroblast line HFW and the human embryonic kidney cell line H293 were cultured in DMEM complete media. The complete media were supplemented with sodium bicarbonate (2.2 %, w/v), L-glutamine (0.03 %, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10 %). CL3 cells were maintained at 37 °C in a humidified incubator containing 5 % CO_2 in air, while HFW and H293 cells were cultured in a 10 % CO_2 incubator.

Treatment

As(III) (Merck, Darmstadt, Germany) and Pb(II) (Merck) was dissolved in MilliQ-purified water (Millipore, Bedford, MA). The G1-enriched CL3 cells were obtained by counterflow centrifugal elutriation using a Beckman J-6M centrifuge equipped with a JE-6B elutriation rotor [34]. Cells in exponential growth or synchronous at the G1 phase were exposed to Pb(II) or As(III) in serum-free media. In experiments to determine the impacts of ERK signaling, cells were pre-treated with specific MKK1/2 inhibitors PD98059 (Calbiochem, San Diego, CA) or U0126 (Calbiochem) for 1 h or a specific PKCα inhibitor Gö6976 (Calbiochem) for 30 min before adding the metals. After treatment, the cells were washes twice with phosphate-buffered saline, harvested immediately or allowed recovery for various times, and then subjected to further analyses.

Transfection

Cells (4×10^5) were plated in a p60-dish one-day before transfection. The MKK1-CA plasmid containing a constitutively active form of MKK1 (ΔN3/S218E/S222D) was carried by liposome and transfected into H293 cells [24]. After incubation for 6 h, the cells were washed with phosphate-buffered saline, kept cultured in complete media for 1 or 2 days, exposed to As(III) or Pb(II), and than subjected to western blotting and NER synthesis assay.

Cell lysate preparation

Cells were rinsed twice with cold phosphate-buffered saline and lysed in a buffer containing 20 mM HEPES, pH 7.6, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 % Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The cell lysate was rotated at 4 °C for 30 min, centrifuged at 10 000 rpm for 15 min, and the precipitates were discarded. The BCA protein assay kit (Pierce, Rockford, IL) was adopted to determine protein concentrations using bovine serum albumin as a standard.

Western blot analysis

Equal amounts of proteins in cell lysate from each set of experiments were subjected to western blot analyses. The polyclonal antibodies specific against phospho-ERK(Thr202/Tyr204) (#9101) and ERK2 (#sc-154) were purchased, respectively, from Cell Signaling (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). Antibody reaction was detected using the enhanced chemiluminescence detection procedure according to the manufacturer's recommendations (NEN, Boston, MA). To

re-probe the membrane with another primary antibody, antibodies in the blot were stripped from membranes by a solution containing 2 % SDS, 62.5 mM Tris-HCl, pH 6.8, and 0.7 % (w/w) β-mercaptoethanol at 50 °C for 15 min. The relative protein intensities on blots were quantitated using a computing densitometer equipped with the ImagQuant analysis program (Molecular Dynamics, Sunnyvale, CA).

NER synthesis

The pUC19 plasmid was prepared by alkaline lysis method and stored at –20 °C. The purified plasmid (250 ng/μl in ddH_2O) was damaged by UV irradiation (254 nm, 400 J/m^2) at intensity of 1–1.5 J/m^2/sec measured with an UVX radiometer (UVP Inc., CA). NER synthesis reaction mixtures (50 μl) contained 60 μg of proteins derived from cell lysate, 250 ng of UV-irradiated or un-irradiated plasmid substrates, 20 μM each of dGTP, dATP, and dTTP, 8 μM dCTP, 2 μCi [α-^{32}P]dCTP (3000Ci/mmol), 2 mM ATP, 45 mM HEPES-KOH, pH 7.5, 60 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 3.4 % glycerol, and 18 μg bovine serum albumin. Reactions were performed at 30 °C for 1 h and terminated by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 20 mM. The samples were then treated with 80 μg/ml RNaseA for 10 min, followed by 190 μg/ml proteinase K and 0.5 % sodium dodecyl sulfate (SDS) for 30 min at 37 °C. The plasmid DNA in the reaction mixtures was purified by phenol/chloroform extraction and ethanol precipitation, linearized with *Bam*HI, and subjected to agarose gel electrophoresis. The plasmid DNA in gel was stained with 0.5 % ethidium bromide and visualized under near-UV transillumination. The gel was then dried and subjected to autoradiography. The band intensities were measured with a computing densitometer equipped with the ImageQuant analysis program.

Cytokinesis-block micronucleus assay

Micronucleus formation was determined by the cytokinesis-block process using cytochalasin B, a microfilament-assembly inhibitor. Briefly, cells were cultured in media containing 1 μg/ml of cytochalasin B for 24–48 h. Next, the cultures were washed with phosphate-buffered saline, treated with 0.05 % KCl for 3 min at room temperature, and then fixed in 3 ml of Carnoy's solution (20:1, methanol: acetic acid, v/v) for 15 min. The dishes were air-dried and stained for 15 min with freshly prepared Giemsa's solution (10 % in 0.1 mM sodium phosphate buffer, pH 6.8). The dishes were blindly coded, and 2000 bi-nucleated cells in each treatment were examined for scoring micronuclei per bi-nucleated cells using an inverted microscope.

Cytotoxicity assay

After As(III) treatment, the cells were washed with phosphate-buffered saline and kept in a CO_2 incubator for another 3 days. The cells were then trypsinized, and a portion of cells were mixed with 0.4 % trypan blue for 15 min. The unstained cells were counted using an inverted microscope and a hemacytometer. Alternatively, the cells were trypsinized immediately after metal treatments and plated at a density of 100–200 cells per a 60 mm-Petri dish in triplicate. Following incubation for 12–14 days, the cell colonies were stained with 1 % crystal violet solution (in 30 % ethanol). The percent of survival was determined to be the number of colonies in the treated cells divided by those obtained in the untreated cells.

Mutagenicity assay

Following metal exposure, the cells were maintained in exponential growth for 7 days to allow for the expression of resistance to 6-thioguanine. One million cells from each treatment were then plated onto

ten 100-mm Petri dishes in a selective medium containing 40 μM 6-thioguanine and incubation for 12–14 days. Plating efficiency of cells at the time of selection was also assayed in a non-selective medium to correct the observed *HPRT* mutant frequency. The mutant frequency was calculated to be the total number of 6-thioguanine resistant colonies divided by the total number of clonable cells at selection time.

Statistical analysis

Results were expressed as mean ± SEM. The statistical significance was determined by one-way analysis of variance followed by Duncan multiple range test or two-way analysis of variance with a statistical package for social science software. Mutation frequency was assessed using the Poisson distribution test. $P < 0.05$ was considered as statistically significant.

RESULTS

Activation of ERK in human cells in response to As(III) and Pb(II)

To compare the ability of As(III) and Pb(II) in inducing ERK signal, CL3 cells were exposed to As(III) or Pb(II) at various concentrations in serum-free medium for 24 h. The activation of ERK was determined by western blotting using a specific antibody against phospho-ERK. As shown in Fig. 1, both As(III) and Pb(II) could increase sustained ERK activation in a dose-dependent manner. Also, similar levels of ERK activation were observed in CL3 cells exposed to 5–10 μM As(III) and 30–100 μM Pb(II) for 24 h (Fig. 1). By contrast, the endogenous ERK2 protein levels were unaltered by these metals. Dose-dependent activation of ERK was also observed in HFW diploid human fibroblasts following As(III) exposure for 3 h (Fig. 2a). The As(III)-elicited ERK activation in HFW and G1-enriched CL3

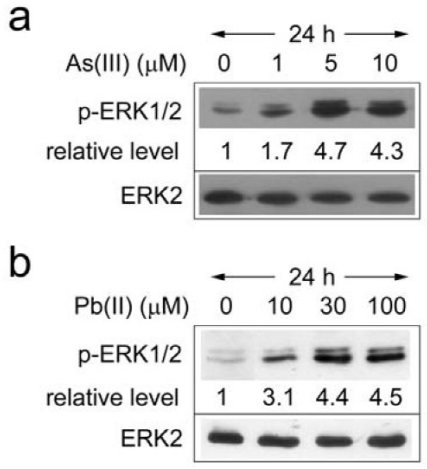

Fig. 1 As(III) and Pb(II) elicit ERK activation in human cells. CL3 lung cancer cells in exponential growth were left untreated or treated with indicated concentrations of As(III) (a) or Pb(II) (b) in serum-free medium for 24 h. The whole cell extract was then harvested and the activation of ERK was determined by western blotting using a phospho-specific antibody. After analysis of the phospho-ERK level, the antibody was stripped and the membrane was reprobed with an anti-ERK2 antibody. The relative phospho-ERK intensities shown under the representative western blots were densitometric results normalized by arbitrarily setting the control level to 1 and calculated by averaging at least three independent experiments. The result in (b) was adopted from [24].

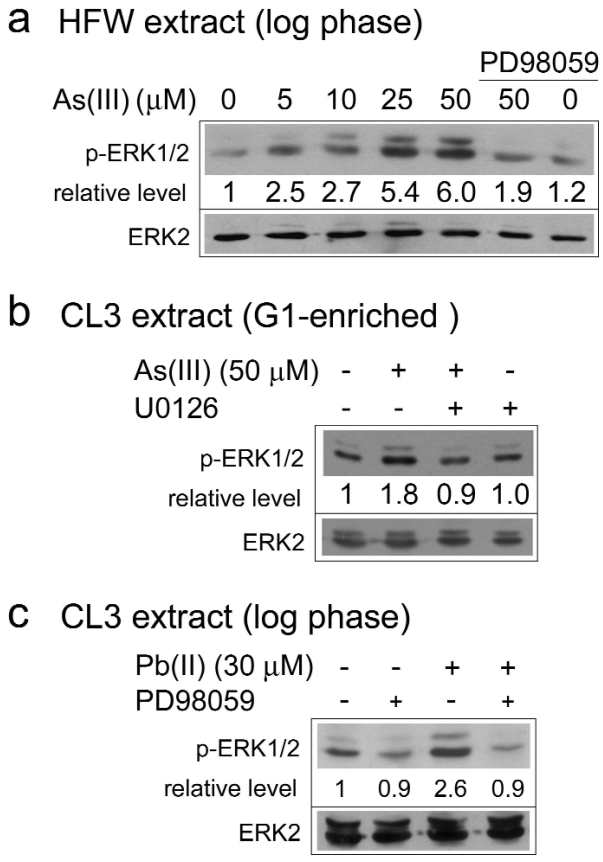

Fig. 2 PD98059 or U0126 block ERK activation by As(III) or Pb(II) in human cells. (a) HFW normal human fibroblasts in exponential growth were treated with As(III) for 3 h in serum-free medium in the presence or absence of PD98059. (b) G1-enriched CL3 cells were treated with As(III) for 3 h in the presence or absence of U0126. (c) CL3 cells in exponential growth were treated with Pb(II) for 24 h in the presence or absence of PD98059. The whole cell extract was then harvested and the ERK activation was determined by western blotting as described in Fig. 1. Results were calculated by averaging at least three independent experiments and data shown in (b) were adopted from [34].

cells could be blocked by two structurally unrelated inhibitors for the ERK upstream kinases MEK1/2, PD98059 and U0126 (Figs. 2a and 2b). PD98059 cotreatment also suppressed the ERK activation by Pb(II) in CL3 cells (Fig. 2c).

Opposing effects of As(III) and Pb(II) on cellular NER activity

It has been hypothesized that As(III) and Pb(II) may impede cellular DNA repair processes as the metals could enhance the genotoxic effects of well-known mutagens such as UV light and alkylating carcinogens [17,19–21]. NER is a major error-free DNA repair system functioning in the removal of a broad variety of DNA lesions caused by environmental carcinogens [36–38]. To explore the impacts of As(III) or Pb(II) on the cellular NER, CL3 cells were exposed to these metals for 24 h and then allowed recovery for 8–12 h before preparation of cell lysates. The proteins in the cell lysates were incubated with UV-irradiated DNA, 4 dNTP, and [α-^{32}P]dCTP for the determination of NER synthesis. Figure 3a shows that the capability of lysates derived from the As(III)-treated cells to incorporate nucleotides into

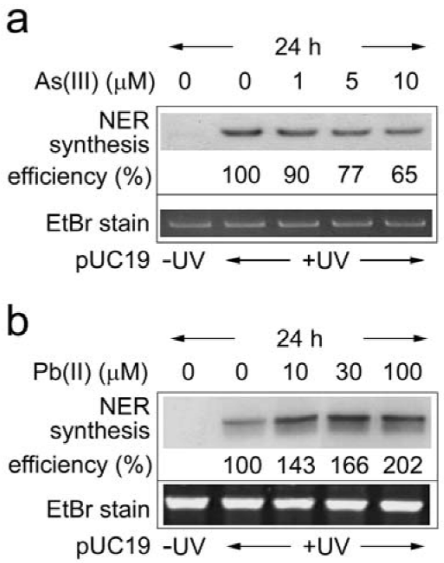

Fig. 3 As(III) decreases while Pb(II) increases NER synthesis efficiency in human cells. CL3 lung cancer cells were exposed to As(III) (a) or Pb(II) (b) as described in Fig. 1. The cells were then kept cultured for 8–12 h before whole cell extraction. The NER synthesis activity of equal amounts of proteins obtained from each treatment was determined by reaction with UV-irradiated pUC19 plasmid (+UV). Un-irradiated pUC19 (-UV) was incubated with the lysate of untreated cells to serve as a negative control. Upper panel: autoradiograph of gels showing the incorporation of [α-^{32}p]dCTP. The relative NER synthesis efficiencies shown below the representative autoradiographs were densitometric results normalized by arbitrarily setting the control level to 100 % and calculated by averaging at least three independent experiments. Lower panel: photograph of the same gels stained with ethidium bromide (EtBr) showing equal amounts of pUC19 used in each reaction. The result in (b) was adopted from [24].

UV-damaged DNA was lower than that derived from the untreated control cells. Conversely, the NER synthesis efficiency increased in protein extracts derived from the Pb(II)-treated CL3 cells (Fig. 3b). The extents of cellular NER activity inhibited by As(III) and stimulated by Pb(II) were dependent on the metal concentrations (Fig. 3). The results indicate that As(III) and Pb(II) exhibit opposing effects on cellular NER activity.

Dual roles of ERK in regulating cellular NER in response to As(III) and Pb(II)

To inspect whether the ERK signal is involved in regulating NER activity, HFW or CL3 cells were exposed to As(III) or Pb(II) in the presence or absence of PD98059 or U0126. As shown in Fig. 4, dose-

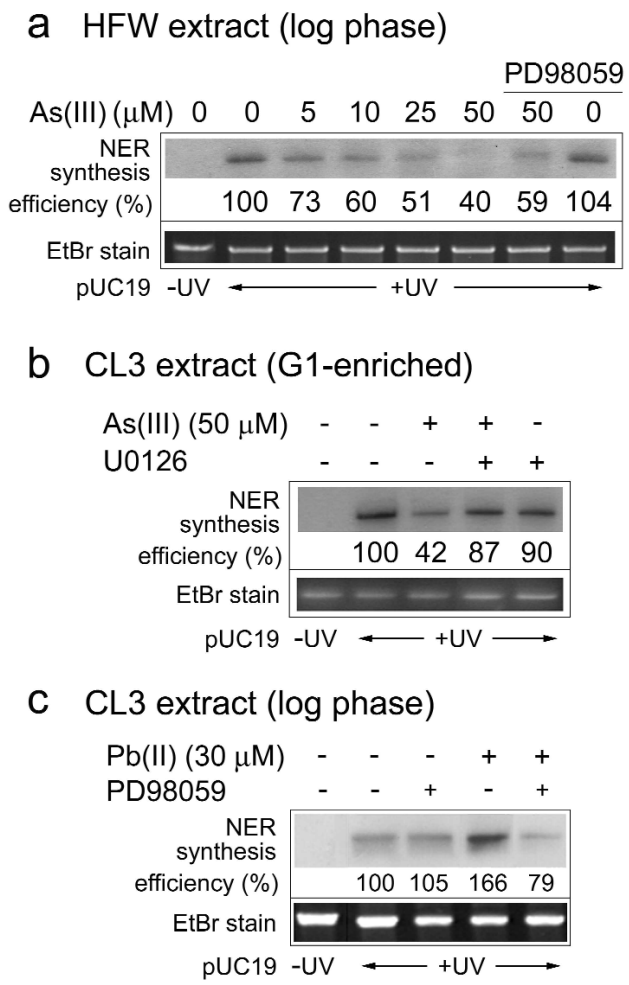

Fig. 4 ERK signaling is involved in As(III)-inhibited and Pb(II)-stimulated NER synthesis in human cells. (a) HFW fibroblasts or (b) G1-enriched or (c) asynchronous CL3 cells were exposed to As(III) or Pb(II) in the presence or absence of ERK signal inhibitors as described in Fig. 2. After recovery for 8–12 h, the cells were subjected to the determination of NER activity as described in Fig. 3. The relative NER synthesis efficiencies were calculated by averaging at least four independent experiments and those shown in (b) and (c) were adopted from [34] and [24], respectively.

dependent inhibition of the NER activity by As(III) also occurred in HFW cells. Intriguingly, the As(III)-inhibited NER activity could be rescued by PD98059 or U0126 cotreatments in HFW or G1-enriched CL3 cells (Figs. 4a and 4b). By contrast, the capability of Pb(II) in stimulating NER activity was blocked under suppression of ERK activation by PD98059 (Fig. 4c). The results indicate that ERK signal could either enhance or inhibit cellular NER synthesis in a stimulus-dependent manner.

As(III) suppresses the induction of NER activity during over-expression of MKK1-CA in human cells

To further examine the role of ERK signal in NER synthesis, we manipulated the ERK activity by introducing MKK1-CA (a constitutive active mutant of MKK1 vector) into H293 cells and allowed expression for 2 days before preparation of cell lysates for the NER synthesis assay. The lysates derived

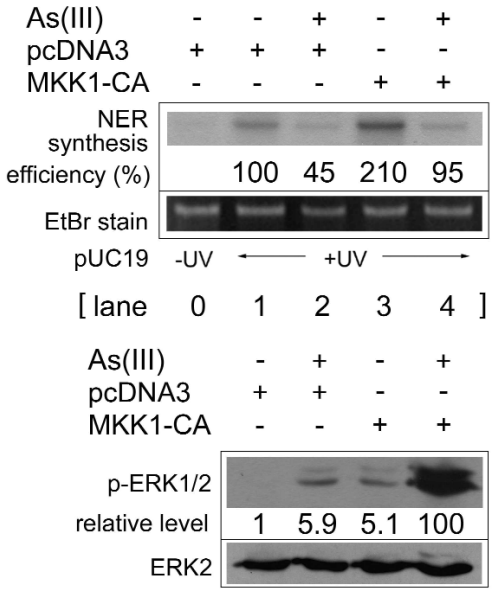

Fig. 5 As(III) suppresses the induction of NER activity during over-expressing MKK1-CA in human cells. H293 cells in exponential growth were transfected with MKK1-CA or pcDNA3 vectors, allowed expression for 33 h, and then left untreated or treated with 50 μM As(III) in serum-free medium for 3 h. After recovery for 12 h, the cells were subjected to the determination of ERK and NER activities as described in Figs. 1 and 3. Results were calculated by averaging at least three independent experiments.

from cells transfected with MKK1-CA had elevated phospho-ERK and NER activity than that derived from cells expression a control vector (Fig. 5, lanes 3 vs. 1). As(III) synergistically increases ERK activation and suppresses the induction of NER activity during enforced expression of MKK1-CA in H293 cells (Fig. 5, lanes 4 vs. 3). The results confirm that ERK signal has dual and opposing roles in regulating the cellular NER activity.

ERK signaling promotes the As(III)-induced micronucleus formation

To explore whether the ERK signal affects As(III) genotoxicity, we adopted a micronucleus formation assay. G1-enriched CL3 cells were exposed to As(III) for 3 h in the presence or absence of PD98059 or U0126, followed by recovery for 24 or 48 h in medium containing cytochalasin B to allow the accumulation of bi-nucleated cells in those underwent complete nuclear division. Microscopic examination showed that 50 μM As(III) induces micronucleus formation ~10-fold and 4-fold higher than the untreated G1 cells when they were recovered, respectively, in cytochalasin B for 24 and 40 h (Fig. 6). Cytochalasin B incubation for 24 h could accumulate 55 % of the untreated cells with bi-nuclei. Only 14 % of the As(III)-treated G1 cells had bi-nuclei following cytochalasin B incubation for 24 h and the

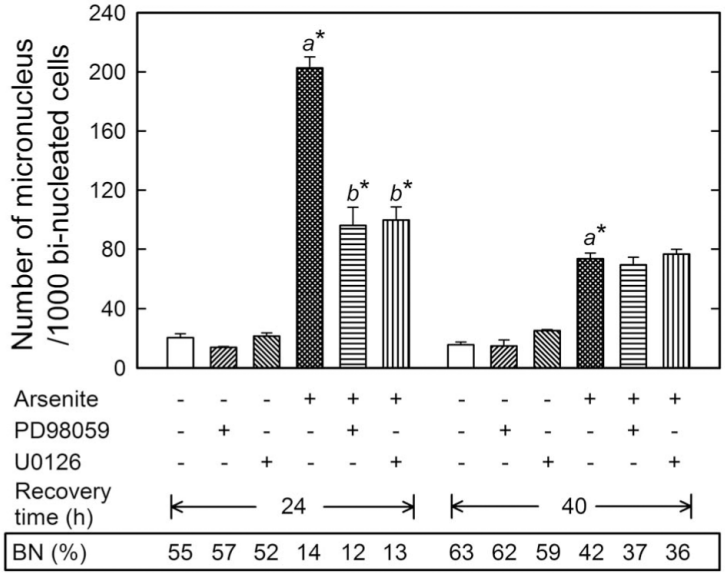

Fig. 6 ERK signaling can promote the As(III)-induced micronucleus formation. G1-enriched CL3 cells were left untreated or treated with 50 μM As(III) in the presence or absence of 50 μM PD98059 or 5 μM U0126. Following treatments, the cells were incubated with cytochalasin B (1 μg/ml) for 24 or 40 h before microscopic examination. Two thousand binucleated (BN) cells in each treatment were examined for scoring the levels of micronucleus formation and the percentage of BN cells using an inverted microscope. Results were obtained by averaging at least three independent experiments (adopted from [34]). Bars represent SEM and * indicates significant difference from the untreated (*a*) or comparable As(III) group in the absence of inhibitors (*b*).

levels increased to 42 % following a prolonged incubation, suggesting As(III) delays cell cycle progression. PD98059 or U0126 significantly lowered the micronucleus levels in As(III)-treated G1 cells when they were recovered in cytochalasin B for 24 h but not 40 h (Fig. 6). The results suggest that ERK activity is involved in micronucleus formation in a population of As(III)-treated G1 cells, possibly those bypassing the delay of cell cycle progression. Yet, after a prolonged recovery period the micronucleus formation in As(III)-treated G1 cells appears to be independent of the ERK signal.

ERK signaling prevents Pb(II) mutagenicity in human cells

To investigate the role of ERK in Pb(II) mutagenicity, HFW or CL3 cells were left untreated or pretreated with PD98059 for 1 h and then exposed to Pb(II) for 24 h in serum-free medium. After treatment, the cells were cultured for 7 days at exponential growth followed by selection for the *HPRT* mutations using 6-thioguanine. As shown in Fig. 7, Pb(II) did not induce the mutation frequency in HFW or CL3 cells. Intriguingly, PD98059 cotreatment dramatically increased the mutation frequency in the Pb(II)-exposed HFW (Fig. 7a) or CL3 cells (Fig. 7b). Pb(II) could activate PKCα as an ERK upstream signaling transducer in CL3 cells [35]. To investigate whether PKCα is involved in the ERK-mediated anti-mutagenicity following Pb(II), CL3 cells were left untreated or pretreated with Gö6976, a PKCα

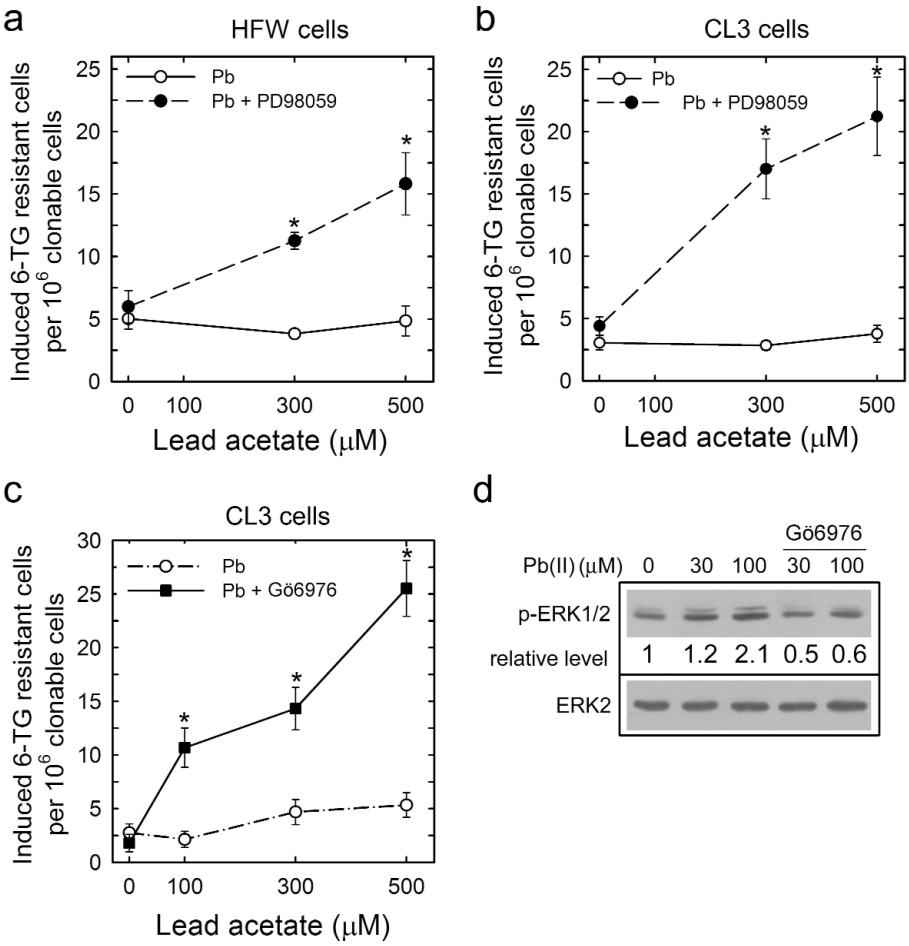

Fig. 7 ERK signaling prevents Pb mutagenicity. (a) HFW fibroblasts or (b) CL3 cells were left untreated or pretreated with PD98059 for 1 h before exposure to Pb(II) for 24 h. (c, d) CL3 cells were left untreated or pretreated with Gö6976 (0.5 μM) for 30 min before exposure to Pb(II) for 24 h. The *HPRT* mutation frequency was determined by the 6-thioguanine (6-TG) resistant assay (a-c) and the activation of ERK was resolved by western blotting (d). Results were obtained from at least four experiments. Bars represent SEM and * indicates significant difference from the comparable Pb(II) group in the absence of inhibitors (adopted from [24] and [35]).

inhibitor, for 30 min and then cotreated with lead acetate for 30 min, followed by two washes with phosphate-buffered saline before exposure to Pb(II) for 24 h. As shown in Fig. 7c, Pb(II) could markedly induce the *HPRT* mutation frequency in Gö6976-pretreated cells, although either the metal or Gö6976 alone exhibited background levels of mutagenicity in CL3 cells. Western blot analysis also confirmed that Gö6976 pretreatment blocked the induction of phospho-ERK by 30–100 μM Pb(II) (Fig. 7d). The results suggest that activation of the ERK signaling prevents human cells from mutagenesis following Pb(II) exposure.

ERK signaling prevents cell death caused by As(III) or Pb(II)

We then compared the impact of ERK signaling on the cytotoxicities caused by As(III) and Pb(II). Treatment of G1-enriched CL3 cells with 50 μM As(III) reduced the cell viability to 40–45 % of the untreated levels as determined using trypan blue exclusion (Fig. 8a) and colony-forming ability assays

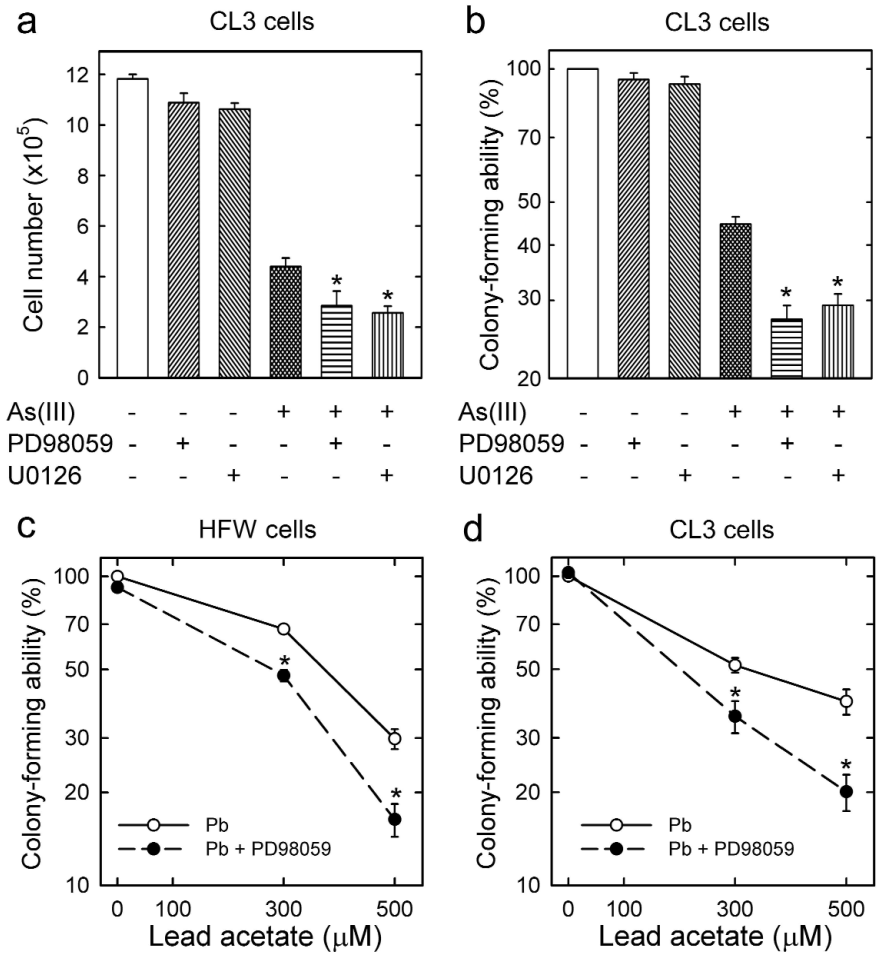

Fig. 8 ERK signaling prevents cell death following As(III) or Pb(II) exposure. (a,b) G1 cells were left untreated or treated with 50 μM arsenite in serum-free medium for 3 h in the presence or absence of 50 μM PD98059 or 5 μM U0126. Following treatment the cells were cultured for another 3 days before cell number determination by trypan blue exclusion analysis (a). Alternatively, after treatments the cells were re-plated for survival determination using colony-forming ability assay (b). HFW fibroblasts (c) or CL3 cells (d) were left untreated or pretreated with PD98059 for 1 h before exposure to Pb(II) for 24 h, and then subjected to colony-forming ability assay. Results were obtained from at least four independent experiments and those shown in (a,b) and (c,d) were adopted from [34] and [24], respectively. Bars represent SEM and * indicates significant difference from the comparable Pb(II) or As(III) groups in the absence of inhibitors.

(Fig. 8b). Coadministering PD98059 or U0126 further decreased the viability of G1-enriched CL3 cells exposed to As(III) (Figs. 8a and 8b). Similarly, blockage of ERK activation reduced the colony-forming ability of Pb(II)-exposed HFW (Fig. 8c) and CL3 cells (Fig. 8d). These results imply that ERK is a pro-survival signaling following As(III) or Pb(II) exposures.

DISCUSSION

It is well documented that As(III) inhibits NER processes in mammalian cells [16–19,22,23]. The capability of As(III) to interfere with cellular NER has been suggested via indirect mechanisms because

it can increase the activities of DNA repair proteins during in vitro reactions [26]. In this report, we demonstrated that the ERK signal can serve as an indirect pathway contributing to the As(III)-elicited NER inhibition in CL3 and HFW cells. This phenomenon is quite the opposite to the fact that Pb(II) stimulates the NER efficiency in an ERK-dependent manner in CL3 cells [24], despite the fact that it has an inhibitory effect on NER in vitro [25]. We further showed that As(III) can suppress the NER syn-

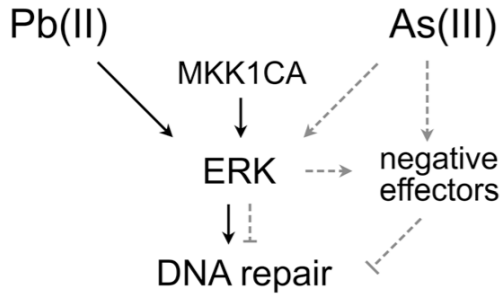

Fig. 9 Scheme depicting the impacts of ERK signaling on DNA repair following As(III) or Pb(II) exposures or MKK1-CA over-expression.

thesis activity induced by constitutive expression of MKK1-CA → ERK signal in H293 cells. More intriguingly, the ERK activation appears to mediate in the As(III)-induced micronucleus formation, whereas the signaling is essential for preventing Pb(II) mutagenicity. On the other hand, the ERK activation counteracts cell death induced by both As(III) and Pb(II). These comparisons suggest that the pro-survival ERK signal has dual and opposing impacts on the cellular NER activity, which subsequently influences genomic stability in a stimulus-dependent manner (Fig. 9).

Why the pro-survival ERK signal exhibits negative and positive effects on the cellular NER activity following As(III) and Pb(II) remains an enigma. The two stimuli may generate distinct arrays of signaling pathways that could be cross-affected. For example, As(III) could elicit sustained activation of two other subfamilies of MAPK: JNK and p38MAPK in CL3 cells (J. P. Li, data not shown); whereas, Pb(II) does not [24]. It is speculated that As(III) may elicit negative effectors regulated by ERK signaling to counteract the ERK impacts on NER stimulation (Fig. 9). Several evidences have also suggested that the ERK signaling pathway may modulate genomic or morphological alterations. ERK activation is required for the cell transformation caused by As(III) in mouse Cl 41 cells [39]. Activation of calcium-dependent PKC, a potential upstream of ERK, has been associated with the As(III)-induced micronuclei in Chinese hamster ovary cells [40]. ERK activation through constitutive expression of Ras greatly enhances chromosome instability [41,42]. On the other hand, ERK signaling has been implicated in the protection of micronucleus induction by cadmium chloride [43] and ionizing radiation [44]. Additionally, the atypical PKCζ was found to increase NER in response to DNA damage by UV or cisplatin [45]. The dual and opposing roles of ERK signaling induced by DNA damage agents and oncoproteins in maintaining genome integrity is obviously an interest issue to be further explored.

ERK phosphorylates and activates numerous substrates involving in nucleotide synthesis, gene transcription, protein synthesis, and cell cycle progression [28,29,46]. ERK may phosphorylate transcription factors to up- or down-regulate the expression of genes involving in NER and thereby alter the DNA repair efficiency. For instance, activation of the Ras-ERK pathway by insulin up-regulates ERCC-1 mRNA expression [47]. Also, ERK activation upon ionizing radiation increases the expression of ERCC-1 and XRCC1 at the transcription and translation levels [44]. Conversely, As(III) decreases the expression of genes encoding NER enzymes, e.g., XPC in human epidermal keratinocytes [48] and XPC, XPD, and DNA ligase-1 in human bronchial epithelial BEAS-2B cells [49]. Moreover, the expression of ERCC1, XPF, and XPB genes of the NER complex is inversely correlated with toenail ar-

senic levels in a case-control study [50]. On the other hand, NER enzymes such as hHR23A, hHR23B, and replication protein A2 have been identified to be posttranslational modified by the ERK pathway [51], suggesting that this signal may regulate the repair efficiency by triggering phosphorylation of NER enzymes. Whether the ERK pathway modulates DNA repair genes under As(III) or Pb(II) exposure and the underlying mechanisms warrant further investigation.

The immediate early gene product c-Fos has been proposed to function as a sensor for ERK signal duration [52]. The expression of c-Fos protein is induced via ERK in Pb(II)-treated CL3 cells [24], implying c-Fos may be a downstream effecter of ERK in cellular defense systems. Mouse fibroblasts lacking c-*fos* are hypersensitive to a wide variety of genotoxic agents, including UV-C light, in the induction of cytotoxicity, apoptosis, and chromosomal breakages [53,54], which has been attributed to a defect in NER, particularly, the re-synthesis of XPF at the transcriptional level via c-Fos/AP-1 [55]. These findings have indicated that c-Fos plays a protective role against DNA damage via inducing NER activity. JNK activation is necessary for the NER induction in UVC-irradiated cells having functional c-Fos [56]; yet, a role of ERK in the c-Fos-mediated NER induction has not been explored. JNK, however, was not associated with the NER inhibition by As(III) (J. P. Li, data not shown) and could not be activated by Pb(II) in CL3 cells [24].

Previous reports have shown that the generation of reactive nitrogen/oxygen species could modulate the induction of oxidative DNA damage and micronuclei as well as the inhibition of NER in As(III)-exposed mammalian cells [57–59]. Pb(II) also causes oxidative stress in vivo [60] and can induce oxidative DNA damage via reactive singlet oxygen in vitro [61]. Cellular NER machinery removes many kinds of base damage including oxidative DNA adducts [36–38]. It is possible that following As(III) exposure the NER inhibition mediated by the pro-survival ERK signal may elevate the accumulation of DNA lesions, which can result in increased micronucleus formation. On the other hand, the Pb(II)-elicited ERK signal may eliminate DNA lesions via enhancing repair efficiency and thereby prevents mutagenesis and cell death.

In summary, this report indicates that As(III) and Pb(II) display distinct cellular effects on DNA repair activity via the pro-survival ERK signal. We show that the ERK activation involves in the NER inhibition and micronuclei induction in As(III)-treated cells, while this signaling pathway is necessary for NER induction and maintaining genome integrity following Pb(II) exposure. Sustained ERK activity is frequently found in cancers during progression stages and thereby has been designed as a therapeutic target [62]. Results obtained here suggest that blockage of the ERK signal pathway may have therapeutic potential to decrease genome instability during arsenite exposure. However, suppression of ERK activation can result in genome instability in lead-exposed populations. These findings provide the concerns regarding careful application of the signaling pathway inhibitors in clinic trials.

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