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Tpl2 is a key mediator of arsenite-induced signal transduction

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Abstract

Arsenite is a well-known human carcinogen that especially targets skin. The *tumor progression locus 2 (Tpl2)* gene encodes a serine/threonine protein kinase that is overexpressed in various cancer cells. However, the relevance of Tpl2 in arsenite-induced carcinogenesis and the underlying mechanisms remain to be explored. We demonstrate that arsenite increased Tpl2 kinase activity and its phosphorylation in mouse epidermal JB6 P+ cells in a dose- and time-dependent manner. Exposure to arsenite resulted in a marked induction of cyclooxygenase (COX)-2 and prostaglandin (PG)E₂, important mediators of inflammation and tumor promotion. Treatment with a Tpl2 kinase inhibitor (TKI) or Tpl2 short hairpin RNA (shRNA) suppressed COX-2 expression and PGE₂ production induced by arsenite treatment, suggesting that Tpl2 is critical in arsenite-induced carcinogenesis. We also found that arsenite-induced phosphorylation of extracellular signal-regulated kinases (ERKs) or c-Jun NH₂-terminal kinases (JNKs) was markedly suppressed by TKI or Tpl2 shRNA. Inhibition of arsenite-induced ERKs or JNKs signaling using a pharmacological inhibitor of ERKs or JNKs substantially blocked COX-2 expression. Furthermore, inhibition of Tpl2 reduced the arsenite-induced promoter activity of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), indicating that NF-κB and AP-1 are downstream transducers of arsenite-triggered Tpl2. Our results demonstrated that Tpl2 plays a key role in arsenite-induced COX-2 expression and PGE₂ production and further elucidated the role of Tpl2 in arsenite signals that activate ERKs/JNKs and NF-κB/AP-1 in JB6 P+ cells.

Keywords

tumor progression locus 2 (Tpl2); arsenite; cyclooxygenase (COX)-2; prostaglandin (PG)E₂

Introduction

Inorganic arsenic, which exists primarily as arsenite (Fig. 1A), is a well-known human carcinogen in the natural environment. Arsenic in drinking water is one of the most potent environmental health threats in the United States and worldwide (1). Chronic intake of arsenic is associated with an increased risk of tumors of the skin, bladder, liver, kidney, lung, and other tissues (2). In particular, arsenic accumulates in the skin and is linked to hyperkeratosis, pigmentation disorders, and skin cancers, including basal cell carcinoma and squamous cell

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carcinoma (3-5). Although evidence of the carcinogenicity of arsenic in humans is strong, the mechanism by which it produces tumors is not completely understood.

The *tumor progression locus 2 (Tpl2)* proto-oncogene, also known as *Cot* or *MAP3K8*, encodes a serine/threonine protein kinase that is activated by provirus integration in retrovirus-induced T-cell lymphomas and mammary carcinomas (6-8). Overexpression of Tpl2 activates the mitogen-activated protein (MAP) kinase pathway (9,10) as well as various transcription factors (11). The activation of Tpl2 is required for the induction of tumor necrosis factor- α by lipopolysaccharide in macrophages (12). Tpl2 is overexpressed in breast cancer (13) as well as in gastric and colon adenocarcinomas (14). Therefore, Tpl2 is a critical component of the signaling pathway that controls tumor development.

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. COX-2 is induced in many tissues, including the epidermis, in response to inflammation. Upregulation of COX-2 has been found in the early stages of carcinogenesis and appears to be a consistent feature of neoplastic development in a wide variety of human and animal epithelial tissues (15). Selective COX-2 inhibitors can reduce the incidence of UV-induced squamous cell carcinoma in hairless mice (16). COX-2-derived bioactive lipids, including prostaglandin (PGE)₂, are potent inflammatory mediators that promote tumor growth and metastasis by stimulating cell proliferation, invasion, and angiogenesis (17). Elevated PGE₂ levels occur in both basal and squamous cell carcinomas of the skin (18) and are associated with increased tumor progression and metastasis. Therefore, high levels of prostaglandins may promote the development of malignancy. Although arsenite induces the expression of COX-2, the precise mechanism underlying these effects remains to be elucidated.

Here we focus on determining whether Tpl2 is obligatory for arsenite-induced signal transduction. We provide evidence that Tpl2 is a mediator of arsenite-induced COX-2 expression and PGE₂ production in mouse epidermal JB6 P+ cells. Furthermore, extracellular signal-regulated kinases (ERKs) and c-Jun NH₂-terminal kinases (JNKs) are the downstream effectors of Tpl2 for the mediation of nuclear factor kappa B (NF- κ B) and activator protein-1 (AP-1) transactivation.

Materials and Methods

Materials

Eagle's minimum essential medium (MEM), gentamicin, and L-glutamine were obtained from Gibco-BRL (Carlsbad, CA). The Tpl2 kinase inhibitor [TKI; 4-(3-chlor-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-1, 7-naphthylidene] was purchased from Calbiochem-Novabiochem (San Diego, CA). The antibody against COX-2 was purchased from Cayman (Ann Arbor, MI) and the antibody against β -actin was from Sigma-Aldrich (St. Louis, MO). The antibody against phosphorylated Tpl2 was obtained from Invitrogen (Carlsbad, CA) and antibodies against total Tpl2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to detect phosphorylated ERKs, total ERKs, phosphorylated JNKs, total JNKs, phosphorylation MEK1, total MEK1, phosphorylated MKK4, and total MKK4 were from Cell Signaling Biotechnology (Beverly, MA). CNBr-Sepharose 4B, glutathione-Sepharose 4B, [γ -³²P]ATP, and the chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA).

Cell culture and shRNA transfection

The JB6 P+ mouse epidermal cell line was purchased from American Type Culture Collection. JB6 P+ cells were cultured in monolayers in 5% fetal bovine serum (FBS)-MEM, 2 mM L-

glutamine, and 25 µg/ml gentamicin at 37°C in a 5% CO₂ incubator. The JB6 P+ mouse epidermal cell line stably transfected with COX-2 luciferase reporter plasmid was a kind gift from Dr. Chauanshu Huang (School of Medicine, New York University). The JB6 mouse epidermal cell lines were stably transfected with an AP-1, NF-κB, or COX-2 luciferase reporter plasmid and maintained in MEM supplemented with 5% FBS containing 200 g/ml G418. Short hairpin RNA (shRNA) constructs against *Mus musculus* Tpl2 were purchased from Origene Company (Rockville MD). For knockdown experiments, the cells were transfected with shRNA targeting *Mus musculus* Tpl2 (catalog number TR512220) or with non-targeting control shRNA (catalog number TR30003) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Western blot assay

For the Western blot assay, cells (1.5×10^6) were cultured in a 10-cm dish for 48 h and then starved in 0.1% FBS–MEM for 24 h to eliminate the FBS activation of MAP kinases. Following treatment, cells were disrupted with lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and a protease inhibitor cocktail tablet). The supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) as described by the manufacturer. Lysate proteins (30 µg) were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). After blotting, the membrane was incubated at 4°C overnight with the specific primary antibody. Protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridization with the horseradish peroxidase-conjugated secondary antibody.

Tpl2 immunoprecipitation and kinase assay

JB6 P+ cells were cultured to 80% confluence and then serum-starved in 0.1% FBS–MEM for 24 h at 37°C. Cells were treated with 20 µM arsenite for different time periods, disrupted with lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, 1 mM sodium orthovanadate [Na₃VO₄], and 1 mM phenylmethylsulfonyl fluoride), and finally centrifuged at 20,000g for 10 min in a microcentrifuge. The lysates containing 500 µg of protein were used for immunoprecipitation with an antibody against Tpl2 and then incubated at 4°C overnight. After the addition of Protein A/G Plus agarose beads, the mixture was continuously rotated at 4°C. The beads were washed 3 times with kinase buffer [20 mM 3-(N-morpholino) propanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na₃VO₄, and 1 mM dithiothreitol], then resuspended in 20 µl of 1× kinase buffer supplemented with 1 µg inactive MEK1, and ERK2, and incubated for an additional 30 min at 30°C. Next, 20 µg myelin basic protein and 10 µl diluted [γ-³²P]ATP solution were added, and the mixture was incubated for 10 min at 30°C. A 20-µl aliquot was transferred onto p81 paper and washed 3 times with 0.75% phosphoric acid for 5 min per wash, followed by a single wash with acetone for 2 min. The radioactive incorporation was determined using a scintillation counter. Experiments were performed in triplicate.

PGE₂ assay

Cells were plated in 24-well dishes and grown to 80% confluence in 500 µl of growth medium for 48 h, then starved in 0.1% FBS–MEM for 24 h. Following treatment, culture medium was collected, centrifuged at 14,000 rpm for 5 min to remove cell debris, and frozen at -80°C prior to analysis. The amounts of PGE₂ released into the medium were measured using the PGE₂ enzyme immunoassay kit (Cayman Chemical). Experiments were performed in triplicate.

Luciferase assay for COX-2, NF- κ B, or AP-1 transactivation

Confluent monolayers of JB6 P+ cells stably transfected with a COX-2, NF- κ B, or AP-1 luciferase plasmid were trypsinized, and 8×10^3 viable cells suspended in 100 μ l of 5% FBS–MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. When cells reached 80–90% confluence, they were starved by culturing in 0.1% FBS–MEM for another 24 h. The cells were treated with TKI for 30 min before they were exposed to 20 μ M arsenite for 24 h. After treatment, cells were disrupted with 100 μ l lysis buffer (0.1 M potassium phosphate buffer pH 7.8, 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA), and the luciferase activity was measured using a luminometer (Luminoskan Ascent, Labsystems, MD).

Statistical analysis

The data are expressed as the mean $\pm$ S.D. A Student's *t*-test was used for single statistical comparisons, with a probability of $p < 0.05$ as the criterion for statistical significance.

Results

Arsenite strongly increases phosphorylation and kinase activity of Tpl2

To determine whether Tpl2 is involved in the cellular response to arsenite (Fig. 1A) exposure, we examined the phosphorylation of Tpl2 induced by arsenite in mouse epidermal JB6 P+ cells. Arsenite exposure caused a substantial elevation of Tpl2 phosphorylation (Fig. 1B) and kinase activity (Fig. 1C) in a time-dependent manner and also induced phosphorylation of Tpl2 in a dose-dependent manner (Fig. 1D). These results indicate that arsenite induces phosphorylation and kinase activity of Tpl2 in JB6 P+ cells.

Arsenite induces COX-2 expression and PGE₂ secretion

We examined whether arsenite could induce COX-2 expression. A time-response study indicated that arsenite exposure led to an increase in COX-2 protein level from 6 to 24 h (Fig. 2A). Arsenite also increased PGE₂ release in a time-dependent manner (Fig. 2B) and arsenite increased COX-2 expression in a dose-dependent manner (Fig. 2C). To further confirm this finding, we measured COX-2 transcription using a gene reporter assay. The promoter activity of COX-2 was significantly increased by arsenite exposure dose-dependently (Fig. 2D). These results indicate that COX-2 expression, COX-2 promoter activity, and PGE₂ production are induced by exposure to arsenite time- and dose-dependently.

TKI suppresses arsenite-induced COX-2 expression

The above data indicated that arsenite induces Tpl2 kinase activity and also increases COX-2 expression and PGE₂ secretion. Therefore, we hypothesized that Tpl2 might play a key role in arsenite-induced COX-2 expression and PGE₂ production and that COX-2 expression would be changed by the inhibition of Tpl2 activation. As expected, we found that TKI, a Tpl2 inhibitor, markedly suppressed arsenite-induced COX-2 expression in a dose-dependent manner (Fig. 3A). TKI also inhibited arsenite-induced COX-2 promoter activity (Fig. 3B) and substantially attenuated arsenite-induced PGE₂ production (Fig. 3C). These results suggest that Tpl2 plays a critical role in arsenite-induced COX-2 induction and PGE₂ production.

Involvement of Tpl2 in COX-2 induction by arsenite

We used shRNA to confirm whether the Tpl2-dependent pathway is involved in the expression of COX-2 in the arsenite response. The expression of Tpl2 shRNA specifically reduced the Tpl2 protein level (Fig. 4A, 3rd panel), and arsenite-induced COX-2 expression was inhibited in the Tpl2 shRNA cells compared with the control shRNA cells (Fig. 4A, 1st panel). Tpl2 shRNA cells impaired arsenite-induced COX-2 promoter-driven luciferase activity in JB6 P+

cells stably transfected with a COX-2 luciferase plasmid (Fig. 4B). Tpl2 shRNA cells also significantly inhibited arsenite-induced PGE₂ production (Fig. 4C). Collectively, these results indicate that arsenite induces COX-2 expression and PGE₂ production through a Tpl2-dependent pathway.

Tpl2 regulates ERKs and JNKs activity

Previous studies have indicated that the MAP kinase signaling pathway is involved in arsenite-induced carcinogenesis in JB6 P+ cells (19,20). We found that Tpl2 is involved in arsenite-induced COX-2 expression; however, the signal transduction components mediated by arsenite-induced Tpl2 activation in JB6 P+ cells are unknown. We hypothesized that Tpl2 might be located upstream of the known arsenite-stimulated signal transduction pathway and that Tpl2 could regulate several downstream kinases. To determine the physiological role of Tpl2 in MAP kinase activation induced by arsenite in JB6 P+ cells, we investigated the influence of TKI on arsenite-induced activation of the MKK/ERKs and MKK4/JNKs pathways. Our data showed that TKI substantially inhibited the phosphorylation of MEK and ERKs (Fig. 5A, *left panel*). In addition, TKI blocked phosphorylation of MKK4 and JNKs (Fig. 5A, *right panel*). To confirm these results, we examined arsenite-stimulated phosphorylation of ERKs and JNKs in control shRNA and Tpl2 shRNA expressing cells. Arsenite-induced phosphorylation of ERKs and JNKs was attenuated in the Tpl2 shRNA cells compared with the control shRNA cells (Fig. 5B). We also found that ERKs and JNKs were required for COX-2 induction by arsenite when JB6 P+ cells were treated with kinase inhibitors 30 min before treatment with arsenite for 12 h. Finally, we observed that U0126 and SP600125, pharmacological inhibitors of MEK and JNKs, respectively, blocked arsenite-induced COX-2 expression (Fig. 5C). However, SB203580, a p38 inhibitor, had no effect on arsenite-induced COX-2 expression (data not shown). These findings indicate that Tpl2 is an upstream kinase of MEK/ERKs and MKK4/JNKs and that it is also involved in arsenite-induced COX-2 expression.

Tpl2 regulates NF-κB and AP-1 activity

The COX-2 gene promoter region contains NF-κB and AP-1 binding sites that can be recognized by these transcription factors and, in turn, lead to *cox-2* gene expression (21). We examined whether NF-κB or AP-1 was a Tpl2 downstream transcription factor induced by arsenite. To determine whether the repression of COX-2 expression by TKI involved the inhibition of NF-κB or AP-1 activation, we measured NF-κB and AP-1 transactivation using JB6 cells stably transfected with an AP-1 or NF-κB luciferase reporter plasmid. Pretreatment of cells with TKI markedly inhibited arsenite-induced NF-κB and AP-1 activation (Fig. 6A and B). A Tpl2 shRNA construct was transfected into JB6 P+ cells, which were then stably transfected with an NF-κB or AP-1 luciferase plasmid. Arsenite treatment decreased NF-κB- and AP-1-dependent transcriptional activity in Tpl2 shRNA cells compared with control shRNA cells (Fig. 6C and D). These data show that NF-κB and AP-1 are downstream regulators of Tpl2 for arsenite-induced COX-2 expression.

Discussion

Arsenite is a well-known skin carcinogen that has the ability to activate signaling pathways and gene expression. Here we provide the first direct evidence that arsenite leads to the activation of Tpl2, which plays a critical role in arsenite-induced PGE₂ production through the induction of COX-2 expression. Moreover, Tpl2 appears to function upstream of MEK/ERKs and MKK4/JNKs, which leads to induction of *cox-2* expression through its downstream NF-κB and AP-1 signaling pathways. Our data indicate that the activation of Tpl2 is a major event involved in arsenite-induced carcinogenesis.

The *Tpl2* proto-oncogene encodes a serine/threonine protein kinase that is activated by provirus insertion in T-cell lymphoma induced by Moloney leukemia virus and mouse mammary tumor virus-induced mammary carcinomas (6,7). Investigations of Tpl2 expression in human tumor specimens have shown that Tpl2 is overexpressed in various cancer cells (13,14). We recently reported a link between the phosphorylation of Tpl2 and neoplastic cell transformation (22). Previous studies have shown that arsenite induces COX-2 expression, which is mediated through the IKK β /NF- κ B pathway in JB6 P+ cells (23). However, little is known about the upstream mediators of arsenite-induced COX-2 expression and PGE₂ synthesis. Here we found that arsenite strongly induces phosphorylation and kinase activity of Tpl2 (Fig. 1). Accumulating evidence suggests that oxidative stress occurs in response to arsenic exposure and may be a factor in dermal arsenic carcinogenesis (24). Additionally, reactive oxygen species can act as signaling molecules by activating various cellular processes (25). Thus, the generation of reactive oxygen species by arsenite may be associated with various cellular processes, such as Tpl2 activation.

COX-2, a key enzyme in PGE₂ synthesis, is maintained at low levels in normal cells but is induced by inflammatory tumor promoters, cytokines, growth factors, and oncogenes (26). COX-2 knockout mice developed ~75% fewer chemically induced skin papillomas than control mice (27). The present study provides evidence that arsenite can induce COX-2 expression and PGE₂ production in JB6 P+ cells. Arsenite induced COX-2 expression, COX-2 promoter activity, and PGE₂ production in a time- and dose-dependent manner. Inhibition of Tpl2 dramatically impaired arsenite-induced expression of COX-2. Moreover, knockdown of Tpl2 expression by shRNA abrogated arsenite-induced COX-2 expression and PGE₂ generation. These results strongly suggest that Tpl2 is involved in arsenite-stimulated COX-2 expression and PGE₂ secretion.

Tpl2 is a pivotal member of several intracellular signaling pathways. Previous studies demonstrated that overexpression of Tpl2 leads to activation of MAP kinases (12,28). Our results show that TKI significantly suppresses the phosphorylation of the MEK/ERKs and MKK4/JNKs signaling pathways, and Tpl2 shRNA reduces arsenite-induced ERKs and JNKs phosphorylation. The results indicate that ERKs and JNKs activation depends on Tpl2. Moreover, U0126 and SP600125, which are MEK and JNK inhibitors, respectively, strongly suppressed arsenite-induced COX-2 expression. Overall, these data suggest that Tpl2 functions upstream of MEK/ERKs and MKK4/JNKs and that it is involved in arsenite-induced COX-2 expression.

The MAP kinases are upstream activators of transcription factors such as NF- κ B and AP-1, which are implicated in COX-2 expression (29,30). The *cox-2* gene contains binding sites for a number of important transcription factors, including NF- κ B and AP-1. Increases in NF- κ B-DNA or AP-1-DNA binding immediately precede upregulation of *cox-2* gene transcription in various cell lines. NF- κ B was reported as an upstream mediator of COX-2 expression in mouse epidermal JB6 P+ cells (23). A correlation between AP-1 and COX-2 expression has been suggested. AP-1 has been demonstrated to tumor promoter-induced expression of COX-2 in mouse skin (31). We found that arsenite could induce transactivation of AP-1 in JB6 P+ cells (32). These previous studies provided strong evidence showing that NF- κ B and AP-1 are involved in arsenite-induced COX-2 expression in JB6 P+ cells.

In the present study, we further examined the arsenite-induced Tpl2 activity leading to NF- κ B and AP-1 transactivation. Pretreatment with TKI significantly reduced arsenite-induced transactivation of NF- κ B and AP-1. In Tpl2 shRNA cells, arsenite treatment did not lead to obvious enhancement of NF- κ B or AP-1 transactivation compared to control shRNA cells. Thus, these data indicate that Tpl2 is involved in NF- κ B and AP-1 arsenite-induced transactivation.

Based on these results, we propose a model for arsenite-induced Tpl2 activation and the associated signaling pathways. According to this model, arsenite induces COX-2 expression and PGE₂ synthesis, which is mediated by the activation of Tpl2. In addition, arsenite triggers a Tpl2-dependent MEK/ERKs and MKK4/JNKs pathway that leads to the activation of NF- κ B and AP-1. These findings provide new insight into the molecular mechanisms of the tumor promotion effects of arsenite and suggest that Tpl2 is an excellent target for the development of drugs to fight arsenite-induced skin carcinogenesis.

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Figure 1. Arsenite-induced phosphorylation and activation of Tpl2

(A) Chemical structure of arsenite. (B) Arsenite induces phosphorylation of Tpl2 in a time-dependent manner. Cells were exposed to 20 μ M arsenite and harvested at the indicated time. The levels of phosphorylated Tpl2 or total Tpl2 proteins were determined by Western blot. (C) Arsenite induces Tpl2 kinase activity in a time-dependent manner. Cells were treated with 20 μ M arsenite and harvested at the indicated times. The cells were disrupted and the kinase activity of Tpl2 was determined as described in Materials and Methods. Data are shown as means $\pm$ S.D. of triplicate assays. Asterisks (**) indicate a significant difference from the untreated control ($p < 0.01$). (D) Arsenite induces phosphorylation of Tpl2 in a dose-dependent manner. Cells were harvested 2 h after treatment with the indicated dose of arsenite. The level

of phosphorylated Tpl2 or total Tpl2 protein was determined by Western blot. The relative value of density for p-Tpl2 bands was normalized to Tpl2.

Figure 2. Effect of arsenite on COX-2 expression and promoter activity and PGE₂ production
 (A) Arsenite elevates COX-2 expression in a time-dependent manner. Cells were exposed to 20 μM arsenite and harvested at the indicated time. The levels of COX-2 and β-actin proteins were determined by Western blot. (B) Arsenite stimulates PGE₂ secretion in a time-dependent manner. Cells were treated with 20 μM arsenite for the indicated time. PGE₂ generation was determined using the PGE₂ assay kit as described in Materials and Methods. Data are shown as mean ± S.D. values from triplicate experiments. Asterisks (**) indicate a significant difference from untreated control ($p < 0.01$). (C) Arsenite induces an increased COX-2 expression in a dose-dependent manner. Cells were harvested 12 h after treatment with the indicated dose of arsenite. The levels of COX-2 and β-actin proteins were determined by

Western blot. (D) Arsenite induces COX-2 promoter activity. JB6 P+ cells that were stably transfected with the COX-2 luciferase reporter plasmid were harvested 24 h after treatment with the indicated dose of arsenite. The relative activity was measured by luciferase assay as described in Materials and Methods. Data are shown as mean $\pm$ S.D. values of COX-2 luciferase activities calculated from triplicate determinations. Asterisks (**) indicate a significant difference from untreated control ($p < 0.01$). The relative value of density for COX-2 bands was normalized to β -actin.

Figure 3. Effect of TKI on arsenite-induced COX-2 expression and promoter activity, and PGE₂ production

(A) Arsenite-induced COX-2 expression is attenuated by TKI. Cells were treated with TKI (0, 5, 10, or 20 μM) for 30 min before being stimulated with 20 μM arsenite for 12 h. The level of COX-2 and β-actin proteins was determined by Western blot using specific antibodies as described in Materials and Methods. (B) Arsenite-induced COX-2 promoter activity is attenuated by TKI. JB6 P+ cells, which were stably transfected with the COX-2 luciferase reporter plasmid, were pretreated with TKI (0, 5, 10, or 20 μM) for 30 min before being exposed to 20 μM arsenite for 24 h. The relative activity was measured by luciferase assay as described in Materials and Methods. Data are shown as mean ± S.D. values of COX-2 luciferase activities

calculated from triplicate determinations. Asterisks (**) indicate a significant difference between groups treated with arsenite and TKI together and the group exposed to arsenite alone ($p < 0.01$). (C) Arsenite-induced PGE₂ secretion is impaired by TKI. Cells were pretreated with TKI (0, 5, 10, or 20 μ M) for 30 min before being exposed to 20 μ M arsenite for 24 h. PGE₂ generation was determined using the PGE₂ assay kit as described in Materials and Methods. Mean $\pm$ S.D. values were calculated from triplicate determinations from one experiment. Asterisks (**) indicate a significant difference between groups treated with arsenite and TKI together and the group exposed to arsenite alone ($p < 0.01$). The relative value of density for COX-2 bands was normalized to β -actin.

Figure 4. The role of Tpl2 in arsenite-induced COX-2 expression and PGE₂ production
 (A) Tpl2 shRNA reduces arsenite-induced COX-2 expression. JB6 P+ cells were transiently transfected with Tpl2 shRNA or control shRNA; and at 72 h after transfection, cells were treated with arsenite (10 or 20 μM) for 12 h. The level of COX-2, β -actin, and Tpl2 proteins was determined by Western blot. (B) Tpl2 shRNA inhibits arsenite-induced COX-2 promoter activity. JB6 P+ cells that were stably transfected with the COX-2 luciferase reporter plasmid were transiently transfected with Tpl2 shRNA or control shRNA and, 72 h after transfection, were treated with arsenite (0, 10 or 20 μM) for 24 h. The relative activity was measured by luciferase assay as described in Materials and Methods. Data are shown as mean $\pm$ S.D. values of COX-2 luciferase activities calculated from triplicate determinations. Asterisks (**) indicate

a significant difference between groups ($p < 0.01$). (C) Tpl2 shRNA reduces arsenite-induced PGE₂ secretion. JB6 P+ cells were transfected with Tpl2 shRNA or control shRNA and, 72 h after transfection, were treated with arsenite (0, 10 or 20 μ M) for 24 h. PGE₂ generation was determined using the PGE₂ assay kit as described in Materials and Methods. Mean and S.D. values are calculated from triplicate determinations. Asterisks (**) indicate a significant difference between groups ($p < 0.01$). The relative value of density for COX-2 bands was normalized to β -actin.

Figure 5. Effects of Tpl2 inhibition on arsenite-induced phosphorylation of ERKs and JNKs
 (A) TKI inhibits arsenite-induced phosphorylation of MEK, ERKs, MKK4, and JNKs. JB6 P+ cells were treated with TKI (0, 5, 10, or 20 μM) for 30 min before being stimulated with 20 μM arsenite for 2 h. The levels of phosphorylated and total MEK, ERKs, MKK4, and JNKs proteins were determined by Western blot. (B) Tpl2 shRNA reduces arsenite-induced phosphorylation of ERKs and JNKs. JB6 P+ cells were transiently transfected with Tpl2 shRNA or control shRNA and, 72 h after transfection, were treated with arsenite (0, 10 or 20 μM) for 2 h. The levels of phosphorylated and total ERKs, JNKs, and Tpl2 proteins were determined by Western blot. (C) Arsenite-induced COX-2 expression in JB6 P+ cells is attenuated by ERKs or JNKs inhibitors. Cells were treated with U0126 or SP600125 (0, 10, or 20 μM) for 30 min before being stimulated with 20 μM arsenite for 12 h. The levels of COX-2 and β-actin proteins were determined by Western blot. The intensity of phosphorylated and COX-2 bands was normalized to the intensity of the corresponding nonphosphorylated and β-actin bands, and the ratio was normalized to the control.

Figure 6. Involvement of Tpl2 in arsenite-induced NF-κB and AP-1 activation

(A and B) TKI reduces arsenite-induced NF-κB (A) and AP-1 (B) transcriptional activities in JB6 P+ cells. JB6 P+ cells that were stably transfected with an NF-κB or AP-1 luciferase reporter plasmid were treated with TKI (5, 10, or 20 μM) for 30 min before being exposed to 20 μM arsenite for 24 h. The relative activity was measured by luciferase assay as described in Materials and Methods. Data are shown as mean ± S.D. values of NF-κB or AP-1 luciferase activities calculated from triplicate determinations. Asterisks (**) indicate a significant difference between groups treated with arsenite and TKI together and the group exposed to arsenite alone ($p < 0.01$). (C and D) Tpl2 shRNA inhibits arsenite-induced NF-κB (C) and AP-1 (D) promoter activity. JB6 P+ cells that were stably transfected with the NF-κB or AP-1 luciferase reporter plasmid were transiently transfected with Tpl2 shRNA or control shRNA. At 72 h after transfection, cells were treated with arsenite (0, 10, or 20 μM) for 24 h. The relative activity was measured by luciferase assay as described in Materials and Methods. Data are shown as mean ± S.D. values of NF-κB or AP-1 luciferase activities calculated from triplicate determinations. Asterisks (**) indicate a significant difference between groups ($p < 0.01$).