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Activation of the p38 MAPK/Akt/ERK1/2 Signal Pathways Is Required for the Protein Stabilization of CDC6 and Cyclin D1 in Low-Dose Arsenite-Induced Cell Proliferation

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ABSTRACT

Arsenic trioxide (ATO) is a first-line anti-cancer agent for acute promyelocytic leukemia, and induces apoptosis in other solid cancer cell lines including breast cancer cells. However, as with arsenites found in drinking water and used as raw materials for wood preservatives, insecticides, and herbicides, low doses of ATO can induce carcinogenesis after long-term exposure. At 24 h after exposure, ATO (0.01–1 μM) significantly increased cell proliferation and promoted cell cycle progression from the G1 to S/G2 phases in the non-tumorigenic MCF10A breast epithelial cell line. The expression of 14 out of 96 cell-cycle-associated genes significantly increased, and seven of these genes including *cell division cycle 6* (*CDC6*) and *cyclin D1* (*CCND1*) were closely related to cell cycle progression from G1 to S phase. Low-dose ATO steadily increased gene transcript and protein levels of both CDC6 and cyclin D1 in a dose- and time-dependent manner. Low-dose ATO produced reactive oxygen species (ROS), and activated the p38 MAPK, Akt, and ERK1/2 pathways at different time points within 60 min. Small molecular inhibitors and siRNAs inhibiting the activation of p38 MAPK, Akt, and ERK1/2 decreased the ATO-increased expression of CDC6 protein. Inhibiting the activation of Akt and ERK1/2, but not p38 MAPK, decreased the ATO-induced expression of cyclin D1 protein. This study reports for the first time that p38 MAPK/Akt/ERK1/2 activation is required for the protein stabilization of CDC6 in addition to cyclin D1 in ATO-induced cell proliferation and cell cycle modulation from G1 to S phase. J. Cell. Biochem. 111: 1546–1555, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ARSENITE; CELL PROLIFERATION; CELL CYCLE; DNA REPLICATION; SIGNAL TRANSDUCTION; PROTEIN STABILIZATION

Arsenic trioxide (ATO) is a first-line chemotherapeutic drug used to induce apoptosis in acute promyelocytic leukemia [Platanias, 2009]. In addition, ATO induces apoptosis in several solid cancer cell lines, including breast cancer [Baj et al., 2002; Platanias, 2009]. ATO is a form of inorganic arsenite found in nature and a common byproduct of copper smelting. In addition, ATO is commonly used as a raw material for manufacturing other arsenic compounds used as wood preservatives, insecticides, and herbicides [Shibata et al., 2007]. Although these chemicals have not been commercially manufactured in the United States since 2004, ATO-treated wood is still used in various products (EPA 2003). It has been estimated that more than 350,000 people in the United States may be supplied with water containing more than 50 μg/L arsenic, and more than 2.5 million people may be supplied with water with levels above 25 μg/L. Long-term exposure to low doses of arsenic compounds has been associated with the increased incidence of several cancer types including skin, lung, liver, bladder, and prostate cancers [Smith et al., 1992].

The mechanisms by which low doses of arsenic initiate or promote carcinogenesis remain unclear and are probably multifactorial [Huff et al., 2000]. The major mechanisms that have been proposed include generation of reactive oxidative species (ROS), perturbation of DNA methylation patterns, inhibition of DNA repair, modulation of signal transduction pathways and epigenetic changes

Abbreviations used: ATO, arsenic trioxide; EPA, Environmental Protection Agency; ROS, reactive oxidative species; MAPK, mitogen-activated protein kinase; CDC6, cell division cycle 6; ERK 1/2, extracellular signal-regulated kinases 1/2.

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[Schoen et al., 2004]. Malignant transformation of human urothelial cells by arsenic is associated with epigenetic changes in histone acetylation and DNA methylation in gene promoter regions [Jensen et al., 2008]. Signaling pathways include mitogen-activated protein (MAP) kinases [Lau et al., 2004], ras signaling activation [Benbrahim-Tallaa et al., 2007], c-Myc overexpression [Chen et al., 2001], acquisition of androgen independence [Benbrahim-Tallaa et al., 2005], and aberrant estrogen receptor (ER) signaling [Shen et al., 2007]. Recent reports describe changes in gene expression upon exposure to arsenic in a variety of cell types at different doses and exposure times. Unfortunately, these findings are largely inconclusive due to large deviations and inconsistencies among the different cell lines and arsenic types used [Schoen et al., 2004; Snow et al., 2005].

Aberrant cell proliferation has long been associated with chemical-induced carcinogenesis [Cohen and Ellwein, 1990; Melnick et al., 1993]. Low-dose arsenic-induced proliferation has been reported in both cell culture and animal models [Huff et al., 2000; Simeonova et al., 2000]. Aberrant cell proliferation has been associated with alterations in cell cycle machinery at checkpoints and associated signal pathways that are critical in tumor initiation, development, and progression [Hwang et al., 2006; DuMond and Singh, 2007; Ouyang et al., 2007]. The signal pathways involved include the activation of cyclin D1 mediated by c-Jun/AP-1 [Zhang et al., 2009], NF-κB [Luster and Simeonova, 2004; Ouyang et al., 2006], and PI-3K/Akt [Ouyang et al., 2007]. In addition, oncogenes, tumor suppressor genes and other transcription factors [Huang et al., 2008], Myc [Chen et al., 2001], and Rb/E2F [Lu et al., 2001] are involved in cell cycle regulation and cell transformation.

In the present report, we investigated the molecular effects of low-dose ATO on cell proliferation and cell cycle progression from G1 to S/G2 phases using the non-tumorigenic MCF10A breast epithelial cell line. Low-dose ATO induced the production of ROS and sequentially activated the p38 MAPK, Akt, and ERK1/2 pathways within 60 min of exposure. Small molecular pathway inhibitors of the p38 MAPK, Akt, and ERK1/2 pathways reversed ATO-induced cell proliferation. We used the human Cell Cycle Tox and Cancer StellARray™ qPCR Array to screen for modulations in gene expression levels in cells after exposure to 0.1 μM ATO for 24 h. *CDC6* and *cyclin D1*, genes critical for the initiation of DNA replication and cell cycle transition from G1 to S phase, were significantly elevated. We performed time course studies to monitor the expression of CDC6 and cyclin D1 at both the transcriptional and protein levels. We also tested the influence of kinase inhibitors and siRNA against p38 MAPK, Akt, and ERK1/2 on arsenic-induced protein expression of CDC6 and cyclin D1.

MATERIALS AND METHODS

CELL CULTURE

The immortalized, non-transformed human mammary epithelial MCF10A cell line was grown in a 5% CO_2-humidified incubator at 37°C in complete MCF10A growth medium composed of DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO), 100 ng/ml cholera toxin (Cambrex, Westborough, MA), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). MCF-7, a breast adenocarcinoma cell line, was grown in DMEM media supplemented with 5% FBS and 1% penicillin/streptomycin.

CELL PROLIFERATION ASSAY

MCF10A and MCF-7 cells were seeded in 96-well plates (2×10^4/well) for 24 h and treated with stepwise dilutions of ATO ranging from 0.01 to 100 μM for 72 h. PBS-treated cells were used as controls. Cells were examined under a light microscope daily. Cell viability was measured by MTT assay (OD_{570}). Data were expressed as average percentages of cell viability and standard deviations, and were calculated from three independent assays performed by triplicate. Differences between groups were compared by Kruskal–Wallis one-way ANOVA, followed by Bonferroni's correlation. For the cell clonegenic assay, cells (1×10^5/well) were seeded in 24-well plates and treated with ATO at 0.01, 0.1, and 1 μM for 24 h. PBS-treated cells were used as controls. Cells were harvested, and 1,000 cells were re-seeded in a P100 culture dish. Cell clones were stained with crystal violet 2 weeks after cell re-seeding.

We screened cell proliferation-associated pathways involved in low-dose ATO-induced cell proliferation. MCF10A cells were seeded in 96-well plates (2×10^4/well) and grown for 24 h. Cells were pre-treated with the p38 MAP kinase inhibitor SB203580, ERK1/2 inhibitor PD 98059 and Akt inhibitor API-59CJ-OME (Sigma Aldrich, St. Louis, MO) for 1 h, followed by treatment with or without 0.1 μM of ATO for another 72 h. Viable cells were measured by MTT assay.

DETECTION OF ROS PRODUCTION USING FLOW CYTOMETRY

ROS was detected using the Total ROS/Superoxide detection kit (Enzo Life Science, Plymouth Meeting, PA). MCF10A cells were seeded in 12-well plates (1×10^5/well). Cells were cultured in serum-free medium for 24 h and then treated with 0.1 or 1 μM of ATO for another 6 h. Cells treated with the ROS inducer pyocyanin or with the ROS inhibitor *N*-acetyl-L-cysteine were used as positive or negative controls, respectively. Cells were harvested and stained with oxidative stress detection reagent and analyzed using the Accuri 6 flow cytometer (Accuri Cytometers, Inc., Ann Arbor, MI). The experiments were repeated three times.

CELL CYCLE ANALYSIS

MCF10A and MCF-7 cells were seeded in 6-well plates (2×10^5/well). The cells were cultured in serum-free media for 24 h and followed with or without exposure to 0.1 or 1 μM of ATO for another 24 h. Cells were dissociated with 0.25% trypsin and fixed with 70% ethanol at 4°C for at least 30 min. The cells were washed by PBS twice, treated with ribonuclease (100 μg/ml) (Sigma Aldrich) for 10 min, and stained with propidium iodide (50 μg/ml) at 37°C for 30 min. The cell cycle distribution was determined using the Accuri 6 flow cytometer, and the data were analyzed by BD CellQest Pro software.

HUMAN CELL CYCLE TOX AND CANCER STELLARAY™ qPCR ARRAY

MCF10A cells (1×10^5 per well) were plated in 12-well plates and grown under serum starvation conditions for 24 h, followed by

treatment with 0.1 μM of ATO for an additional 24 h. Cells were harvested and total RNA was prepared by the RiboPure™ kit (Applied Biosystems, Foster City, CA). Total RNA (500 ng) was reverse transcribed to cDNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Twenty-five nanograms of cDNA was used for each well of 96-well plates in the Human Cell Cycle Tox and Cancer StellARray™ qPCR Array (LONZA, Allendale, NJ). The assay was performed under standard PCR conditions in an ABI 7500 Real Time-PCR system (Applied Biosystems, Framingham, MA). Each sample was done in three replicates in individual plates, and the data were analyzed following the manufacturer's protocol using the Global Pattern Recognition™ (GPR) Analysis Tool (www.lonza.com).

GENE EXPRESSION ASSAY BY REAL-TIME RT-PCR

MCF10A cells (1×10^5 per well) were seeded in 24-well plates. The cells were treated with 0.1 and 1 μM of ATO for 24 or 72 h. Total RNA was extracted and reverse-transcribed to cDNA as described above. One hundred nanograms of cDNA was used in a qPCR reaction (ABI 7500 Real Time-PCR system). The primers for CDC6, cyclin D1, and β-actin were: CDC6: 5′-GGGTGAAGGCTGCGGGTTCC-3′ (forward) and 5′-GCCCAGACGTTTCCTGGGGC-3′ (reverse); cyclin D1: 5′-CGTGCCCGTGTGCATGTCCT-3′ (forward) and 5′-GTGGCCTTTCCCGACCCTGC-3′ (reverse); and β-actin: 5′-CTGGGACGACATGGAGAAAA-3′ (forward) and 5′-AAGGAAGGCTGGAAGAGTGC-3′ (reverse). Statistical analyses were performed using Student's *t*-test.

WESTERN BLOTTING ANALYSIS

MCF10A cells (4×10^6) were plated in 100-mm culture dishes and grown under serum-starvation conditions for 24 h before treatment with 0.1 or 1 μM ATO. The cells were harvested at 15, 30, and 60 min after the ATO exposure. Equal amounts of proteins (40 μg) were separated by SDS–PAGE and transferred to a nitrocellulose membrane by electroblotting. The antibodies against p38 MAPK, phosphorylated p38 MAPK, ERK1/2, phosphorylated ERK1/2, Akt and phosphorylated Akt were purchased from Cell Signaling (Danvers, MA). Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Cell Signaling) and Supersignal West Pico Chemiluminescent Substrate (Pierce).

MCF10A cells were treated with 0.1 or 1 μM of ATO for 24, 48, and 72 h; or MCF10A cells were pre-treated with 10 μM of p38 MAPK inhibitor SB 203580 or ERK1/2 inhibitor PD98059, or 5 μM of Akt inhibitor API-59CJ-OME for 1 h, followed by exposure to 0.1 μM of ATO for another 24 h. The cells were lysed and tested for expression of CDC6 and cyclin D1 (Cell Signaling). The experiments were repeated three times.

MCF10A cells were transfected with the siRNAs to p38 MAPK, ERK1/2, and Akt as well as a control siRNA (Cell Signaling) using a DharmaFECT transfection reagent (Dharmacon, Inc., Lafayette, CO). The cells were cultured with 0.1 μM ATO for 24 h following transfection. Proteins were harvested and p38, ERK1/2, Akt, CDC6, and cyclin D1 expression was determined by Western blotting. The relative intensity of the bands observed by Western blotting was measured using pixel information obtained with Adobe Photoshop CS4 software. The experiments were repeated three times.

RESULTS

ATO WAS CYTOTOXIC IN CANCER CELLS, BUT PROMOTED CELL PROLIFERATION AT LOW DOSES IN NON-TUMORIGENIC EPITHELIAL CELLS

ATO has been shown to induce apoptosis in breast cancer cells [Baj et al., 2002]. We were interested in testing the response of non-tumorigenic breast epithelial cells to low-dose ATO. In the present study, we first tested cell growth in the MCF-7 breast cancer cell line and the non-tumorigenic MCF10A breast epithelial cell line following exposure to different doses of ATO (0.01–100 μM). Consistent with a previous report [Ye et al., 2005], ATO decreased the viability of MCF-7 cells, even at very low doses (0.01 μM) (Fig. 1A). However, low levels of ATO (0.01 and 1 μM) significantly promoted cell proliferation in MCF10A (~30–40% above control levels), while higher doses of ATO (10 μM) induced cytotoxicity, which increased with increasing doses (Fig. 1C). The cell clonogenic assay also consistently confirmed the results of low-dose ATO-promoted cell proliferation (Fig. 1B and 1D). From these results, we determined that doses ranging from 0.01 to 1 μM of ATO promote cell proliferation. These doses were thus used in the following experiments.

LOW-DOSE ATO PROMOTED CELL CYCLE PROGRESSION FROM G1 TO S AND G2/M PHASES

To further elucidate the mechanism by which low doses of ATO promote cell proliferation, we analyzed the influence of low-dose ATO on cell cycle regulation. The average cell population at the G0/G1 phase was 93.94%, and S and G2/M phases were only 4.39% or 0.8% when the cells were starved of serum for 24 h. The cell populations at G0/G1 phase decreased to 27.3%, while the cell population at S phase increased to 11.87% and the cell population at G2/M phase increased to 60.04% when MCF10A cells were cultured together with 0.1 μM of ATO without serum. Cell populations at G0/G1 phase decreased to 18.55%, while the cell population at S phase increased to 25.65% and at G2/M phase increased to 55.21% when MCF10A cells were cultured together with 1 μM of ATO (Fig. 2A). Low-dose ATO promoted cell cycle progression from G1 to S and G2/M phases in MCF10A cells. The results suggest that low-dose ATO promoted DNA replication and DNA synthesis at 24 h after exposure to ATO. In MCF-7 cells, 0.1 or 1 μM ATO increased the average cell population at G0/G1 phases from 85.27% to 88.75% or 90.43%, respectively, and decreased the average cell population at G2/M phase from 8.16% to 5.91% or 3.55%, which suggested that low doses of ATO induced cell cycle arrest at G0/G1 (Fig. 2B). The cell cycle arrest at G0/G1 blocked or delayed the progression of cells from G0/G1 phase into S phase, and induced cells toward apoptosis. These results are consistent with the cell proliferation in Figure 1.

LOW-DOSE ATO INFLUENCED THE EXPRESSION OF GENES CRITICAL FOR CELL CYCLE PROGRESSION FROM THE G1 TO G2/S PHASES

To determine the profile of the cell cycle-regulated genes involved in low-dose ATO-induced cell proliferation and cell cycle progression

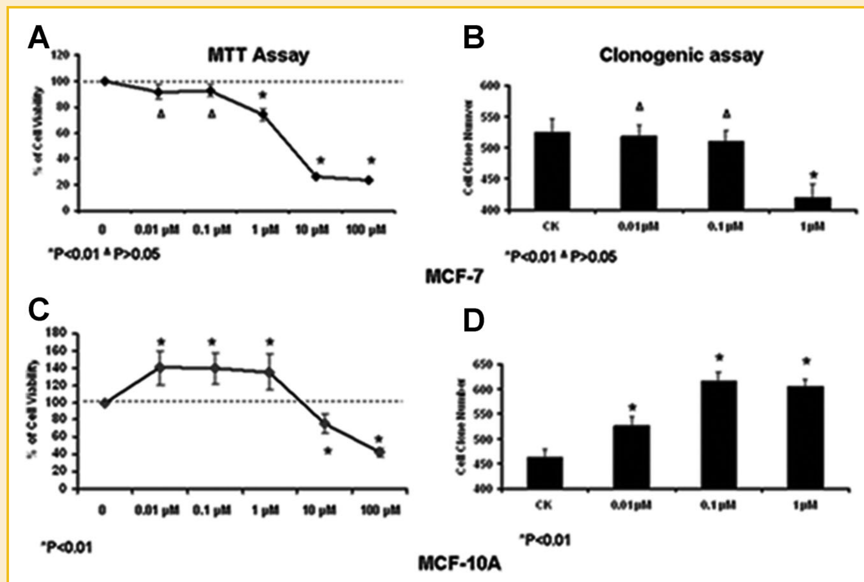

Fig. 1. ATO was cytotoxic at low doses in the MCF-7 cancer cell line, but promoted cell proliferation at low doses in the non-tumorigenic MCF10A epithelial cell line. Cells (2×10^4/well) were seeded in 96-well plates for 24 h and treated with serial dilutions of ATO for 72 h. MTT staining was performed to detect cell viability, followed by the OD reading at 570 nM. Data were expressed as average percentages of cell viability and standard deviations, and were calculated from three independent assays performed by triplicate. $^*P < 0.01$, $^{\Delta}P > 0.05$, n = 8. The differences were identified by Kruskal–Wallis one-way ANOVA on ranks and Dunnett's test. Cell clonogenic assays were performed to confirm the MTT results. The cells (1×10^5/well) were seeded in 24-well plates and treated with ATO for 24 h. One thousand cells were re-seeded in p100 culture dishes and cultured for another 2 weeks. A crystal violet assay was used to stain the cell clones. Data were expressed as mean ± SD, $^*P < 0.01$, $^{\Delta}P > 0.05$, n = 4. *T*-test was used for the difference between control and treated cells. A: MTT assay in MCF-7; (B) clonogenic assay in MCF-7; (C) MTT assay in MCF10A; (D) clonogenic assay in MCF10A.

from the G1 to S and G2/M phases, we monitored changes in gene expression 24 h post-exposure to 0.1 μM of ATO using a Human Cell Cycle Tox and Cancer StellARay™ qPCR Array. This array was specifically designed to profile the expression of cell cycle genes important in both toxicology and cancer research, including cyclins, cyclin-dependent kinases (CDK) and phosphatases, cell cycle inhibitors, and genes important during DNA replication, cycle checkpoints and cell cycle arrest. In contrast to control, expression levels of 14 out of 96 tested genes from ATO-exposed cells increased more than twofold. *HADC1* was excluded because the folds of change were unreasonable in contrast to other data in this experiment (Supplement Table I). Seven of these 14 genes are essential cell cycle regulators at the G1, S, or G2 phase and are required for DNA replication and progression from the G1 to the S and G2 phases. Impressively, the gene expression level of *CDC6* increased approximately 10-fold, *CDKN2C* increased approximately 4-fold, *CDK2* increased approximately 2.6-fold, *CDC2* increased approximately 2.4-fold, *E2F3* increased approximately 2.2-fold, while *cyclin D1* increased approximately 2-fold (Table I).

LOW-DOSE ATO STEADILY INCREASED THE EXPRESSION OF CDC6 AND CYCLIN D1 AT THE TRANSCRIPTION AND PROTEIN LEVELS

Low-dose ATO increased the gene transcription levels of *CDC6*, *CDKN2C*, *CDK2*, *CDC2*, *E2F3*, and *cyclin D1* in the Human Cell Cycle Tox and Cancer StellARay™ qPCR Array. We monitored the dynamic changes in gene and protein expression following exposure to ATO at 24, 48, and 72 h. Exposure to 0.1 μM of ATO led to steady increases in the transcription levels of *CDC6* and *cyclin D1* from 24 to 72 h. In addition, both 0.1 and 1 μM ATO steadily increased the protein levels of CDC6 and cyclin D1 in a time- and dose-dependent manner (Fig. 3).

LOW-DOSE ATO ACTIVATED THE P38 MAPK, AKT, AND ERK1/2 SIGNAL PATHWAYS AND INDUCED THE PRODUCTION OF REACTIVE OXIDATIVE SPECIES (ROS)

It is well known that mitogen-activated protein kinase (MAPK) is activated by environmental stresses, such as ultraviolet irradiation, heat and osmotic shock, genotoxic agents, anisomycin and toxins, as well as growth factor and inflammatory cytokine stimulation [Wagner and Nebreda, 2009]. The MAPK cascade pathways play a critical role in the pleiotropic effects of ATO [Huang et al., 1999a,b; Dong, 2002]. Activation of the p38 MAPK, Akt, and ERK1/2 pathways by ATO has been implicated in the regulation of cell proliferation [Ouyang et al., 2007; Zhang et al., 2009], but the exact molecular mechanism is not clear. We tested whether low-dose ATO activated the p38 MAPK, Akt, and ERK1/2 pathways in MCF10A cells. The activation of MAP kinase cascades and the Akt pathway was tested using phosphorylated antibodies in Western blots following cell exposure to low-dose ATO (0.1 and 1 μM). We observed a sequential phosphorylation of p38 MAPK at 15 min, Akt at 30 min, and ERK1/2 at 60 min following exposure to ATO (Fig. 4A).

Several studies have suggested that the ATO-induced activation of MAPK pathways [El Mchichi et al., 2007; Kang and Lee, 2008; Sanchez et al., 2009] and the Akt pathway [Bornhauser et al., 2007] might result from ROS production after high doses of ATO. We

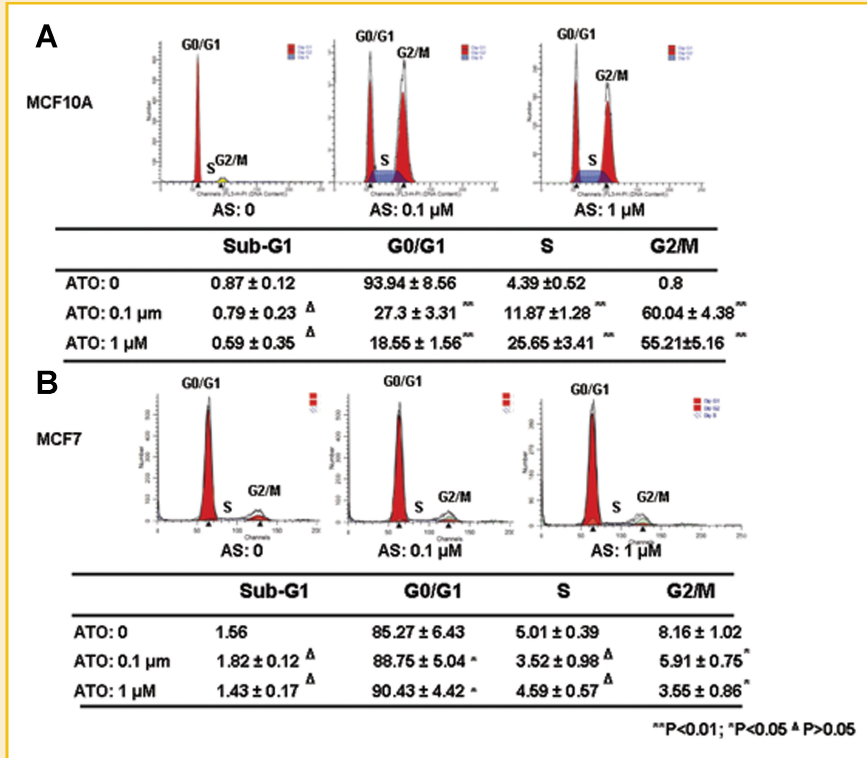

Fig. 2. Low-dose ATO promoted cell cycle progression from G1 to S/G2 phases, and induced cell cycle arrest at G2/M phases. MCF10A cells in 6-well plates were cultured in serum-free media for 24 h, followed by exposure to ATO for another 24 h. Cells were dissociated and fixed with 70% ethanol at 4°C for at least 30 min. The cells then were treated with ribonuclease to degrade RNA and the DNA was stained with propidium iodide. The cell cycle was determined by DNA content as measured by flow cytometry. The data were expressed as means ± SE. $^{**}P < 0.01$, $^{*}P < 0.01$, $^{\Delta}P > 0.05$, n = 3. The differences were identified by Kruskal–Wallis one-way ANOVA on ranks and Dunnett's test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

wanted to clarify if low-dose ATO also induced ROS production under the same conditions in which the p38 MAPK, Akt, and ERK1/2 pathways were activated. ATO (0.1 µM) increased the production of ROS from 10.6% in control cells to 22.7%, and 1 µM of ATO increased the production of ROS to 25.4% (Fig. 4B). These results show that ROS production can be induced even at low doses of ATO, which suggests that the activation of the p38 MAPK, Akt, and ERK1/

TABLE I. Low Doses of ATO Increase the Expression of Cell Cycle G1/S-Associated Genes

Rank	Gene name	*P*-value	Fold change
3	CDC6	0.114	9.62106
4	CDC25A	0.246	5.04739
6	CDKN2C	0.123	3.86191
10	CDK2	0.242	2.60711
11	CDC2	0.342	2.36529
13	E2F3	0.149	2.15532
14	CCND1	0.321	1.93116

MCF10A cells (1×10^5/well) were plated in 12-well plate and the cells were grown at serum starvation media for 24 h, and followed by the exposure of 0.1 µM of arsenic trioxide for another 24 h. The cells were harvested and total RNA was prepared and then was synthesized to cDNA. cDNA was used for real-time RT-PCR in Human Cell Cycle Tox and Cancer StellARay™ qPCR Array. The data were analyzed by using Global Pattern Recognition™ (GPR) Analysis Tool.

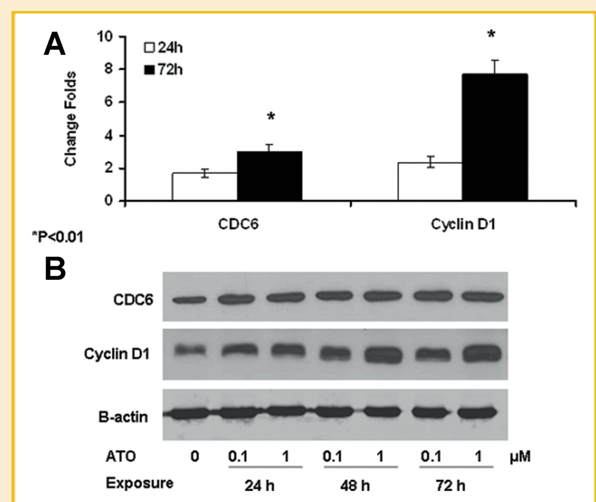

Fig. 3. Low-dose ATO steadily increased the expression of CDC6 and cyclin D1 at both the transcription and protein levels. A: MCF10A cells were treated with ATO after serum starvation for 24 h. The cells were collected 24 or 72 h after treatment, and the total RNA was prepared for real-time qRT-PCR. Data were expressed as mean ± SD, $^{*}P < 0.01$, n = 3. *T*-test was used for the difference between control and treated cells. B: MCF10A cells were treated with ATO. The cells were collected 24, 48, or 72 h after treatment, and the protein was collected for Western blot.

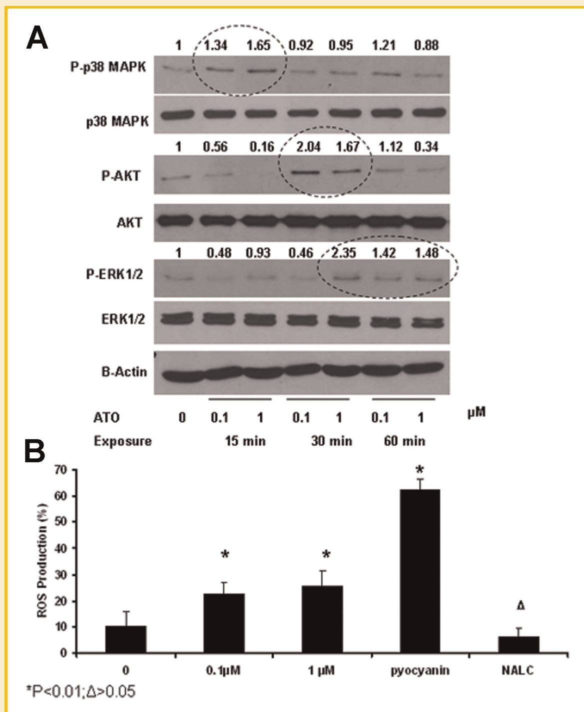

Fig. 4. Low-dose ATO activated the p38 MAPK/Akt/ERK1/2 pathways and induced ROS production. A: MCF10A cells were grown under serum starvation conditions for 24 h before treatment with 0.1 or 1 μM of ATO. The cells were collected at 15, 30, and 60 min after exposure to ATO. The cells were assayed by Western blot to test protein expression and activation of the p38 MAPK, Akt, and ERK1/2 pathways. B: Low-dose ATO induced the production of ROS. MCF10A cells were seeded in 12-well plates. The cells were cultured in serum-free medium for 24 h and then treated with ATO for another 6 h, with the PBS-treated cells used as controls. Cells treated with the ROS inducer pyocyanin or with the ROS inhibitor *N*-acetyl-L-cysteine were used as positive or negative controls. The cells were collected, stained with an oxidative stress detection reagent, and analyzed by flow cytometry. The data were expressed as mean ± SD, $^{*}P < 0.01$, n = 3. *T*-test was used for the difference between control and treated cells.

2 pathways by low doses of ATO might be mediated by ROS production.

INHIBITION OF THE P38 MAPK/AKT/ERK1/2 SIGNAL PATHWAYS REVERSED THE INCREASED CELL PROLIFERATION INDUCED BY LOW-DOSE ATO

To study whether the activation of p38 MAPK, Akt, and ERK1/2 pathways are involved in low-dose ATO-induced cell proliferation, we tested the cellular response when MCF10A cells were co-treated by low-dose ATO and small molecular inhibitors of the p38 MAPK, Akt, and ERK1/2 pathways. The p38 MAPK inhibitor SB203580, Akt inhibitor API-59CJ-OME and ERK1/2 inhibitor PD098059 were used alone at different doses or in combination with 0.1 μM of ATO. As expected, 0.1 μM of ATO increased cell viability, and the inhibitors of p38 MAPK, Akt, and ERK1/2 decreased the cell viability of MCF10A at the tested doses. Interestingly, the increase in cell proliferation induced by low-dose ATO was completely reversed when the inhibitors of p38 MAPK, Akt, and ERK1/2 were used at 10 or 5 μM, respectively (Fig. 5).

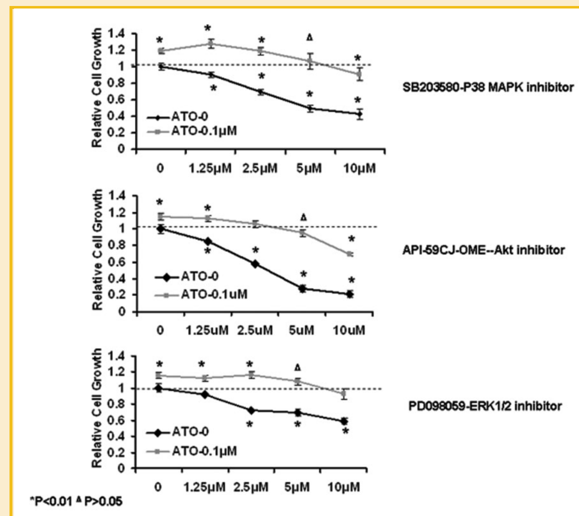

Fig. 5. P38 MAPK/Akt/ERK1/2 signal pathways are involved in low-dose ATO-induced cell proliferation. MCF10A cells were plated in 96-well plates and grown under serum starvation conditions for 24 h. Cells were pre-treated by p38 MAPK inhibitor SB203580, Akt inhibitor API-59CJ-OME and ERK1/2 inhibitor PD098059 for 1 h, followed by treatment with 0.1 μM of ATO for another 72 h. Cell viability was evaluated by MTT assay at OD570. Data were expressed as mean ± SD, $^{*}P < 0.01$, $^{\Delta}P > 0.05$, n = 8. *T*-test was used for the difference between control and treated cells.

ACTIVATION OF THE MAPK AND AKT SIGNAL PATHWAYS IS REQUIRED FOR THE PROTEIN STABILIZATION OF CDC6 AND CYCLIN D1

There was a correlation between up-regulation of CDC6 and cyclin D1 and induction of cell proliferation and cell cycle progression from G1 to S and G2 phases by low-dose ATO. In order to elucidate if elevation of the protein expression of CDC6 and cyclin D1 was due to the activation of the p38 MAPK, ERK1/2, and Akt signal pathways, we tested the influence of the inhibitors of p38 MAPK, ERK1/2, and Akt on the protein expressions of CDC6 and cyclin D1. The doses of inhibitors that can completely reverse ATO-increased cell proliferation are shown in Figure 5. The p38 MAPK inhibitor SB 203580, ERK1/2 inhibitor PD 98059, and Akt inhibitor API-59CJ-OME significantly decreased the ATO-induced increase in the protein expression of CDC6 when cells were treated by inhibitors alone or in combination with ATO. The ERK1/2 inhibitor PD 98059 and Akt inhibitor API-59CJ-OME significantly decreased the ATO-induced increase in the protein expression of cyclin D1, but the p38 MAPK inhibitor SB 203580 did not diminish the ATO-induced cyclin D1 increase when the cells were treated with the inhibitors alone or in combination with ATO (Fig. 6A).

The results were confirmed by inhibiting the endogenous p38 MAPK, ERK1/2, and Akt with siRNA. The cells transfected with siRNAs of p38MAPK (Fig. 6B-1), ERK1/2 (Fig. 6B-2), and Akt (Fig. 6B-3) showed the decreased protein levels compared to the cells

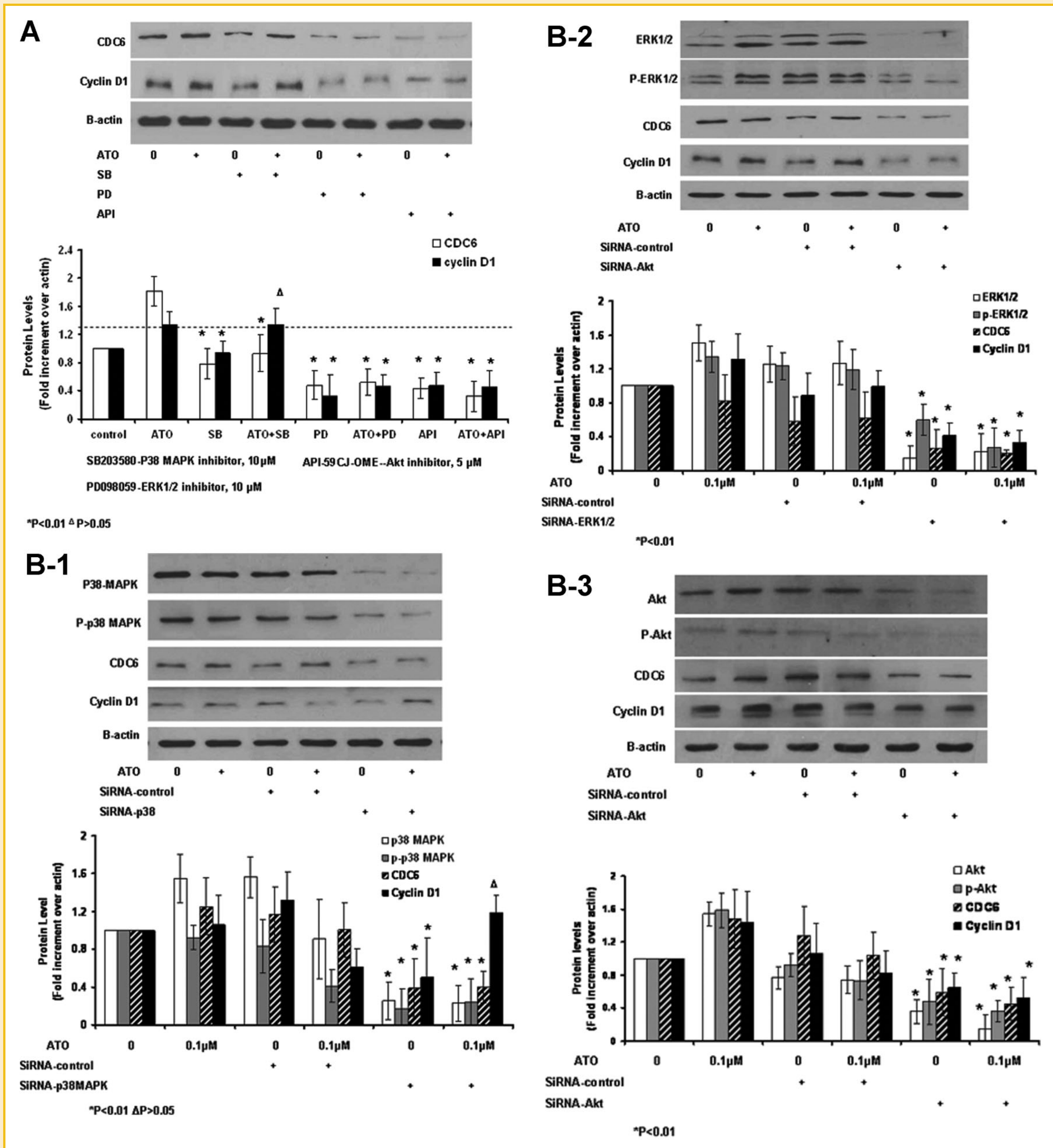

Fig. 6. Activation of the p38 MAPK/Akt/ERK1/2 signal pathways is required for the protein stabilization of CDC6 and cyclin D1. A: MCF10A cells were plated in 6-well plates, and the cells were treated with the p38 MAPK inhibitor SB 203580, ERK1/2 inhibitor PD 98059 and Akt inhibitor API-59CJ-OME for 1 h, and followed by treatment with 0.1 μM of ATO for another 24 h. The cells were collected and lysed for PAGE and Western blot. B-1: MCF10A cells were transfected with the siRNAs to p38 MAPK, ERK1/2, or Akt as well as a control siRNA. The cells were cultured with 0.1 μM of ATO 24 h after transfection. Proteins were harvested and tested for the expression of p38, ERK1/2, Akt, CDC6, and cyclin D1 by Western blot. The experiments were repeated three times. B-1: SiRNA of p38 MAPK; (B-2): SiRNA of ERK1/2; (B-3): SiRNA of Akt. $^{*}P < 0.01$, $^{\Delta}P > 0.05$, n = 8. *T*-test was used for the difference between control and treated cells.

with the transfection of control siRNAs. The down-regulation of p38 MAPK, ERK1/2, and Akt decreased the protein expression of CDC6. Down-regulation of ERK1/2 and Akt decreased the protein expression of cyclin D1, but down-regulation of p38 MAPK did not decrease the protein expression of cyclin D1, which was consistent with the effects of the inhibitors. The data indicated that activation of the p38 MAPK, ERK1/2, and Akt pathways is required for the protein stabilization of CDC6, and activation of the ERK1/2 and Akt pathways, but not the p38 MAPK pathway, is required for the protein stabilization of cyclin D1.

DISCUSSION

The molecular mechanisms by which low doses of arsenic species induce carcinogenesis are complex [Schoen et al., 2004]. Aberrant

cell proliferation has long been associated with carcinogenesis [Cohen and Ellwein, 1990; Fong et al., 1996]. In non-tumorigenic MCF10A cells, low-dose ATO activated the p38 MAPK, ERK1/2, and Akt pathways at different time points after ATO exposure, and the activation might be associated with the production of ROS. low-dose ATO increased cell proliferation, and promoted cell cycle progression from G1 to S and G2/M phases. On the other hand, low doses of ATO induced cell cycle arrest at the G2/M phases and inhibited cell differentiation. However, this biological response to ATO is different in breast cancer MCF-7 cells, where the same doses of ATO induced cell cycle arrest at the G0/G1 phases, and resulted in apoptosis. The promotion of cell cycle progression from the G1 to S and G2/M phases and cell cycle arrest at G2/M will amplify genetic mutations during DNA replication. In several previous reports, low doses of arsenic were reported to promote cell proliferation [Hwang et al., 2006; Trouba et al., 2000a], or inhibit cell differentiation [Kachinskas et al., 1994; Trouba et al., 2000b] individually. For cells to undergo differentiation, cells must exit the cell cycle and enter into mitogenically quiescent states, and cell proliferation is halted [Salnikow and Cohen, 2002]. Therefore, the cells have to undergo cell cycle arrest at G2/M to inhibit cell differentiation when low doses of ATO promote cell proliferation. We did not observe the detected cytotoxicity in ATO-treated cells at 24 h, thus it was believed that the cells arrested at G2/M re-entered into cell cycle. The continuous effects of ATO in cell cycle progression caused the increase of cell number at 72 h.

Aberrant cell proliferation results from alterations in cell cycle machinery at checkpoints and activation of cell proliferation-associated signaling pathways. Cell cycle dysregulation leads to genomic rearrangement of both regular and neoplastic transcription programs [Prindull, 2008]. Therefore, we hypothesize that low doses of ATO might initiate or promote carcinogenesis by altering cell cycle machinery at checkpoints. Cell cycle re-entry by quiescent cells from the G0 to G1 phases and the progression of G1/S transition in actively proliferating cells are regulated by the cell-cycle-associated genes [Johnson and Walker, 1999; Sherr, 2000]. Exposure to low-dose ATO (0.1 μM) in MCF10A cells for 24 h increased the gene expression of 14 out of 96 cell-cycle-associated genes, 7 of which were associated with cell cycle progression at the G1 and S phases. Importantly, CDC6 and cyclin D1 expression increased in a dose- and time-dependent manner from 24 to 72 h at both the transcriptional and translational levels.

CDC6, a licensing factor of the pre-replication complex, is essential for the initiation of DNA replication. It functions as a cell cycle regulator in the early steps of DNA replication by promoting assembly of pre-replicative complexes. This process is tightly regulated to ensure that proper origin licensing occurs once per cell cycle [Borlado and Mendez, 2008]. CDC6 mRNA expression peaks during the G1/S phase and is required for cell cycle transition from G1 to S phase [Hateboer et al., 1998; Yan et al., 1998]. CDC6 is very important for promotion of cell proliferation, as its down-regulation inhibits cell proliferation [Luo et al., 2006]. In the present study, low-dose ATO promoted cell proliferation, and this corresponded with increased expression of CDC6. ATO exposure leading to up-regulation of CDC6 expression is both dose and time dependent. These results suggest that initiation of DNA replication via the regulation of CDC6 might be involved in low-dose ATO-promoted cell proliferation. To our knowledge, this is the first report to show that ROS produced by low-dose ATO induced the activation of p38 MAPK, ERK1/2, and Akt, which are required for the protein stabilization of CDC6. Both genome and epigenome are synthesized at the replication fork in each cell cycle, so DNA replication during S-phase is critical to maintain the complex structure of chromatin and the epigenetic state of the cell. Interestingly, high levels of CDC6 have been recently reported in around 50% of non-small cell lung carcinomas [Karakaidos et al., 2004], brain cancer [Ohta et al., 2001] and a subset of mantle cell lymphomas [Pinyol et al., 2006], which suggests that CDC6 has oncogenic properties. DNA replication stress initiated by CDC6 may play a critical role in low-dose arsenic-induced carcinogenesis.

Cyclin D1 functions as a mitogenic sensor, and relays signals from the extracellular environment to the core cell cycle machinery [Tashiro et al., 2007]. Cyclin D1 forms a complex with, and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. The increase in gene transcription of cyclin D1 is accompanied by enhanced translation and reduced proteolysis, which is triggered through a distinct Ras-mediated pathway involving phosphatidylinositol-3-kinase (PI3K) and Akt, further potentiating the induction of cyclin D1 [Ouyang et al., 2006, 2007]. In the present study, the transcription and protein levels of cyclin D1 increased with increased exposure dose and time. The inhibition of ERK1/2 and Akt, but not p38 MAPK, was significantly correlated with a reduction of the ATO-induced increase in the protein expression of cyclin D1. These results are consistent with previous reports that p38 MAPK negatively regulates cyclin D1 expression [Page et al., 2001].

In summary, low-dose ATO promoted cell cycle progression from G1 to S/G2 phases and induced cell cycle arrest at G2/M in the non-tumorigenic MCF10A breast epithelial cell line. We detected significant increases in CDC6 and cyclin D1 transcription when the cells were exposed to 0.1 μM of ATO for 24 h. Expression of CDC6 and cyclin D1 at both the transcription and protein levels steadily increased with dose and time. We also observed activation of p38 MAPK, Akt, and ERK1/2 at discrete time points following exposure to ATO, and production of ROS. Inhibition of p38 MAPK, ERK1/2, and Akt by both small molecular inhibitors and siRNAs reversed ATO-increased cell proliferation, and decreased the expression levels of CDC6 and cyclin D1. Taken together, these findings suggest that activation of p38 MAPK, ERK1/2, and Akt is required for the protein stabilization of CDC6 and cyclin D1. Certainly, many other signaling pathways such as cyclins, CDCs, and cell-dependent kinases (CDKs), together with oncogenes and transcription factors, are involved in cell cycle progression from G1 to S and G2 phases after low-dose ATO. The expression of CDC6 and cyclin D1 is regulated by transcription factors, and many transcription factors are activated along with p38 MAPK, ERK1/2, and Akt. Future investigations will explore the critical transcription factors involved in the regulation of CDC6 and cyclin D1 expression.

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