Arsenic Trioxide-Induced Growth Arrest of Breast Cancer MCF-7 Cells Involving FOXO3a and I κ B Kinase β Expression and Localization

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Abstract

Currently, arsenic has been clinically investigated as a therapeutic agent for a variety of solid malignancies, including breast cancer. However, the exact underlying molecular mechanisms through which arsenic trioxide (As₂O₃) induces cell growth arrest and apoptosis in solid tumors have not been clearly understood. The aim of our study was to gain an insight into the effect of As₂O₃ on the human breast cancer MCF-7 cell line and investigate cell growth inhibition, apoptosis, and the molecular mechanism after As₂O₃ treatment in MCF-7 cells. Expression of FOXO3a, nuclear-FOXO3a, caspase-3, and I κ B kinase β (IKK β) mRNA levels in MCF-7 cells was determined by reverse transcription–polymerase chain reaction (RT-PCR). The protein expression was examined by the Western blot analysis and immunocytochemical staining. The distribution of apoptotic cells was assessed by flow cytometry, and the morphology of the apoptotic cells was investigated by Hoechst33258 staining. Our results showed that As₂O₃ significantly induced the apoptosis of MCF-7 cells tested in this study in a dose-dependent manner. As₂O₃ induced the decrease of IKK β expression and the increase of total as well as nuclear FOXO3a expression, which triggered the phosphorylation of cytoplasmic FOXO3a at the Thr32 residue decrease. RT-PCR, Western blot analysis, and immunocytochemistry revealed that the expression of IKK β in MCF-7 cells was upregulated when As₂O₃ was combined with tumor necrosis factor- α (TNF- α), whereas the expression of FOXO3a was downregulated in comparison with the As₂O₃-alone group. These findings indicated a specific molecular mechanism by which MCF-7 cell lines were susceptible to the As₂O₃ therapy through FOXO3a expression and localization. This FOXO3a accumulation may be well correlated with the As₂O₃-induced reduction of active IKK β , which may provide new insights into As₂O₃-related signaling activities.

Key words: arsenic trioxide (As₂O₃), breast cancer, cell apoptosis, FOXO3a localization, I κ B kinase β (IKK β)

Introduction

Breast cancer is a common malignancy among women, with a drastically increased incidence of breast cancer over the past several decades. Treatment of breast cancer includes surgery, radiation, and drugs (hormone therapy and chemotherapy), but there is an increased interest in the discovery of new agents for breast cancer treatment. Arsenic

trioxide (As₂O₃) is one of the arsenic compounds found in nature. Arsenic agents have long been used as anticancer drugs in the traditional Chinese medicine. It has recently been used to treat patients suffering from promyelocytic leukemia.¹ Furthermore, As₂O₃ is of potential value for the therapeutics of other promyelocytic malignancies and a number of solid tumors, including breast cancer.^{2–4} Previous studies have shown that induced differentiation, cell cycle arrest, and

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apoptosis are the principal modalities involved in the anti-tumor effects of As₂O₃. However, its molecular mechanisms of the antitumor effects remain to be further elucidated.

The Forkhead Box Class O (FOXO) transcription factors, namely the downstream targets for the PI3K/Akt pathway, are a large group of proteins sharing a common conserved 100-amino-acid DNA-binding domain, which is termed as a winged-helix or fork head domain after the founding member of the group, the fork head gene in *Drosophila*.^{5,6} FOXO proteins play a pivotal role in biological processes, such as apoptosis, cell cycle control, differentiation, stress response, DNA damage repair, and glucose metabolism.⁷ FOXO3a, a member of the family of fork head transcription factors, has been shown to mediate apoptosis by activating proapoptotic genes in a variety of cells, and was phosphorylated by the proto-oncogene *Akt* (protein kinase B) and/or serum- and glucocorticoid-induced kinase (SGK) in mammals.^{8,9} Phosphorylation of FOXO3a can result in their release from the DNA and the translocation from the nucleus to the cytoplasm and the inactivation of FOXO3a, which inhibit the expression of FOXO-regulated genes.^{8,10}

It has been also reported that the upregulation of FOXO3a proteins can induce apoptosis and cell cycle arrest through the regulation of multiple pro-apoptotic proteins, including Bim, Puma, Fas ligand, p21^{Cip1}, p27^{Kip1}, cyclin D1/2, and TRAIL.^{11–15} Meanwhile, FOXO proteins have been associated with DNA damage repair via the upregulation of GADD45a or the interaction with ATM to promote DNA repair via the downstream mediators of FOXO proteins.^{16,17} The upregulation of FOXO3a by paclitaxel has been reported to increase the Bim mRNA and the Bim protein level with the subsequent induction of apoptosis in breast cancer cells.¹⁸ FOXO3a also contributes to the nerve growth factor deprivation-induced apoptosis of developing sympathetic neurons by the activation of Bim.¹⁹ However, recent statistics provides information on the novel aspects of FKHL1, notably the fact that it is regulated not only by *Akt* but also by other molecules such as nitric oxide-ROCK (in breast cancer), FSH-IGF-I (in ovarian follicle cells), TRAIL and PTEN (in prostate cancer), and p66shc (in neuronal cells).^{15,20–23} Thus, FOXO3a has generally been considered as an inducer of apoptosis. It is not clear whether the FOXO3a-regulated apoptosis also applies to breast cancer MCF-7 cells or other tumor cells. Despite the recent studies that have suggested that As₂O₃ interferes with a variety of cellular processes to induce the apoptosis of tumor cells, the detailed mechanisms of its anticancer effects and the signal pathway in MCF-7 have not been clarified. In this study, we examined the effects of FOXO3a on the survival and apoptosis of MCF-7 cells *in vitro*. We found that during the process of apoptosis by As₂O₃, the expression of the FOXO3a level was upregulated, with the IκB kinase (IKK)/FOXO3a pathway involved. To investigate the role of the IKK/FOXO3a pathway in the treatment of MCF-7 cells with As₂O₃, the cells were treated with the addition of tumor necrosis factor-α (TNF-α) (IκB kinase β [IKKβ] agonist).²⁴

Materials and Methods

Cell culture and cell treatment

The human breast cancer cell line MCF-7 (CCTCC; Wuhan University) was cultured in an RPMI-1640 medium, sup-

plemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO₂ at 37°C. Cells were added in 24-well plates and 96-well plates (3 × 10⁵ per well). The medium was replaced 24 hours later with a fresh medium containing various concentrations of As₂O₃ (Sigma-Aldrich). MCF-7 cells suspending in a medium containing dimethyl sulfoxide or a free medium were used as the negative control.

Morphology of the apoptotic cells

To determine whether apoptosis was induced by As₂O₃ at various concentrations, Hoechst33258 staining was performed. MCF-7 cells were cultured in a six-well plate using the coverslip culture method. At the end of treatment with As₂O₃ (0, 2.0, 4.0, and 8.0 μM) for 24 hours, all the coverslips were taken out. Cells in the control group were treated with the culture medium alone. The cell samples were rinsed twice with phosphate-buffered saline (PBS) and fixed by methanol:glacial acetic acid (3:1) for 30 minutes. After the wash in PBS, cells were incubated in 1 μg/mL Hoechst33258 solution for 10 minutes in the dark at 37°C. The cells were analyzed by a fluorescence microscope (Olympus BX-51).

Flow cytometric analysis

The apoptosis of MCF-7 cells was determined using propidium iodide (PI) staining by a flow cytometric analysis. The MCF-7 human breast cancer cells (1 × 10⁶) were cultured with or without As₂O₃ at various concentrations (0, 2.0, 4.0, and 8.0 μM), respectively. Three days later, the cells were harvested and rinsed in cold PBS (pH 7.4). The cell pellets were fixed in 70% cold alcohol for over 24 hours at 4°C, and then rinsed in cold PBS. Thereafter, Annexin V-fluorescein isothiocyanate/PI was added to the MCF-7 cells, according to standard procedures. The MCF-7 apoptotic cells were analyzed by flow cytometry.

Reverse transcription–polymerase chain reaction

MCF-7 cells were seeded at 5 × 10⁵ cells/well in six-well plates. Cells were treated with As₂O₃ (0, 2.0, 4.0, and 8.0 μM) for 24 hours, 4 μM As₂O₃ for 0, 6, 12, 24, and 48 hours respectively or cotreated with TNF-α for 2 hours. Controls were prepared by incubation with the experimental medium alone. After the treatment, total and nuclear mRNA was isolated using the TRIzol reagent (Invitrogen), according to the instructions by the manufacturer.²⁵ cDNA was synthesized from total RNA by using oligo(dT) primers. The newly synthesized cDNA was the template for polymerase chain reaction (PCR). The products of reverse transcription (RT) were stored at –20°C in the following PCR reaction mixture: 2 μL reverse transcription product (template), 1 μL for each pair of primers (choosing β-actin as an internal reference), the upstream primer sequence: 5'-TGA CGT GGA CAT CCG CAA AG-3', and the downstream primer sequence: 5'-CTG GAA GGT GGA CAG CGA GG-3', which yielded a 205-bp amplified fragment. The FoxO3a upstream primer sequence was 5'-AGG GAA GTT TGG TCA ATC AGAA-3'; the downstream primer sequence was 5'-TGG AGA TGA GGG AAT CAA AGTT-3', and the amplified fragment was 369 bp. The IKKβ upstream primer sequence was 5'-GTT TGA GAA CTG CTG TGG TCTG-3'; the downstream primer sequence

was 5'-AAC TTC ACC GTT CCA TTC AAGT-3'; and the amplified fragment was 435 bp. The Caspase-3 upstream primer sequence was 5'-CAG ACA GTG GTG TTG ATG AGTA-3'; the downstream primer sequence was 5'-TAG CGT CAA AGG AAA AGG ACTC-3'; and the amplified fragment was 243 bp. The reaction system used was as follows: 2.5 μ L of 10 $\times$ buffer, 2 μ L of 25 mM MgCl₂, 2 μ L of 10 mM dNTP mixture, and 0.5 μ L Taq enzyme; the total volume was 25 μ L with deionized water. Reaction conditions used were the same as described above.²⁵ Samples (10 μ L) were mixed with 2 μ L 6 $\times$ loading dye, followed by electrophoresis in a 1% agarose gel. The band intensities of PCR products were analyzed by Image Quant TL™ (Amersham Biosciences).

Western blot analysis

MCF-7 cells treated with As₂O₃ at different time points and various concentrations were collected. In addition, the nuclear and cytosolic protein fractions were isolated from MCF-7 cells. Cells were promptly homogenized in a homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% sodium dodecyl sulfate (SDS), 0.5% sodium pyrophosphate, 20 mM β -glycerophosphate, 0.5 M ethylenediaminetetraacetic acid, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 1 mM PMSF, followed by centrifugation at 13,000 *g* for 10 min to collect the resulting supernatant. Protein concentrations were determined with a Bio-Rad protein assay (BioRad). The supernatant was diluted in 2 $\times$ SDS-loading buffer and boiled. Proteins were separated with SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filter membranes (Millipore). The membranes were blocked with 5% skim milk powder in TBST (20 mM Tris, 150 mM NaCl, and 0.05% Tween-20, pH=8.0). Two hours later at room temperature, the filters were thrice rinsed by TBST, followed by the addition of monoclonal anti-rabbit antibodies for incubation overnight as described below: the primary antibodies were anti-FOXO3a (1:1000; Cell Signaling Technology, Inc.) and anti-(Thr32) p-Foxo3a (1:1000; Cell Signaling Technology, Inc.); anti-IKK β (1:1000; Santa Cruz Biotechnology); anti- β -actin (1:4000; Sigma-Aldrich); anti-caspase-3 (1:1000; Cell Signaling Technology, Inc.); and horseradish peroxidase-linked immunoglobulin G as the secondary antibodies. Finally, a horseradish peroxidase-conjugated secondary antibody was added for an additional 2 hours, and the blots were developed using an NBT/BCIP color solution. Values from at least three independent reactions were recorded. Gray values of target protein were analyzed by ImageJ image analysis software.

Immunocytochemistry

In six-well plates (1 $\times$ 10⁷–10⁹ per well), MCF-7 cells in two wells (first group as control) received nothing, and the cells in the other four wells (second and third groups) were treated with 4.0 μ M of As₂O₃. The cells in each group were cultured for 24 hours, followed by the addition of TNF- α (10 ng/mL) in the third group for cell stimulation for 2 hours. The cells in each group were rinsed twice in PBS for 40 minutes of fixation in 4% paraformaldehyde. The PBS buffer replacing the primary antibody was applied as a positive control. According to the PV kit instructions, the cells were incubated with anti-

FOXO3a (1:50) or anti-IKK β (1:150) antibody for 24 hours, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour followed by colorimetric detection using diaminobenzidine. For evaluation of FOXO3a- and IKK β -positive fractions, at least 200 cells were counted from six different regions to determine the average.

Statistical analysis

Experimental values were expressed as mean $\pm$ standard deviation. Statistically significant differences among the groups were compared by using a one-way analysis of variance and the SNK method between the two groups. A *p*-value of <0.05 was considered statistically significant.

Results

Apoptotic cell detection by flow cytometry

As shown in Figure 1, large numbers of apoptotic MCF-7 cells were visible among the cells treated with As₂O₃ (2.0, 4.0, and 8.0 μ M), compared to those untreated cells (*p*<0.05). In addition, As₂O₃ induced the apoptosis of MCF-7 cells in a dose-dependent manner. The data indicated that As₂O₃ could remarkably induce the apoptosis of the human breast cancer MCF-7 cell line.

Morphological characterization of apoptotic cells

Hoechst33258 staining revealed the presence of a large number of apoptotic cells after As₂O₃ treatment. As₂O₃ significantly induced the apoptosis of MCF-7 cells. Apparent typical morphological changes of apoptotic cells were observed, with characteristic karyopyknosis, chromatin condensation, and apoptotic bodies, whereas the cells in the control group showed nuclear integrity, with no apoptotic morphological changes (Fig. 2).

Expression of FOXO3a, nuclear FOXO3a, caspase-3, and IKK β mRNA levels after As₂O₃ treatment

To explore the mechanism of the anticancer activity of As₂O₃, the variations in the expression levels of FOXO3a, nuclear FOXO3a, and IKK β mRNA in MCF-7 cells during As₂O₃ exposure were assessed by RT-PCR. Total RNA samples from the untreated control and groups with As₂O₃ treatment were isolated and analyzed by RT-PCR. As shown in Figure 3, As₂O₃ treatment significantly induced the up-regulated expression of total FOXO3a mRNA after As₂O₃ treatment at 2.0, 4.0, and 8.0 μ M for 24 hours. More importantly, As₂O₃ treatment resulted in a significant increase in the expression level of active caspase-3 (FOXO3a target gene) for 24 hours (Fig. 3A), compared with the PBS-treated control groups (*p*<0.05; Fig. 3B). The levels of nuclear FOXO3a mRNA began to increase 24 hours later and remained at an elevated level until 72 hours in a time-dependent manner (Fig. 3C), compared with the untreated control groups (*p*<0.05; Fig. 3D). The expression of the IKK β mRNA level was decreased after As₂O₃ treatment; when we plugged TNF- α to stimulate cells for 2 hours, the expression of the IKK β mRNA level was increased, whereas the expression of the total FOXO3a mRNA level was suppressed (Fig. 3E), and the FOXO3a expression level in MCF-7 cancer cells treated with TNF- α was lower than that in the untreated cells

FIG. 1. As₂O₃-induced apoptosis of breast cancer MCF-7 cells. The human breast cancer MCF-7 cells were treated with As₂O₃ (2.0, 4.0, and 8.0 μ M), or PBS for 24 hours followed by the flow cytometric analysis. **(A, B)** Apoptosis analysis was conducted using Annexin V-FITC and PI double staining. The Annexin V-positive cells in the total cell population represented apoptotic cells. The percentage of apoptotic cells was significantly higher in 2.0, 4.0, and 8.0 μ M groups than that in the PBS group (* p < 0.05, compared with the PBS groups, respectively). Results are representative of three independent experiments. As₂O₃, arsenic trioxide; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide.

(p < 0.05; Fig. 3F), indicating that IKK β is capable of efficiently inhibiting the expression of FOXO3a in human breast cancer MCF-7 cells.

Expression of FOXO3a, nuclear FOXO3a, P-FOXO3a, caspase-3, and IKK β protein levels after As₂O₃ treatment

In addition to the RNA levels, we examined the protein levels of FOXO3a, nuclear FOXO3a, P-FOXO3a, caspase-3, and IKK β in MCF-7 cells by the Western blot analysis. The phosphorylation level of FOXO3a at the Thr²⁶ residue (a

known Akt phosphorylation site) was decreased after As₂O₃ treatment at 2.0, 4.0, and 8.0 μ M for 24 hours (Fig. 4A). With the rising concentrations of As₂O₃, the level of total FOXO3a began to increase gradually. As₂O₃ treatment resulted in a significant elevation of the level of active caspase-3, compared with the PBS-treated control groups (p < 0.05) (Fig. 4A). β -Actin was determined as a control for equal loading of the gels, with scanty variations. To further investigate the mechanism of the anticancer activity of As₂O₃, we examined nuclear and cytoplasmic protein extracts isolated from MCF-7 cells. As shown in Figure 4B, As₂O₃ treatment at 4 μ M at different time points increased the expression of nuclear

FIG. 2. Microscopic examination of Hoechst-stained nuclei of breast cancer MCF-7 cells in the untreated group **(A)** and groups treated with As₂O₃ 2.0 μ M **(B)**, As₂O₃ 4.0 μ M **(C)**, and As₂O₃ 8.0 μ M **(D)**. After treatment with As₂O₃ for 24 hours, MCF-7 cells of visible apoptosis were detected by Hoechst33258 staining (arrows: the apoptotic cells).

FIG. 3. mRNA expression of FOXO3a, caspase-3, nuclear FOXO3a, and IKK β in MCF-7 cells after As₂O₃ treatment. Total RNA was isolated, transferred to cDNA, and amplified with FOXO3a, caspase-3, nuclear FOXO3a, and IKK β primers. **(A)** RT-PCR analysis of FOXO3a (369 bp) and caspase-3 (243 bp) mRNA levels in MCF-7 cancer cells treated with 2.0, 4.0, and 8.0 μ M As₂O₃ for 24 hours, β -actin (205 bp) was used as an internal control for normalization. **(B)** Densitometric analysis of the amplified FOXO3a and caspase-3 PCR products after normalization with β -actin (* p < 0.05, compared with the PBS group cells). **(C)** RT-PCR analysis of nuclear FOXO3a (369 bp) mRNA level in MCF-7 cancer cells treated with 4.0 μ M As₂O₃ at different time points. **(D)** Densitometric analysis of the amplified nuclear FOXO3a PCR products after normalization with β -actin (* p < 0.05, compared with control group cells). **(E)** RT-PCR analysis of FOXO3a (369 bp) and IKK β (435 bp) mRNA levels in MCF-7 cancer cells treated with 4.0 μ M As₂O₃ for 24 hours and with TNF- α for 2 hours. **(F)** Densitometric analysis of the amplified FOXO3a and IKK β PCR product after normalization with β -actin (* p < 0.05, compared with control group cells; ** p < 0.01, compared with 4.0 μ M As₂O₃ group). Data were representative of three independent experiments. IKK β , I κ B kinase β ; RT-PCR, reverse transcription–polymerase chain reaction; TNF- α , tumor necrosis factor- α .

FOXO3a, which remained at an elevated level until 48 hours, whereas the expression of cytoplasmic-p-FOXO3a was decreased and remained at a lower level until 48 hours. The equivalence of nuclear and cytoplasmic components was verified by the expression of β -actin, with statistically significant differences in each group (p < 0.05) (Fig. 4B). These results were consistent with the observations of those mRNA levels following As₂O₃ treatment. It is noteworthy that the expression level of IKK β was decreased following As₂O₃ treatment at a mol of 4.0 μ M, for 24 hours. When we plugged TNF- α to stimulate cells for 2 hours, the expression of IKK β was upregulated, whereas FOXO3a expression was significantly lower than that in the nonstimulated group, which might account

for the association between the expression variations of FOXO3a and the activity of IKK β (Fig. 4C), with statistically significant differences in each group (p < 0.01). These results indicated that As₂O₃ induced the nuclear accumulation of FOXO3a via the reduction of the phosphorylation level of FOXO3a. IKK β , that is, a FOXO3a upstream target gene, might regulate the intracellular localization of FOXO3a.

Expression of FOXO3a and IKK β protein levels after As₂O₃ and TNF- α treatment

To further evaluate the expression of FOXO3a and IKK β and their interactions in MCF-7 cells after As₂O₃ treatment,

FIG. 4. Protein expression of FOXO3a, nuclear FOXO3a, P-FOXO3a, caspase-3, and IKKβ in MCF-7 cells after As₂O₃ treatment was analyzed by Western blot. **(A)** The expression of phosphorylated and total FOXO3a (91 and 79 kDa) and active caspase-3 (17 kDa) protein levels was analyzed in MCF-7 cells treated with 2.0, 4.0, and 8.0 μM As₂O₃ for 24 hours, respectively; also shown is a blot for β-actin as a loading control, with statistically significant differences in each group (**p* < 0.05; ***p* < 0.01, compared with the PBS group). **(B)** The expression of nuclear and cytoplasmic p-FOXO3a protein levels was evaluated in MCF-7 cells treated with 4.0 μM As₂O₃ at different time points, with statistically significant differences in each group (**p* < 0.05; ***p* < 0.01, compared with the untreated group). **(C)** The expression of FOXO3a and IKKβ (89 kDa) protein levels was examined in MCF-7 cancer cells treated with 4.0 μM As₂O₃ for 24 hours, followed by combination with TNF-α for 2 hours, with statistically significant differences in each group (**p* < 0.05; ***p* < 0.01, compared with the 4.0 μM As₂O₃ group). Data were representative of three independent experiments.

immunocytochemical staining was performed. Previous studies have shown that FOXO3a exists in many normal cells and tumor cells, and regulates the transcription of genes involved in metabolism, apoptosis, immunity, DNA damage repair, and oxidative stress.¹⁰ When stimulated by external factors, such as PKB/Akt and SGK, FOXO3a is activated, and nuclear translocation occurs. To understand the molecular mechanism of the IKKβ and FOXO3a activity, we investigated the expression variations of FOXO3a and IKKβ proteins after 24 hours of As₂O₃ treatment (4 μM) and the subsequent combination of As₂O₃ and TNF-α for 2 hours. As expected, the expression level of cytoplasmic IKKβ was lower than that in the PBS group, with FOXO3a remarkably upregulated and largely located in the cell nuclei after As₂O₃ treatment (***p* < 0.01, compared with the PBS group) (Fig. 5B, E). When MCF-7 cells were exposed to TNF-α stimulation for 2 hours, the cytoplasmic expression of IKKβ was increased and was higher than in the As₂O₃ group, whereas the expression level of nuclear FOXO3a was downregulated and was lower than in the As₂O₃ group (**p* < 0.05, compared with the As₂O₃ group), which indicated the exclusion of FOXO3a from the nuclei of MCF-7 cells (Fig. 5C, F). Thus, IKKβ can suppress FOXO3a transcription activity in the process of As₂O₃-treated MCF-7 cells. Moreover, IKKβ can regulate intracellular localization of the FOXO3a, which is consistent with the results of Western blotting and RT-PCR analyses.

Discussion

Despite the long history of the application of As₂O₃ as an anticancer drug in cancer therapeutics, the mechanisms underlying its antitumor effects still remain obscure. To date, there is little literature available as to the mechanisms of the development of resistance to As₂O₃ in chemotherapy. Thus, As₂O₃ represents a novel agent worthy of further investigation. Previous studies have confirmed that As₂O₃ could alter the cellular redox status, open mitochondrial permeability transition pore, activate caspase family, inhibit the *bcl-2* gene, increase the gene expressions of *p53*, *bax*, and *FasL*, and thus could induce the apoptosis of tumor cells.^{27,28} With the increased incidence of breast cancer, in addition to the current chemotherapeutic drugs and endocrine therapy, other new therapeutics are still required. The therapeutic efficacy of As₂O₃ in breast cancer has caused a widespread concern. However, further explorations are still required as to its possible underlying mechanisms. In view of the significance of FOXO3a in cell metabolism, differentiation, proliferation, cell death, and oxidative stress,^{8,10} FOXO3a has been considered to be involved in the development of breast tumors.²⁹ Thus, it is of great necessity to explore the role of FOXO3a in antitumor therapy with As₂O₃. In this study, we evaluated the expression variations of FOXO3a, intracellular localization of FOXO3a, and other important cell cycle molecules in the consequences of As₂O₃-induced MCF-7 cell

FIG. 5. Expression levels of FOXO3a and IKK β proteins in MCF-7 cells after As₂O₃ and TNF- α treatment were analyzed by immunocytochemical staining (A, D). Immunocytochemical analyses of IKK β and FOXO3a expression and cytoplasmic and nuclear staining in normal MCF-7 cells (B, E). Cytoplasmic staining of IKK β and nuclear staining of FOXO3a in MCF-7 cancer cells were performed following As₂O₃ treatment (4 μ M) for 24 hours ($p < 0.01$, compared with the PBS group) (C, F). The expression variations of nuclear FOXO3a and cytoplasmic IKK β in MCF-7 cells were evaluated after 24 hours of As₂O₃ treatment (4 μ M) and subsequent 2 hours of combination with TNF- α ($p < 0.05$, compared with the As₂O₃ [4 μ M] group). Data were representative of three independent experiments.

apoptosis. This is the first study that demonstrates an important role of FOXO3a in the apoptosis of As₂O₃-induced MCF-7 cell line. We have shown that the accumulation of nuclear FOXO3a in As₂O₃-treated MCF-7 cells at various concentrations and at different time points from the mRNA level and the protein level. Hoechst33258 fluorescent staining showed the morphological changes of apoptotic MCF-7 cells from the complete nucleus to karyopyknosis, chromatin condensation, and apoptotic bodies. Apoptotic MCF-7 cells were detected by flow cytometry, showing that the percentage of apoptotic cells was significantly higher in other three groups than in the PBS group ($p < 0.05$), which fully indicated that As₂O₃ could significantly inhibit the proliferation of breast cancer MCF-7 cells and induce cellular apoptosis.

IKK β is one of the catalytic subunit of the IKK complex as a serine kinase. In the case of the stimulation from external signals (TNF- α , cytokines, infections, etc.) and through the complex signal transduction, the IKK complex is activated. IKK β is phosphorylated, and I κ B is degraded, so that NF- κ B is activated and translocated into the nucleus. Thus, NF- κ B regulates the expression of many genes in the inflammatory responses, immune responses, cell proliferation, apoptosis, and the transcriptional activation of angiogenesis genes of a variety of biological processes.^{26,30} Hu et al.³¹ surprisingly discovered that FOXO3a was excluded from the nuclei of some tumors lacking Akt-p, suggesting an IKK β -independent mechanism of regulating FOXO3a localization. They provide evidence for such a mechanism by showing that IKK physically interacts with FOXO3a, phosphorylates, and inhibits FOXO3a independent of Akt. Moreover, cytoplasmic FOXO3a correlates with the expression of IKK β or Akt-p in many tumors and associates with poor survival in breast cancer. In this study, we have shown that As₂O₃ treatment also resulted in the accumulation of FOXO3a in the nuclei and the

decrease of the IKK β and FOXO3a levels in the cytoplasm after As₂O₃ treatment. Two hours after cells were exposed to TNF-stimulation, the expression of IKK β was upregulated, whereas the nuclear expression of FOXO3a was down-regulated. This indicated that IKK β could regulate the expression and localization of FOXO3a in As₂O₃-inhibited MCF-7 cells, which was confirmed by the results of RT-PCR, Western blot analysis, and immunocytochemical staining, suggesting that the nuclear accumulation of FOXO3a was well correlated with the As₂O₃-induced reduction of IKK β activity, and the inhibition of IKK β by As₂O₃ caused the reduction of the levels of FOXO3a phosphorylation, leading to nuclear accumulation of FOXO3a.

A model depicting the FOXO-shuttling system was initially described to explain the mechanism of transcriptional regulation by this family member of transcription factors.³² This shuttling system changes the intracellular localization of FOXO through phosphorylation of three Akt sites in the fork head domain, primarily at Ser253.^{8,33} The phosphorylation of FOXO3a at Thr32 was reported to be important in determining the intracellular localization of FOXO3a.^{27,34} After phosphorylation of FOXO3a, the complex that includes 14-3-3 proteins, a small GTPase Ran, and chromosomal region maintenance protein 1 is transported through the nuclear pore into the cytosol. This shuttling system provides the cells with the double-negative regulation of FOXO factors.³⁵ This is consistent with our results of treatment of MCF-7 cells with As₂O₃ resulted in the FOXO3a accumulation in the nuclei. Western blot analysis showed the phosphorylation levels of FOXO3a at the Thr²⁶ residue and cytoplasmic p-FOXO3a were decreased at the end of As₂O₃ treatment at various concentrations and at different time points. Moreover, this nuclear accumulation was well correlated with the As₂O₃-induced reduction of IKK β activity, with statistically significant differences in each group ($p < 0.05$).

It is apparent that As₂O₃ interferes with a variety of cellular processes by targeting numerous different intracellular molecules. The present study may suggest a specific molecular mechanism by which the breast cancer MCF-7 cell line is susceptible to the As₂O₃ therapy through FOXO3a expression and localization. In summary, our data have demonstrated that As₂O₃ treatment can induce the upregulated expression of FOXO proteins in MCF-7 cells. Despite the numerous reports focused on the nuclear exclusion of FOXO3a in human primary tumors lacking Akt activity, the independent mechanism of IKK β contributing to the nuclear exclusion of FOXO3a has not been elucidated. To our delight, our study suggested that patients with high levels of functional FOXO3a responded more favorably to As₂O₃ regimens, and that I κ B/IKK β activation might be a therapeutically desirable end point in human mammary cancer cells. It is the first demonstration that the I κ B/IKK β /FOXO3a signaling pathway regulates the FOXO3a function and the sensitivity of MCF-7 cells to As₂O₃. FOXO3a might be expected to become a novel prognostic biomarker for cancer survival. In addition, FOXO3a might also serve as a new target gene for the therapeutic or preventive intervention in breast cancer as well as other cancer typologies.

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Disclosure Statement

No potential conflicts of interest were disclosed.

References

1. Evens AM, Tallman MS, Gartenhaus RB. The potential of arsenic trioxide in the treatment of malignant disease: Past, present, and future. *Leuk Res* 2004;28:891.
2. Ye J, Li A, Liu Q, et al. Inhibition of mitogen-activated protein kinase enhances apoptosis induced by arsenic trioxide in human breast cancer MCF-7 cells. *Clin Exp Pharmacol Physiol* 2005;32:1042.
3. Chow SK, Chan JY, Fung KP. Inhibition of cell proliferation and the action mechanisms of arsenic trioxide (As₂O₃) on human breast cancer cells. *J Cell Biochem* 2004;93:173.
4. Plataniias LC. Biological responses to arsenic compounds. *J Biol Chem* 2009;284:18583.
5. Weigel D, Jürgens G, Küttner F, et al. The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell* 1989;57:645.
6. You H, Pellegrini M, Tsuchihara K, et al. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* 2006;203:1657.
7. Huang H, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007;120:2479.
8. Tang ED, Nunez G, Barr FG, et al. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem* 1999;274:16741.
9. Burgering BM, Medema RH. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J Leukoc Biol* 2003;73:689.
10. Accili D, Arden KC. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell* 2004;117:421.
11. Rosas M, Birkenkamp KU, Lammers JW, et al. Cytokine mediated suppression of TF-1 apoptosis requires PI3K activation and inhibition of Bim expression. *FEBS Lett* 2005;579:191.
12. Chen J, Yusuf I, Andersen HM, et al. FOXO transcription factors cooperate with delta EF1 to activate growth suppressive genes in B lymphocytes. *J Immunol* 2006;176:2711.
13. Segrelles C, Moral M, Lara MF, et al. Molecular determinants of Akt-induced keratinocyte transformation. *Oncogene* 2006;25:1174.
14. Yang JY, Xia W, Hu MC. Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. *Int J Oncol* 2006;29:643.
15. Chen YJ, Hsiao PW, Lee MT, et al. Interplay of PI3K and cAMP/PKA signaling, and rapamycin-hypersensitivity in TGFbeta1 enhancement of FSH-stimulated steroidogenesis in rat ovarian granulosa cells. *J Endocrinol* 2007;192:405.
16. Tsai WB, Chung YM, Takahashi Y, et al. Functional interaction between FOXO3a and ATM regulates DNA damage response. *Nat Cell Biol* 2008;10:460.
17. Sakoe Y, Sakoe K, Kirito K, et al. FOXO3A as a key molecule for all-trans retinoic acid-induced granulocytic differentiation and apoptosis in acute promyelocytic leukemia. *Blood* 2010;115:3787.
18. Sunter A, Fernández de Mattos S, et al. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 2003;278:49795.
19. Hughes R, Kristiansen M, Lassot I, et al. NF-Y is essential for expression of the proapoptotic bim gene in sympathetic neurons. *Cell Death Differ* 2010;18:937.
20. Radisavljevic Z. Nitric oxide suppression triggers apoptosis through the FKHRL1 (FOXO3A)/ROCK kinase pathway in human breast carcinoma cells. *Cancer* 2003;97:1358.
21. Smith WW, Norton DD, Gorospe M, et al. Phosphorylation of p66Shc and forkhead proteins mediates Abeta toxicity. *J Cell Biol* 2005;169:331.
22. Cornforth AN, Davis JS, Khanifar E, et al. FOXO3a mediates the androgen-dependent regulation of FLIP and contributes to TRAIL-induced apoptosis of LNCaP cells. *Oncogene* 2008;27:4422.
23. Dubrovskaya A, Kim S, Salamone RJ, et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc Natl Acad Sci U S A* 2009;106:268.
24. Ghosh S, Karin M. Missing pieces in the NF- κ B puzzle. *Cell* 2002;109:S81.
25. Zagzag D, Salnikow K, Chiribogal L, et al. Downregulation of major histocompatibility complex antigens in invading glioma cells: Stealth invasion of the brain. *Lab Invest* 2005;85:328.
26. Jackson-Bernitsas DG, Ichikawa H, Takada Y, et al. Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-kappaB activation and proliferation in human head and neck squamous cell carcinoma. *Oncogene* 2007;26:1385.
27. Mann KK, Colombo M, Miller WH, Jr. Arsenic trioxide decreases AKT protein in a caspase-dependent manner. *Mol Cancer Ther* 2008;7:1680.

28. Ho SY, Chen WC, Chiu HW, et al. Combination treatment with arsenic trioxide and irradiation enhances apoptotic effects in U937 cells through increased mitotic arrest and ROS generation. *Chem Biol Interact* 2009;179:304.
29. Jin G-S, Kondo E, Miyake T, et al. Expression and intracellular localization of FKHRL1 in mammary gland neoplasms. *Acta Med Okayama* 2004;58:197.
30. Bu DX, Erl W, de Martin R, et al. IKKbeta-dependent NF-kappaB pathway controls vascular inflammation and intimal hyperplasia. *FASEB J* 2005;19:1293.
31. Hu MC, Lee DF, Xia W, et al. I κ B kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* 2004;117:225.
32. Van Der Heide LP, Hoekman MF, Smidt MP. The ins and outs of FoxO shuttling: Mechanisms of FoxO translocation and transcriptional regulation. *Biochem J* 2004;380:297.
33. Rena G, Woods YL, Prescott AR, et al. Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. *EMBO J* 2002;21:2263.
34. Chong ZZ, Maiese K. Erythropoietin involves the phosphatidylinositol 3-kinase pathway, 14-3-3 protein and FOXO3a nuclear trafficking to preserve endothelial cell integrity. *Br J Pharmacol* 2007;150:839.
35. Wang Y, Oh SW, Deplancke B, et al. *C. elegans* 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. *Mech Ageing Dev* 2006;127:741.