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JWA enhances As$_2$O$_3$-induced tubulin polymerization and apoptosis via p38 in HeLa and MCF-7 cells

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Abstract Arsenic trioxide (As$_2$O$_3$) has potential anti-cancer activity against a wide range of carcinomas via apoptosis induction or oncoprotein degradation. The mechanisms involved are not fully elucidated. Here, we demonstrated that As$_2$O$_3$ induced-apoptosis in HeLa and MCF-7 cancer cells was in part triggered by tubulin polymerization. High expression of JWA promoted tubulin polymerization and increased the sensitivity of the cancer cells to As$_2$O$_3$. The activation of the p38 MAPK (mitogen-activated protein kinases) signaling pathway was found to contribute to JWA-promoted tubulin polymerization. Our results suggest that JWA may serve as an effective enhancer of microtubule-targeted As$_2$O$_3$ anti-cancer therapy.

Keywords Apoptosis · Arsenic trioxide · JWA · Tubulin · p38

Introduction

Arsenic is a naturally occurring substance that has been used as a medicinal agent for more than 2,400 years [1]. Recent studies have shown that As$_2$O$_3$ has potential to be

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effective in solid tumor cells [2, 3]. Thus, there was an increasing interest in discovering the full range of arsenic's potential as a chemotherapeutic agent and several clinical trials have been initiated to test the efficacy of arsenic in treating solid tumours. We chose two cell lines HeLa (relatively sensitive to As$_2$O$_3$) and MCF-7 (relatively resistant to As$_2$O$_3$) to investigate the molecular mechanisms involved in As$_2$O$_3$-induced apoptosis. Published data has shown that reactive oxygen species (ROS) may be involved in the molecular mechanisms by which As$_2$O$_3$ induces apoptosis, but the process is far from fully elucidated.

Microtubules as key components of cellular cytoskeleton are composed of long filamentous and tube-shaped protein polymers that are essential in all eukaryotic cells. They are crucial in the development and maintenance of cell shape, transportation of vesicles, mitochondria and other components throughout cells, in cell signaling, division and mitosis [4]. Recent studies have shown that microtubules may act as a potential target of arsenic [2, 3, 5]. The precise role of microtubules in mediating cell apoptosis is an area of intense investigation, and the degree to which the rapid dynamics of microtubules contribute to As$_2$O$_3$-induced cell death is an interesting open question.

The JWA gene, also known as ARL6ip5, was initially cloned from human tracheal bronchial epithelial cells [6]. Subsequent studies indicated that JWA is a structurally novel microtubule-binding protein, which regulates cancer cellular migration via mitogen-activated protein kinases (MAPK) cascades [7] and mediates differentiation in multiple leukemic cells [8, 9]. More recent data has shown that JWA is required for As$_2$O$_3$-induced apoptosis in HeLa and MCF-7 cells via ROS and mitochondria linked signal pathway [10]. The further question was raised regarding whether JWA contributes to dynamic reorganization of

microtubules and serves as an independent event in mediating As_2O_3-induced apoptosis.

MAPK are a family of widely expressed serine-threonine kinases regulating important cellular processes. In mammals, three major MAPK family subgroups were identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal of stress-activated protein kinases (JNK) and the p38 group of protein kinases [11]. Both JNK and p38 are key mediators of stress signals and seem to be responsible mainly for protective responses, stress-dependent apoptosis and inflammatory responses. Conversely, the ERK pathway plays a major role in regulating cell proliferation, differentiation and provides a protective effect against apoptosis [11]. The p38 MAPK was recently identified to mediate microtubule polymerization [12] or depolymerization [13] under certain situations.

In the present study, we aimed at examining the role of JWA in As_2O_3-induced apoptosis and microtubule reorganization in HeLa and MCF-7 cells.

Materials and methods

Cell culture

Both HeLa and MCF-7 cells were purchased from the Institute of Biochemistry and Cell Research, China Life Science Academy (Shanghai, China). Both cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies/Gibco, Gaithersburg, MD, USA). For experiments, cells were grown at 37°C in a humidified incubator with 5% CO_2 (HERA Cell, Heraeus, Germany).

Reagents

A stock solution of As_2O_3 was prepared by dissolving As_2O_3 powder (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS, pH 7.4) to a concentration of 10 mM. The reagents used were paclitaxel (Sigma St Louis, MO, USA), colchicine (Biosh Biological Technology, Shanghai, China), U0126 (Cell Signaling Technology, Beverly, MA, USA), SB203580 (Alexis, Nottingham, United Kingdom), SP600125 (Calbiochem-Novabiochem, San Diego, California, USA) and tubulin polymerization inhibitor (Merck chemicals, Darmstadt, Germany).

Plasmids and transfection

FLAG vector and FLAG-JWA were kindly provided by Professor Gang Li (University of British Columbia, Canada). RNA interference of siRNA was performed using double-stranded RNA molecules. The sequence for the JWA-siRNA was 5′-AAGACCAUGACUCCUCCAAA-CAUGG-3′ and a none-related control siRNA (Invitrogen #12935300) was used as a control. Signalsilence p38 MAPK siRNA and its corresponding control siRNA were purchased from Cell Signaling Technology (#6564 and #6568 respectively). The plasmid pcDNA3-MKK6b(E): dominant active MKK6b, which is upstream enzyme of p38, was kindly provided by Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA, USA). The plasmid DNA or siRNA was transfected into cells with Lipofectamine 2000 according to manufacturer's instruction.

Indirect immunofluorescence microscopy

Cells were grown on 35 mm glass bottom dish (Shenyou Technology, Zhejiang, China), treated as indicated. For harvesting cells, the cell culture dishes were rinsed in PBS and fixed with 4% formaldehyde in PBS for 20 min at 4°C. Cells were further permeabilized with PBS containing 0.01% Triton X-100 and 0.05% SDS, pH 7.4 for 5 min at 4°C. Permeabilized cells were rinsed in PBS supplemented with 0.5% Tween-20 (PBST), and incubated with the blocking solution (0.1% saponin and 0.2% BSA in PBS, pH 7.4) for 1 h. After then, the cells were incubated with either anti-FLAG mouse monoclonal antibody (Sigma) or anti-JWA rabbit polyclonal antibody (Research Genetics Inc., S.W. Huntsville, AL) or anti-p38 rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) or anti-HA rabbit polyclonal antibody (Santa Cruz, CA) at a 1:200 dilution in PBST overnight at 4°C. After rinsing with PBST, the dishes were further incubated with either Texas red-conjugated anti-mouse IgG secondary antibody or Texas red-conjugated anti-rabbit IgG secondary antibody (KPL, Inc. USA) at a 1:200 dilution in PBST for 1 h at 37°C, and monoclonal anti-α-tubulin-FITC antibody (1:100 dilution in PBST; Sigma) for 45 min. Cell nuclei were counterstained with 5 μg/ml 40, 60-diamidino-2-phenylindole (DAPI) (Beyotime, Jiangsu, China). The intracellular distributions of the target proteins were analyzed using confocal fluorescent microscopy. The confocal images of cells were sequentially acquired with Zeiss AIM software on a Zeiss LSM 510 confocal microscope system with excitation at 488 nm (for FITC), 543 nm (for Texas red) and 340 nm (for DAPI).

Isolation of polymerized and depolymerized (soluble) tubulin

A method was modified to quantitate tubulin polymerization, which was originally described by Minotti et al. [14]. Treated cells were washed twice with PBS, scraped and centrifuged at $1,000 \times g$ for 5 min to obtain cell pellets. 100 μl of hypotonic buffer (1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40, 1.3% cocktail, 1 mM orthovanadate and 20 mM Tris–HCl,
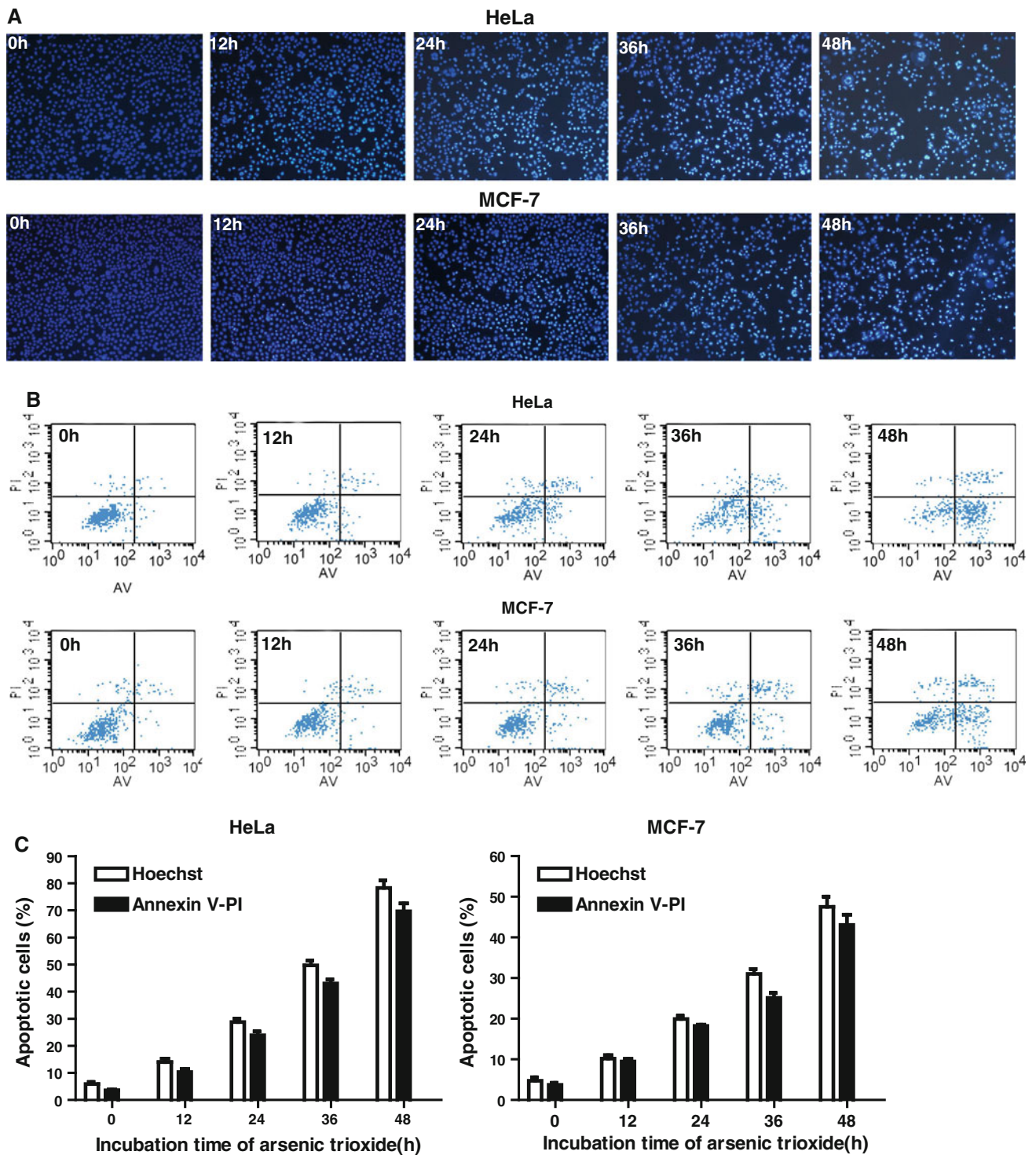
Fig. 1 As$_2$O$_3$ induces apoptosis in HeLa and MCF-7 cells. HeLa and MCF-7 cells were incubated with 5 or 10 μM As$_2$O$_3$, respectively for indicated time. The cellular apoptosis was examined by Hoechst 33258 staining assay (**A**) and Annexin V-PI assays (**B**). The quantitative data showed that As$_2$O$_3$ induced apoptosis in HeLa and MCF-7 cells in a time-dependent manner, the quantitative data were as follows (%): HeLa Hoechst 33258 staining: 5.9 ± 1.3, 14.0 ± 2.2, 28.8 ± 2.2, 50.0 ± 3.1, 78.2 ± 4.9, Annexin V-PI assays: 3.6 ± 0.6, 10.3 ± 2.0, 23.9 ± 2.5, 43.0 ± 2.6, 69.7 ± 5.0; MCF-7 Hoechst 33258 staining: 4.7 ± 1.5, 10.2 ± 1.4, 20.0 ± 1.4, 31.0 ± 2.1, 45.8 ± 6.9, Annexin V-PI assays: 3.7 ± 0.9, 9.5 ± 1.1, 18.2 ± 0.6, 25.1 ± 2.1, 43.1 ± 4.3 (**C**)

pH 6.8) were added, and cells were lysed at 37°C for 5 min in the dark room. After a brief but vigorous vortex, the samples were centrifuged at 14,000×g for 10 min. The 100 µl supernatants containing soluble (cytosolic) tubulin and the pellets containing polymerized (cytoskeletal) tubulin were separated. The pellets were resuspended in 100 µl of hypotonic buffer, and the respective lysates were subjected to western blot analysis. Bandscan 5.0 software was employed to obtain the quantitative data of soluble or polymerized tubulin in cells. The relative percentages of polymerized or soluble tubulin were determined by dividing the densitometric values of polymerized or soluble tubulin by the total tubulin content (the sum of P plus S).

Apoptosis assay

Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (bisbenzimide). Cells were washed in PBS (pH 7.4) and then fixed with 4% paraformaldehyde for 10 min at room temperature. Following fixation, cells were rinsed 3 times in PBS (pH 7.4) and then stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China). Stained nuclei were observed under a fluorescence microscope (IX70, Olympus, Japan). Five fields were randomly sampled from each coverslip for fluorescence microscopy observation. All the cells stained with Hoechst dye in each field were counted as either pyknotic or viable. The number of pyknotic cells was counted in the 5 sampled regions and expressed as % apoptotic cells per coverslip = (total number of pyknotic cells)/(total number of pyknotic cells + total number of viable cells) × 100%. To confirm the results of morphologic analysis, apoptosis was further determined by Annexin V-PI assay (KeyGen, Jiangsu, China). Cells were washed twice with PBS, harvested by trypsinization, and collected by 1,000 rpm for 5 min. Cell pellets were incubated with 50 µl binding buffer and the subsequent Annexin V-PI incubation reagent. The fluorescent signals of Annexin V and PI were detected at channels of fluorescence intensity FL1 and FL2 (Cytomics FC500; Beckman Coulter, USA).

Western blot

Total cell lysates were prepared with a detergent lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF]. Western blots were performed as previously reported [10]. For each treatment group, three parallel samples were applied, and equal amounts of proteins from the parallel samples were mixed and used for blots. The antibodies used were the polyclonal anti-α-tubulin (1:1,000), polyclonal anti-

Fig. 2 As$_2$O$_3$ induces tubulin polymerization in HeLa and MCF-7 ▶ cells. HeLa and MCF-7 cells were treated with paclitaxel (3 µM for HeLa, 6 µM for MCF-7) or colchicine (2 µM for HeLa, 4 µM for MCF-7) for 12 h, respectively. The soluble or polymerized tubulin was isolated and detected by Western blot (**A**). The relative percentages of polymerized or soluble tubulin in paclitaxel or colchicine treated cells (**B**). As$_2$O$_3$ induced tubulin polymerization in HeLa (5 µM) or MCF-7 (10 µM) cells in a time-dependent manner (**C**). The relative percentages of polymerized or soluble tubulin in As$_2$O$_3$ treated HeLa and MCF-7 cells (**D**). Immunofluorescence assay was employed to detect changes of microtubule network in cells treated with As$_2$O$_3$ (5 µM for HeLa, 10 µM for MCF-7). Paclitaxel (3 µM for HeLa, 6 µM for MCF-7) or colchicine (2 µM for HeLa, 4 µM for MCF-7) was used as polymerization or depolymerization agents, respectively (scale bar: 20 µM, for all immunofluorescent images) (**E**). Tubulin polymerization inhibitor, TPI (10 µM) attenuated As$_2$O$_3$-induced polymerization of tubulin (**F**) and apoptosis (**G**) in HeLa and MCF-7 cells, respectively. Data represented as the mean ± standard deviation of three independent experiments. *$P < 0.05$, indicating significant differences by Student's t-test. Quantitative data for apoptosis assay of G: HeLa (%): 10.8 ± 0.8, 30.1 ± 1.7, 22.6 ± 1.5, $P = 0.004$ by Student's t-test. MCF-7 (%): 5.8 ± 0.7, 15.8 ± 1.6, 10.7 ± 1.0, $P = 0.008$

phospho-p44/42 MAPK (Thr202/Tyr204) (1:1,000) (sigma), anti-p44/42 MAPK (ERK1/2) (1:1,000), polyclonal anti-phospho-p38 (Thr188/Tyr182) (1:1,000), anti-p38 (1:1,000) (Cell Signaling Technology, Beverly, MA, USA); polyclonal anti-phospho-JNK (1:1,000), anti-JNK (1:1,000) (Bioworld Technology, Barrie, ON, Canada); polyclonal goat anti-JWA (1:1,000) (Imgenex, San Diego, CA, USA); polyclonal rabbit anti-HA (1:500) (Santa Cruz, CA) and polyclonal anti-β-Actin (1:1,000) (Boster Biotechnology, Wuhan, China). Each blot was repeated three times.

Detection of cellular ROS

2,7-Dichlorofluorescein diacetate (DCFH-DA) binds specific to intracellular H$_2$O$_2$, and can be used to detect the intracellular levels of the H$_2$O$_2$. It was dissolved in DMSO at 2 mM and kept at −20°C. DCFH-DA was added into the cell culture medium in each well 15 min before the treatments were completed, and the staining was carried out at 37°C. The final DMSO concentration in the cell culture media was 0.1%. In the case of cellular imaging, the cells were placed onto glass coverslips in 6-well plates. After being stained, the cells were washed in PBS (pH 7.4) and fixed with 10% buffered paraformaldehyde. The coverslips were mounted on glass slides and examined with fluorescence microscope (IX70, Olympus, Japan) fitted with an argon-ion laser.

Results

As$_2$O$_3$ induces apoptosis in HeLa and MCF-7 cells

The Hoechst 33258 staining assay showed that 5 or 10 µM of As$_2$O$_3$ treatment induces significant morphological
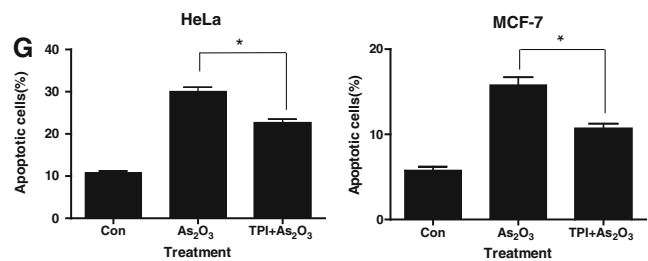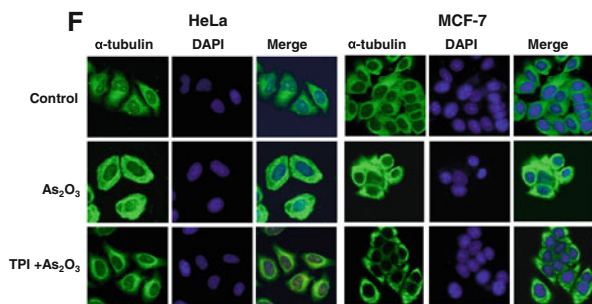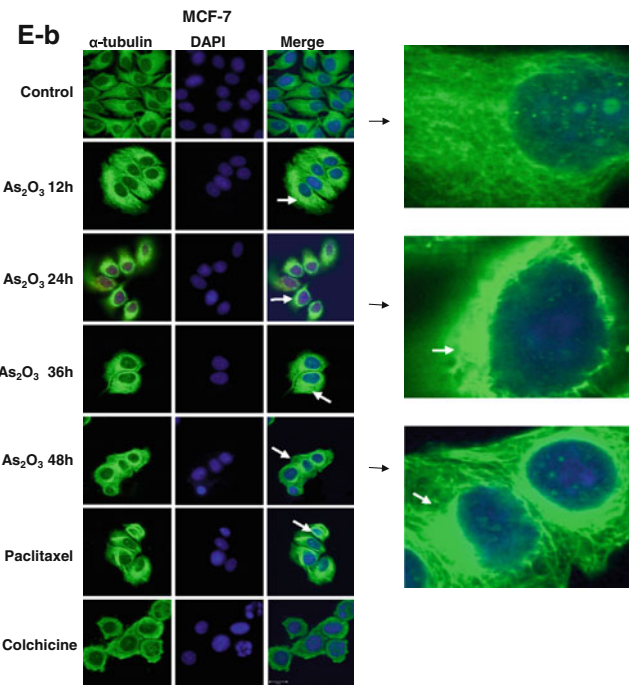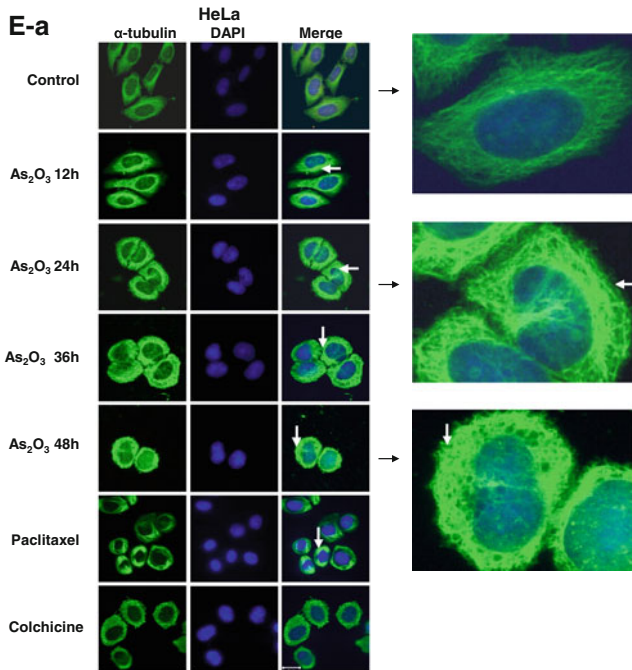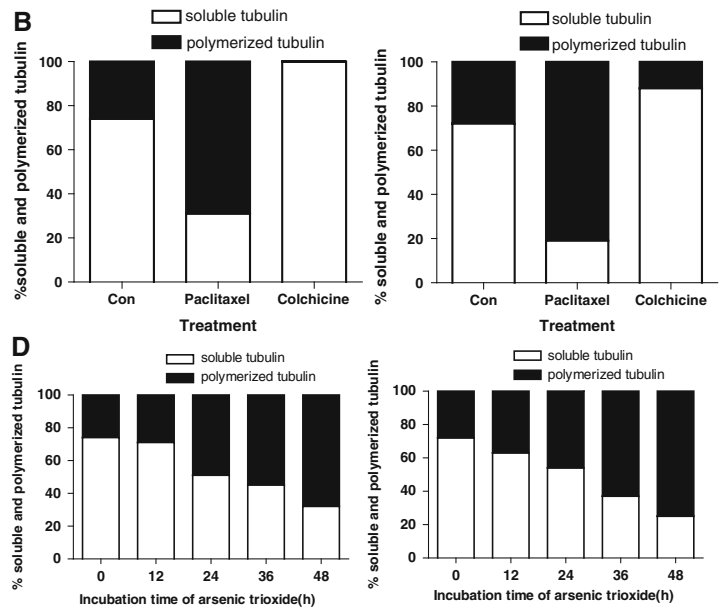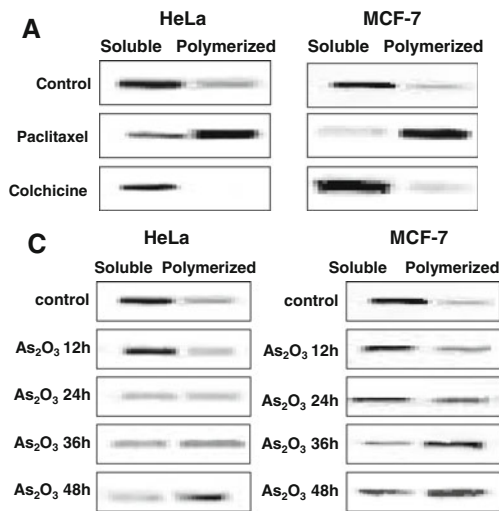
apoptosis in HeLa or MCF-7 cells, respectively, in a time-dependent manner (Fig. 1A). For HeLa cells, treatment with 5 μM As_2O_3 for 48 h induced almost 80% apoptosis in cells; however, 10 μM As_2O_3 for 48 h induced only about 50% apoptosis in MCF-7 cells. Similarly, Annexin V-PI assays confirmed the differential sensitivities between HeLa and MCF-7 cells to As_2O_3 (Fig. 1B). Quantitative data were shown in Fig. 1C. In conclusion, HeLa cells exhibited higher sensitivity to As_2O_3 treatment, while MCF-7 was relatively resistant.

## As_2O_3-induced tubulin polymerization mediates apoptosis in HeLa and MCF-7 cells

The paclitaxel (microtubule polymerizing agent) and colchicine (microtubule depolymerizing agent) was used to test tubulin polymerization. As shown in Fig. 2A, in untreated cells, the majority of tubulin was found in the soluble form, and the ratios of polymerized (P) to soluble (S) tubulin were similar between two cell lines (Fig. 2B). Cells exposed to paclitaxel exhibited obviously increased tubulin polymerization. Conversely, colchicine treatment induced an increase of tubulin in the soluble form. These results indicated that this method could be used to detect the microtubule amount in polymerized or depolymerized fraction. Results of Fig. 2C showed that the percentage of polymerized microtubule was increased by As_2O_3 treatment. The amount of polymerized tubulin was increased from 26% in untreated HeLa cells and up to 68% in As_2O_3 treated cells; or 28–75% in MCF-7 cells (Fig. 2D). These results were further confirmed by indirect immunofluorescence microscopy. The microtubule network in control cells exhibited normal organization. As image characteristics, cells in paclitaxel group exhibited microtubule polymerization, showing with an increased density of cellular microtubules and formation of long thick microtubule bundles surrounding the nucleus. As_2O_3 treatment resulted in findings similar to those of paclitaxel-induced microtubule changes, such as thickening and increased density of microtubules. The presence of brightly staining tubulin around nucleus indicates that microtubule has reorganized into polymer structures, with the polymerized microtubule increased as incubation time prolonged. In contrast, colchicine treatment caused cellular microtubule depolymerization, and short microtubules were shown in cytoplasm (Fig. 2E). Interestingly, 10 μM As_2O_3 induced more tubulin polymerization in MCF-7 cells than 5 μM As_2O_3 in HeLa cells. Comparing with results of Fig. 1 that 10 μM As_2O_3 in MCF-7 cells couldn't induce as much apoptosis as 5 μM As_2O_3 in HeLa cells, we postulated that apoptosis could be induced at least in part by tubulin cytoskeleton reorganization. Co-treatment cells with tubulin polymerization inhibitor effectively blocked As_2O_3-induced

microtubule polymerization (Fig. 2F) and cell death (Fig. 2G). These results indicated that microtubules might be a target of As_2O_3 in inducing cell apoptosis.

JWA participates in As_2O_3-induced apoptosis by enhancing tubulin polymerization

As_2O_3 is a well established anti-proliferative or pro-apoptotic chemical [15]. We have previously identified that JWA is required for As_2O_3 via ROS-mitochondria linked signal pathway induced apoptosis in HeLa and MCF-7 cells [10]. In the present study, JWA expression was increased during As_2O_3-induced cellular apoptosis (Fig. 3A). With these results, we postulated that JWA, as a cytoskeleton associated protein, might enhance As_2O_3-induced apoptosis by promoting tubulin polymerization. To test this hypothesis, HeLa and MCF-7 cells were transfected with FLAG-JWA plasmids and exposed to As_2O_3 for the indicated time. Immunofluorescence microscopy was employed to observe microtubule network. Transfection with FLAG vector alone was used to eliminate the possibility that microtubule organization can be affected by transfection itself. As shown in Fig. 3B, in FLAG vector transfected HeLa and MCF-7 cells, no different extent of tubulin polymerization between cells was observed following As_2O_3 treatment. In FLAG-JWA group, however, tubulin polymerized more obviously in cells with higher expression of FLAG-JWA than those with lower FLAG-JWA expression after As_2O_3 treatment. The differential microtubule networks were shown between the cells due to different levels of JWA expression (Fig. 3C). Similar images were also observed in MCF-7 cells. These results suggested that overexpression of JWA triggered more microtubule polymerization in addition of that induced by As_2O_3 treatment alone. As shown in Fig. 3D, the percentage of cells with obvious microtubule polymerization was increased from 36.5 to 76.8% by transfection with FLAG-JWA in HeLa cells, and 42.6–85.4% in MCF-7 cells, indicating microtubule polymerization induced by As_2O_3 were effectively strengthened by high expression of JWA. Therefore, we have demonstrated over expression of JWA could positively regulate microtubule polymerization and apoptosis. We further want to know if JWA siRNA could block or inhibit these events. As a result, JWA expression was significantly reduced by transfection of siJWA (Fig. 3E). As shown in Fig. 3F, cells with lower expressions of JWA exhibited less microtubule polymerization than those with normal JWA expression, indicating microtubule polymerization was inhibited. As we predicted, As_2O_3-induced apoptosis was also significantly reduced by transfection with JWA siRNA (Fig. 3G).
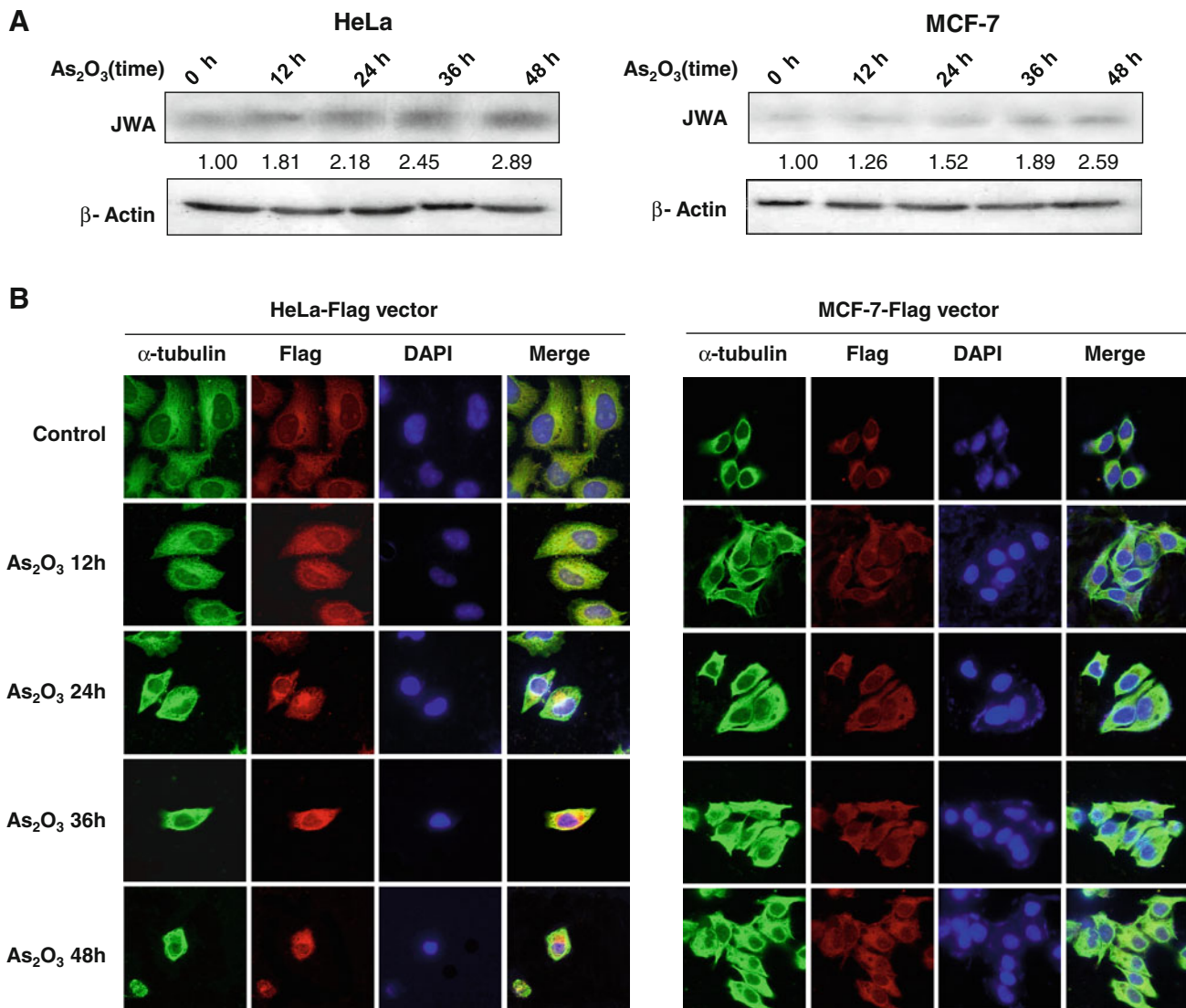
Fig. 3 JWA positively regulates tubulin polymerization and cell apoptosis in As_2O_3 treated HeLa and MCF-7 cells. The expression of JWA in HeLa and MCF-7 cells was time-dependently increased after exposure to As_2O_3 (**A**). HeLa and MCF-7 cells were transiently transfected with FLAG vector (**B**) or FLAG-JWA (**C**); the cells were treated with As_2O_3 for the indicated time, intracellular tubulin (*green*), FLAG-JWA (*red*) or cell nuclei (*blue*) were detected by immunofluorescence microscopy; FLAG-JWA positive cells showed more polymerized tubulin and earlier apoptosis than those FLAG-JWA negative cells (**C**). Data was displayed as the percentage of cells with obvious microtubule polymerization through macroscopical counting in all pictures we took (**D**). Quantitative data: HeLa: 36.5,

76.8%, $P = 0.000$; MCF-7: 42.6, 85.4%, $P = 0.000$, by χ^2 tests. Cells were transfected with either JWA siRNA or control siRNA, and treated with or without As_2O_3, Western blot (**E**) and indirect immunofluorescence (**F**) was employed to detect target protein expression and microtubule network, respectively. Apoptosis was detected by flow cytometry (**G**). Group 1: control cells, Group 2: cells treated with As_2O_3, Group 3: cells transfected with control siRNA and treated with As_2O_3. Group 4: cells transfected with JWA siRNA and treated with As_2O_3. Detailed data for apoptosis assay: HeLa (%): 6.1 ± 2.1, 30.4 ± 5.0, 32.8 ± 2.2, 16.9 ± 1.2, *$P = 0.000$; MCF-7 (%): 4.9 ± 3.9, 22.2 ± 1.9, 26.6 ± 0.7, 16.8 ± 3.7, *$P = 0.011$, by Student's *t*-test

P38 regulates As_2O_3-induced tubulin polymerization and apoptosis

P38 was reported to be involved in microtubule reorganization [16–18]. We further investigated whether p38 plays an important role in As_2O_3-induced microtubule polymerization and apoptosis. As shown in Fig. 4A, expression of p38 and phosphorylated p38 was significantly inhibited by

p38 siRNA. The p38 siRNA effectively inhibited As_2O_3-induced microtubule polymerization (Fig. 4B) and apoptosis compared with the cells transfected with control siRNA (Fig. 4C). To understand whether microtubule reorganization was affected by increased phosphorylation of p38, the cells were transfected with pcDNA3-MKK6b. As shown in Fig. 4D, transfection of pcDNA3-MKK6b in cells significantly increased p-p38, indicating p38 was
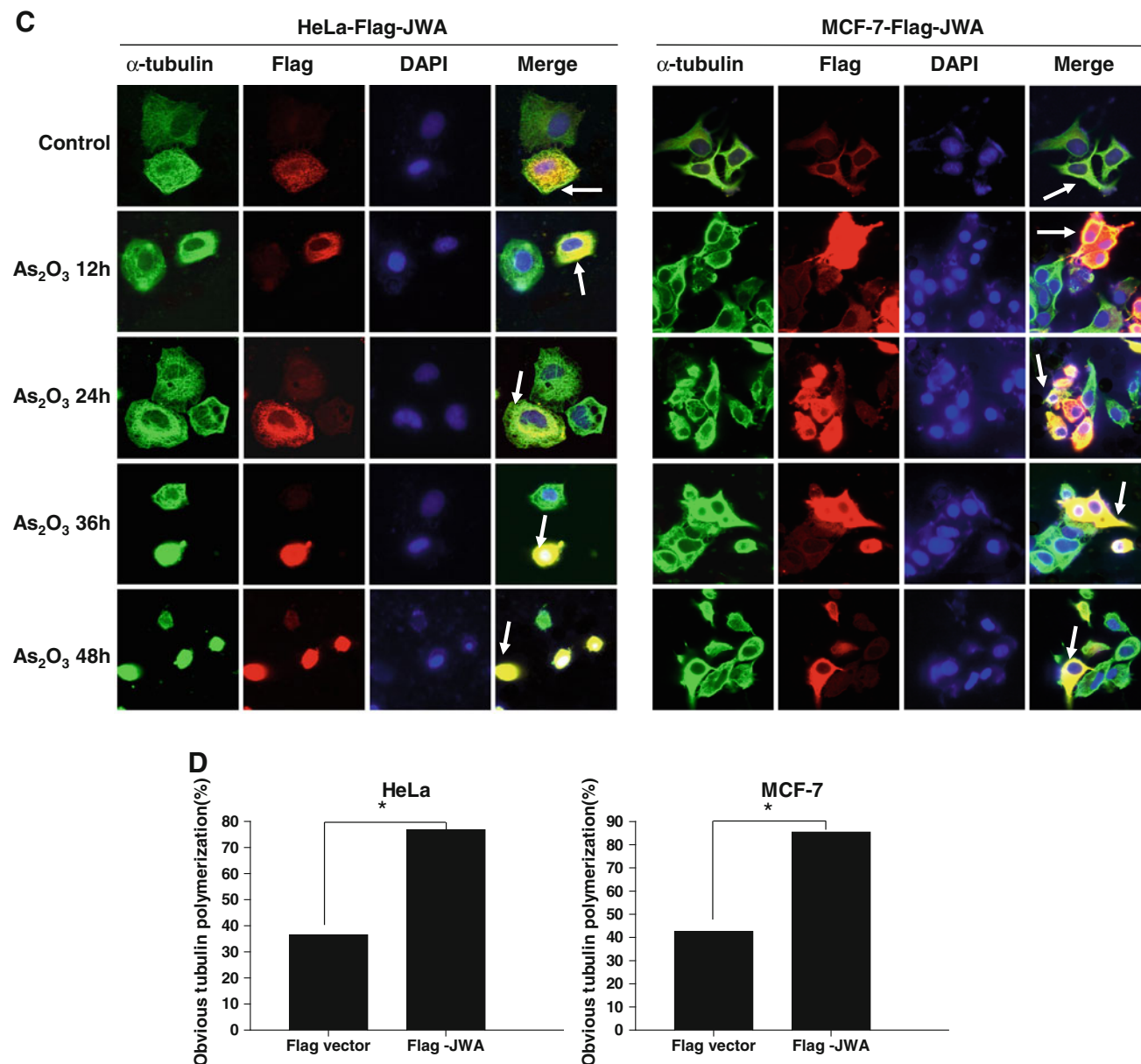
Fig. 3 continued

effectively phosphorylated by MKK6b. Immunofluorescence assay was employed to detect the expression level of HA and microtubule network. Results from Fig. 4E showed cells with higher expression of HA-MKK6b, exhibited more tubulin polymerization, indicating phosphorylation of p38 effectively promoted microtubule polymerization in response to As_2O_3. Consequently, As_2O_3 induced much more apoptosis in response to As_2O_3 in cells transfected with MKK6b (Fig. 4F), compared with those transfected with empty vector. Taken together, these data clearly reveal a critical role of p-p38 in determine the sensitivity of cells to As_2O_3-induced microtubule polymerization and apoptosis.

JWA regulates tubulin polymerization and cell apoptosis via p38

Based on the above and previous results [7], we postulated that JWA may serve as an upstream molecule of p38 and regulates microtubule polymerization and cell apoptosis. To confirm our hypothesis that other members of MAPK signaling are not involved in this process, we detected the expression of p38, ERK and JNK and investigated their efficacy with their respective inhibitors. As shown in Fig. 5A, As_2O_3 treatment upregulated the phosphorylations of ERK, JNK and p38 in HeLa cells, and of ERK, p38 in MCF-7 cells. These activations of MAPK signal molecules
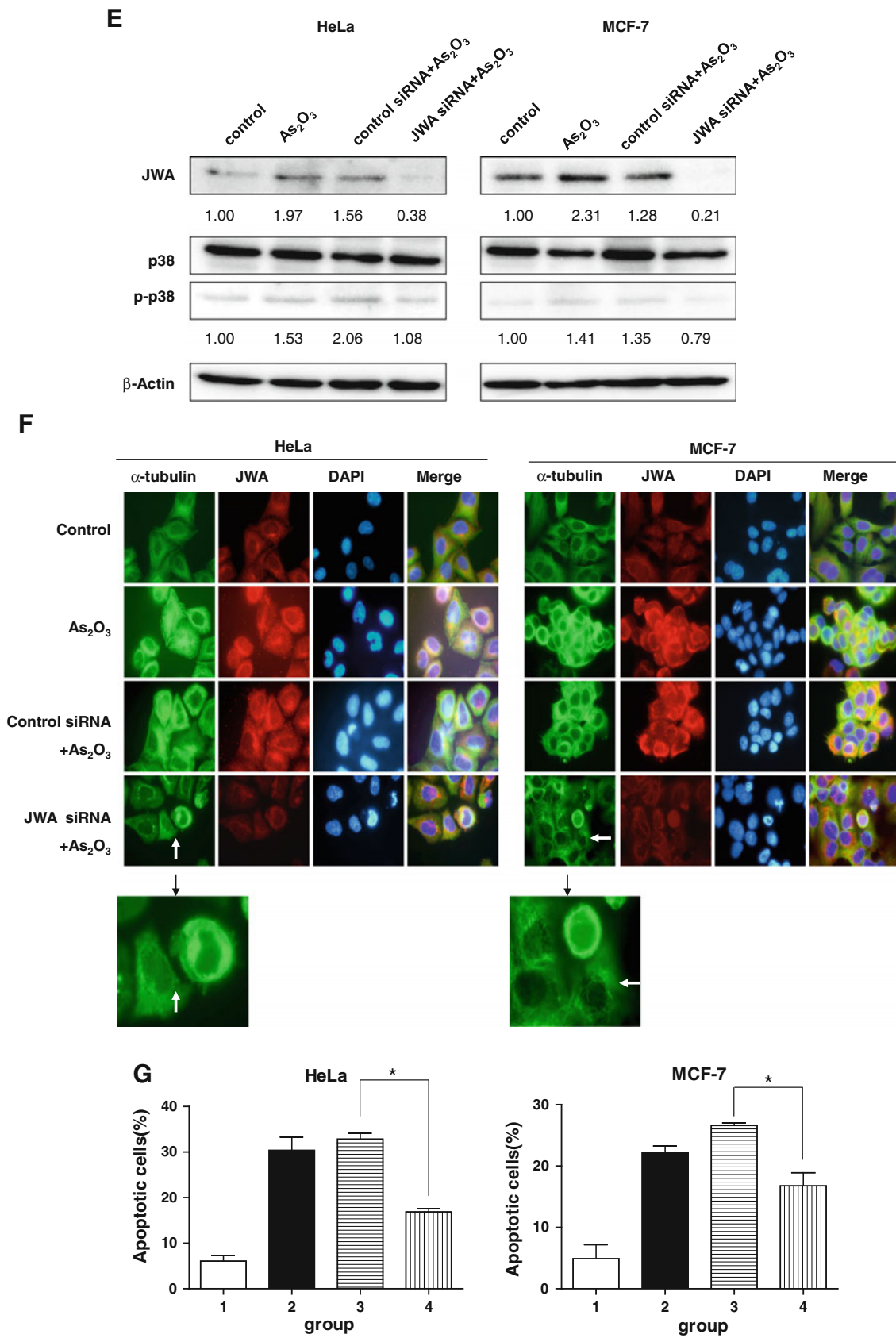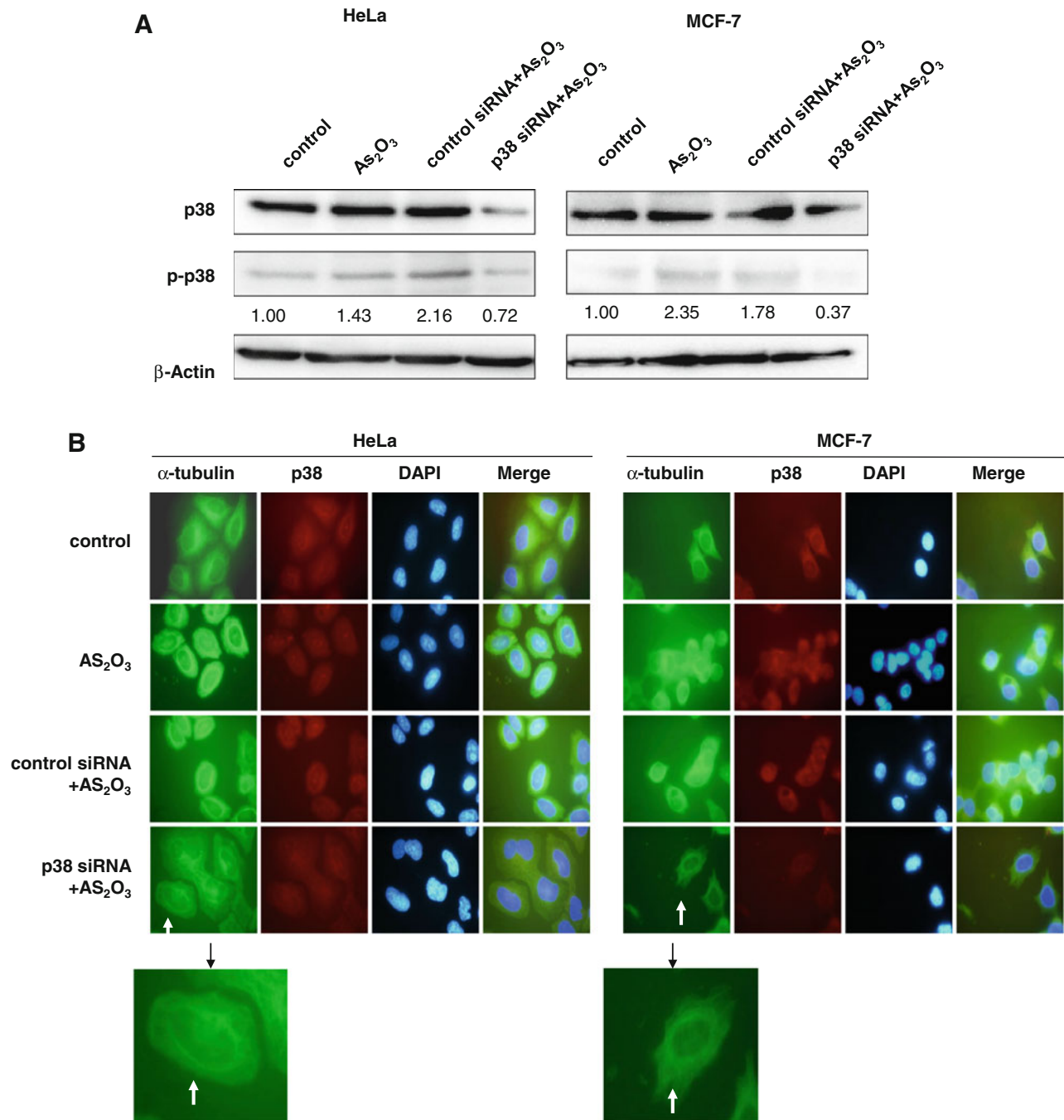
Fig. 3 continued

Fig. 4 P38 knockdown inhibits As_2O_3-induced microtubule polymerization and cell apoptosis. Cells were transfected with either p38 siRNA or control siRNA, and treated with or without As_2O_3. Target proteins in whole-cell lysates were detected by immunoblotting using antibodies against p38, p-p38 (**A**). Indirect immunofluorescence (**B**) was employed to detect microtubule network (**B**). Apoptosis was detected by flow cytometry (**C**). Group 1: control cells, Group 2: cells treated with As_2O_3, Group 3: cells transfected with control siRNA, and treated with As_2O_3. Group 4: cells transfected with p38 siRNA and treated with As_2O_3. Detailed data for apoptosis assay: HeLa (%): 3.1 ± 0.3, 29.4 ± 3.7, 32.4 ± 3.2, 23.7 ± 1.1, *$P = 0.012$; MCF-7 (%): 5.3 ± 0.7, 20.0 ± 2.4, 21.7 ± 2.1, 15.4 ± 2.6, *$P = 0.029$, by Student's t-test. HeLa and MCF-7 cells were transfected with either empty vector

(pcDNA3) or expression plasmid pcDNA3-MKK6b and (or) treated with or without As_2O_3 for 24 h. Cell lysates were analyzed for levels of the proteins of interest by Western blotting (**D**). Immunofluorescence assay (**E**) and flow cytometry (**F**) were used to detect microtubule network and cell apoptosis, respectively. Group 1: control cells; Group 2: cells treated with As_2O_3 alone; Group 3: cells transfected with empty vector; Group 4: cells transfected with empty vector and treated with As_2O_3; Group 5: cells transfected with MMK6b; Group 6: cells transfected with MMK6b and treated with As_2O_3. Quantitative data for apoptosis assay: HeLa (%): 3.1 ± 0.3, 25.7 ± 2.6, 17.8 ± 0.6, 49.4 ± 1.7, 33.2 ± 2.1, 75.4 ± 2.7, *$P = 0.000$; MCF-7 (%): 4.8 ± 0.2, 21.3 ± 2.7, 11.2 ± 1.1, 30.0 ± 1.9, 18.1 ± 2.9, 41.6 ± 1.7, *$P = 0.001$, by Student's t-test
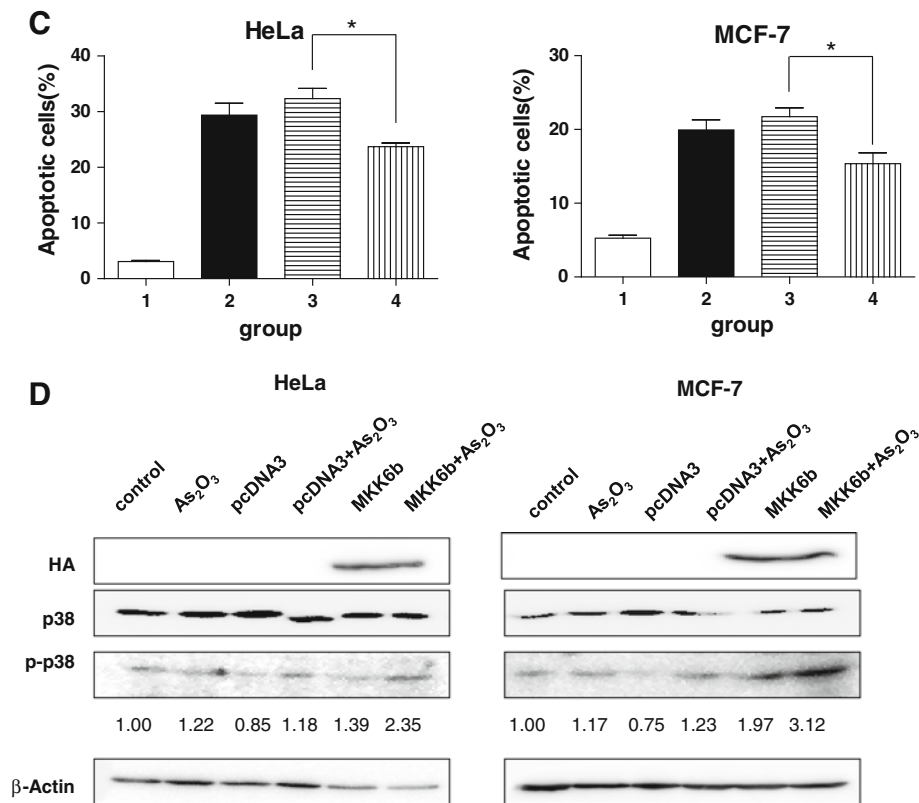
Fig. 4 continued

by As_2O_3 were further enhanced in FLAG-JWA cells, indicating that JWA seems to synergistically activate these proteins with As_2O_3 treatment. Interestingly, FLAG-JWA HeLa cells exhibited a marked increase of phosphorylated JNK compared with their FLAG vector controls in response to As_2O_3 treatment. In MCF-7 cells, however, JNK was phosphorylated in FLAG vector cells and p-JNK levels were little affected by transfection with FLAG-JWA or As_2O_3 treatment. As predicted, these MAPK molecules can be inactivated by use of separate inhibitors (SB203580 to p38, SP600125 to JNK, and U0126 to MEK) respectively (Fig. 5B). To ascertain that JWA is an upstream regulator of p38, further studies in Fig. 5C showed that JWA expression level was almost unaffected by p38 inhibitor SB203580. Among three inhibitors, only p38 inhibitor could prevent JWA-promoted additional tubulin polymerization after As_2O_3 treatment (Fig. 5D). Further more, p38 inhibitor effectively blocked JWA-enhanced cell death (Fig. 5E). These results demonstrated that p38 may function as a downstream key molecule of JWA in mediating microtubule polymerization and cell death during As_2O_3 treatment.

As Bogatcheva et al. [13] reported that tubulin network remodeling affects p38 activation. In the present study, we confirmed that when HeLa and MCF-7 cells were prior exposed to tubulin polymerization inhibitor, As_2O_3-induced p38 activation was significantly attenuated (Fig. 6).

Oxidative stress may not contribute to As_2O_3-induced microtubule polymerization

ROS are known to affect mitochondria membrane potential, therefore trigger a series of mitochondria-associated events including apoptosis. As_2O_3 was found to induce the formation of ROS in a wide variety of cells. Our previous study showed that use of catalase significantly inhibited As_2O_3-induced and ROS mediated apoptosis [10]. We hypothesized that As_2O_3-induced microtubule polymerization may be due to the role of ROS. To test this, the generation of intracellular ROS (H_2O_2) was detected in cells exposed to As_2O_3 for 24 h (Fig. 7A). This phenomenon was completely suppressed by the addition of the antioxidant catalase (10,000 U/ml) (Fig. 7B). As a linked consequence, however, As_2O_3-induced microtubule polymerization was almost unaffected, suggesting As_2O_3 may induce microtubule polymerization via ROS independent signal pathway.
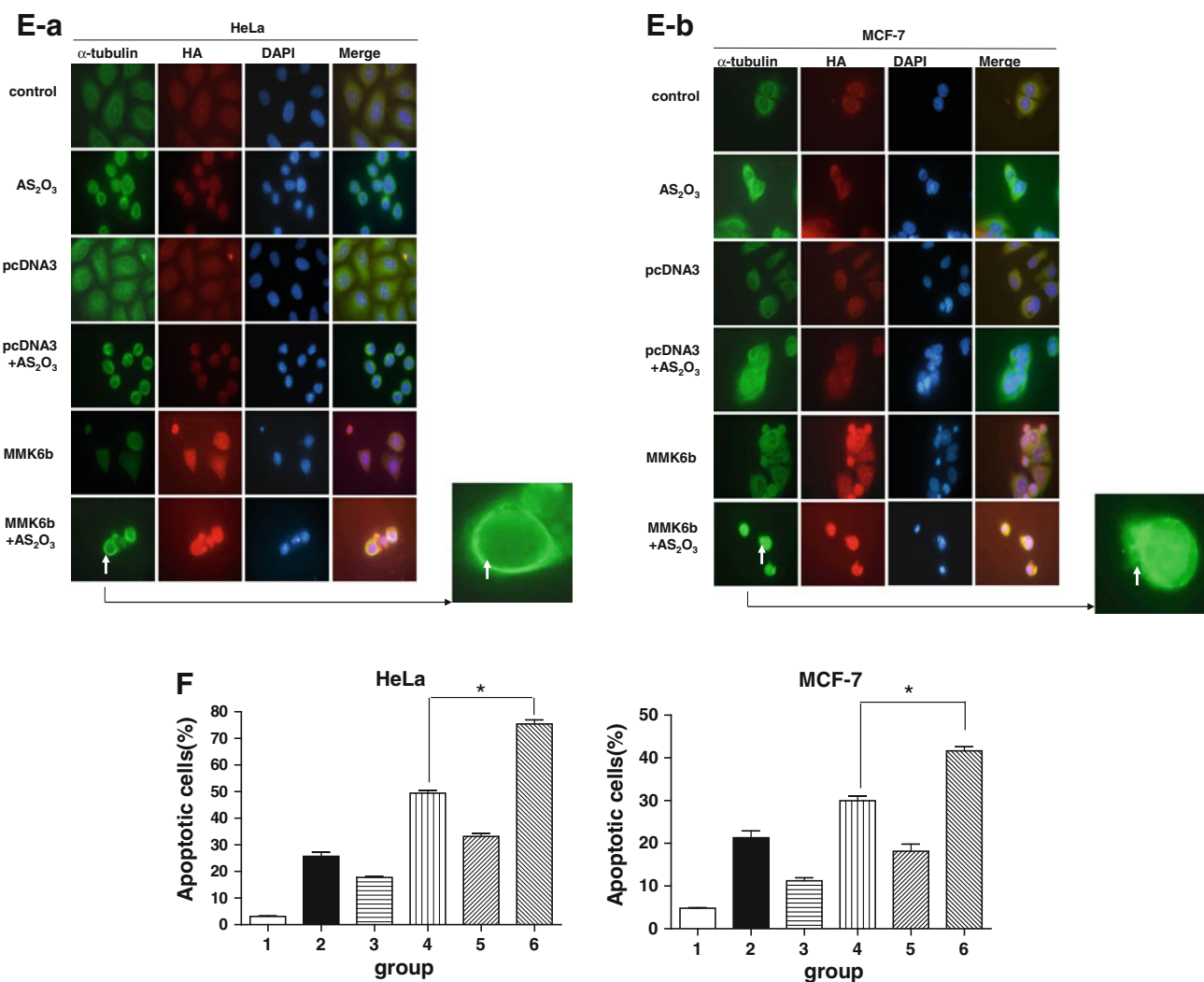
E-a

E-b

F

Fig. 4 continued

Discussion

As$_2$O$_3$ was firstly used as a chemotherapeutic agent in acute promyelocytic leukaemia (APL). Recently, there are increasing results indicating that it can be used in a variety of cancers, including solid cancers [2, 3]. However the precise mechanisms of arsenic-dependent induction of apoptosis on neoplastic cells have not been completely elucidated. Microtubules are reported to be a potential target of arsenic [2–4]. Although it was previously shown that microtubules polymerized [3] or depolymerized [5] following As$_2$O$_3$ treatment, the possible involvement of microtubule in apoptosis has not been investigated.

In the present study, HeLa and MCF-7 cells exhibited different susceptibilities to As$_2$O$_3$. Ling et al. [3] found that As$_2$O$_3$ was a poor substrate for transport by P-glycoprotein or by MRP, suggesting that pumping out intracellular As$_2$O$_3$ may not be a major factor in explaining the different

susceptibilities to As$_2$O$_3$ of different cell lines. These differences remain unexplained. One possibility could be due to different cellular components and/or thresholds in different cell lines for apoptotic signaling.

Further more, we demonstrated that As$_2$O$_3$-induced apoptosis in HeLa and MCF-7 cells was linked to microtubule polymerization. In HeLa cells, the microtubule poison nocodazole partially blocks accumulation of condensed chromatin within surface blebs, and confocal imaging revealed that microtubules associate closely with chromatin within late apoptotic surface blebs [19, 20]. However, Taylor et al. [21] reported that arsenite-induced mitotic death was independent of tubulin polymerization. Beside arsenic, a number of anti-cancer drugs produce pro-apoptotic effects by their microtubule-toxicity. Well established microtubule targeting drug include: paclitaxel, vinblastin and colchicine and so on. Microtubule depolymerization was demonstrated to be involved in gambogic
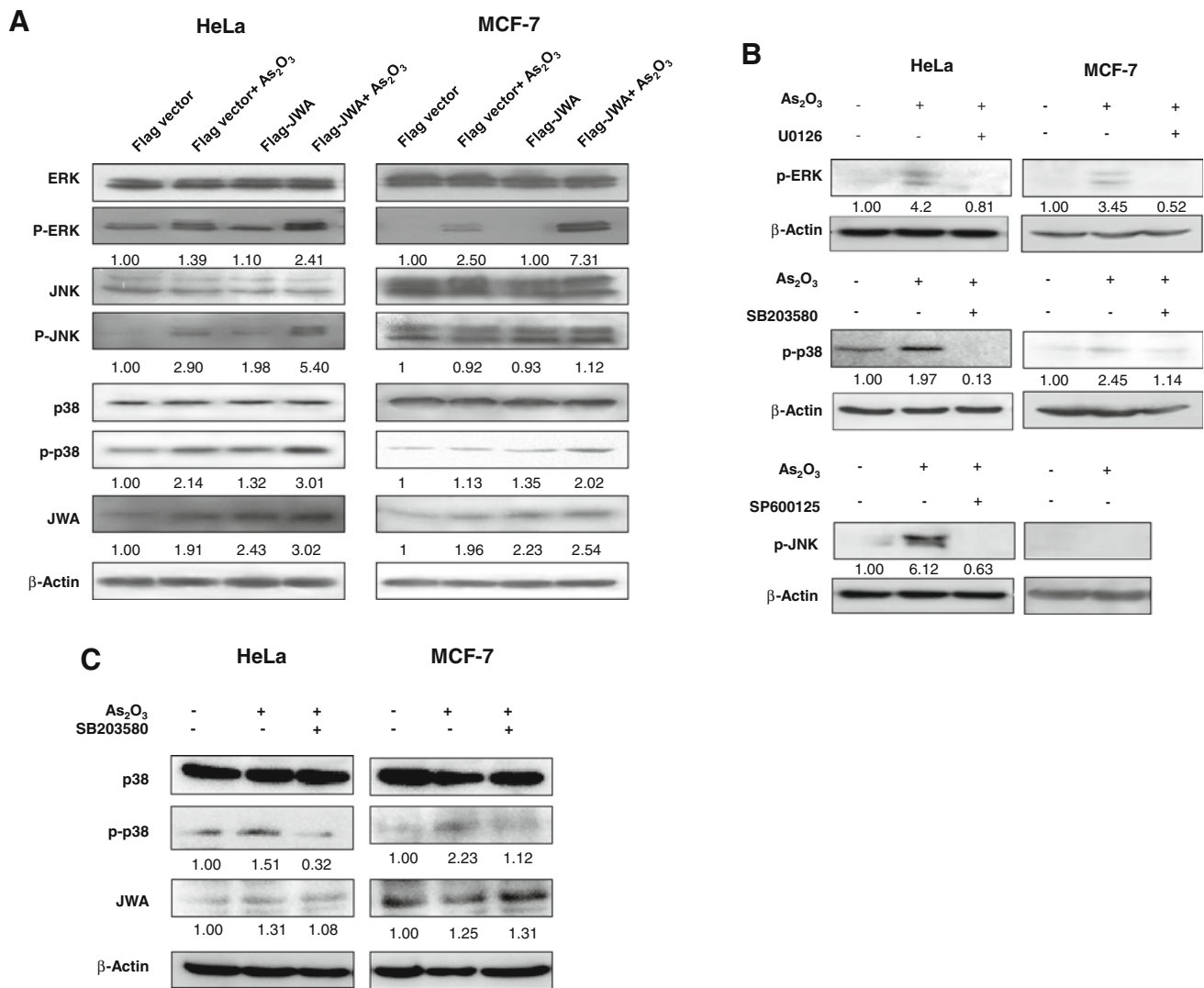
Fig. 5 JWA via p38 promotes As_2O_3-induced tubulin polymerization and apoptosis in HeLa and MCF-7 cells. HeLa and MCF-7 cells were transfected with FLAG vector or FLAG-JWA, and treated with or without As_2O_3. The target MAPK pathway proteins in whole-cell lysates were detected by immunoblotting using respective antibodies (**A**). Cells were treated with As_2O_3 and (or) specific inhibitors, activation of MAPK proteins was analyzed by immunoblotting with respective antibodies (**B**). Cells were treated with SB203580 and (or) As_2O_3, then JWA proteins in whole-cell lysates were detected by immunoblotting (**C**). Cells were transfected with FLAG vector or FLAG-JWA, treated with As_2O_3 and (or) specific inhibitors, then immunofluorescence assay was employed to detect changes of microtubule network (**D**). Apoptosis was detected by flow cytometry in different groups (**E**). Group 1: control cells; Group 2: cells treated with As_2O_3 alone; Group 3: cells transfected with FLAG vector and treated with As_2O_3; Group 4: cells transfected with FLAG JWA and treated with As_2O_3; Group 5: cells transfected with FLAG-JWA and treated with SB203580 before As_2O_3 treatment. Data represent as the mean $\pm$ standard deviation of three independent experiments. *$P < 0.05$, indicating significant differences by Student's t-test. HeLa (%): 5.1 ± 0.2, 30.6 ± 3.0, 35.6 ± 1.7, 46.3 ± 2.8, 40.6 ± 2.0, Group 4 versus Group 3, $P = 0.005$; Group 5 versus Group 4, $P = 0.046$; MCF-7 (%): 6.7 ± 1.8, 19.7 ± 1.3, 21.9 ± 1.2, 33.8 ± 1.4, 26.0 ± 1.8, Group 4 versus Group 3, $P = 0.000$; Group 5 versus Group 4, $P = 0.004$

acid induced cell cycle arrest and apoptosis in human breast carcinoma MCF-7 cells [22]. Therefore, it is reasonable to postulate that microtubules are important targets for a series of drugs to induce apoptosis in cancer cells.

JWA was originally recognized as an all-trans-retinoic acid responsive and cytoskeleton-associated gene [6]. Later Zhu et al. [23, 24] noted that JWA was actively regulated by environmental stressors such as heat shock and

oxidative stress, suggesting that JWA might be a functional gene. JWA may play different biological functions under differential stress conditions due to its subcellular localization. Our recent studies [10] have shown that JWA is a pro-apoptotic gene since it is required for As_2O_3-induced apoptosis in HeLa and MCF-7 cells via ROS and mitochondria linked signal pathway. We further confirmed our results by siRNA technology and found that JWA
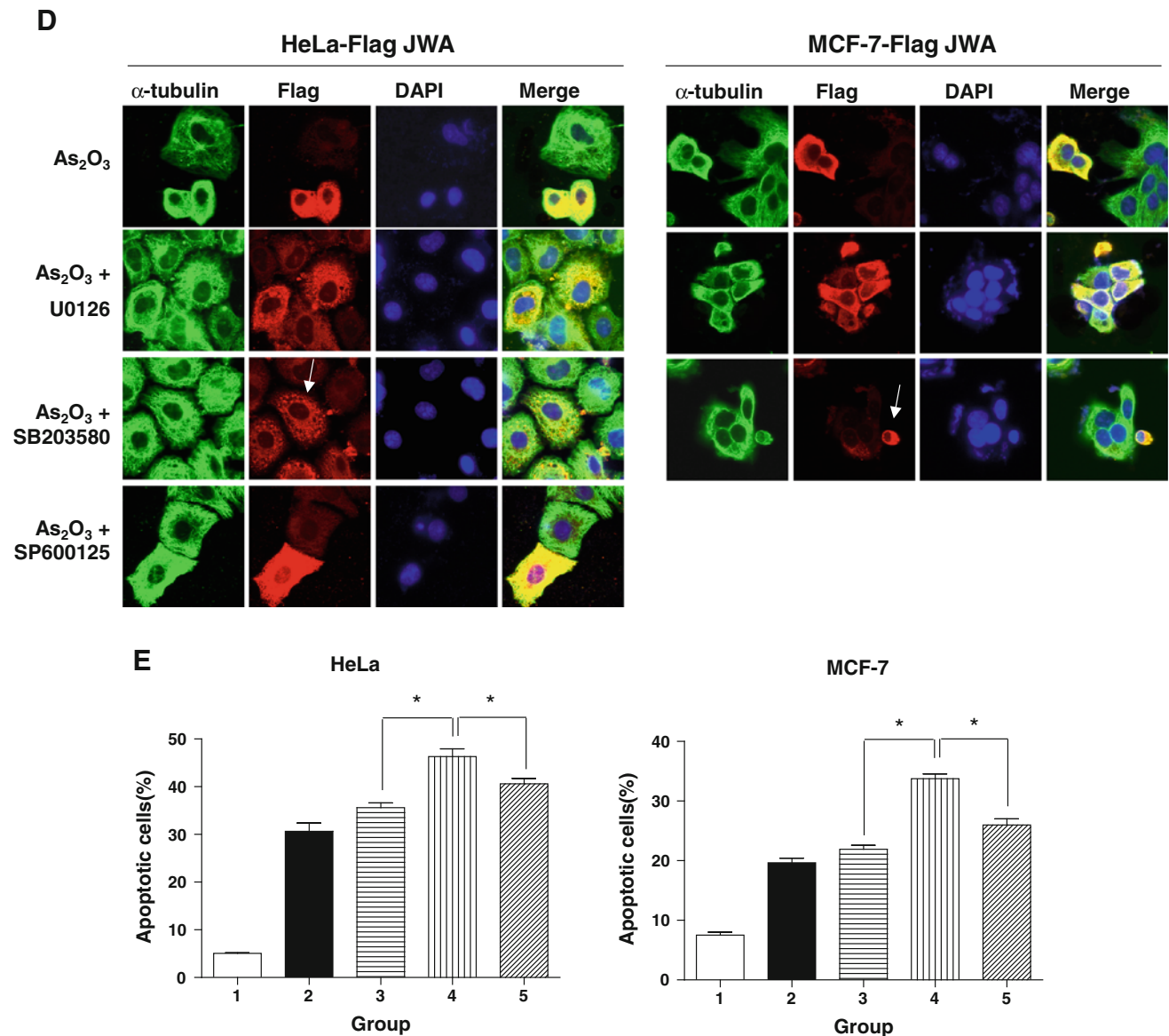
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knockdown could inhibit As_2O_3-induced microtubule polymerization and cell apoptosis. Moreover, microarray analysis revealed that genes in microtubule cytoskeleton organization set showed a decreasing trend in JWA deficient spleens (unpublished data), indicating JWA functions as a positive regulator of microtubule organization. The exact mechanisms involved are under our further investigation.

JWA was also shown to act as a functional molecule to regulate cancer cell migration via MAPK cascades and actin filament [7]. In this study, we further confirmed that JWA, a microtubule associated protein, enhances As_2O_3-induced cell death by promoting tubulin polymerization via p38 activation. The cross-talk between p38 pathway and

microtubule dynamics have recently become an area of intensive research [16–18]. Vogl et al. [25] reported that the activation of p38 MAPK signal pathway induces tubulin polymerization during cell transmigration. Activation of p38 modulates cytoskeletal dynamics during adhesion by unknown mechanisms [26, 27]. Moreover, p38 and JNK MAPK, but not ERK1/2 MAPK, play an important role in colchicine-induced cortical neurons apoptosis, while the microtubule system is the main target of colchicine [28]. In the present study, we found that JNK MAPK was activated in HeLa cells, which was identical with results of Kang et al. [29]. These activations were further enhanced by transfection with FLAG-JWA. But in MCF-7 cells, JNK was phosphorylated in FLAG vector group, and was not
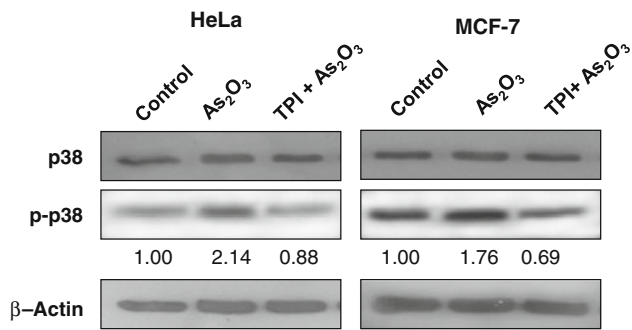
Fig. 6 Tubulin polymerization conversely promotes As$_2$O$_3$-induced p38 phosphorylation in HeLa and MCF-7 cells. HeLa and MCF-7 cells were treated with As$_2$O$_3$ alone or both TPI and As$_2$O$_3$. Phosphorylation of p38 was analyzed by immunoblotting

affected by As$_2$O$_3$ treatment or transfection of FLAG-JWA, indicating JNK was likely not involved in As$_2$O$_3$-induced apoptosis. We found that inhibition of p38 pathway attenuates microtubule polymerization in As$_2$O$_3$ treated JWA overexpression cells and vice versa, inhibition of microtubule polymerization with TPI significantly attenuates activation of p38 cascade. As noted by Birukova et al. [18] that microtubule network was tightly related to p38

MAPK, we hypothesize that microtubule polymerization initiates activation of p38 cascade, which, in turn, facilitates further microtubule polymerization. However, the precise role of p38 pathway and delineation of p38 cascade substrates leading to microtubule rearrangement still need to be further elucidated.

How microtubules are reassembled also remains uncertain. Besides p38, caspase cleavage of the regulatory c-terminus of α-tubulin might be important because this cleavage increased the capacity of tubulin to assemble into polymers [30]. Other studies revealed some negative influences, including microtubule destabilizing factors such as Op18 or stathmin [31], severing proteins such as katanin [32] and catastrophe-promoting motors such as he kinesins MCAK [33] or XKCM1 [34].

This paper extends the work of Zhou et al. [10] by examining the effects of ROS on microtubule polymerization. Our results showed that catalase can not effectively block As$_2$O$_3$-induced microtubule polymerization, suggesting ROS may not be involved in regulating As$_2$O$_3$-induced microtubule polymerization. Kligerman et al. [35] also found that ROS is unlikely to be involved in trivalent arsenicals caused inhibition of tubulin polymerization.

Fig. 7 As$_2$O$_3$-triggered tubulin polymerization in HeLa and MCF-7 cells are independent of ROS. HeLa and MCF-7 cells were treated with catalase and (or) As$_2$O$_3$, H$_2$O$_2$ staining (**A**) was employed to detect intracellular ROS products and immunofluorescence microscopy (**B**) was employed to examine microtubule network
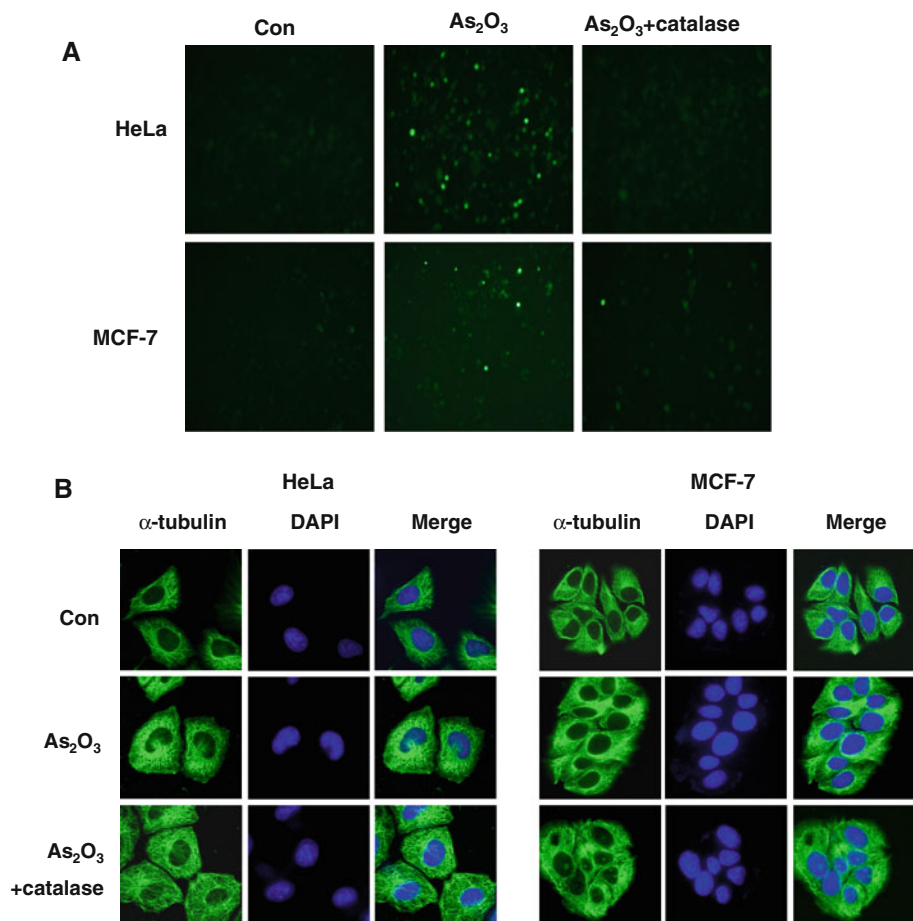
Further investigations are needed to explore the exact mechanisms.

In conclusion, we demonstrated that As_2O_3-induced apoptosis in both HeLa and MCF-7 cells is accompanied with microtubule polymerization. Furthermore, using tubulin polymerization inhibitor, we confirmed that the microtubule polymerization is required for apoptosis in both cell types. High expression of JWA renders microtubules more vulnerable to As_2O_3 and makes both cancer cells hypersensitive to this chemical, and is therefore involved in enhancing As_2O_3-induced apoptosis in both HeLa and MCF-7 cells. Finally, we found that p38 is a downstream molecule of JWA and participates in JWA-regulated tubulin polymerization. Our present study has shown that JWA as a microtubule associated protein is an important regulator during arsenic-induced apoptosis by promoting microtubule polymerization. Our cumulative data suggest that JWA may via both targeting to tubulin and ROS independent pathways to mediate As_2O_3-triggered apoptosis in HeLa and MCF-7 cells.

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