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JWA IS REQUIRED FOR THE ANTIPROLIFERATIVE AND PRO-APOPTOTIC EFFECTS OF ALL-*TRANS* RETINOIC ACID IN HELA CELLS

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SUMMARY

1. All-*trans* retinoic acid (ATRA) is known to inhibit cellular proliferation and induce differentiation and apoptosis. It usually activates gene expression by binding to a nuclear receptor that interacts with retinoic acid-response elements (RARE) and then activates the mitogen-activated protein kinase signal pathway. *JWA*, a newly identified ATRA-responsive gene, has recently been proposed as an important molecule for cellular differentiation induced by some chemicals, including ATRA.

2. To investigate the possible involvement of *JWA* in the inhibition of cellular proliferation and induction of apoptosis by ATRA, HeLa cells were stably transfected with sense or antisense *JWA* to establish cell lines that overexpressed or were deficient in *JWA*; ATRA (0.05–10 μmol/L) was used to induce cellular differentiation and apoptosis.

3. Western blot analysis revealed that ATRA caused increased expression of *JWA* in HeLa cells in a dose- and time-dependent manner, accompanied by activation of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. However, ERK1/2 phosphorylation induced by ATRA was inhibited in *JWA*-deficient HeLa cells. In *JWA*-overexpressing HeLa cells, ATRA showed more significant antiproliferative effects and induced more apoptosis.

4. The reporter gene assay showed that ATRA (5 μmol/L) enhanced the transcriptional activity of *JWA* by interacting with its promoter in the region from –194 to +107 bp ($P < 0.01$). Bioinformatic analysis indicated that the *JWA* promoter did not contain RARE, but did contain two CCAAT boxes in this fragment spanning –194 to +107 bp, which may be responsive to the ATRA-activated nuclear transcription factor CCAAT/enhancer binding proteins (C/EBP) or interacting proteins. Therefore, ATRA-inhibited cellular proliferation and -induced apoptosis in HeLa cells may be dependent on *JWA* transactivation via its C/EBP-binding motifs.

5. These data indicate that the inhibition of proliferation and the induction of apoptosis by ATRA are dependent on *JWA* expression in HeLa cells. The findings may represent a novel mechanism by which the effects of ATRA in regulating cellular proliferation and apoptosis are mediated, at least in part, by *JWA* expression.

Key words: apoptosis, all-*trans* retinoic acid, cell proliferation, extracellular signal-regulated kinase, *JWA*.

INTRODUCTION

Carcinogenesis is a chronic and multistep process resulting from mutagenic damage to growth-regulating genes and their products that ultimately leads to the development of invasive or metastatic cancers.[1] Retinoids are a class of compounds structurally related to vitamin A. In many cases, retinoids display antitumoral activities. Both natural (e.g. all-*trans* retinoic acid (ATRA) and 13-*cis*-retinoic acid (13-*cis*-RA)) and synthetic retinoids (e.g. *N*-(4-hydroxyphenyl) retinamide (4-HPR)) have been used as the most important chemopreventive and therapeutic agents, experimentally as well as clinically, for the treatment of patients with breast carcinomas, head and neck squamous cell carcinomas, acute leukaemia and cervical carcinomas.[2–5]

Retinoids have demonstrated inhibition of cellular proliferation and the induction of differentiation and apoptosis in various cancers.[6–9] However, the critical molecular mechanisms behind these effects are still poorly understood. Retinoids are usually thought to exert most of their effects by regulating gene expression, primarily through two classes of nuclear receptors, namely retinoic acid receptors (RAR) and retinoid X receptors (RXR), both composed of three subtypes (α, β and γ). The RAR and RXR modulate the expression of target genes by interacting as either homodimers or heterodimers with the retinoic acid response element (RARE) located in the promoter regions of target genes.[10] Furthermore, ATRA may also exert its effects via a non-receptor pathway by activating nuclear factors, such as the CCAAT/enhancer binding proteins (C/EBP).[11–14] It had been shown that C/EBPα is involved in the activation of *Hp* gene expression by ATRA in human monocytic cells.[15] The coactivator activating signal cointegrator-2 (ASC-2) plays an important role in the differentiation of HL-60 cells into granulocytes by mediating C/EBPα-induced gene transcription.[16] Recent evidence has also shown that cell growth inhibition by ATRA involved mitogen-activated protein kinases (MAPK).[17–19]

JWA (AF070523), a newly identified ATRA-responsive gene, was initially cloned from primary human tracheal bronchial epithelial cells by Zhou *et al*. in 1997.[20] Although it was thought that *JWA*

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could involved in several intracellular signal transduction pathways, few studies have investigated its functions. However, some *JWA* homologues may give us new insights that *JWA* may be a potentially important functional gene. JM4, a *JWA* homologous protein, has been indicated to be a novel CC chemokine receptor 5 (CCR5)-interacting protein that binds to the CCR5 receptor with a potential role in regulating chemokine-mediated reactions.[21] It has been suggested that JM4 functions in the trafficking and membrane localization of the CCR5 receptor, as well as possibly other receptors or amino acid transporters, which regulates glutamate uptake by interacting with the 10 transmembrane-spanning excitatory amino acid carrier 1 (EAAC1).[21–25] *ARL-6* (AF133912), a member of the Ras superfamily that interacts with the SEC61 β-subunit has also been identified as a homologous gene to *JWA*.[26] Another *JWA* homologue gene (AB097051) was found to encode a putative MAPK-activating protein.[27] Recent studies have also provided evidence that *JWA* may be an important molecule for regulating intracellular amino acids homeostasis, as well as directional differentiation of leukaemia cells induced by some chemicals, including ATRA, arsenic trioxide (As_2O_3), cytosine arabinoside (Ara-C) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[28–33] In the present study, we investigated whether *JWA* was involved in the antiproliferative and pro-apoptotic effects of ATRA and the potential association with the MAPK signal pathway in a cervical carcinoma (HeLa) cell lines.

METHODS

Chemicals and antibodies

RPMI 1640 cell culture medium, 10% fetal calf serum (FCS), L-glutamine (200 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL) and trypsin-EDTA were purchased from Life Technologies (Paisley, UK). All-*trans* retinoic acid was purchased from Sigma-Aldrich (St Louis, MO, USA) and a 1 mmol/L stock solution was prepared in dimethylsulphoxide (DMSO). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical (St Louis, MO, USA). The JWA protein c-terminal 20 amino acid peptide and the anti-JWA polypeptide sera were prepared by Research Genetics (Huntsville, AL, USA). Rabbit polyclonal against total extracellular signal-regulated kinase (ERK), mouse monoclonal against phospho-ERK1/2 and β-actin monoclonal antibodies were purchased from Sigma-Aldrich. Secondary antibodies conjugated with horseradish peroxidase (HRP) against rabbit were obtained from Cell Signalling Technology (Beverly, MA, USA). The inhibitor of MAPK kinase (MEK), namely PD 98059, was obtained from Calbiochem (La Jolla, CA, USA). Working solutions were made according to the manufacturer's instructions.

Cell culture

The HeLa cells were purchased from Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (1×10^6 cells/100 mm dish) supplemented with 10% FCS (containing 100 units/mL penicillin and streptomycin) and grown at 37°C with 5% CO_2 in a humidified incubator (HERA CELL, Heraeus, Germany). When cells were determined to yield 60–70% confluent cultures, they were treated with the concentrations of ATRA specified and harvested at the times indicated. As an untreated solvent control, cells were treated with DMSO (Sigma Chemical) at a final concentration of < 0.1%.

Stable transfection

To construct *JWA*-overexpressing and -deficient vectors, the pEGFP-C1 expression vector was purchased from Clontech (Palo Alto, CA, USA). The full-length sense *JWA* coding region sequence (567 bp) and antisense *JWA* coding region sequence (from 409 to 71 bp; 339 bp) was inserted into the vector using both *Bam*HI and *Hin*dIII restriction endonucleases, respectively. The ligated plasmids were transformed into *Escherichia coli* (DH5α) and the transformed clones were then further confirmed by automated DNA sequencing. Transfections of pEGFP-C1-sense/antisense *JWA*, pEGFP-C1 vector DNA plasmids into HeLa cells were performed using Polyfect Transfection Reagent (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The empty vector pEGFP-C1 was used as a control. Transfected cells were selected in the presence of 1000 mg/mL G418. The single cell-derived clones were expanded sufficiently for *in vitro* experiments. The transfected cells had a stable deficiency of *JWA* expression (herein termed as*JWA*-HeLa), *JWA* overexpression (*JWA*-HeLa) and control-vector transfected cells (vector-HeLa).

Cell proliferation assays

Cell proliferation was measured using the MTT assay. The HeLa cells, vector-HeLa cells, *JWA*-HeLa and as*JWA*-HeLa cells were seeded onto 96-well plates (2×10^4 cells/well) and were cultured overnight to allow for cell attachment. Cells were then treated with increasing concentrations of ATRA (0.05–10 μmol/L) for 24, 48 and 72 h. At the conclusion of the experiment, 20 μL MTT solution (5 mg/mL in phosphate-buffered saline (PBS) buffer) was added to each well. Water-insoluble formazan was formed during incubation. After incubation for 4 h at 37°C, the culture medium containing MTT was removed and 150 μL/well DMSO was added to solubilize the formazan formed by viable cells. After 10 min shaking, plates were read by an Ultra Microplate Reader (EL808; Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. All assays were performed in triplicate. Cell viability was expressed as a percentage ratio of exposed cells to control cells.

Apoptosis assay

Cell apoptosis was ascertained using staining with the fluorescent DNA-binding dye Hoechst 33258 (Beyotime, Jiangsu, China) and observed under a fluorescence microscope and by flow cytometric sub-G_1 DNA detection.

Fluorescent DNA-binding dyes were used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells grown on coverslips were washed with PBS and fixed in methanol : acetic acid (3 : 1) at 4°C for 5 min. After fixation, cells were stained for 10 min with the fluorescent DNA-binding dye Hoechst 33258 (8 mg/mL) and nuclear morphology was examined by fluorescence microscopy. Individual nuclei were visualized at ×200 to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells. To quantify apoptosis, 500 nuclei from random microscopic fields were analysed and the percentage of apoptotic cells was calculated as the (number of apoptotic cells/total number of cells) × 100. Each experiment was conducted in triplicate.

For the flow cytometric assay, the vector-HeLa, *JWA*-HeLa and as*JWA*-HeLa cells were seeded onto 100 mm dishes (1×10^6 cells/dish). Cells were treated with 5 μmol/L ATRA for 24, 48 and 72 h, harvested, fixed in 70% ethanol, centrifuged (1000 *g* for 10 min) and stored at 4°C until analysis. Cell pellets were then resuspended in 400 μL PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_47H_2O$, 1.4 mmol/L KH_2PO_4; pH 7.3) containing 2 mg/mL RNase (Sigma) and stained with 400 μL of 0.1 mg/mL propidium iodide (PI; Sigma). The DNA content was analysed by flow cytometry using excitation at 488 nm and emission at 610 nm (red) with a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA). Data were analysed using Phoenix software (Coulter).

Western blots

Western blots were performed as reported previously.[30] Briefly, harvested cells were washed with PBS twice and lysed by scraping into 100–200 μL keratin extraction buffer (1% Triton-X 100, 0.02 mmol/L Tris, 0.6 mol/L KCl

and 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), pH 7.0). Samples were centrifuged at 12 000 *g* for 20 min at 4°C and pellets were discarded. After detecting the protein concentration of samples using the Bradford assay (Bio-Rad, Hercules, CA, USA), aliquots of protein were then separated on 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 15 V for 1 h and membranes were incubated with 2% non-fat-dried milk for 1 h and then washed and further incubated for 2 h at 37°C with the first antibody (1 : 1000 dilution). After washing in PBS solution containing 0.05% Tween-20 (PBST; 5 min, five times), membranes were then incubated in HRP-labelled secondary antibody (1 : 1000 dilution) at 37°C for 1 h. After washing in PBST (5 min, five times), each membrane was developed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

For each treatment, three parallel samples were applied and an equal amount of proteins from the parallel samples was mixed and used for the blots. Each blot was repeated three times. The immunoreactive bands were scanned for semiquantification and normalized to the β-actin bands of the same membrane.

Reporter plasmids and chloramphenicol acetyl transferase assay

Wild-type −1680/+107 (−1680PCAT^{wt}), −595/+107 (−595PCAT^{wt}), −494/+107 (−494PCAT^{wt}), −257/+107 (−257PCAT^{wt}), −194/+107 (−194PCAT^{wt}) and −27/+107 (−27PCAT^{wt}) bp fragments of the human *JWA* promoter were cloned into the pCAT3-basic vector (Promega, Madison, WI, USA) at the *Sca*I (3′) and *Xho*I (5′) restriction sites. All constructs were confirmed prior to use by restriction enzyme digestions and DNA sequencing.

For transfection experiments, HeLa cells were seeded onto six-well, 35 mm diameter plates (2.5×10^5 cells/well). Each well was transfected with 3.2 μg reporter vector plasmid DNA, using Polyfect Transfection Reagent (Qiagen). For cotransfections, 0.8 μg empty β-galactosidase (β-gal) vector was added to each reaction. After transfection, the HeLa cells were treated with ATRA (5 μmol/L) or 0.1% DMSO in RPMI 1640 plus 10% FCS for 48 h. Cell extracts were then prepared and each sample was assayed in triplicate using a CAT-ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Absorbance was measured using a spectrophotometric microtitre plate reader at a wavelength of 405 nm. Chloramphenicol acetyl transferase (CAT) activity was normalized with β-gal activity for transfection efficiency.

Statistical analysis

Data are expressed as the mean±SD. Two-way analysis of variance (ANOVA) and the Dunnett's *t*-test were used to assess differences within treatment groups. Differences were considered significant at $P < 0.05$.

RESULTS

All-*trans* retinoic acid caused a dose- and time-dependent increase in the expression of JWA proteins in HeLa cells

To investigate whether *JWA* is involved in the effect of ATRA on HeLa cells, we designed an ATRA-treated cell culture model investigating the effects of ATRA over time and according to dose. The HeLa cells were treated with ATRA (5 μmol/L) for 1, 3, 6, 12, 24 and 48 h. Western blot analysis showed obviously increased *JWA* expression within 1 h and up to 3, 6, 12 and 48 h. The expression of *JWA* was increased up to 4.5-fold of control at the 48 h time point. All-*trans* retinoic acid caused a time-dependent increase in the expression of *JWA* (Fig. 1a). Based on these data, we further exposed cells to 0.05–10 μmol/L ATRA for 48 h to determine whether *JWA*

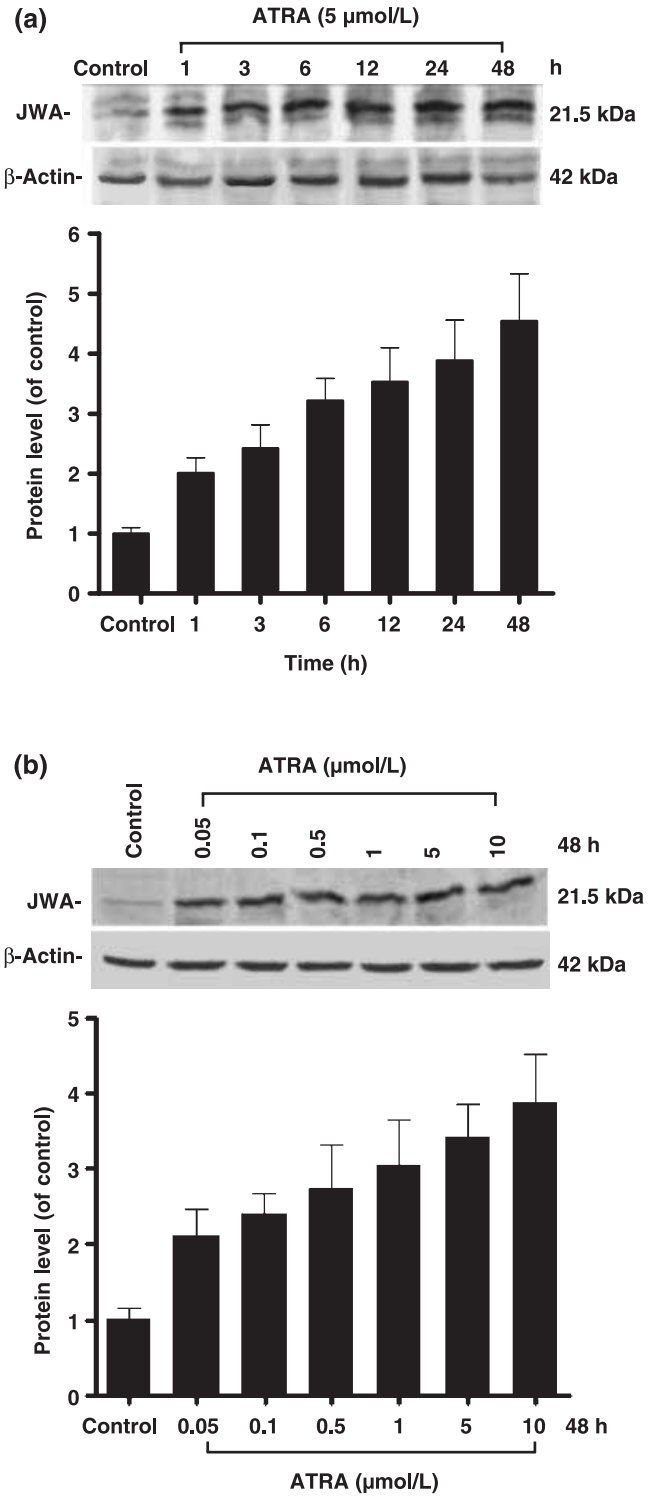

Fig. 1 Effects of all-*trans* retinoic acid (ATRA) on the expression of *JWA* proteins. (a) HeLa cells were treated with 0.1% dimethylsulphoxide (DMSO; control) or ATRA (5 μmol/L) for the time indicated (1–48 h). Proteins were extracted and examined for the expression of *JWA* by western blotting. The expression level of JWA protein was gradually increased with time of ATRA treatment. (b) HeLa cells were treated with 0.1% DMSO (control) or with the indicated concentrations of ATRA for 48 h. Proteins were extracted and examined for the expression of *JWA* by western blot. The expression of JWA protein increased gradually with ATRA treatment (0.05–10 μmol/L). β-Actin was used as an internal control for protein loading. Data are the mean ± SD ($n = 3$).

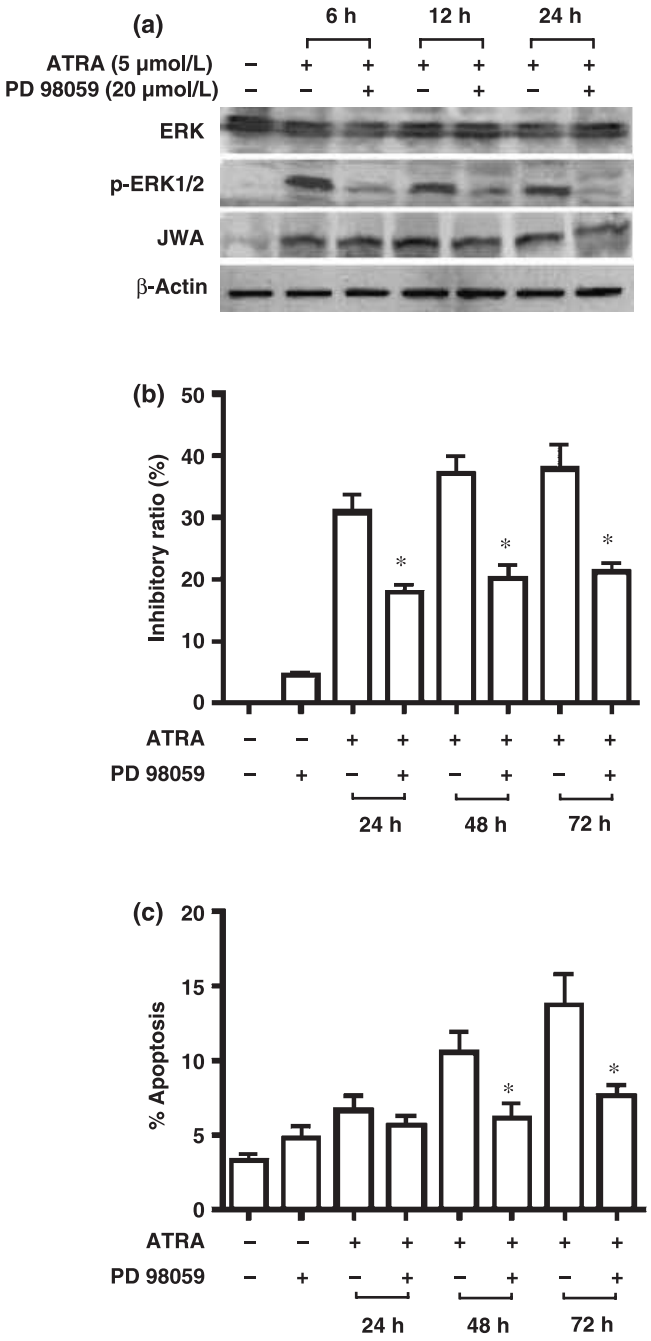

Fig. 2 All-*trans* retinoic acid (ATRA) activated extracellular signal-regulated kinase (ERK) phosphorylation and PD 98059 prevented the antiproliferartive or pro-apoptotic effects of ATRA in HeLa cells. HeLa cells were incubated for 12 h in the absence (–) or presence (+) of 20 μmol/L PD 98059. (a) After treatment with PD 98059, HeLa cells were stimulated with 5 μmol/L ATRA for 6, 12 or 24 h. Proteins were extracted and examined for the expression of total ERK, phosphorylated (p-) ERK1/2 and *JWA* by western blotting. (b) After treatment with PD 98059, HeLa cells were stimulated with 5 μmol/L ATRA for 24, 48 or 72 h. Cellular viability was determined using the MTT assay after treatment with 0.1% dimethylsulphoxide (DMSO; control) or the treatments indicated. The cell inhibitory ratio (%) was calculated using the formula $(1 - A_{\text{treated group}}/A_{\text{control group}}) \times 100$. (c) After treatment with PD 98059, HeLa cells were stimulated with 5 μmol/L ATRA for 24, 48 or 72 h. Cells were examined by the Hoechst 33258 assay. Apoptotic cells showed condensed, segregated chromatin revealed by Hoechst 33258 staining. Results represent the mean ± SD of three independent experiments. *$P < 0.01$ compared with control.

was induced by ATRA in a dose-dependent manner. As predicted, ATRA caused an increase in *JWA* expression in a dose-dependent manner (Fig. 1b). In the present study, the response of *JWA* to ATRA treatment seemed to be sensitive and ATRA-induced *JWA* expression could be detected even with concentrations as low as 0.05 μmol/L ATRA.

PD 98059 blocked ATRA-induced antiproliferartive or pro-apoptotic effects in HeLa cells

In order to determine whether the antiproliferative and pro-apoptotic effects of ATRA in HeLa cells were mediated by the MAPK signal pathway, especially by triggered cascades involved in phosphorylation events of down-stream molecules such as MEK and ERK, the MEK-specific inhibitor PD 98059 (20 μmol/L, 12 h) was used to block ATRA (5 μmol/L for 6, 12, 24 h)-induced ERK phosphorylation. Western blot analysis was performed to examine the expressions of total ERK and phosphorylated ERK1/2 (p-ERK1/2) proteins during this process. As shown in Fig. 2, ATRA induced significant phosphorylation of ERK1/2. The maximal phosphorylation of ERK1/2 was observed at the 6 h time point. PD 98059 effectively blocked phosphorylation of ERK1/2 in HeLa cells treated with ATRA, but had no effect on the expression of total ERK and JWA proteins.

To determine whether PD 98059 also blocked the antiproliferative and pro-apoptotic effects of ATRA in HeLa cells, we treated cells with ATRA (5 μmol/L) for 24, 48, 72 h with or without PD 98059 (20 μmol/L). As shown in Fig. 2b, treatment of cells with PD 98059 (20 μmol/L) alone for 12 h resulted in a slight, but not significant, antiproliferartive effect. Treatment with ATRA (5 μmol/L for 24, 48 and 72 h) induced significant antiproliferative effects in HeLa cells. The effects were effectively prevented by cotreatment with PD 98059 (20 μmol/L; $P < 0.01$). Similarly, the Hoechst 33258 staining assay indicated that PD 98059 partially blocked the pro-apoptotic effects of ATRA in HeLa cells ($P < 0.05$; Fig. 2c).

Effect of *JWA* on phosphorylation of ERK1/2 induced by ATRA in HeLa cells

Based on the above data, we speculated that *JWA* may be involved in the regulation of an ATRA-activated MAPK-associated signal pathway in HeLa cells. To test this hypothesis, *JWA*-overexpressing or -deficient HeLa cells were used to investigate whether *JWA* expression is required for ERK1/2 phosphorylation induced by ATRA treatment. As shown in Fig. 3, 0.05–10 μmol/L ATRA for 48 h induced the expression of phosphorylated ERK1/2 proteins in both vector-HeLa cells and *JWA*-HeLa cells. However, the effect was inhibited in as*JWA*-HeLa cells (Fig. 3).

JWA is required for ATRA-induced antiproliferative effects in HeLa cells

Because the antiproliferative effect of ATRA in HeLa cells was mostly dependent on activating the MAPK pathway and this process was further reliant on *JWA* expression, we predicted that *JWA* expression was required for the antiproliferative effects of ATRA. To confirm this, the MTT assay was performed. After HeLa cells had been treated with 0.05, 0.1, 0.5, 1, 5 and 10 μmol/L ATRA for 24, 48 and 72 h, a more significant antiproliferative effect was observed

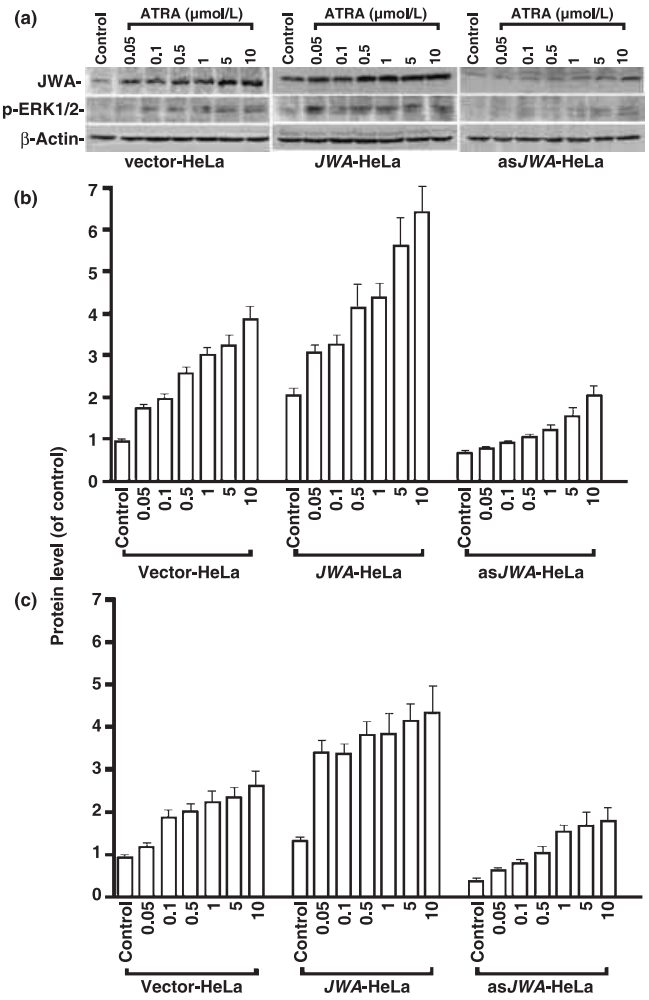

Fig. 3 All-*trans* retinoic acid (ATRA)-induced expression of JWA and phosphorylated (p-) extracellular signal-regulated kinase (ERK) proteins. *JWA*-HeLa cells, as*JWA*-HeLa cells and vector-HeLa cells were treated with 0.1% dimethylsulphoxide (DMSO; control) or with the indicated concentrations of ATRA for 48 h. Proteins were extracted and examined for expression of JWA and p-ERK1/2 proteins by western blot. The expression level of JWA proteins increased gradually with increasing concentrations of ATRA (0.05–10 μmol/L) in *JWA*-HeLa cells and vector-HeLa cells ($P < 0.05$). In as*JWA*-HeLa cells, ATRA-induced JWA expression was effectively inhibited and ERK phosphorylation (p-ERK1/2) was also blocked. β-Actin was used as an internal control for protein loading. Data are the mean ± SD ($n = 3$).

Table 1 Percentage apoptosis detected by the Hoechst 33258 assay for *JWA*-HeLa, as*JWA*-HeLa and vector-HeLa cells treated with all-*trans* retinoic acid (5 μmol/L) at 24, 48 and 72 h

		% Apoptosis after		
Group	Control	24 h	48 h	72 h
vector-HeLa	3.35 ± 0.45	5.30 ± 1.12	8.11 ± 2.23	11.03 ± 1.63
JWA-HeLa	5.82 ± 0.40	7.04 ± 0.45	11.56 ± 1.42	17.34 ± 0.93*
as*JWA*-HeLa	3.27 ± 0.57	3.28 ± 0.14*	3.80 ± 1.48†	4.79 ± 1.15†

Data are the mean ± SD from five samples. *$P < 0.05$, †$P < 0.01$ compared with the vector control.

Exponentially growing cultures of vector-HeLa cells, *JWA*-HeLa cells and as*JWA*-HeLa cells were treated with 0.1% dimethylsulphoxide (control) or 5 μmol/L all-*trans* retinoic acid (ATRA). After treatment for 24, 48 and 72 h, cells were examined by the Hoechst 33258 assay.

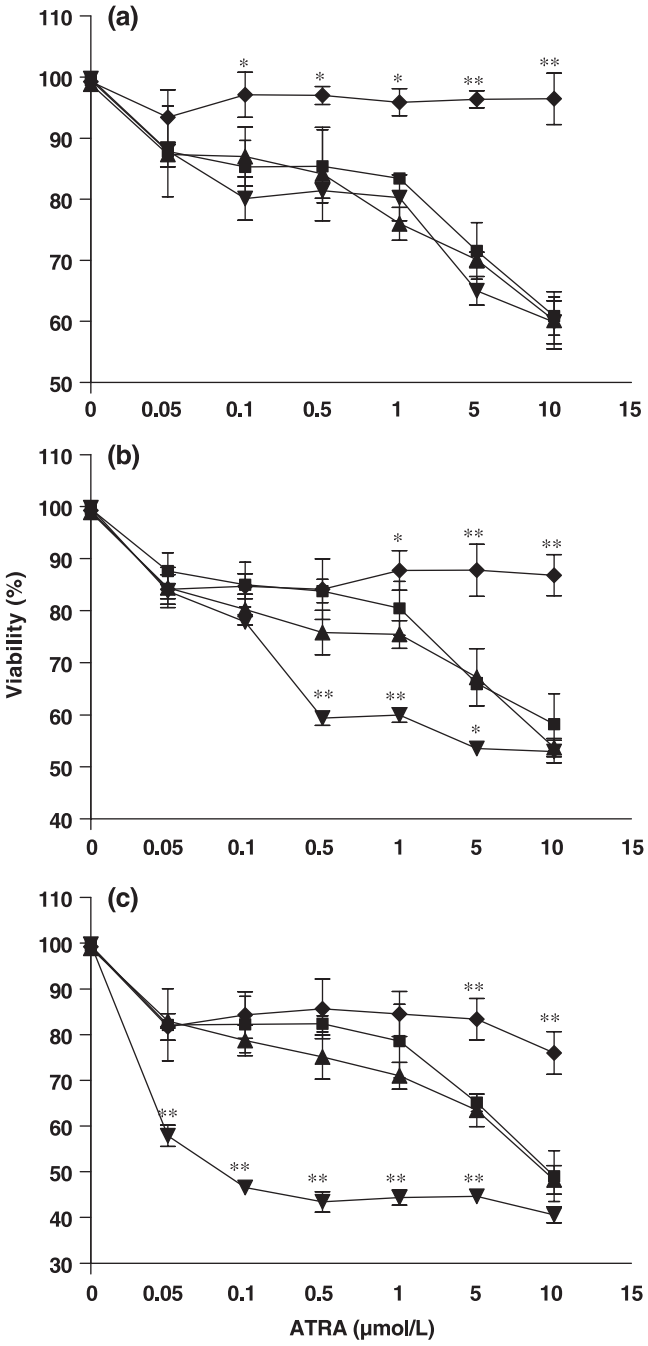

Fig. 4 Effects of all-*trans* retinoic acid (ATRA) on the viability of HeLa cells (■), vector-HeLa cells (▲), *JWA*-HeLa cells (▼) and as*JWA*-HeLa cells (◆). Cellular viability was determined using the MTT assay after treatment with 0.1% DMSO (*Control*) or the indicated concentrations of ATRA for the indicated time periods (24, 48 and 72 h, respectively). Cellular viability was expressed as percentage of control. Results are mean ± SD from four samples. Significant differences from control values are indicated by *$P < 0.05$, **$P < 0.01$.

in *JWA*-HeLa cells than in vector-HeLa cells and control HeLa cells when cells were exposed to ATRA at concentrations ranging from 0.05 to 5 μmol/L for 72 h ($P < 0.01$), and even at 0.5 μmol/L ATRA for 48 h ($P < 0.01$). In contrast, the as*JWA*-HeLa cells showed a resistance to ATRA treatment (Fig. 4). In addition, 10 μmol/L ATRA markedly inhibited cellular proliferation in all HeLa cell lines except in the as*JWA*-HeLa cells.

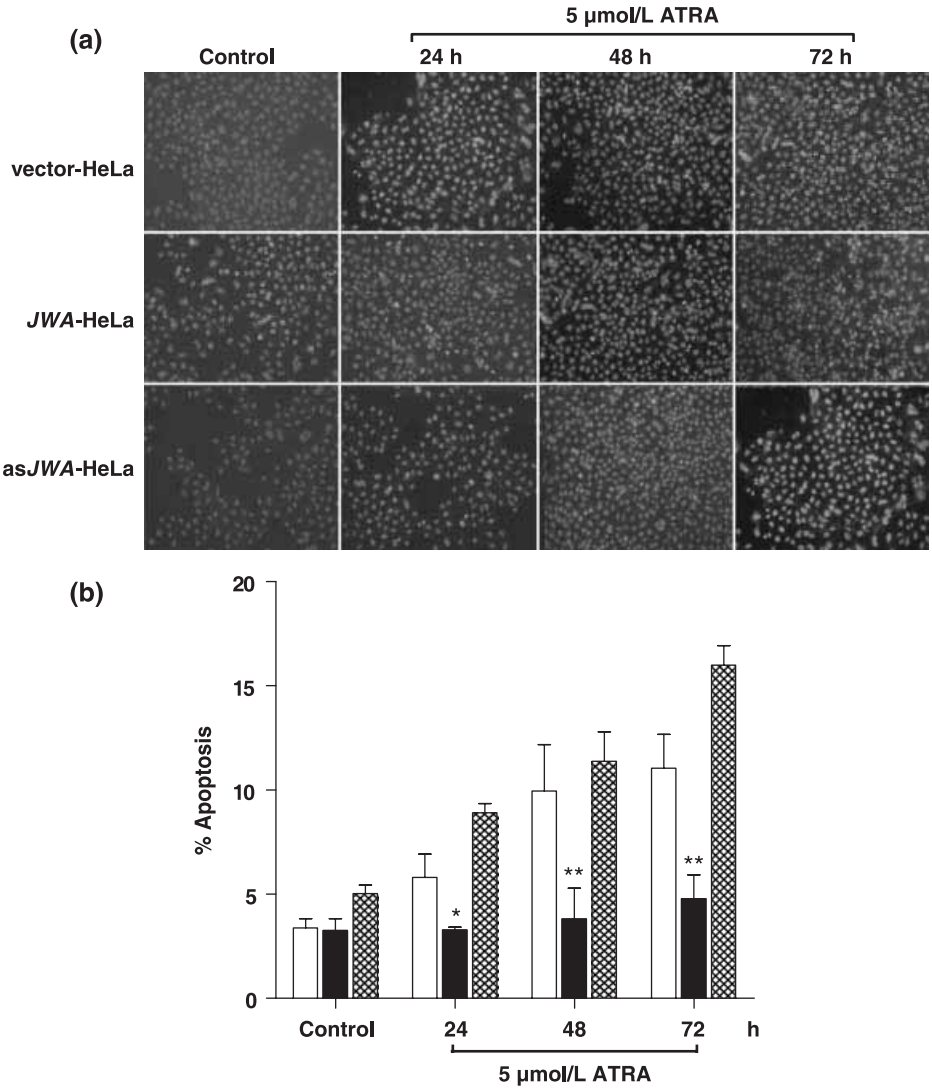

Fig. 5 Effects of *JWA* on ATRA-induced cell apoptosis. Exponentially growing cultures of vector-HeLa cells (□), *JWA*-HeLa cells (▨) and as*JWA*-HeLa cells (■) were treated with 0.1% dimethylsulphoxide (DMSO; control) or 5 μmol/L ATRA. After treatment for 24, 48 and 72 h, cells were examined using the Hoechst 33258 assay (a). Apoptotic cells showed condensed, segregated chromatin revealed by Hoechst 33258 staining (original magnification ×200). (b) Numbers of apoptotic cells were calculated. Results are the mean ± SD from five samples. *$P < 0.05$, **$P < 0.01$ compared with the vector control.

JWA is required for ATRA-induced apoptosis in HeLa cells

All-*trans* retinoic acid has been reported to induce apoptosis in human cancer cells.[6,8] To determine whether *JWA* is also involved in ATRA-induced apoptosis, both Hoechst 33258 staining and flow cytometry assays were conducted. As indicated in Table 1, 5 μmol/L ATRA treatment had a time-dependent pro-apoptotic role in *JWA*-HeLa cells and vector-HeLa cells; *JWA*-HeLa cells also showed a higher percentage of apoptosis than vector-HeLa cells ($P < 0.05$) after ATRA exposure (Fig. 5). In contrast, as *JWA*-HeLa cells almost lost their response to the ATRA-induced pro-apoptotic effect. In the present study, as the solvent control, DMSO did not have any significant effects on apoptosis. In addition, data from the flow cytometry assay were almost completely in line with those obtained using Hoechst staining (data not shown).

All-*trans* retinoic acid-induced *JWA* promoter transactivation in HeLa cells

As shown in Fig. 6a, two C/EBP sites were bioinformatically found in the 5′ flank region of the *JWA* gene. Some nuclear factors, such as C/EBP, have been reported to control a target gene's transactivation by binding with these CCAAT motifs.[13–15] To determine whether ATRA regulated transactivation of *JWA* through this mechanism, a reporter gene assay was designed using *JWA* CAT constructs (−1680PCAT^{wt}). In order to identify which promoter region(s) was responsive to ATRA, a series of reporter gene constructs with the 5′ deletion of promoter were used in the analysis. After transfection of the reporter plasmids into HeLa cells, cells were treated with 5 μmol/L ATRA for 48 h and CAT activity was then determined in cell extracts. The data revealed that ATRA had a substantial effect on *JWA* promoter activity. The region spanning from −194 to +107 bp showed the best response to ATRA ($P < 0.01$; Fig. 6b).

DISCUSSION

Retinoids are effective in suppressing tumour development in many animal models of carcinogenesis and are being evaluated in clinical trials for the prevention and treatment of cancers.[34,35] Retinoids are also known to regulate gene expression, leading to inhibition of cellular proliferation, induction of differentiation and apoptosis in a variety of cancer cells.[36–39] Whether *JWA*, as a newly identified

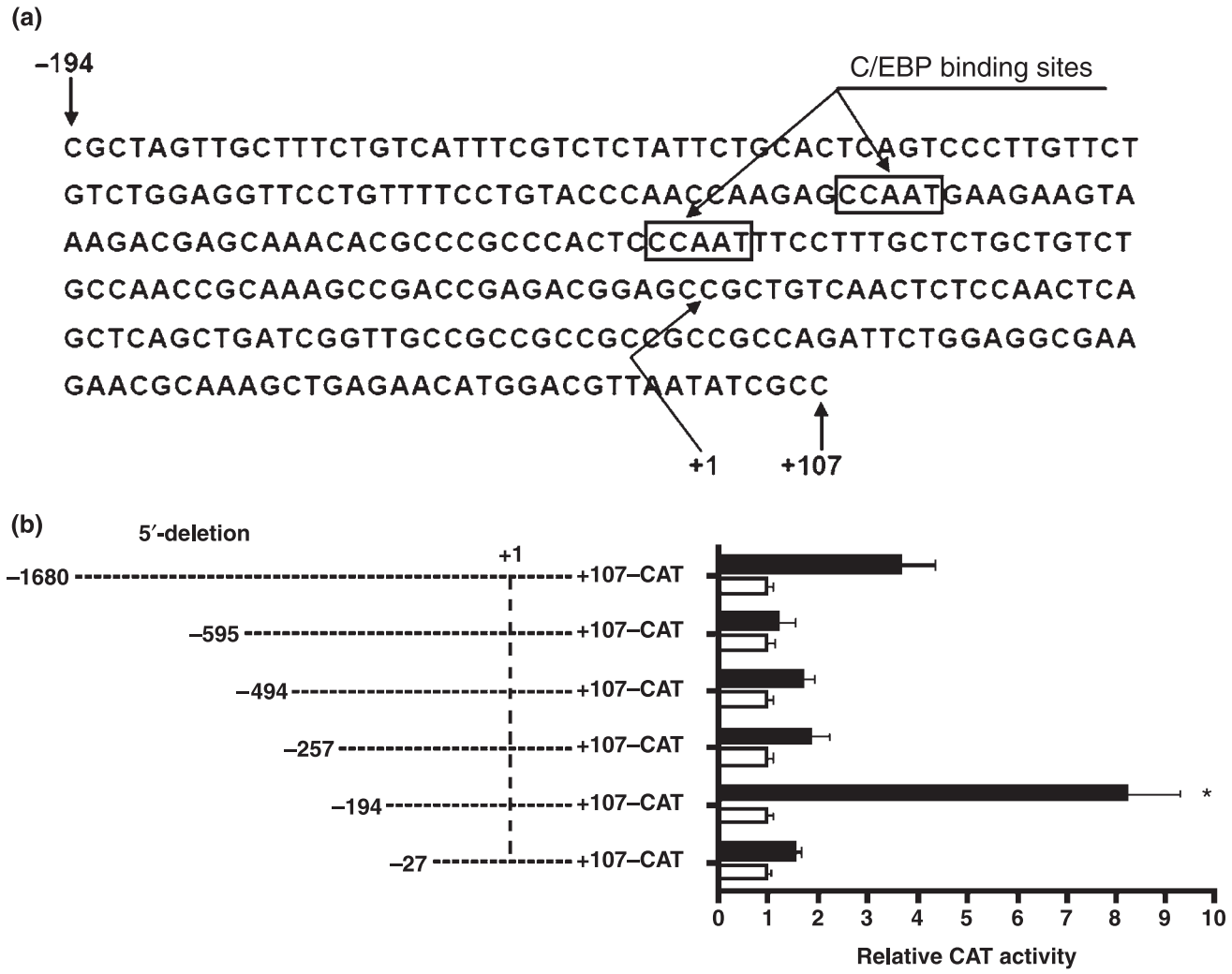

Fig. 6 Effects of all-*trans* retinoic acid (ATRA) on the transcriptional activity of *JWA* in HeLa cells. Transient transfection assays were performed with a chloramphenicol acetyl transferase (CAT) reporter plasmid containing series *JWA* promoter fragments. The CAT activity was determined in cells incubated with ATRA (5 μmol/L) for 48 h as described in the Methods. The distal sequence from –194 to +107 containing the CCAAT box element was able to confer responsiveness to ATRA (a). The lines and numbers indicate the *JWA* promoter sequence (in bp) contained in each promoter–reporter construct. The chart on the right side indicates the relative CAT activity level for each romoter-reporter constructs (b). (□), –ATRA; (■), +ATRA. All transient transfection studies were conducted in triplicate on three separate occasions. The chart reports the means of three experiments.

ATRA-responsive gene, is associated with cellular proliferation and apoptosis and its possible mechanisms of action remain unclear. In order to provide further molecular evidence for the antiproliferation and pro-apoptosis induced by ATRA, the role of *JWA* was investigated in the present study.

Although the actions of ATRA are known to be mediated by binding to nuclear receptors (RAR), increasing evidence indicates that nuclear receptors can mediate extragenomic effects that stimulate signalling pathways by mechanisms that are still not well defined and are independent of receptors but are involved in binding to DNA response elements.[40] The results of present study provide interesting new insights for the understanding of the regulatory mechanisms of the antiproliferation and pro-apoptosis caused by ATRA in HeLa cells. All-*trans* retinoic acid increased the expression of JWA proteins in a dose- and time-dependent manner in HeLa cells and also stimulated transactivation of *JWA* via a 5′ flank region. The antiproliferative or pro-apoptotic effects of ATRA also relied on upregulated *JWA* expression in HeLa cells. In contrast, the *JWA*-deficient HeLa cells were obviously resistant to ATRA exposure. This evidence suggests that increased intracellular expression of *JWA* appears to be important for the processes of cell proliferation and apoptosis triggered by ATRA in HeLa cells.

The present study has shown that the effect of ATRA on cell growth, differentiation and apoptosis is dependent on the concentration of and exposure time to ATRA.[6,41,42] A high concentration (10 μmol/L) of ATRA and a relatively long exposure time is required to induce cell apoptosis.[43] In the present study, we also found that when cells were exposed to ATRA at a high concentration (10 μmol/L), a similar inhibition of proliferation was observed between vector-HeLa cells and *JWA*-overexpressing HeLa cells. However, ATRA at a low concentration (0.05–1 μmol/L) markedly inhibited the proliferation of *JWA*-overexpressing HeLa cells compared with HeLa cells or vector-HeLa cells. Previous studies have also demonstrated that *JWA* may be involved ATRA-induced differentiation in

several leukaemia cell lines.[30–33] Therefore, we speculate that the mechanism of upregulated *JWA* expression involved in the antiproliferation may be partly through the induction of differentiation following treatment of HeLa with lower concentrations of ATRA, thus inhibiting cell proliferation, whereas *JWA* may be involved in apoptotic pathways in HeLa cells induced by high concentrations of ATRA.

Unlike many other cytotoxic agents that rapidly induce cell damage and cell death, we found that ATRA-induced apoptosis in HeLa cells seemed to require a relatively high concentration (5 μmol/L). At 5 μmol/L, ATRA caused a time-dependent induction of apoptosis in *JWA* overexpressing HeLa cells, which had a higher apoptosis rate than vector-HeLa cells. These results further indicate that upregulated *JWA* may be involved in the pro-apoptosis induced by ATRA at high concentrations (5 μmol/L) in HeLa cells. Furthermore, the findings show that the antiproliferative or pro-apoptotic effects of ATRA are prevented in *JWA*-deficient HeLa cells. These results further suggest that *JWA*, through an interaction with the ERK pathway, plays an important role in the control of the susceptibility of cells to ATRA-induced differentiation and apoptosis. We are working on the proliferative and apoptotic mechanism to further confirm this hypothesis.

All-*trans* retinoic acid may also exert its effects via a non-receptor pathway by activating nuclear factors, such as C/EBP.[11–16] Because the *JWA* proximal promoter region does not contain RARE but does contains two CCAAT boxes, which C/EBP may bind to, the expression of *JWA* may be mediated by ATRA-activated C/EBP, which binds to CCAAT boxes of the *JWA* promoter, and this may critical for the antiproliferation and pro-apoptosis role of ATRA; however, direct evidence of this needs to be provided.

The MAPK signal pathway is obviously activated in a variety of cell types by diverse extracellular stimuli and participates in a wide range of cellular programmes, including proliferation, differentiation and apoptosis.[44–47] Despite several studies have shown that ATRA activates MAPK pathways in different cellular models,[18,19,48] the precise mechanism and the context of signal molecules by which ATRA regulates these pathways remain obscure. In the present study, we found that ATRA-activated phosphorylation of ERK was necessary for its antiproliferative and pro-apoptotic effects in HeLa cells. *JWA* expression seems vital to this MAPK phosphorylation-based context linkage. In fact, ATRA-induced antiproliferative and pro-apoptotic effects via ERK phosphorylaion could be almost completely blocked in *JWA*-deficient HeLa cells, suggesting that *JWA* functions as a key molecule in this context. Similarly, *JWA* partially affected ATRA-induced antiproliferation and pro-apoptosis. This evidence further indicates that ATRA-activated ERK phosphorylation may be dependent mostly on *JWA*. Therefore, *JWA* may play an important role in the ATRA-triggered MAPK signal transduction pathway. In addition, although PD 98059 could effectively inhibit ERK phosphorylation, it failed to affect *JWA* expression.

In conclusion, *JWA* may act as a novel regulator of the MAPK signalling pathway, functionally involved in the antiproliferation and pro-apoptosis caused by ATRA in HeLa cells and via a non-receptor mechanism. Although the potential clinical applications of *JWA* expression in tumour cells need to be investigated further, modification of *JWA* expression in tumour cells may be beneficial as a adjuvant chemotherapy for patients with carcinomas.

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