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INHIBITORY EFFECTS OF DIARSENIC TRIOXIDE (As2O3) ON HEPATOCELLULAR CARCINOMA CELLS EXERTED BY REGULATION OF PROMYELOCYTIC LEUKEMIA PROTEIN LEVELS

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Abstract

Previous Chinese research revealed that diarsenic trioxide (As_2O_3) inhibits acute promyelocytic leukemia (PML) cell proliferation and initiates apoptosis through degradation of the PML-retinoic acid receptor protein. This study was to analyse whether As_2O_3 also had an effect on hepatocellular carcinoma (HCC) cells. As_2O_3 effects on various HCC cell lines and primary HCC cells were investigated in time and dose series, including measurements of cell growth, PML mRNA and protein expression, xenografted tumor formation, and the self-renewal Oct4 and hepatocyte marker expressions in mouse model xenografts or cells treated with PML siRNA. The results were analyzed by immunocytochemistry, quantitative reverse transcription PCR and western blotting as well as indocyanine green and Periodic Acid Schiff staining. As_2O_3 inhibited HCC cell and HCC cell-derived xenograft tumor formation in a time-dependent manner and reduced PML protein expression in HCC cells, but had limited effects on PML mRNA levels in cell nuclei. The HCC cell line HuH7 treated with As_2O_3 showed a decreased expression of alpha-fetoprotein and increased expression and transcription of mature hepatocyte markers, indicating differentiation of HCC cells into hepatocytes. Cytokeratin 18 protein and mRNA levels as well as tyrosine aminotransferase and apolipoprotein B mRNA transcriptions were enhanced by As_2O_3 as were the numbers of indocyanine green and Periodic Acid Schiff stained cells. In addition, As_2O_3 downregulated the expression of *Oct4*. In conclusion, since As_2O_3 inhibited HCC cell proliferation and HCC cell-derived xenograft tumor formation it is suggested that an appropriate concentration of As_2O_3 might be a promising therapy to treat HCC.

Keywords: As_2O_3. Hepatocellular carcinoma. Promyelocytic leukemia (PML). Transcription factor 4.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the highest mortality cancers in the world and is the fifth most common malignancy (Golabi et al. 2017). According to a report of 2013, about 45.7% of HCC cases in China were attributed to hepatitis B virus infection and 37.8% to viral hepatitis C infections (Wang et al. 2017). Unfortunately, most *cases of* HCC present in the clinic after the disease has markedly progressed, and surgical resection rates are below 50% (Ferlay et al. 2015; Torre et al. 2015). Postoperative relapse *and* metastasis are common in HCC patients, with the survival rate of patients typically being 10-30% after 5 years (Ferlay et al. 2015; Torre et al. 2015).

The main genetic abnormality in most acute promyelocytic leukemia (APL) patients is an aberration of chromosome t (Alimoghaddam 2014). In 95% of APL cases, promyelocytic leukemia (PML) protein is involved in the production of a PML-retinoic acid receptor alpha (PML-RaRα) oncoprotein, which contributes to the development of APL by blocking the differentiation of granulocytes and through other mechanisms as well (Yoshida et al. 1996). Disruption of this fusion gene or its signaling pathways could potentially inhibit the progression of APL.

Diarsenic trioxide (As_2O_3) has been shown to cure APL in about 95% of cases treated in China (Jeanne et al. 2010; Zhang et al. 2010). A number of suggestions have been put forward to explain the mechanism(s) of action of As_2O_3 on APL cells. For example, sumoylation is triggered when arsenic binds to PML protein, which initiates the degradation of PML-RaRα, leading to the apoptosis of leukemia-initiating and APL cells (Jeanne et al. 2010; Zhang et al. 2010). Higher concentrations of As_2O_3 (0.5-2.0 mmol/L) can induce apoptosis through direct cytotoxic effects, or indirectly by actions on a number of pathways that regulate the activity of leukemic cells (Woo et al. 2002; Alimoghaddam 2014). The anti-angiogenesis effects of As_2O_3 are also considered to be important during leukemia transformation (Alimoghaddam et al. 2006). Previous studies have found that the dissociation of arsenite into arsenic (III) ions triggers cell apoptosis and inhibits the growth and development of HCC cells (Liu et al. 2011; Qu et al. 2011; Wei et al. 2014). However, identifying the exact mechanisms involved in reducing the survivability and proliferation of HCC cells requires further investigation. We have speculated that As_2O_3 may induce differentiation and inhibition of HCC cell proliferation through interaction with PML protein. The aims of our research were to determine whether As_2O_3 affects PML protein expression levels in HCC cells and to establish the relationship between As_2O_3 treatment and its inhibiting effect on HCC cells through PML protein.

2. Material and Methods

Cell lines

P19 cells (ATCC, Virginia, USA) were grown inα-MEM medium containing 2.5% FBS, 7.5% calf serum, and 1% streptomycin-penicillin in a cell culture incubator with 5% CO2 and 37 °C temperature. HuH7, HepG2, Hep3B and SMCC-7721 were used as hepatocarcinoma cell lines to observe whether As_2O_3 affects the morphological and functional changes of these hepatocarcinoma cells lines while L02 human hepatocytes were used as control. All hepatic cell lines were provided as a gift from the Southwest Center for Cancer Research (Chongqing, China) in China. HepG2, Hep3B, HuH7, L01 and the endocervical adenocarcinoma SMMC-7721 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY, USA) containing streptomycin-penicillin (1%) and 10% fetal bovine serum. Human acute promyelocytic leukemia cell lines HL60 and NB4 were purchased from the Medical University of Chongqing and cultivated in 10% fetal bovine serum containing RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA).

Collection and processing of HCC specimens

Thirty-two HCC specimens randomly obtained from 205 patients who had liver resections from June 1, 2013 to June 20, 2017 in 3 hospitals of the Third Army Medical University were analyzed. All patients provided written informed consent. Fresh tumor specimens were cut into 1 mm^3 pieces and immersed into liquid with Liberase for 5-10 min in cell culture incubator. After passing the supernatant from the specimen through a 100 μM cell filter, the cell suspension was obtained. The protocol used in this study was approved by the Ethics Committee of the Yongchuan Hospital of the Chongqing Medical University.

Cell sorting and cell transfection

Control and HuH7 cells were treated with anti-human CD133, anti-human CD13, and conjugated monoclonal antibodies, at 4°C for 30 min. A flow cytometer (Accuri C6, BD Biosciences, San Jose, CA, USA) and CFlow software (allophycocyanin (APC) fluorescence 630 nm, phycoerythrin (PE) fluorescence 488 nm)

(BD Biosciences, San Jose, CA, USA) were used for flow cytometry analysis. For siRNA and ectopic expression of PML protein experiments, siRNAs and cytomegalovirus - *promyelocytic leukemia* protein (CMV-PML) vectors, as well as their controls (scrambled siRNA and empty CMV vector), were transfected into cells using HiPerfect transfection reagent (QIAGEN, Duesseldorf, Germany) for a total of 96 h. PML protein-silencing RNAs (PML siRNA) were used in the experiments (sc-36284; Santa Cruz Biotechnology, CA, USA):

5'-UCUGGGUCUCAAUGGCUUUCC-3'
5'-AAAGCCAUUGAGACCCAGACC-3'
5'-GCUGUUCUUCGUAGUGUAUUU-3'
5'-AUACACUACGAACAACAGCUU-3'

The changes in the expression levels of the indicators were measured by Western blotting, and the relative transcription rates of the relevant indicators in the total RNA of cells was detected by the qRT-PCR method.

Cell proliferation assays

Hepatocarcinoma cell lines HepG2, Hep3B, HuH7, hepatic cell line L01 and the endocervical adenocarcinoma SMMC-7721 cell assays were carried out on 96-well microtiter plates containing high-glucose/DMEM culture medium. The cell proliferation assessment was used by a Cell-Counting Kit-8 purchased from Dojindo Laboratories in Japan. For proliferation assays, 3×10^3 cells were seeded into each well and As_2O_3 concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 µg/mL were added to appropriate wells as required. The degree of proliferation was measured every 24 h for 1 week after drug exposure. For each dilution, 3 independent measurements were performed in triplicate at each time point.

Immunohistochemistry and immunofluorescence

A cryostat was used to cut 4 µm-thick specimens, which were fixed for 15 min in 4% paraformaldehyde. After 1 h of blocking, sections were exposed to appropriate antibodies in a humidified chamber (*vide supra*) overnight at 4°C. Sections of human HCC specimens were embedded in paraffin and immunohistochemical analysis was carried out to detect PML protein using primary antibodies raised to detect PML protein (sc-5621, Santa Cruz Biotechnology, CA, USA). Immunofluorescence was performed using primary antibodies generated against PML protein; Hoechst 33342 (ThermoFisher Scientific, MA, USA) was used to stain cell nuclei.

Western blotting analyses

Western blot analysis was carried out as previously described (Chen et al. 2017), utilizing specific primary PML protein antibodies (sc-5621, Santa Cruz Biotechnology, CA, USA), cytokeratin 18 (CK18) (sc-32329, Santa Cruz Biotechnology, CA, USA), alpha-fetoprotein (AFP) (sc-8399, Santa Cruz Biotechnology, CA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-32233, Santa Cruz Biotechnology, CA, USA), β-actin (sc-47778, Santa Cruz Biotechnology, CA, USA), Octamer-binding transcription factor 4 (Oct4) (ab18976, Abcam, Cambridge, UK) and albumin (ALB) (sc-271605, Santa Cruz Biotechnology, CA, USA). Briefly, cell lysates were spun for 10 min at 12,000 × g at a temperature of 4°C. Next, the supernatant was harvested to measure the protein concentrations using a BCA assay kit (Pierce, Promega, Madison, WA, USA). Fifteen-µg aliquots of protein were ran on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation, relocated to polyvinylidene fluoride membranes, and incubated with primary antibodies, then with HRP-conjugated secondary antibodies. Specific proteins were detected by Western blot reagents (ECL) (Santa Cruz Biotechnology, CA, USA). Internal controls were GAPDH and β-actin expressions and 3 independent measurements were performed in triplicate.

Assay of tumor spheres

Six-well, ultra-low attachment, culture dishes (Corning) were used to seed the cells in medium containing no serum. The DMEM/F12 (1:1) medium contained the following additives: 1% streptomycin-penicillin, 1% sodium pyruvate, 2% stem supplement, epidermal growth factor (20 ng/mL), L-glutamine (2 mM), B27 supplement (2%), basic fibroblast growth factor (10 ng/mL), insulin (5 µg/mL), and heparin (50 ng/mL). A total of 5,000 cells per well were cultured, and maintained at 37°C in a humidified atmosphere (including 5% CO_2). After culturing the cells for 7 to 15 days, the numbers of spheres were counted and photographed with the aid of a light microscope (Nikon, Japan).

***In vivo* tumorigenesis assay**

In vivo xenograft assays were carried out as previously described (Haraguchi et al. 2010). This study received approval from the Medical Ethics Committee of Chongqing Medical University and was in line with the Guide for the Care and Use of Laboratory Animals developed by the Chongqing Medical University. In this study, nude mice (ages 4-5 weeks) raised in a sterile, constant-temperature, ventilated feeding cabinet, obtained from the animal center of Daping Hospital Affiliated Third Military Medical University (temperature 25-26°C, humidity 40-60%) were used for the *in vivo* assay. First, 0.1 mL of cell suspension (primary HCC and the HuH7 cell line) at final concentrations of 1.0×10^6 /mL) was injected into the ventral forelimb of nude mice subcutaneously, and then every other day with 2 to 3 µg of As_2O_3 prepared on phosphate-buffer saline (PBS, Gibco, MD, USA) using 100 µL to 150 µL of 0.10 mM As_2O_3 stock solution, subcutaneously (7 injections in total). As_2O_3 stock solutions (0.10 mM) were prepared using phosphate-buffered saline (PBS, Gibco, MD, USA) and kept at -20°C. Stock solutions were serially diluted with PBS to the required final concentration immediately prior to each experiment. The control group received only saline injections. Post-transplantation, from the date of injection, each mouse was thoroughly examined by ultrasound, using a scanner fitted with a 10 MHz transducer (Sequoia 512, Acuson, Mountain View, CA, USA) for signs of tumor growth for 30-60 days. The mice were sacrificed by cervical dislocation.

Quantitative RT-PCR

The RNA contained in HCC cells and tissue was extracted using Trizol reagent. RT-PCR was carried out on the total RNA with a BioRT cDNA synthesis kit (first strand: BSB09M1). For RT-PCR analysis, Promega GoTaq qPCR Master Mix (A60012; Promega, Madison) was used, and PCR was performed on a STRATAGNE mx3000P Stratagene thermocycler. The relative values were normalized to GAPDH and presented as Ct methods (Liu et al. 2011). For each determination, 3 independent measurements were performed in triplicate. Table 1 shows the PCR primers used in the amplification process.

Table 1. Primers of the target genes (Invitrogen).

Genes	Primers
PML protein	F: 5'-GGCTCGAGAAGGATGTGGTC-3' R: 5'-GAAGTGAGGGCTCCCATAGC-3'
AFP	F: 5'-ACGAGGAAAGCCCCTCAG-3' R: 5'-GCCATTCCCTCACCACAG-3'
ALB	F: 5'-CCAGACATTCCCCAATGC-3' R: 5'-CAAGTTCCGCCCTGTCAT-3'
CK18	F: 5'-GCCATTCCCTCACCACAG-3' R: 5'- ACAGAGCCACCCCAGACA-3'
TAT	F: 5'-ACCTTCAATCCCATCCGA-3' R: 5'-TCCCGACTGGATAGGTAG-3'
ApoB	F: 5'-CATGTGATCCCCACAGCA-3' R: 5'-TCCCAGGACCATGGAAAA-3'
GAPDH	F: 5'-CCCACTCCTCCACCTTTGAC-3' R: 5'-CCACCACCCTGTTGCTGTAG-3'

AFP, alpha fetoprotein; ALB, albumin; ApoB, apolipoprotein B; CK18, cytokeratin 18; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PML, promyelocytic leukemia; TAT, tyrosine aminotransferase.

Indocyanine green (ICG) and periodic acid-Schiff (PAS) staining test

After 6 to 9 days of treatment with 0.5 μg/mL of As_2O_3, HuH7 cells were washed 3 times with PBS, 1 mg/mL of ICG 200 μL (a fluorescent dye for photometric hepatic function diagnostics) was added, and cells were maintained for 60 min at 37°C in a humidified atmosphere (plus 5% CO_2 (Ishizawa et al. 2014). Finally, PAS staining was performed using the periodic acid-Schiff test (Ikeda et al. 2014).

Statistical analyses

Data analysis was conducted using SPSS ver.13.0, (SPSS Inc., USA). Data are presented as mean ± SD, and a one-way ANOVA methos was used to analysis for groups comparisons. $P < 0.05$ was considered statistically significant between groups.

3. Results

Effects of As_2O_3 on HCC cell proliferation

In this study, As_2O_3 was proved that have an inhibitory effect on SMMC-7721, HuH7, HepG2, and Hep3B cell growth after they were exposed to 0.1–1.0 μg/mL of As_2O_3 for 2, 3, and 4 days, determined by measuring the proliferation of the 4 cell lines using cell-counting Kit 8. The inhibitory actions of As_2O_3 on the 4 cell lines were both dependent on the concentration and the time of exposure, but the effects were diverse.

Briefly, a significant inhibiting effect of As_2O_3 on SMMC-7721 cells was detected at a low concentration of 0.2 μg/mL for 96 h of treatment ($P < 0.05$), as well as 0.4 μg/mL for 48 h ($P < 0.01$) (Figure 1A). For HepG2 and Hep3B cells, 0.4 μg/mL of As_2O_3 for at least 72 h was essential to significantly inhibit cell growth ($P < 0.01$) (Figure 1 B, C). HuH7 cells were found to be more resistant to As_2O_3 treatments, since a concentration of at least 0.8 μg/mL for at least 72 h was the threshold level that significantly inhibited cell growth (Figure 1 D).

As_2O_3 inhibits tumor sphere formation and xenograft tumors

The xenograft tumor assay revealed that only 1 mouse formed xenograft tumors in the HuH7 cell group (1/10) after injection with As_2O_3. No mice formed xenograft tumors in the primary HCC cell group (0/10). These findings revealed that As_2O_3 inhibits the *formation* of HuH7 cell-derived and primary HCC cell-derived xenograft tumors (Table 2).

Nude mice were injected subcutaneously with As_2O_3 at doses of 3-5 μg every other day for 7 consecutive treatments.

Table 2. Effects of As_2O_3 on xenografted hepatocytes in nude mice.

Name of xenografted cells	Number of mice	Number of tumors in the control group	Number of tumors in the treatment group
HuH7 cells	20	19	1
Primary HCC cells	20	16	0

As_2O_3 induced the maturation and differentiation of HuH7 cells

Primary HCC cells and HuH17 cells developed tumor spheres after 14 and 9 days, which could be effectively inhibited by treatment with As_2O_3 (0.5 μg/mL) (Figure 2A).

Compared with control cells, HuH7 cells exposed to 0.5 μg/mL As_2O_3 treatment (0.5 μg/mL) significantly enhanced the transcriptions of ALB, CK18, tyrosine aminotransferase (TAT), and ApoB mRNAs, as well as the expressions of ALB and CK18 proteins, whereas AFP transcriptions and expressions were significantly reduced (Figure 2 B, C) which showed differentiation of HCC cells into hepatocytes because AFP was recognized as a molecule marker produced by HCC cells. The degrees of ICG- and PAS-stained

HuH7 cells significantly increased after 10 d exposure to 0.5 μg/mL As_2O_3 (Figure 2D, 2E). These results indicate that As_2O_3 treatment induced HuH7 cell maturation and differentiation.

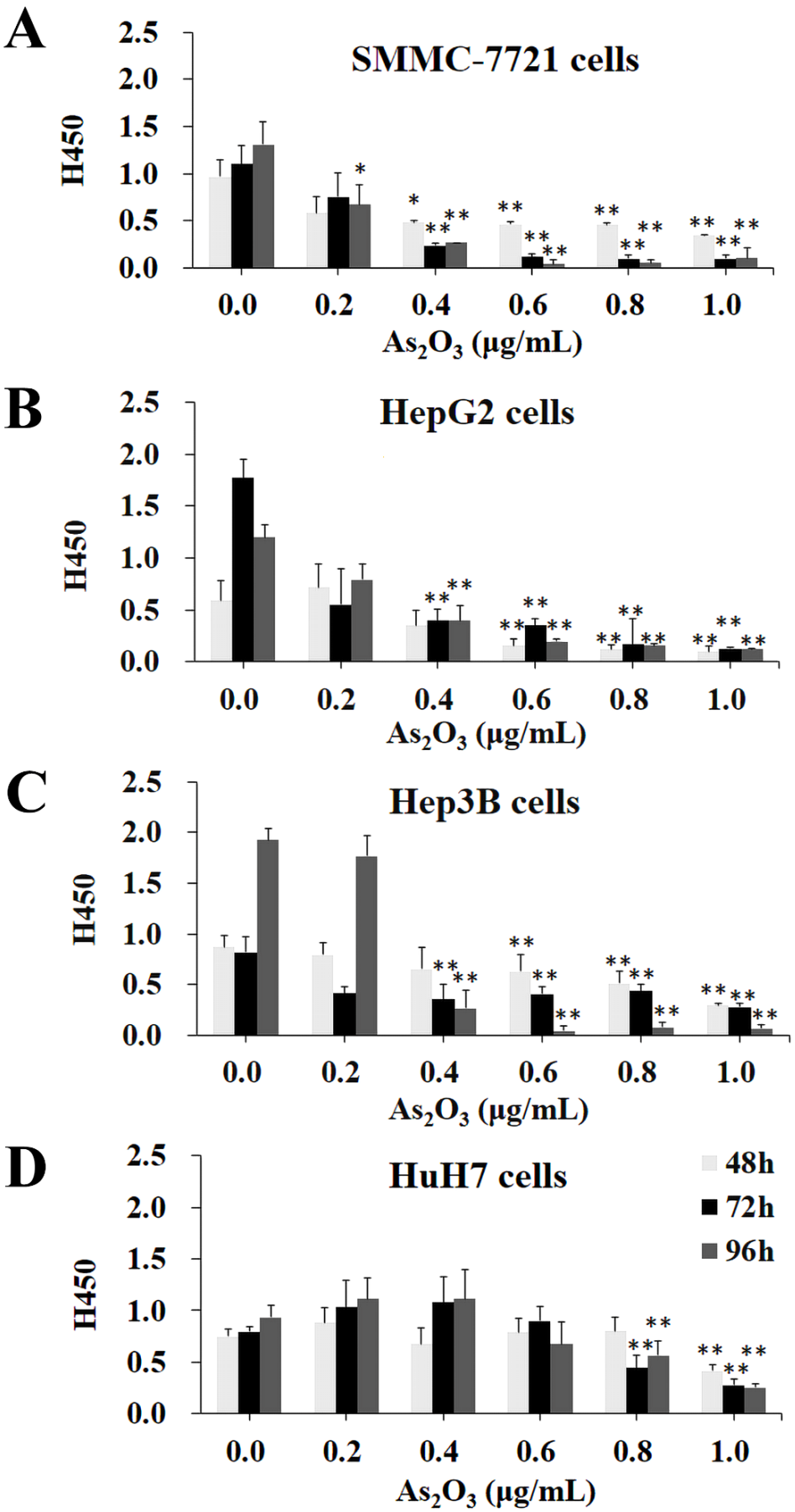

Figure 1. The inhibitory effects of As_2O_3 (0.1–1.0 μg/mL) on the proliferation of A - SMMC-7721, B - HepG2, C - Hep3B, and D - HuH7cells. Note: $^{*}P < 0.05$, $^{**}P < 0.01$. The reference for comparison with each cell line was 0.0 μg/mL As_2O_3).

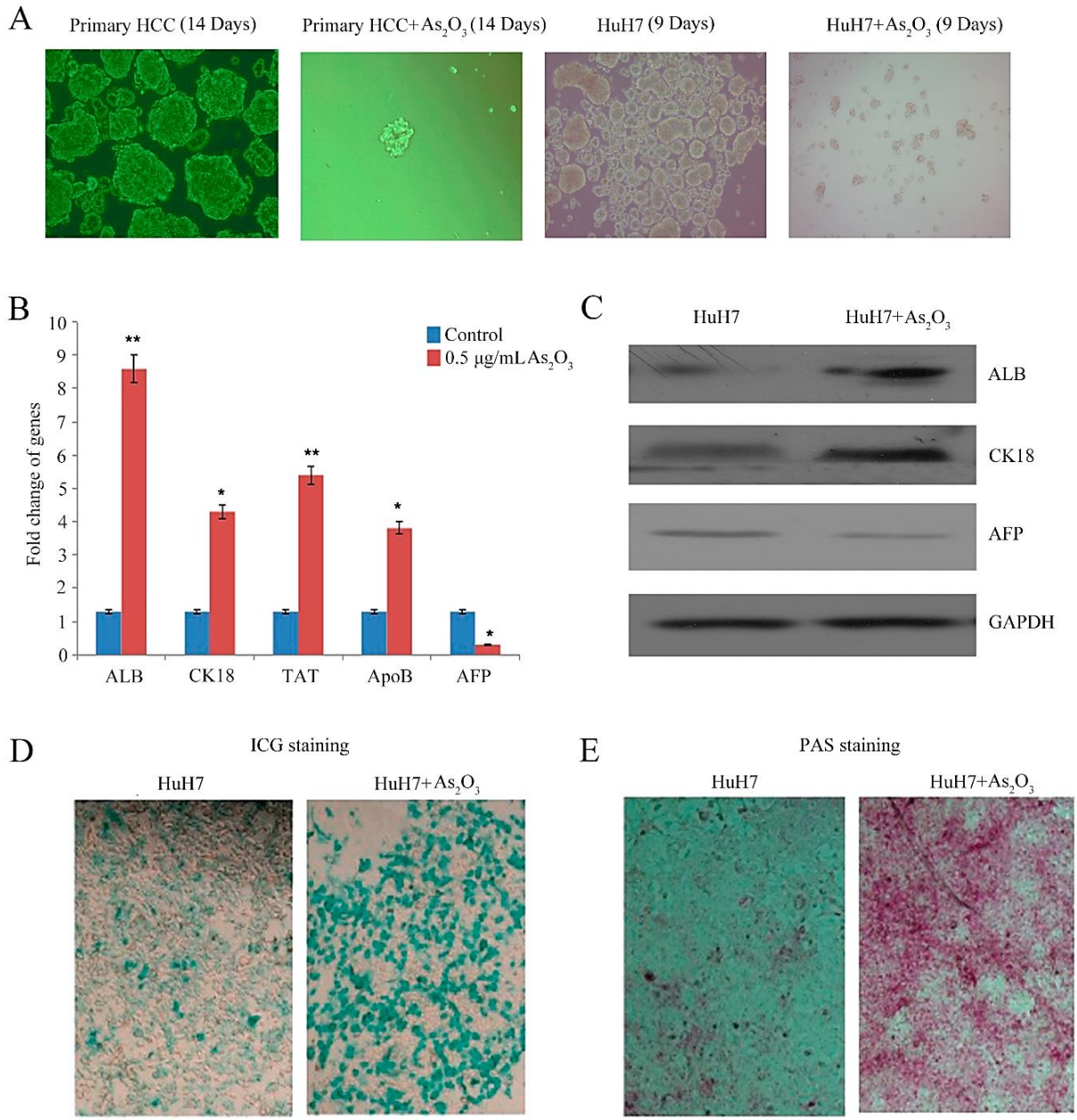

Figure 2. Effect of As_2O_3 on cell growth, transcription and expression of hepatic markers, metabolism and functionality of HuH7 cells. A - Tumor sphere formation assay of HuH7 cells cultured with or without 0.5 μg/mL As_2O_3 (9 days), and primary HCC cells cultured with or without 0.5 μg/mL As_2O_3 (14 days). B - RNA transcriptions of hepatic-specific markers in As_2O_3-induced HuH7 cells detected by RT-PCR (*$P < 0.05$; **$P < 0.01$ versus the control). C - Protein expression of hepatic-specific markers in As_2O_3-induced HuH7 cells detected by western blotting. D - ICG uptake and E - glycogen storage function of As_2O_3-induced HuH7 cells (original magnification: × 100).

The relative protein expression of PML in human tissue specimens and HCC cells

Patients with HCC (n = 205) were divided into well-, moderately- and poorly differentiated groups. Five HCC tissue samples in every group were selected randomly to undergo immunohistochemical analysis for PML protein. Figure 3A shows that PML protein was expressed in the nuclei of tumor tissue samples in the 3 groups but at varying levels. The PML protein gene was also transcribed in all of the 4 cell lines examined (Figure 3B), as well as in all samples of the 32 cases of HCC examined (Figure 3C).

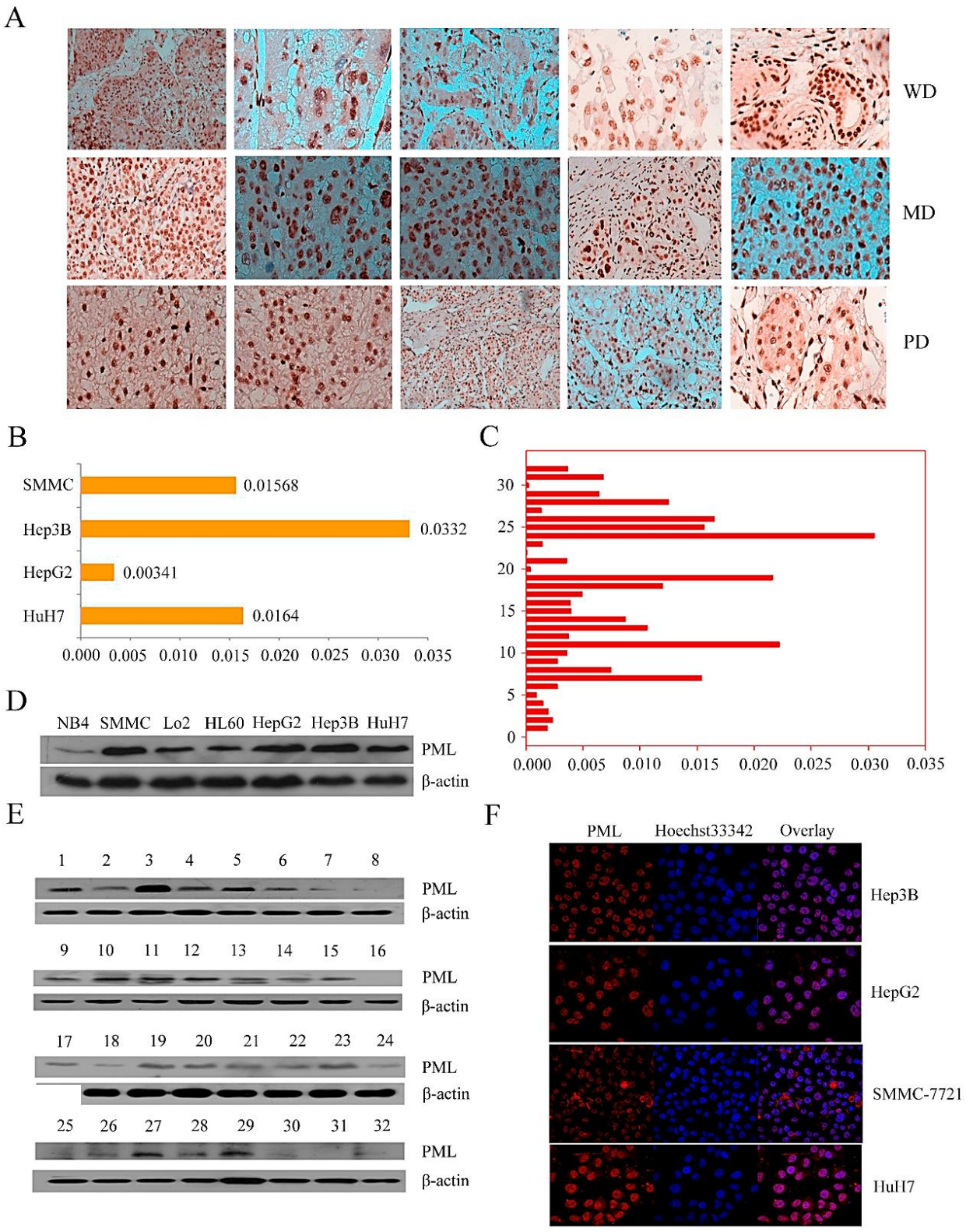

Figure 3. Expression levels of PML protein in HCC specimens and HCC cell lines. A - Expression of PML (red stain) in well- (WD, n = 5), moderately- (MD, n = 5), and poorly-differentiated (PD, n = 5) specimens using immunohistochemical techniques. B - mRNA expression of PML protein in SMMC-7721, Hep3B, HepG2, and HuH7 cells. C - mRNA transcription of PML protein in tumor specimens obtained from HCC cases (n = 32) using qRT-PCR. D - The PML protein in SMMC-7721, HepG2, Hep3B, HuH7 cells, as well as in NB4 (positive control), HL60 (positive control), and L02 cells (human hepatocyte control) cells. E - PML expression in tumor tissue specimens of HCC (n = 32). F - PML expression in SMMC-7721, HepG2, HuH7, and Hep3B cells using immunofluorescence.

Western blot analysis revealed that all SMMC-7721, HuH7, Hep3B and, HepG2 cells (Figure 3D) and cells from the 32 tumor samples expressed PML protein to various degrees (Figure 3E). To exclude false-positive results, we used HL60 and NB4 cells as the positive PML protein controls, whereas L02 cells served as human hepatic cell control in Figure 3D. The expression of PML protein visualized by immunofluorescence in SMMC-7721, HuH7, Hep3B, and HepG2 cells are shown in Figure 3F. PML protein was particularly expressed in the nuclei of all 4 cell lines examined and displayed a punctate nuclear distribution.

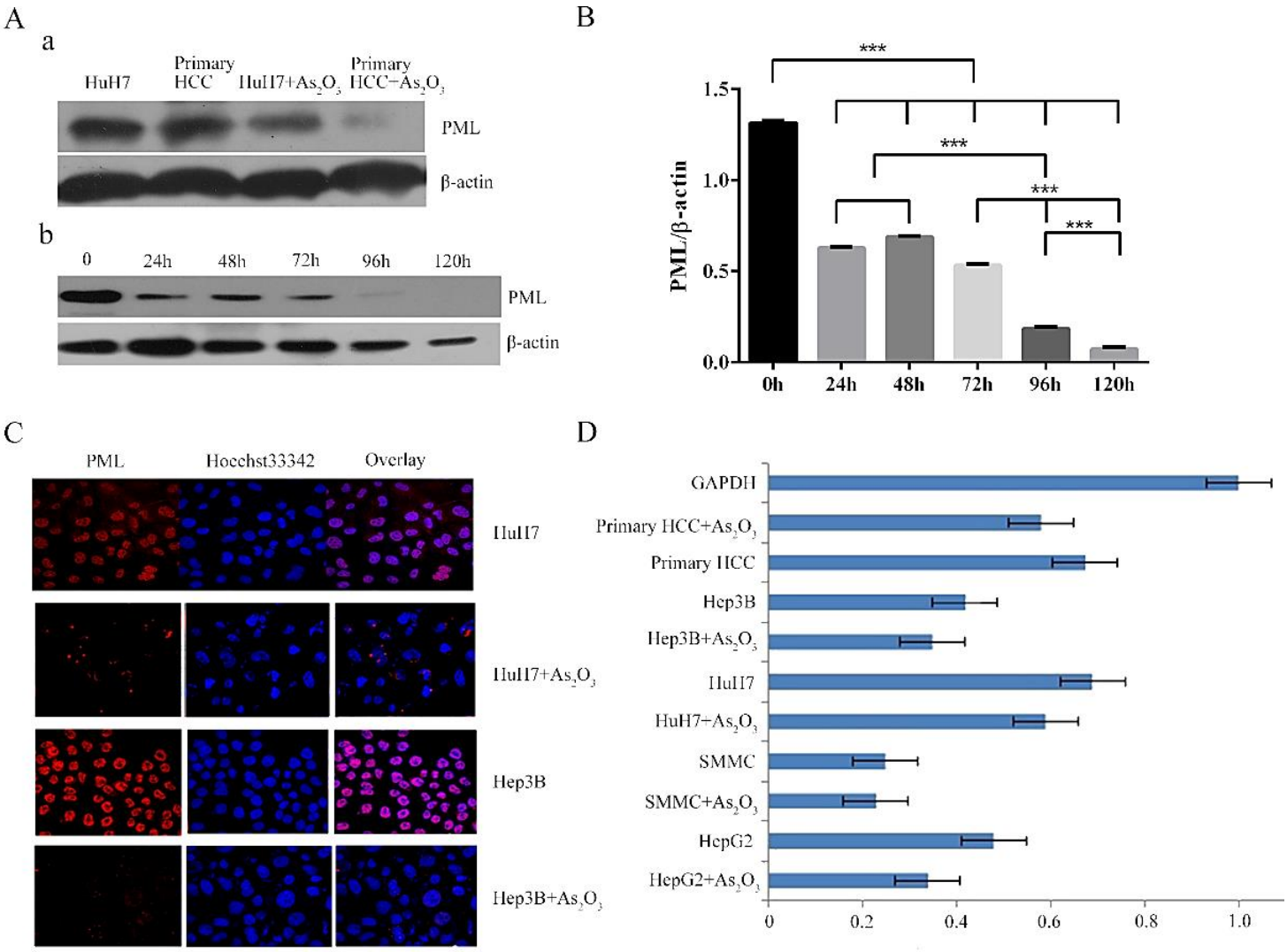

Figure 4. Effect of As_2O_3 on transcription and expression of PML in hepatic cell lines. A - a: Reduced PML protein in HuH7 and primary HCC tissue treated with 0.5 µg/mL As_2O_3 for 5 days; A - b: Time course of PML protein levels in HuH7 cells treated with 0.5 µg/mL As_2O_3 for 120 h evaluated by Western blotting; B - Statistical analysis of relative PML protein levels in HuH7 cells treated with 0.5 µg/mL As_2O_3 for 120 h, based on the bands from A-b Western blot test by Image J software analysis; C - PML protein levels in HuH7 and Hep3B cells cultured with or without 0.5 µg/mL As_2O_3 for 5 d, assessed by immunofluorescence using an anti-PML protein primary antibody (red fluorescence) and Hoechst 33342 nuclear staining (blue fluorescence); D - PML protein mRNA transcription in HuH7, HepG2, Hep3B, SMMC-7721 cells, and primary HCC cells cultured with 0.5 µg/mL As_2O_3 for 5 days, assessed by qRT-PCR. ***P-value < 0.001.

As_2O_3 reduced the expression of PML protein

To determine if As_2O_3 treatment could change PML protein levels in HCC cells, HuH7 and primary HCC cells were treated for 5 days with 0.5 µg/mL As_2O_3, and any changes in PML protein were measured. The results demonstrated that PML protein levels in both cell types clearly declined after treatment with a

low concentration of As_2O_3 (Figure 4A). To further establish whether the expression of PML protein levels in HuH7 cells exposed to As_2O_3 decreased as a function of exposure time, expressions were assessed after the application of 0.5 µg/mL As_2O_3 for 0, 24, 48, 72, 96 and 120 h. We found that PML protein levels in HuH7 cells gradually decreased as a function of the exposure time (Figure 4Ba, Figure 4Bb). Immunofluorescence stained particles in the HuH7 and Hep3B cell nuclei exposed to a concentration of 0.5 µg/mL As_2O_3 also decreased significantly (Figure 4C). In contrast, As_2O_3 did not affect the PML mRNA level in the nuclei of primary HCC cells and the other cell lines ($P > 0.05$; Figure 4D), indicating that a low concentration of As_2O_3 only markedly decreased cytosolic PML protein levels in primary HCC cells and HCC cell lines.

Reduction of PML protein suppressed downstream gene Oct4 expression

Levels of Oct4 and PML proteins in $HuH7^{CD133+\ CD13+}$ and P19 embryonic carcinoma cells were reduced by As_2O_3 treatment and PML protein silencing (Figure 5, A-B). In comparison with the siRNA-scrambled control, As_2O_3 and PML protein silencing both decreased Oct4 mRNA levels in $HuH7^{CD133+\ CD13+}$ cells (Figure 5C). Ectopic PML protein expression in $HuH7^{CD133+\ CD13+}$ cells was reduced with concomitant PML protein silencing via siPML, also resulting in decreased Oct4 protein expression (Figure 5D). The normal (CMV) and, to a limited extend, the ectopic (CMV-PML) PML protein expressions were reduced in $HuH7^{CD133+\ CD13+}$ cells treated with As_2O_3, which led to reduced Oct4 expressions (Figure 5E). It is noteworthy that the ectopic-increased expression of CMV-PML protein in $HuH7^{CD133+\ CD13+}$ cells could reverse the effects of As_2O_3 and siPML only to a limited extent. However, the findings indicate that a decrease in PML protein led to reduced expression of its downstream gene *Oct4*.

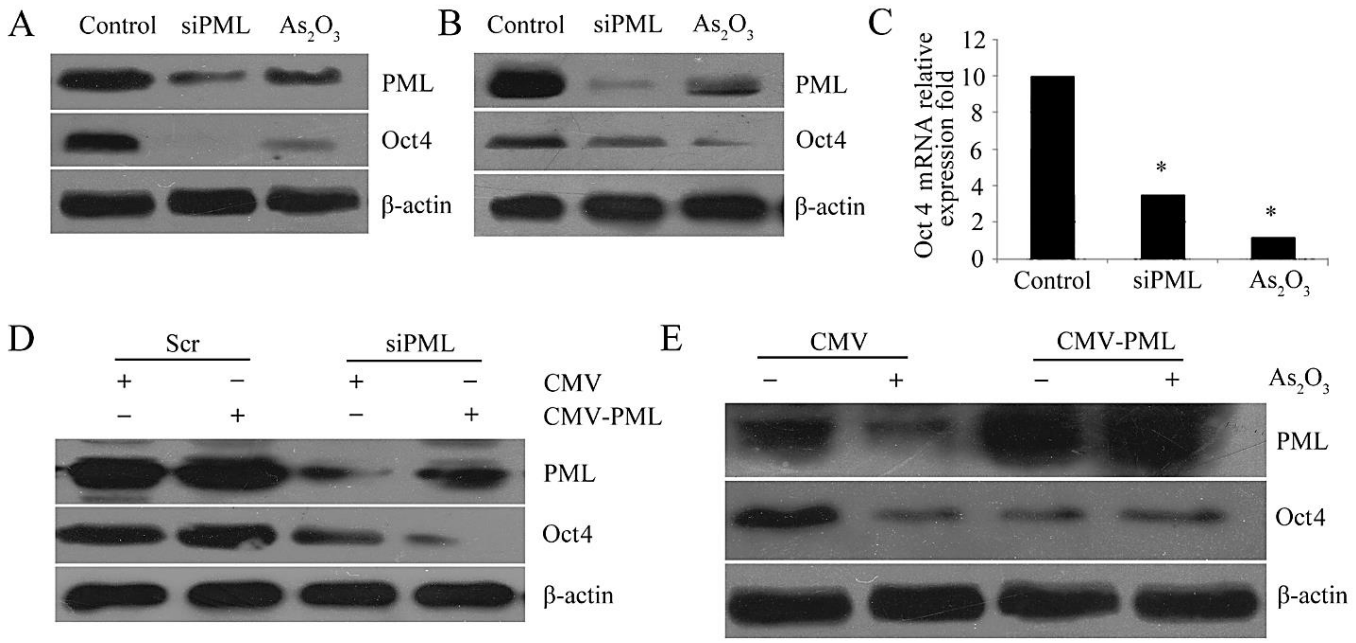

Figure 5. Effects of PML silencing and overexpression on Oct4 transcription and expression in As_2O_3 treated $HuH7^{CD133+\ CD13+}$ cells. A - The PML protein levels in P19 EC cells, cultured with or without siPML or 0.5 µg/mL As_2O_3 for 96 h, assessed by Western blotting; B - PML protein in $HuH7^{CD133+\ CD13+}$ cells cultured with or without siPML or 0.5 µg/mL As_2O_3; C - qRT-PCR was used to assess *Oct4* mRNA transcription in $HuH7^{CD133+\ CD13+}$ cells treated with siPML or 0.5 µg/mL As_2O_3. D - Western blot analysis of PML protein and Oct4 levels in $HuH7^{CD133+\ CD13+}$ cells in the presence or absence of ectopically PML protein expression and transfected with siPML or a scrambled control RNA (Scr). E - PML protein and Oct4 protein levels in $HuH7^{CD133+\ CD13+}$ cells with and without ectopic PML protein expression and with or without 0.5 µg/mL As_2O_3 for 96 h. *P-value < 0.05.

4. Discussion

As_2O_3 is the main element of a hypertoxic Chinese medicine proven to produce an alleviation rate of circa 95% for APL. In APL cells, it has been demonstrated that As_2O_3 binds directly to zinc fingers (containing cysteine residues) located within the RING finger, B boxes, and coiled-coil region (RBCC) domain of PML-RAR alpha (Zhang et al. 2010). When As_2O_3 binds, it triggers oligomerization of PML-RAR, which then interacts with SUMO-conjugating enzyme UBC9, resulting in an increase in sumoylation and subsequent degradation (Zhang et al. 2010). Research has revealed that PML-RAR alpha and PML protein are degraded after they are sumoylated, through the action of As_2O_3 and their specificity for APL (Ito et al. 2008; Zhang et al. 2010; Liu et al. 2011; Qu et al. 2011; Wei et al. 2014). Previous studies have reported that As_2O_3 prevents HCC cell proliferation, induces their apoptosis, and also has inhibitory actions on liver cancer stem cells (Ito et al. 2008; Liu et al. 2011; Qu et al. 2011; Nakahara et al. 2014; Wei et al. 2014). The direct binding of As_2O_3 to PML-RARα protein leading to its degradation has been widely studied in APL cells (Jeanne et al. 2010; Zhang et al. 2010; Vitaliano-Prunier et al. 2014; Wang et al. 2015; Bai and Zheng 2017).

Our results have further confirmed that both HCC tissue and HCC cell lines can express PML protein (Figure 3). The treatment of HuH7 cells with As_2O_3 occurred in a time-dependent manner for both cell growth (Figure 1) and PML protein levels (Figure 4B). These findings imply that As_2O_3-induced inhibition of HCC cells is potentially related to its binding effect on PML protein, further leading to the degradation of PML protein, which also showed a similar mechanism in APL cells.

A time course assay of As_2O_3 treatment revealed substantial effects of As_2O_3 on PML protein levels (Figure 4C). However, there were no significant effects on PML protein mRNA in primary HCC cells, or the other cell lines investigated (Figure 4D), indicating that As_2O_3 only regulates PML protein levels, possibly via direct binding with PML protein to stimulate the degradation process. In addition,

The PML gene was found to fuse with the retinoic acid receptor α (RARα) gene during a chromosome translocation of acute promyelocytic leukemia (APL). PML was consistently localized to the nucleus, although a minority of cells (approximately 20%) were found to be PML positive in the cytoplasm of cells with *in vitro* and *in vivo* experiments. The nuclear staining type varied based on non-APL (speckled) or APL cells (micropunctate). Although both physiologically expressed PML isoforms could be detected only by immunocytochemistry or predominantly in the cytoplasm of transfected cells, the cytoplasmic localization of PML was also the PML isoform that was predominantly localized to the nucleus. The results of immunohistological analysis showed that PML expression is different in the different tissue, with the highest expression postmitotic differentiated cells including, endothelial cells, epithelial cells, and macrophages, especially for activated cells (Flenghi et al. 1995).

In previous studies, it has been found that CD133 is a stem cell marker for ovary (Ferrandina et al. 2008), brain (Singh et al. 2004), prostate (Collins et al. 2005), liver (Suetsugu et al. 2006), colon (O'Brien et al. 2007; Ricci-Vitiani et al. 2007), and pancreatic (Hermann et al. 2007) cancers, while CD133 is a marker for semiquiescent hepatic cancer stem cells (Haraguchi et al. 2010). In addition, Oct4 protein, encoded by the *Pou5f1* gene, is an important factor necessary for the maintenance of undifferentiated states and pluripotency of mouse and human embryonic stem cells, in addition to embryonic cells at an early stage (Kellner and Kikyo 2010; Zeineddine et al. 2014). It is frequently used as a marker for undifferentiated cells (Niwa et al. 2000). In our study, the expression levels of PML protein were decreased by As_2O_3 treatment and in siPML knockdowns, further leading to the suppression of *Oct4* gene expression (Chuang et al. 2011; Koo et al. 2015).

The reduction of PML protein in $HuH7^{CD133+\ CD13+}$ cells with siPML and As_2O_3 led to concomitant diminished Oct4 expression, suggesting that in HuH7 cancer stem cells, PML protein acts a differentiation inhibitor, which also has been postulated for APL (Yoshida et al. 1996). This hypothesis is also supported by enhanced expressions of the mature hepatocyte markers ALB, CK18, TAT, and ApoB and reduced expression of AFP (Cui et al. 2016).

Taken together, the findings of the present study suggest that As_2O_3 inhibits uncontrolled HCC cell proliferation and induces HCC cells to differentiate and mature via triggering the degradation of PML protein.

5. Conclusions

As_2O_3 treatment can inhibit HCC cell proliferation and repress tumor formation in a time-dependent and concentration-dependent manner. The inhibitory actions of As_2O_3 were closely associated with PML protein levels, despite the limited effect of As_2O_3 on PML As_2O_3 mRNA levels. Furthermore, the downregulation of PML protein led to a decreased expression of the undifferentiated cell marker *Oct4* gene. Overall, it is suggested that a low concentration of As_2O_3 (0.5 μg/mL) can be employed as a promising therapy to treat HCC patients.

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References

ALIMOGHADDAM, K., et al. Anti-leukemic and anti-angiogenesis efficacy of arsenic trioxide in new cases of acute promyelocytic leukemia. *Leukemia & lymphoma.* 2006, **47**(1), 81-88. https://doi.org/10.1080/10428190500300373

ALIMOGHADDAM, K. A review of arsenic trioxide and acute promyelocytic leukemia. *International Journal of Hematology-Oncology and Stem Cell Research.* 2014, **8**(3), 44-54.

BAI, D.M. and ZHENG, X.F. PML-RARA mutations confer varying arsenic trioxide resistance. *Protein & Cell.* 2017, **8**(4), 296-301. https://doi.org/10.1007/s13238-016-0356-4

CHEN, D.C., et al. CBFA2T2 is associated with a cancer stem cell state in renal cell carcinoma. *Cancer Cell International.* 2017, **17**(103. https://doi.org/10.1186/s12935-017-0473-z

CHUANG, Y.S., et al. Promyelocytic leukemia protein in retinoic acid-induced chromatin remodeling of Oct4 gene promoter. *Stem Cells (Dayton, Ohio).* 2011, **29**(4), 660-669. https://doi.org/10.1002/stem.623

COLLINS, A.T., et al. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Research.* 2005, **65**(23), 10946-10951. https://doi.org/10.1158/0008-5472.can-05-2018

CUI, J., et al. All-trans retinoic acid inhibits proliferation, migration, invasion and induces differentiation of hepa1-6 cells through reversing EMT in vitro. *International Journal of Oncology.* 2016, **48**(1), 349-357. https://doi.org/10.3892/ijo.2015.3235

FERLAY, J., et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer.* 2015, **136**(5), E359-386. https://doi.org/10.1002/ijc.29210

FERRANDINA, G., et al. Expression of CD133-1 and CD133-2 in ovarian cancer. *International Journal of Gynecological Cancer : Official Journal of the International Gynecological Cancer Society..* 2008, **18**(3), 506-514. https://doi.org/10.1111/j.1525-1438.2007.01056.x

FLENGHI, L., et al. Characterization of a new monoclonal antibody (PG-M3) directed against the aminoterminal portion of the PML gene product: immunocytochemical evidence for high expression of PML proteins on activated macrophages, endothelial cells, and epithelia. *Blood.* 1995, **85**(7), 1871-1880.

GOLABI, P., et al. Mortality assessment of patients with hepatocellular carcinoma according to underlying disease and treatment modalities. *Medicine.* 2017, **96**(9), e5904. https://doi.org/10.1097/MD.0000000000005904

HARAGUCHI, N., et al. CD13 is a therapeutic target in human liver cancer stem cells. *The Journal of Clinical Investigation.* 2010, **120**(9), 3326-3339. https://doi.org/10.1172/JCI42550

HERMANN, P.C., et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell.* 2007, **1**(3), 313-323. https://doi.org/10.1016/j.stem.2007.06.002

IKEDA, S., NAGATA, C. and SUZUKI, K. Periodic acid Schiff staining-positive spermatocytic seminoma. *Pathology International.* 2014, **64**(4), 192-194. https://doi.org/10.1111/pin.12150

ISHIZAWA, T., et al. Mechanistic background and clinical applications of indocyanine green fluorescence imaging of hepatocellular carcinoma. *Annals of Surgical Oncology.* 2014, **21**(2), 440-448. https://doi.org/10.1245/s10434-013-3360-4

ITO, K., et al. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature.* 2008, **453**(7198), 1072-1078. https://doi.org/10.1038/nature07016

JEANNE, M., et al. PML/RARA oxidation and arsenic binding initiate the antileukemia response of As2O3. *Cancer Cell.* 2010, **18**(1), 88-98. https://doi.org/10.1016/j.ccr.2010.06.003

KELLNER, S. and KIKYO, N. Transcriptional regulation of the Oct4 gene, a master gene for pluripotency. *Histology and Histopathology.* 2010, **25**(3), 405-412. https://doi.org/10.14670/HH-25.405

KOO, B., et al. Oct4 is a critical regulator of stemness in head and neck squamous carcinoma cells. *Oncogene.* 2015, **34**(18), 2317.

LIU, Z.M., et al. Suppression of TG-interacting factor sensitizes arsenic trioxide-induced apoptosis in human hepatocellular carcinoma cells. *The Biochemical Journal.* 2011, **438**(2), 349-358. https://doi.org/10.1042/BJ20101653

NAKAHARA, F., WEISS, C.N. and ITO, K. The role of PML in hematopoietic and leukemic stem cell maintenance. *International Journal of Hematology.* 2014, **100**(1), 18-26. https://doi.org/10.1007/s12185-014-1518-x

NIWA, H., MIYAZAKI, J. and SMITH, A.G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics.* 2000, **24**(4), 372-376. https://doi.org/10.1038/74199

O'BRIEN, C.A., et al. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature.* 2007, **445**(7123), 106-110. https://doi.org/10.1038/nature05372

QU, F.L., et al. [Multicenter phase II clinical trial of arsenic trioxide injection in the treatment of primary hepatocarcinoma]. *Zhonghua Zhong Liu Za Zhi [Chinese Journal of Oncology].* 2011, **33**(9), 697-701.

RICCI-VITIANI, L., et al. Identification and expansion of human colon-cancer-initiating cells. *Nature.* 2007, **445**(7123), 111-115. https://doi.org/10.1038/nature05384

SINGH, S.K., et al. Identification of human brain tumour initiating cells. *Nature.* 2004, **432**(7015), 396-401. https://doi.org/10.1038/nature03128

SUETSUGU, A., et al. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochemical and Biophysical Research Communications.* 2006, **351**(4), 820-824. https://doi.org/10.1016/j.bbrc.2006.10.128

TORRE, L.A., et al. Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians.* 2015, **65**(2), 87-108. https://doi.org/10.3322/caac.21262

VITALIANO-PRUNIER, A., et al. Clearance of PML/RARA-bound promoters suffice to initiate APL differentiation. *Blood.* 2014, **124**(25), 3772-3780. https://doi.org/10.1182/blood-2014-03-561852

WANG, Q.Q., et al. Methylated arsenic metabolites bind to PML protein but do not induce cellular differentiation and PML-RARalpha protein degradation. *Oncotarget.* 2015, **6**(28), 25646-25659. https://doi.org/10.18632/oncotarget.4662

WANG, M., et al. Contribution of hepatitis B virus and hepatitis C virus to liver cancer in China north areas: Experience of the Chinese National Cancer Center. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases.* 2017, **65**(15-21. https://doi.org/10.1016/j.ijid.2017.09.003

WEI, J., et al. All-trans retinoic acid and arsenic trioxide induce apoptosis and modulate intracellular concentrations of calcium in hepatocellular carcinoma cells. *Journal of Chemotherapy (Florence, Italy).* 2014, **26**(6), 348-352. https://doi.org/10.1179/1973947814Y.0000000200

WOO, S.H., et al. Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in HeLa cells. *International Journal of Oncology.* 2002, **21**(1), 57-63.

YOSHIDA, H., et al. Accelerated degradation of PML-retinoic acid receptor alpha (PML-RARA) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. *Cancer Research.* 1996, **56**(13), 2945-2948.

ZEINEDDINE, D., et al. The Oct4 protein: more than a magic stemness marker, *Am J Stem Cells.* 2014, **3**(2), 74-82.

ZHANG, X.W., et al. Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. *Science (New York, N.Y.).* 2010, **328**(5975), 240-243. https://doi.org/10.1126/science.1183424

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