

# Molecular Targeting Therapy Against Promyelocytic Leukemia Protein Using Arsenic Acids in Experimental Intracranial Medulloblastoma

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## Abstract

Our previous study using human Daoy medulloblastoma cells showed that the promyelocytic leukemia (PML) gene was significantly upregulated (2.5-fold) in cells positive to prominin-1 antigen (CD133), a possible marker for cancer initiating cells. Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) is known to degrade PML protein and has been used for the treatment of patients with acute PML. In the present study, the effect of PML targeting therapy with As<sub>2</sub>O<sub>3</sub> and cytarabine (Ara-C) on Daoy medulloblastoma cell proliferation was investigated. Daoy cells were pretreated with As<sub>2</sub>O<sub>3</sub> for 6 weeks. The As<sub>2</sub>O<sub>3</sub>-pretreated Daoy cells were cultured in medium containing Ara-C and cell viability was examined. Next, the As<sub>2</sub>O<sub>3</sub>-pretreated Daoy cells were inoculated into the nude mouse brain and the effect of Ara-C on the tumor size was evaluated. A significant increase in chemosensitivity to Ara-C was observed in the As<sub>2</sub>O<sub>3</sub>-pretreated Daoy cells in both *in vitro* and *in vivo* conditions. PML and CCND1 (cyclin D1) protein expression of Daoy medulloblastoma cells was downregulated by As<sub>2</sub>O<sub>3</sub> treatment. PML has been proposed as a novel therapeutic target to eradicate quiescent leukemia-initiating cells, and PML-expressing CD133-positive cells are similarly a potential therapeutic target of treatment for medulloblastoma.

Key words: medulloblastoma, promyelocytic leukemia, arsenic trioxide, Ara-C, cancer initiating cell

## Introduction

The use of multidisciplinary approaches including radical surgical resection followed by radiation and chemotherapy has substantially improved the survival rates of patients with medulloblastoma, the most common malignant brain tumor in childhood, but many survivors suffer from neurologic and endocrinologic sequelae.<sup>5,17</sup> Therefore, more effective and less toxic treatment modalities must be developed to improve the quality of life of patients with medulloblastoma, through understanding of the molecular properties and fundamental signaling pathways involved in the tumorigenesis.

Our previous study isolated cells positive to prominin-1 antigen (CD133), a possible marker for cancer initiating cells,<sup>13</sup> from human Daoy medulloblastoma cells by using the CD133 magnetic beads

cell-sorting method and transcript analysis using deoxyribonucleic acid microarrays. We successfully identified upregulated genes that might be involved in both medulloblastoma oncogenesis and stem cell proliferation, such as Wnt target genes and Notch target genes in the CD133-positive cell-enriched fractions of human Daoy medulloblastoma cells.<sup>6</sup> In addition, we found a significant upregulation (2.5-fold increase) of the promyelocytic leukemia (PML) gene transcripts in the CD133-positive cell enriched fractions in comparison with control unsorted Daoy cells.<sup>6</sup>

PML was originally found to be involved in the chromosomal translocation seen in acute promyelocytic leukemia (APL), encoding for a protein localized in the PML nuclear body.<sup>3</sup> PML has since been proposed as a novel therapeutic target to eradicate quiescent leukemia-initiating cells, as exemplified by the use of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>), which is known to degrade PML protein.<sup>9</sup> Furthermore, As<sub>2</sub>O<sub>3</sub> has also been used in the treatment of relaps-

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ing APL previously treated with all-trans retinoic acid or 13-cis retinoic acid. Recent *in vitro* studies revealed that such modulation of retinoic acid signaling pathway also resulted in apoptosis and decreased cell proliferation in medulloblastoma cells.<sup>4,7,12,21</sup> Therefore, we considered that As<sub>2</sub>O<sub>3</sub> treatment might also degrade the upregulated PML protein in CD133-positive medulloblastoma cells, the possible initiating cells of medulloblastoma, and then increase their chemosensitivity.

In the present study, we first performed immunohistochemical studies which revealed coexpression of CD133 and PML in a few cells of Daoy cells and of specimens obtained from patients with medulloblastoma. Consequently, we investigated the effects of combination therapy with As<sub>2</sub>O<sub>3</sub> and Ara-C, a well-known chemotherapeutic agent, and found a significant proliferation inhibition of the human Daoy medulloblastoma cells in both *in vitro* and *in vivo* conditions.

## Materials and Methods

### I. Cell culture

The human Daoy medulloblastoma cell line was purchased from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under 5% CO<sub>2</sub>.

### II. Immunohistochemistry

Daoy cells were plated on 3-cm glass-bottom dishes (IWAKI; Asahi Glass Co., Ltd., Tokyo), and incubated overnight at 37°C under 5% CO<sub>2</sub>. The cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes at room temperature, permeabilized with 0.1% Tween 20 in PBS for 5 minutes, and blocked with 10% FBS in PBS for 1 hour at room temperature. The blocked cells were incubated with primary antibodies (anti-CD133, 1:100 (v/v), anti-PML 1:100 (v/v); Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) for 1 hour. Subsequently, the primary antibodies were stained with Alexa488-conjugated donkey anti-goat immunoglobulin G (IgG) and Alexa594-conjugated goat anti-mouse IgG (Invitrogen®; Life Technologies Corp., Carlsbad, California, USA) for 1 hour, for detecting CD133 and PML. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kamimashiki-gun, Kumamoto) and observed under a BX-51 fluorescence microscope (Olympus Corp., Tokyo).

### III. Fluorescent immunohistochemical analysis

Formalin-fixed, paraffin-embedded sections of human medulloblastomas (thickness, 5 µm) were deparaffinized, rehydrated, and irradiated in a microwave oven for 30 minutes in 10 mM sodium citrate buffer (pH = 6.0). The sections were blocked with 10% FBS in PBS for 1 hour at room temperature and incubated overnight with the primary antibodies anti-CD133 1:100 (v/v) and anti-PML 1:100 (v/v). The primary antibodies were detected using Alexa488-conjugated donkey anti-goat IgG and Alexa594-conjugated goat anti-mouse IgG. The sections were stained with DAPI and observed under an AX-80 fluorescence microscope (Olympus Corp.).

### IV. *In vitro* Ara-C sensitivity assay after As<sub>2</sub>O<sub>3</sub> treatment

Daoy cells were cultured in a complete medium containing As<sub>2</sub>O<sub>3</sub> (Kanto Chemical Co., Inc., Tokyo; final concentration of 0.1 µM) for 6 weeks. As<sub>2</sub>O<sub>3</sub>-treated Daoy cells or control Daoy cells were plated in 96-well plates and cultured for 1 week in a complete medium with or without containing 0.1 µM Ara-C (Sigma-Aldrich, Inc.). The cell viability was analyzed using a tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay).<sup>14</sup>

### V. *In vivo* Ara-C combination therapy after As<sub>2</sub>O<sub>3</sub> treatment

Animal experiments were performed in accordance with the ethical guidelines for animal experiments in Hamamatsu University School of Medicine. Twenty BALB/c nude mice (6 weeks old, 18–22 g) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka) and divided into 4 groups (As<sub>2</sub>O<sub>3</sub>(–) Ara-C(–), As<sub>2</sub>O<sub>3</sub>(–) Ara-C(+), As<sub>2</sub>O<sub>3</sub>(+) Ara-C(–), As<sub>2</sub>O<sub>3</sub>(+) Ara-C(+), n = 5 for each). Daoy cells (5 × 10<sup>4</sup>) with or without As<sub>2</sub>O<sub>3</sub> exposure for 6 weeks were intracranially inoculated. Mice were subsequently treated with daily intraperitoneal administration of Ara-C (100 mg/kg/day, Ara-C(+)) or saline (Ara-C(–)) for 1 week. Eight weeks after inoculation, the mice were anesthetized with Equithesin (0.3 ml/100 g) and perfused with 20% formalin. The fixed brains were removed and quickly frozen in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo). Serial sections of each sample were stained with hematoxylin and eosin and the tumor size was measured using ImageJ version 1.33 (public domain, Java-based image processing program developed at the National Institutes of Health; available at <http://rsb.info.nih.gov/ij/>). The tumor volume was calculated by adding the cross-sectional areas.

## VI. Effect of As<sub>2</sub>O<sub>3</sub> treatment on PML and CCND1 expression

To examine the effect of As<sub>2</sub>O<sub>3</sub> treatment on Daoy cells, the expression of PML and cell cycle-related gene CCND1 (cyclin D1) was examined in the As<sub>2</sub>O<sub>3</sub>-treated Daoy cells using Western blotting analysis, because both these factors were upregulated in the CD133-enriched Daoy cells in our previous study.<sup>6)</sup> As<sub>2</sub>O<sub>3</sub>-treated Daoy cells were lysed using lysis buffer (Bio-Rad Laboratories, Hercules, California, USA) containing complete protease inhibitors. The lysates were cleared by centrifugation, resolved on Ready gels J (Bio-Rad Laboratories) and electrophoretically transferred to poly-vinylidene fluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 5% (wt/v) skim milk (BD Biosciences, San Jose, California, USA) containing 0.1% Tween 20 at 4°C overnight. The blocked membrane was incubated with the primary antibodies (anti-PML, 1:500 and anti-CCND1, 1:1000, Santa Cruz Biotechnology, Inc.; and anti-β-actin, 1:10000, Sigma-Aldrich, Inc.) for 1 hour at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 30 minutes. The bound antibodies were detected using the ECL Plus Western blotting detection system (GE Healthcare UK Ltd., Buckinghamshire, UK).

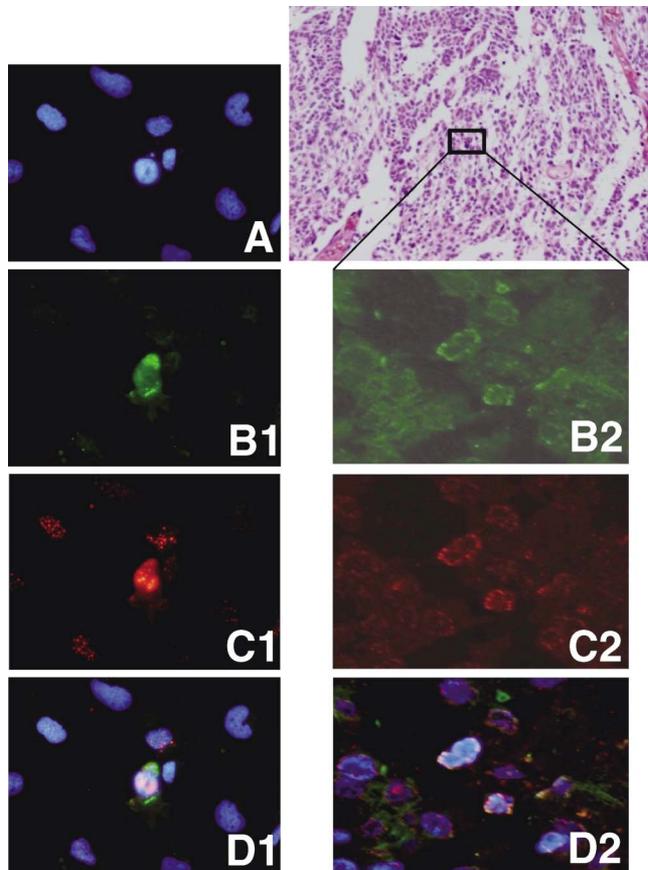
## VII. Statistical analysis

All values are shown as mean ± standard deviation and one way analysis of variance was used to analyze the differences. The statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, North Carolina, USA).

## Results

### I. PML expression in the CD133-positive Daoy medulloblastoma cell line and human medulloblastoma specimens

PML is known to be upregulated in the CD133-positive cell-enriched fractions (2.538-fold increase).<sup>6)</sup> We used PML as the target molecule because As<sub>2</sub>O<sub>3</sub> is well known to degrade and knock-down the PML protein, and furthermore As<sub>2</sub>O<sub>3</sub> is used in APL therapy. Therefore, we performed immunohistochemical analysis of the Daoy medulloblastoma cell line and specimens collected from 2 patients with medulloblastoma, a 14-year-old boy and a 5-year-old girl. Both CD133 and PML were highly coexpressed but restricted to a few cells of both the Daoy cell line and the human medulloblastoma specimens (Fig. 1).



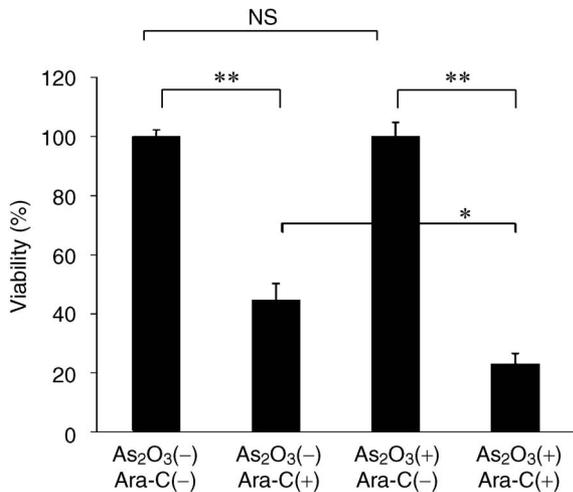
**Fig. 1** Immunohistochemical analyses of the Daoy medulloblastoma cells (left column) and clinical medulloblastoma specimen (right column). Upper right panel: hematoxylin and eosin staining of the clinical sample. Original magnification  $\times 100$ . A: 4,6-Diamidino-2-phenylindole; B1 and B2: CD133; C1 and C2: PML; D1: merged image of A, B1, and C1; D2: merged image of A, B2, and C2.

### II. In vitro chemosensitivity of As<sub>2</sub>O<sub>3</sub>-treated Daoy cells to Ara-C

In vitro treatment with 6-week low-dose (0.1  $\mu$ M) exposure to As<sub>2</sub>O<sub>3</sub> showed no cytotoxic effect (As<sub>2</sub>O<sub>3</sub>(-) Ara-C(-) vs As<sub>2</sub>O<sub>3</sub>(+) Ara-C(-) in Fig. 2). Treatment with Ara-C (0.1  $\mu$ M), a chemotherapeutic alkylating agent, significantly reduced the cell viability compared to the corresponding controls ( $p < 0.01$ , As<sub>2</sub>O<sub>3</sub>(-) Ara-C(-) vs As<sub>2</sub>O<sub>3</sub>(-) Ara-C(+) and As<sub>2</sub>O<sub>3</sub>(+) Ara-C(-) vs As<sub>2</sub>O<sub>3</sub>(+) Ara-C(+) in Fig. 2). The antitumor effect of Ara-C was significantly higher (more than 20%) in As<sub>2</sub>O<sub>3</sub>(+) Ara-C(+) than in As<sub>2</sub>O<sub>3</sub>(-) Ara-C(+) ( $p < 0.05$ , Fig. 2).

### III. In vivo therapeutic effect of Ara-C on As<sub>2</sub>O<sub>3</sub>-treated Daoy cells

The therapeutic effect of combined therapy with Ara-C and As<sub>2</sub>O<sub>3</sub> was examined in an intracranial

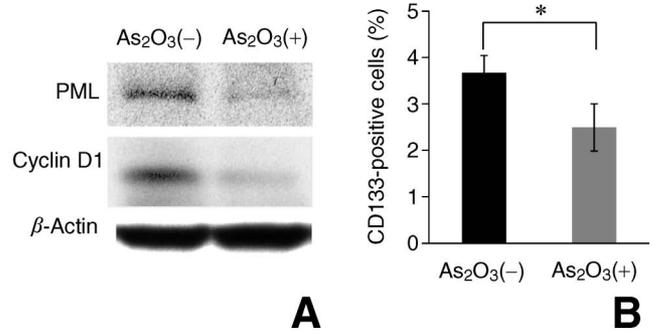


**Fig. 2** In vitro effect of cytarabine (Ara-C) in the medium (0.1 μM) on the viability of the Daoy cells pretreated with/without arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) (0.1 μM) for 6 weeks. \*p < 0.05, \*\*p < 0.01, NS: not significant.

Daoy cell inoculation model in nude mice. The tumor size of mice in the fourth group (Ara-C administration combined with As<sub>2</sub>O<sub>3</sub> treatment) was significantly smaller than in other groups (p < 0.01, Fig. 3).

**IV. Effect of As<sub>2</sub>O<sub>3</sub> treatment on PML expression and cell cycle progression**

To elucidate the mechanisms underlying enhanced chemosensitivity in As<sub>2</sub>O<sub>3</sub>-treated Daoy cells, PML and CCND1 expression were examined. Western blot analysis showed that the PML and CCND1 expression was downregulated by As<sub>2</sub>O<sub>3</sub> treatment compared to the controls (Fig. 4A). These findings revealed that As<sub>2</sub>O<sub>3</sub> treatment influenced

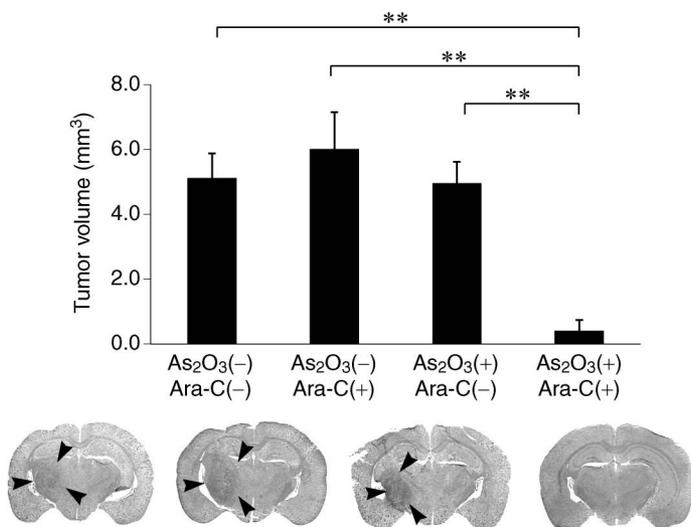


**Fig. 4** A: PML and CCND1 (cyclin D1) proteins were downregulated by 6-week arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) treatment. B: CD133-positive cells were reduced by 6-week As<sub>2</sub>O<sub>3</sub> treatment (\*p < 0.05).

the cell proliferation signals and/or cell cycle phase. The CD133-positive cells were also significantly reduced by As<sub>2</sub>O<sub>3</sub> treatment compared to the control cells (Fig. 4B).

**Discussion**

Our previous study found expression changes in retinoic acid signal-related genes in CD133-positive Daoy cells; significant upregulation (2.5-fold increase) of the PML gene transcripts and suppression of RARRES2, RBP1, and RAI16 in comparison with control unsorted Daoy cells. PML protein is highly enriched in germinal areas including ventricular zone of the neocortex suggesting that PML is associated with the immature state of neural progenitors.<sup>18)</sup> PML is also highly enriched in immature hemopoietic progenitors and leukemia-initiating cells.<sup>9)</sup> Our results indicated that PML might be highly expressed in the tumor-initiating cells of medulloblastoma. Although some reports have described the function of PML in leukemia stem cells,<sup>9,15)</sup> the expression and function of PML in medulloblastoma have not been described to date. It is strongly suspected that the same molecular mechanisms of PML



**Fig. 3** In vivo therapeutic effect of cytarabine (Ara-C) (100 mg/kg/day for 1 week) on the inoculated Daoy cells pretreated with/without arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) in nude mice. Upper row: Calculated tumor volumes of the 4 groups. The tumor volume of the As<sub>2</sub>O<sub>3</sub>(+) Ara-C(+) group was significantly smaller than in any other group (\*\*p < 0.01). Lower row: Representative images of hematoxylin and eosin staining brain sections from mice of the 4 groups. Arrowheads indicate tumor.

in leukemia-initiating cells act also in medulloblastoma, because fundamental housekeeping genes such as cell cycle genes are highly conserved from yeast to mammals. The present study demonstrated coexpression of CD133 and PML in a few Daoy cells and in specimens obtained from patients with medulloblastoma, which might correspond to cancer initiating cells. These findings indicate that the same molecular mechanisms of PML might operate in the cancer initiating cells of medulloblastoma.

To investigate whether degradation of PML by  $As_2O_3$  could increase the effect of chemotherapy, as in the case of APL, we examined the antitumor effect of Ara-C in Daoy cells following pre-treatment with  $As_2O_3$ . After culturing in the medium containing  $0.1 \mu M$  Ara-C for 1 week, the viability of the cells pretreated with  $As_2O_3$  was significantly lower than that of the cells not pretreated with  $As_2O_3$  (Fig. 2). Furthermore,  $As_2O_3$ -pretreated or control Daoy cells was injected into the nude mouse brain and then Ara-C systemically administered for 1 week. At 8 weeks after the tumor inoculation, the tumor size in the  $As_2O_3$ -pretreated group was significantly smaller than in the control groups ( $As_2O_3(+)$  Ara-C(+) vs  $As_2O_3(-)$  Ara-C(+) in Fig. 3). Interestingly, no in vivo effect of Ara-C was observed on control Daoy cell proliferation ( $As_2O_3(-)$  Ara-C(-) vs  $As_2O_3(-)$  Ara-C(+) in Fig. 3), whereas the in vitro effect was highly significant ( $As_2O_3(-)$  Ara-C(-) vs  $As_2O_3(-)$  Ara-C(+) in Fig. 2). This difference is probably because Ara-C only temporarily inhibits the proliferation of Daoy cells without  $As_2O_3$  treatment and tumor regrows during 7 weeks after Ara-C withdrawal. Because of the toxicity of longer Ara-C treatment, we administered Ara-C for only 1 week in the present experiment.

$As_2O_3$  ( $0.1 \mu M$ ) treatment for 6 weeks decreased PML protein expression of Daoy cells, a finding that parallels with the previous report that  $As_2O_3$  ( $0.15 \mu M$ ) treatment for 4 weeks decreased PML protein expression of hematopoietic stem cells.<sup>9)</sup> To elucidate the effect of  $As_2O_3$  treatment on Daoy cells, PML protein expression as well as cell cycle-related gene CCND1 were examined using Western blotting analysis because both of these proteins are upregulated in the CD133-enriched Daoy cells.<sup>6)</sup> Reduction of cyclin D1 protein was observed by exposure to a low concentration of  $As_2O_3$  (Fig. 4A). Cyclin D1 downregulation is important for permanent cell cycle exit and initiation of the differentiation induced by anchorage-deprivation in human keratinocytes.<sup>16)</sup>  $As_2O_3$  treatment also decreased CCND1 and caused cell cycle changes in glioblastoma and other types of tumor.<sup>1,8,22)</sup> In chronic myeloblastic leukemia, the  $As_2O_3$  treatment induced the release from quies-

cence of leukemia-initiating cells and contributed to increased efficacy of Ara-C anti-tumor effect.<sup>9)</sup> However, the cell cycle changes induced by  $As_2O_3$  may differ among different cell types.<sup>8)</sup>

As previously found in immunohistochemical studies using Daoy cells and specimens obtained from patients with medulloblastoma, we observed coexpression of CD133 and PML in a small fraction of tumor cells. We consider that those cells may correspond to cancer initiating cells. We also performed immunohistochemical analysis of CD133 and PML expression in various cancers, such as lung, liver, and breast cancers and, to our surprise, CD133 and PML coexpressing cells were also observed in some of these tumor types (2 of 4 lung cancers, 1 of 3 liver cancers, 2 of 4 breast cancers) (data not shown). These observations suggest that PML may be commonly expressed in CD133-positive cancer cells and involved in the maintenance of cancer initiating cells. The number of CD133-positive Daoy cells, possible cancer initiating cells, were also reduced by  $As_2O_3$  treatment (Fig. 4B). The increased chemosensitivity of  $As_2O_3$ -pretreated cells might be attributable to the decreased numbers of cancer initiating cells.

Recently,  $As_2O_3$  was demonstrated to inhibit human cancer cell growth including medulloblastoma and tumor development in mice by blocking the Hedgehog/GLI pathway.<sup>2,10)</sup> Only  $As_2O_3$  inhibited the growth of Hedgehog (Hh) activity-induced medulloblastoma allografts.<sup>10)</sup> However, the concentrations of  $As_2O_3$  required for full inhibition of the Hh pathway activity in an in vitro cell-based signaling assay is about  $4-8 \mu M$ , which is much higher than the concentration ( $0.1 \mu M$ ) used in this study. Viability and GLI1/2 expression of the HepG2 cells are not inhibited by this low concentration, so blocking the Hh pathway may not be the main cause in our study.<sup>2)</sup> Further investigations are needed to elucidate changes in the signaling pathway of tumor cell inhibition caused by  $As_2O_3$ .

In summary, targeting therapy against PML using  $As_2O_3$  is a candidate for a new sophisticated therapy targeting the cancer initiating cells. At present, we can speculate that  $As_2O_3$  probably mediates its action by PML degradation,<sup>19)</sup> which has been the rationale for its use in treatment of APL.<sup>11,20)</sup> Further understanding of the molecular mechanisms underlying the effect of  $As_2O_3$  treatment is needed for the development of more refined clinical protocols.

### Conflict of Interest

The authors have no conflict of interest or any financial disclosures to make.

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