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Original Article

Arsenic trioxide inhibits cancer stem-like cells via down-regulation of Gli1 in lung cancer

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Abstract: Cancer stem cells (CSCs) are responsible for the tumorigenesis and recurrence, so targeting CSCs is a potential effective method to cure cancers. Activated Hedgehog signaling pathway has been proved to be implicated in the maintenance of self-renewal of CSCs, and arsenic trioxide (As_2O_3) has been reported to inhibit Gli1, a key transcription factor of Hedgehog pathway. In this study, we evaluated whether As_2O_3 has inhibitory effects on cancer stem-like cells (CSLCs) in lung cancer and further explored the possible mechanism. CCK8 assay and colony formation assay were performed to demonstrate the ability of As_2O_3 to inhibit the growth of NCI-H460 and NCI-H446 cells, which represented non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively. Tumor sphere formation assay was carried out to evaluate the effects of As_2O_3 on stem cell-like subpopulations. The expression of stem cell biomarkers CD133 and stem cell transcription factors such as Sox2 and Oct4 were detected. Moreover, the effects of As_2O_3 on expression of Gli1 and its target genes were observed. We found that As_2O_3 inhibited the cell proliferation and reduced the colony formation ability. Importantly, As_2O_3 decreased the formation of tumor spheres. The expression of stem cell biomarker CD133 and stem cell transcription factors such as Sox2 and Oct4 were markedly reduced by As_2O_3 treatment. Furthermore, As_2O_3 decreased the expression of Gli1, N-myc and GAS1. Our results suggested that As_2O_3 is a promising agent to inhibit CSLCs in lung cancer. In addition, the mechanism of CSLCs inhibition might involve Gli1 down-regulation.

Keywords: Arsenic trioxide, cancer stem-like cells, lung cancer, Gli1

Introduction

Lung cancer is one of the most aggressive malignant tumors throughout the world, claiming millions of death each year [1]. Despite advances in chemotherapy and targeted drugs, the overall five-year survival rate among patients with advanced lung cancer remains poor, and the recurrence rate is high. It is reported that lung cancer stem cells (LCSCs) are closely correlated to the poor prognosis and unsuccessful clinical outcomes in lung cancer [2].

Cancer stem cells (CSCs) or cancer initiating cells are small subpopulations of cancer cells, which can differentiate and generate heterogeneous cell populations to constitute the tumor [3]. CSCs thus play a key role in the initiation and progression of malignant tumors [3]. CSCs are highly resistant to conventional chemotherapy and ionizing radiation [4]. This suggests that while many chemotherapeutic agents kill the tumor bulk population, CSCs can survive to generate new tumor and cause tumor recurrence. It is reported that CSCs form spherical colonies when they are cultured in serum-free medium in the presence of specific growth factors [5, 6]. Besides, some researchers proposed that $CD133^+$ cells have the characteristics of CSCs because they are more proliferative, clonogenic and tumorigenic than $CD133^-$ counterparts and express genes associated with stemness [7, 8]. Oct4 and Sox2, initially known as key transcription factors for embryonic stem cells, are also involved in the maintenance of CSCs [9, 10].

Aberrant Hedgehog signaling pathway is implicated in the initiation and progression of vari-

ous types of tumors, including myeloid leukaemia [11], multiple myeloma [12], basal-cell carcinoma [13], glioma [14] as well as lung cancer [15, 16]. Inhibition of Hedgehog signaling impedes clonogenic growth and tumor initiation ability of glioma stem cells [14]. It has been reported that GANT-61 (Smo antagonist) is able to reduce CSCs and profoundly impede pancreatic cancer metastatic spread [17]. In lung cancer, Gli1 expression is associated with poor overall survival [18]. Depletion of Gli1 significantly abolishes the growth of stem-like side populations from NSCLCs [19].

Arsenic trioxide (As_2O_3) has been used as a traditional remedy in China for thousands of years [20]. In recent decades, As_2O_3 has been proved to induce complete remission in acute promyelocytic leukemia (APL) with minimal toxicity [21], and it also strongly inhibited the self renewal of APL stem cells [22]. Furthermore, it was found that the combination of As_2O_3 and PI3K inhibitor PI-103 strongly diminishes acute myeloid leukemia stem cells while sparing normal hematopoietic stem cells [23]. Owing to its effects on patients with leukemia, researchers have turned their attention to utilization of As_2O_3 for treatment of various solid tumors. Studies have shown that As_2O_3 inhibits the cancer stem-like cells in gliomas via deregulation of Notch activation [24]. As_2O_3 has been found to induce differentiation of human hepatocellular carcinoma stem cells and prolong survival after hepatectomy in a mouse model [25]. Our team has previously demonstrated that As_2O_3 induces apoptosis and arrests cell cycle in lung cancer cells [26]. Additionally, As_2O_3 significantly inhibits the growth of lung cancer xenograft tumors and the formation of malignant pleural effusion in a mice model as a result of its anti-angiogenic effects [27, 28]. Moreover, As_2O_3 is clinically effective in the treatment of lung cancer complicated with malignant pleural effusion. Our findings suggest that As_2O_3 might be a new approach for the treatment of lung cancer. However, it is unclear whether As_2O_3 has the ability to inhibit cancer stem-like cells (CSLCs) in lung cancer.

We hypothesized that As_2O_3 could inhibit CSLCs in lung cancer through down-regulation of Gli1. In our studies, we employed two human lung cancer cell lines, NCI-H460 and NCI-H446, representing non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively.

Materials and methods

Cell culture and reagents

NCI-H460 cell line was obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NCI-H446 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 (HyClone, Logan City, Utah, USA) plus 10% fetal bovine serum (FBS) (HyClone, Logan City, Utah, USA) and 1% penicillin-streptomycin (HyClone, Logan City, Utah, USA). The cells were maintained at 37°C in a humidified incubator containing 5% CO_2. As_2O_3 was dissolved in PBS and diluted into indicated concentrations when needed.

Cell viability assay

Cell viability was determined by the CCK8 assay (beyotime, Shanghai, China). 3×10^3 cells were seeded per well in 96-well culture plates. After adhesion, the cells were treated with 1, 2, 4, 8, 16 and 32 μM As_2O_3, respectively. Cells treated with vehicle were used as controls. After incubation at 37°C for 24, 48 or 72 hours, the absorbance was measured by spectrophotometer at a wavelength of 450 nm. The cell viability was calculated as a percentage of the OD value in the control cells. Dose-response curves were made. The IC_{50} which represents the drug concentration that inhibits 50% of the growth of vehicle-treated control cells was calculated.

Colony formation assay

NCI-H460 and NCI-H446 cells were treated with 1, 2 and 4 μM As_2O_3 for 72 hours. Subsequently, 2×10^3 cells per well were counted and seeded in 6-well culture dishes. The cells were not exposed to As_2O_3 at this stage. After incubation at 37°C for 14 days, colonies were stained by Coomassie brilliant blue dying and photographed by a camera. Macroscopic colonies of each well were counted.

Tumor sphere formation assay

NCI-H460 and NCI-H446 cells were treated with different concentrations of As_2O_3 (1-4 μM) for 72 hours. Subsequently, 1×10^4 cells per well were counted and seeded in low-adherent 6-well culture plates (Corning, NY, USA) under serum-free conditions consisting of DMEM/F-12 (Life Technologies, Rockville, MD, USA), 20

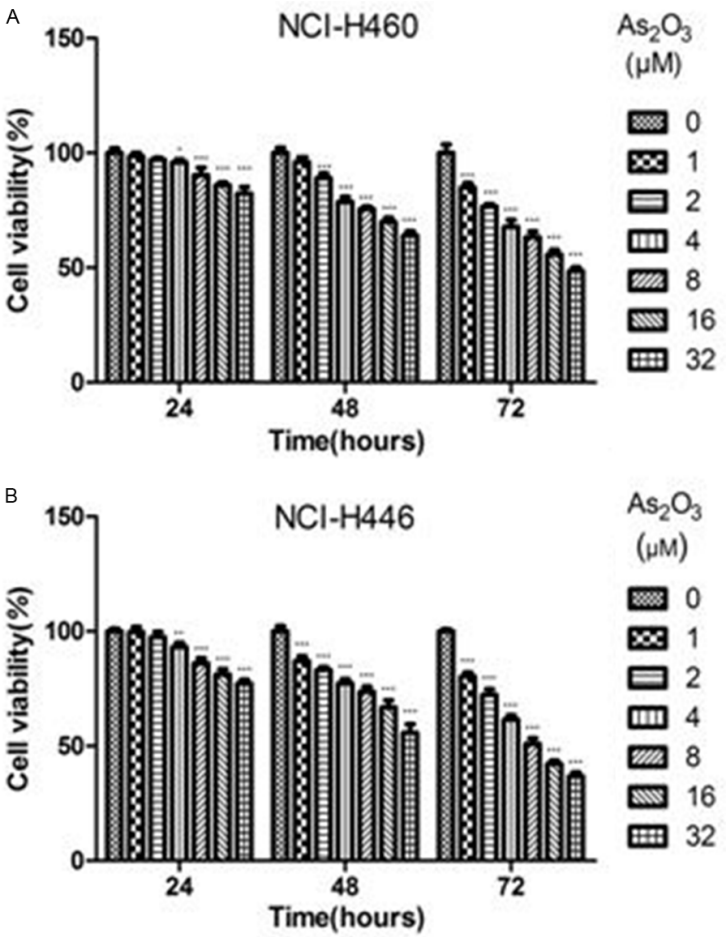

Figure 1. Inhibitory effects of As_2O_3 on lung cancer cell growth. NCI-H460 and NCI-H446 cells were treated with different concentrations of As_2O_3 for 24, 48 or 72 h. CCK8 assay was used to determine the cell viability. The control group was treated with PBS. A. Concentration- and time-dependent growth inhibitory effects of As_2O_3 on NCI-H460 cells. B. As_2O_3 inhibited the growth of NCI-H446 cells in a concentration- and time-dependent manner. *Columns*, mean; *Error bars*, SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the control.

μl/ml B27 (Life Technologies, Rockville, MD, USA), 20 ng/ml epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA, USA), 20 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (HyClone, Logan City, Utah, USA). After incubation at 37°C for 5 days, pictures were taken under a microscope and the number of tumor spheres was counted in five separated 50× fields.

Quantitative real-time PCR (qPCR)

NCI-H460 and NCI-H446 cells were treated with different concentrations of As_2O_3 (1-4 μM) for 72 hours. The total RNA was extracted and then reverse transcribed into cDNA. RT-PCR analysis was performed using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan). The following primers were used: CD133 forward 5'-TTTCAAGGACTTGCGAACTCTC-3'; CD133 reverse 5'-TGCTATTCAGCTGGCTTAGAGAC-3'; Oct-4 forward 5'-CGACCATCTGCCGCTTTGAG-3'; Oct4 reverse 5'-CCCCCTGTCCCCCATTCCTA-3'; Sox2 forward 5'-GCGAACCATCTCTGTGGTCT-3'; Sox2 reverse 5'-GGAAAGTTGGGATCGAACAA-3'; Gli1 forward 5'-ATGAAACTGACTGCCGTTGG-3'; Gli1 reverse 5'-CTTCTCGCCAGTGTGTCTGC-3'; N-myc forward 5'-ACCACAAGGCCCTCAGTACC-3'; N-myc reverse 5'-GTGCATCCTCACTCTCCACG-3'; GAS1 forward 5'-CGGAGCTTGACTTCTTGGAC-3'; GAS1 reverse 5'-CCCAACCCTTCAAATTGCTA-3'; β-actin forward 5'-CCTGGCACCCAGCACAAT-3'; β-actin reverse 5'-GGGCCGGACTCGTCATACT-3'.

Western blotting

NCI-H460 and NCI-H446 cells were treated with various concentrations of As_2O_3 (1-4 μM) for 72 hours. Proteins were extracted, electrophoretically separated, and then transferred onto PVDF membranes. The membranes were blocked with a solution containing 5% skim milk for 1 h, and were incubated overnight at 4°C with the primary antibodies. The primary antibodies were as follows: CD133 (1:1000, ABclonal, Cambridge, MA, USA), Sox2 (1:1000, Cell Signal technology, Danvers, MA, USA), Oct4 (1:000, Cell Signal technology, Danvers, MA, USA), Gli1 (1:1000, ABclonal, Cambridge, MA, USA), N-myc (1:1000, ABclonal, Cambridge, MA, USA), GAS1 (1:1000, Abcam, Cambridge, UK), and β-actin (1:1000, ABclonal, Cambridge, MA, USA). Finally, the membranes were incubated with the appropriate secondary antibody and visualized using the ECL detection reagents.

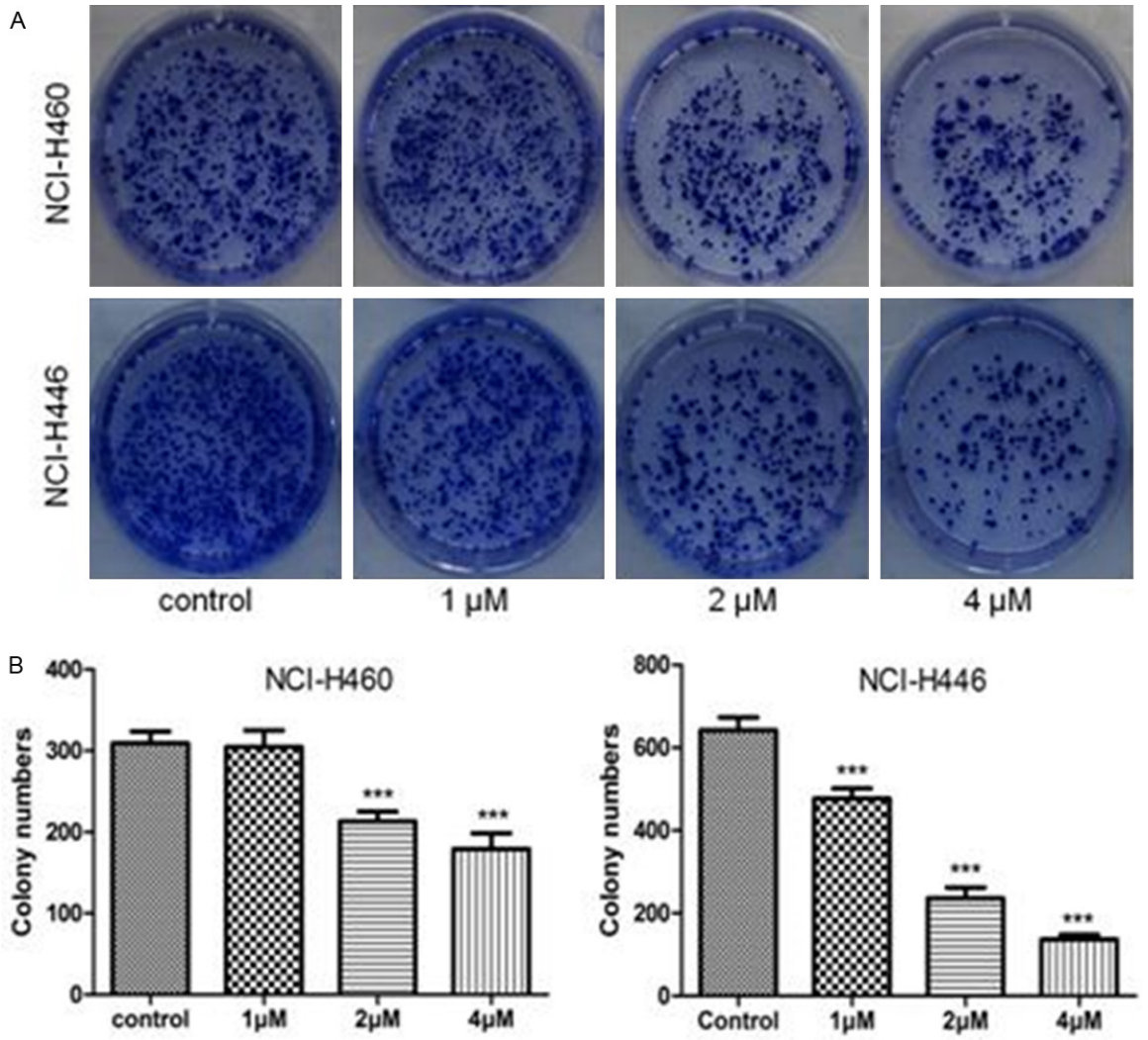

Figure 2. As_2O_3 inhibited the colony formation of lung cancer cells. NCI-H460 and NCI-H446 cells were treated with 1-4 μM As_2O_3 for 72 h. A. Images revealed that As_2O_3 could inhibit colony formation in NCI-H460 and NCI-H446 cells. B. The clonony numbers were significantly reduced by As_2O_3 treatment. *Columns*, mean; *Error bars*, SD. ***P<0.001 compared to the control.

Statistical analysis

All data were analyzed using the SPSS 22.0 software. The measured data were presented as the means ± SD, and were analyzed by a one-way ANOVA, followed by Dunnett's test. A value of P<0.05 was considered to be statistically significant.

Results

As_2O_3 inhibited lung cancer cell growth

Firstly, we used CCK8 assay to examine the growth inhibitory effects of As_2O_3 on NCI-H460 and NCI-H446 cells. Cells were treated with 1, 2, 4, 8, 16 and 32 μM As_2O_3 for 24, 48 or 72 h. Our data showed that As_2O_3 could inhibit the proliferation of NCI-H460 and NCI-H446 cells in a concentration- and time-dependent manner (**Figure 1**). The IC_{50}s of NCI-H460 and NCI-H446 cells after treatment for 72 h were 5.610 and 4.252 μM, respectively.

Colony formation analysis

To determine whether As_2O_3 could inhibit the colony formation capacity, we subsequently performed the colony formation assay. In NCI-H460 cells, control group, 1 μM As_2O_3-treated group, 2 μM As_2O_3-treated group and 4 μM As_2O_3-treated group formed an average of 309,

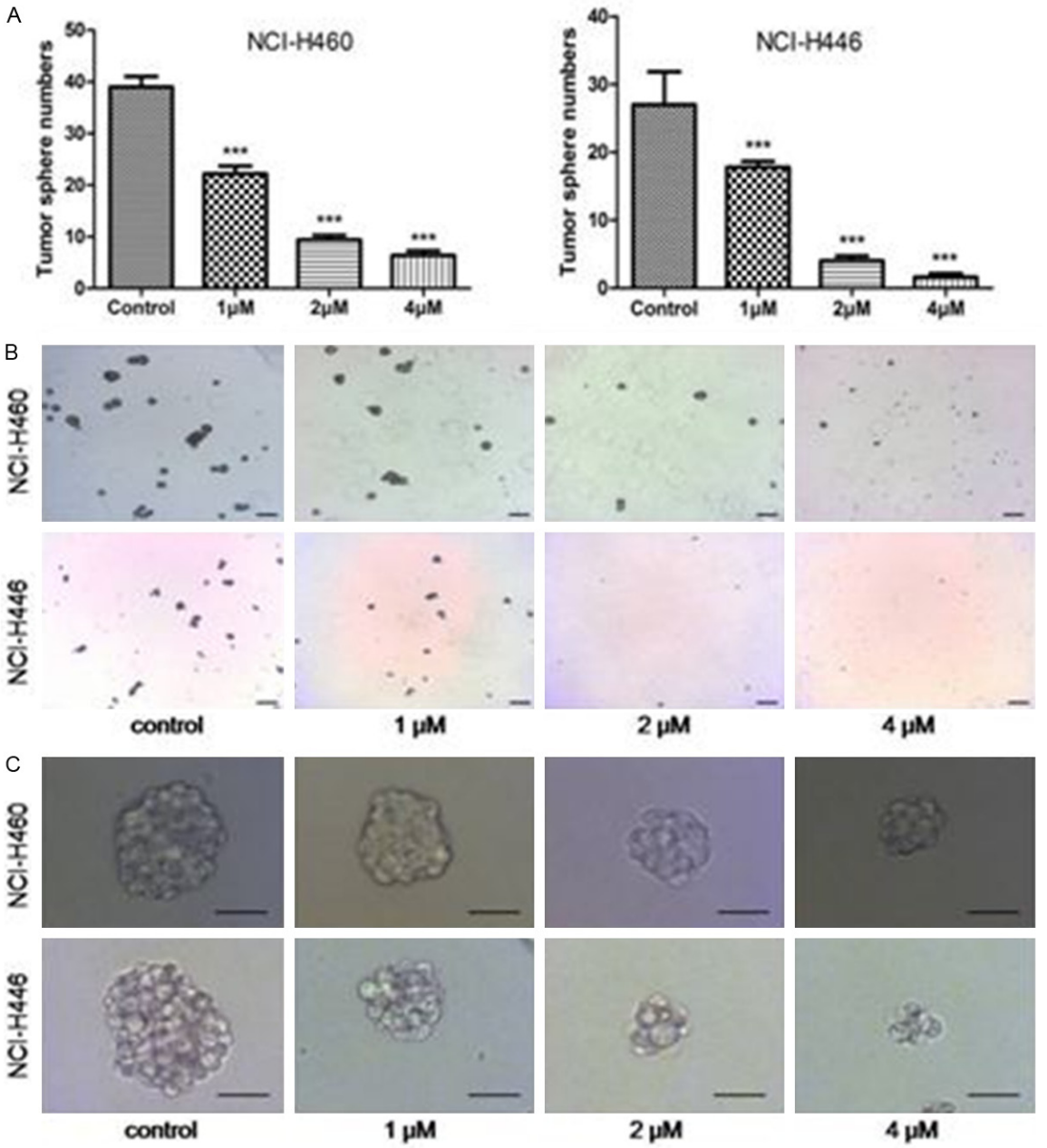

Figure 3. The effects of As_2O_3 on the tumor sphere formation. Equal numbers of viable NCI-H460 and NCI-H446 cells were cultured in serum free conditions following 72 h of As_2O_3 treatment. A. The number of tumor spheres was significantly decreased following As_2O_3 treatment. B. Representative images revealed that As_2O_3 could reduce the number of tumor spheres. *Bar*, 200 μm. C. Typical images showed that the size of tumor spheres in As_2O_3-treated group was smaller than that in control group. *Bar*, 50 μm. *Columns*, mean; *bars*, SD. $^{***}P<0.001$ compared to the control.

304, 213 and 179 colonies, respectively (**Figure 2**). In NCI-H446 cells, control group formed an average of 642 colonies. However, these numbers decreased to 477, 236 and 137 when treated with 1 μM, 2 μM and 4 μM As_2O_3, respectively (**Figure 2**). Our data showed that the clonogenic capacity of NCI-H460 and NCI-H446 cells could be remarkably inhibited by As_2O_3 treatment in a dose-dependent manner.

As_2O_3 reduced the formation of tumor spheres

Tumor spheres maintain stem cell-like subpopulations [5, 6]. Therefore, we investigated the

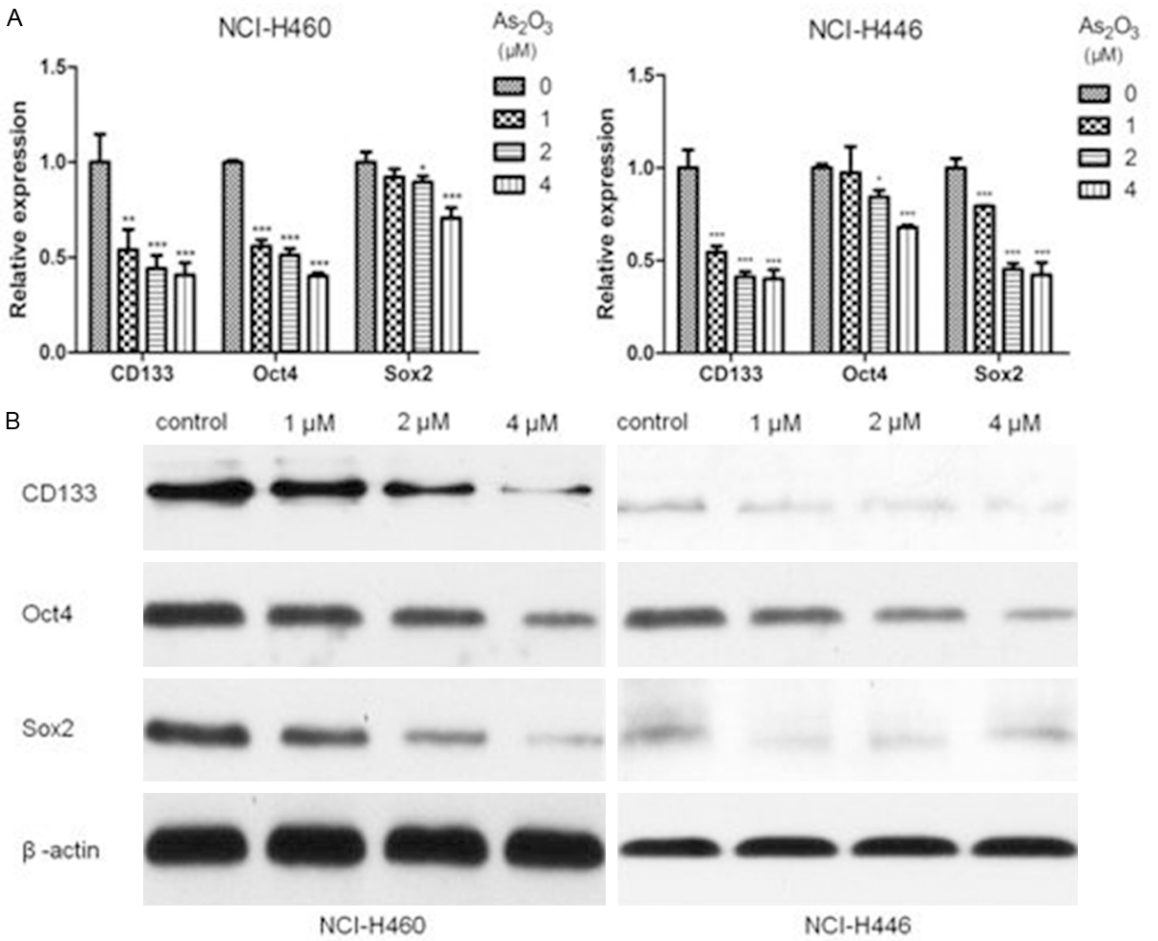

Figure 4. As_2O_3 treatment down-regulated the expression of stem cell markers. NCI-H460 and NCI-H446 cells were treated with different concentrations of As_2O_3 (1 μM, 2 μM and 4 μM) for 72 h. A. qPCR analysis showed that As_2O_3 significantly suppressed CD133, Oct4 and Sox2 mRNA expression in a dose-dependent manner. B. Western blot showed that reductions in CD133, Oct4 and Sox2 at protein levels were seen in As_2O_3 treated group. β-actin served as internal control. *Columns*, mean; *Error bars*, SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the control.

effects of As_2O_3 on the formation of tumor spheres. As expected, As_2O_3 was able to significantly decrease the number and the size of the tumor spheres. Cells treated with vehicle formed more numerous and larger tumor spheres. However, As_2O_3-treated cells merely formed smaller and lesser tumor spheres. Additionally, the growth inhibitory effects of As_2O_3 on tumor spheres were concentration-dependent (**Figure 3**). These results suggested that the stem cell-like subpopulations were sensitive to As_2O_3 treatment.

The expression of CD133, Sox2 and Oct4 were reduced by As_2O_3 treatment

To further examine the effects of As_2O_3 on CSLCs, we used qPCR and Western blot analysis to measure the expression levels of CD133 after treatment with As_2O_3 for 72 h. As_2O_3 significantly suppressed the expression of CD133 at both mRNA and protein levels in a dose-dependent manner (**Figure 4A** and **4B**). Besides, we detected the effects of As_2O_3 on Sox2 and Oct4 which are very important transcription factors in regulating self-renewal and multipotency of CSCs. As shown in **Figure 4A** and **4B**, the expression levels of Sox2 and Oct4 were also decreased by As_2O_3 treatment.

As_2O_3 down-regulated Gli1 and its target genes expression

Aberrant activated Hedgehog signaling is implicated in the initiation and propagation of lung cancer [15, 18, 29-32]. Blockade of Hedgehog signaling leads to a reduction in stem cell-like subpopulations [19, 33]. To further explore the

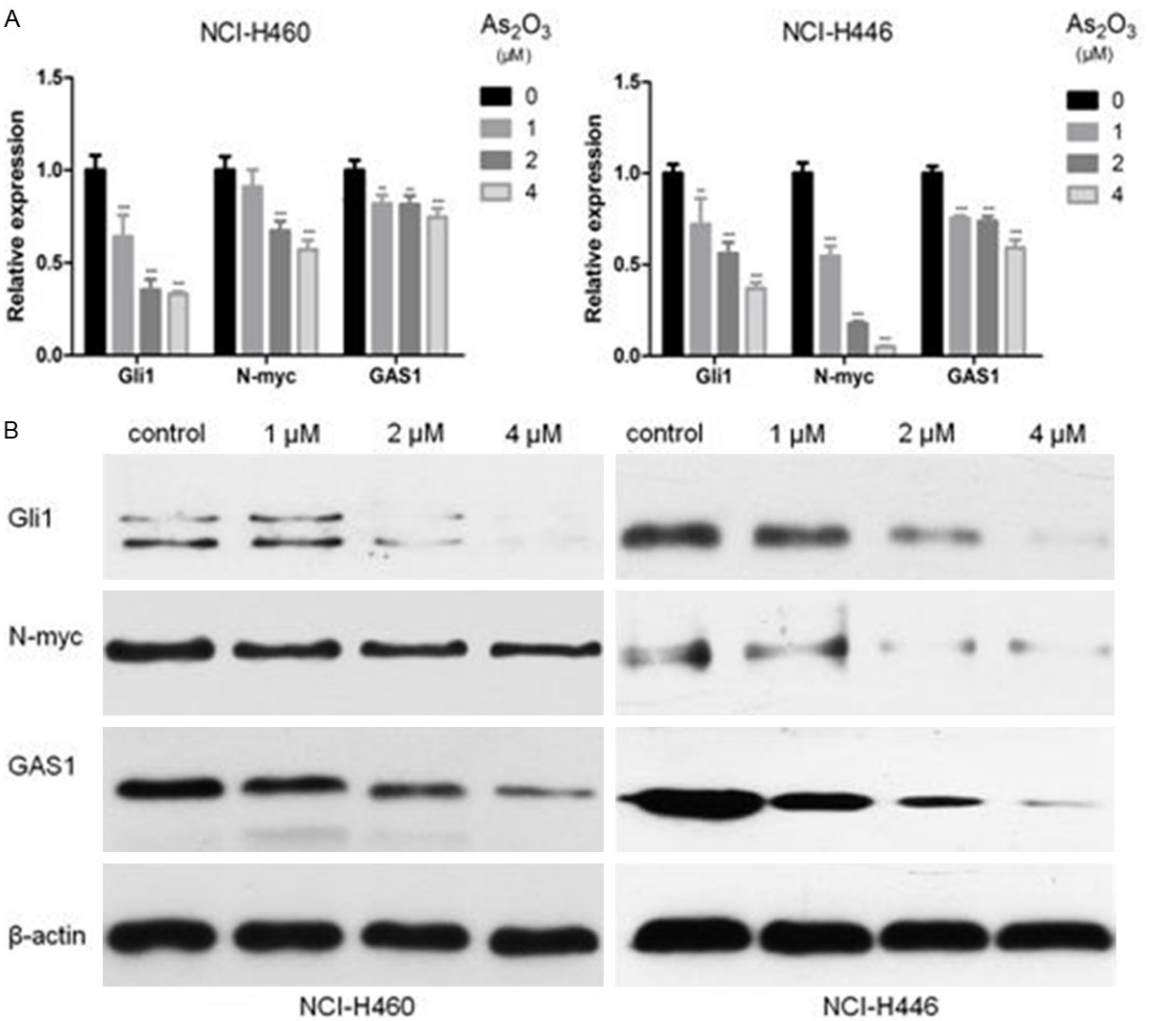

Figure 5. As_2O_3 inhibited the expression of Gli1 and its target genes such as N-myc and GAS1. NCI-H460 and NCI-H446 cells were treated with 1-4 μM As_2O_3 for 72 h. A. As_2O_3 decreased the Gli1, N-myc and GAS1 mRNA levels in NCI-H460 and NCI-H446 cells. B. As_2O_3 also influenced the expression of Gli1, N-myc and GAS1 at protein levels. *Columns*, mean; *Error bars*, SD. $^{**}P<0.01$, $^{***}P<0.001$ compared to the control.

underlying mechanism of the inhibitory effects of As_2O_3 on CSLCs, we used qPCR analysis to measure the expression levels of Hedgehog signaling molecules after treatment with As_2O_3 at the indicated concentrations. The Gli1 mRNA levels were significantly decreased by As_2O_3 in a concentration-dependent manner. Additionally, downstream genes of Gli1 such as N-myc and GAS1 were reduced by As_2O_3 treatment (**Figure 5A**). These results were confirmed at protein levels by Western blot (**Figure 5B**).

Discussion

The CSC theory indicates that malignant tumor is made up of heterogeneous neoplastic cells, among which a subpopulation exhibits unlimited self-renewal capacity and cell division potential to generate heterogeneous offspring [34]. This group of cancer cells is known as cancer stem cells or cancer initiating cells which propagate cancer initiation, development, invasion and metastasis [35]. Differing from the bulk population of tumor cells, CSCs display particular resistance to multiple cytotoxic chemotherapy drugs and are responsible for unsatisfied clinical outcomes and tumor relapse [35, 36]. Therefore, a novel therapy that targets CSCs has the potential to be a more powerful cancer therapeutic strategy. So far, the best example involving inhibition of CSCs is treatment of APL with As_2O_3 which could effectively eradicate APL-derived stem cells and lead to improved overall survival [22, 37, 38]. Moreover, research-

ers found that As_2O_3 was effective in inhibition of CSCs in various solid tumors such as gliomas [24, 39, 40], hepatocellular carcinoma [25] and pancreatic cancer [41]. Our data showed that As_2O_3 significantly inhibited the survival of lung cancer cells in a dose- and time-dependent manner. In colony formation assay, NCI-H460 and NCI-H446 cells were treated with different concentrations of As_2O_3 for 72 hours; then equal numbers of viable cells per well were counted and seeded in 6-well culture plates. After incubation for 14 days, we observed that number of macroscopic colonies of As_2O_3-treated group was lesser than control group. So we speculated that As_2O_3 might inhibit CSLCs in NSCLC and SCLC cell lines. It is reported that CSCs has the ability of forming spherical colonies when cultured in serum-free medium, termed tumor spheres. Tumor sphere cells show increased proliferation, clonogenic potential, tumorigenic capacity as well as drug-resistant properties compared with monolayer cells [5, 6]. Therefore, we performed sphere formation assay to validate our hypotheses. Our results revealed that low dose of As_2O_3 could dramatically decrease the size and the number of the tumor spheres, indicating the effects of As_2O_3 on stem cell-like subpopulations. Some researchers found that $CD133^+$ lung cancer cells also display a spectrum of features consistent with CSCs, including clonogenic ability, tumorigenic capacity, multipotency and multidrug-resistant properties [7, 42]. Patients with $CD133^+$ tumors have shorter median progression-free survival and higher recurrence risks than patients with $CD133^-$ tumors [43-45]. We found that the expression of CD133 was significantly suppressed by As_2O_3 treatment. Additionally, As_2O_3 significantly decreased the expression of stem cell transcription factors, Oct4 and Sox2, which play crucial roles in the maintenance of multipotency and self-renewal of CSCs [9, 46]. In a word, we found that low dose of As_2O_3 could inhibit stem cell-like subpopulations in lung cancer. It is very exciting for the potential clinical use of As_2O_3 in lung cancer treatment because of this drug's minimal toxicity and lower economic burden for patients.

Hedgehog signaling pathway specifies the proliferation, differentiation and migration of normal stem cell [47, 48]. Aberrant activated Hedgehog signaling pathway also plays an important role in the initiation and development of lung cancer and is required for the maintenance of LCSCs [15, 18, 29-32]. This pathway is composed of Hh ligands, Hh receptors (Ptch), Smoothened (Smo) and Gli protein [49]. Gli1 protein is the vital transcription factor of Hedgehog signaling pathway and contributes to activation of Hedgehog downstream genes. At present, a majority of Hedgehog antagonists exert its effects by binding to Smo. However, they displayed some limitations because constitutively activated mutations in Smo [13] and mutations in the downstream of Smo such as inactivated mutations in the inhibitory factor Sufu [50] or increased Gli expression [51, 52] can lead to loss-of-function of these Hedgehog antagonists. Therefore, Gli1 may be a more effective target for blockade of Hedgehog signaling pathway. A previous study reported that As_2O_3 could block Hedgehog signaling by directly interaction with Gli1 protein [53]. Consistent with these results, our data showed that As_2O_3 down-regulated the expression levels of Gli1 and its target genes such as N-myc and GAS1. Besides, we showed that As_2O_3 caused degradation of Gli1 protein. A previous study established that As_2O_3 could directly bind to cysteine residues in the zinc fingers and induce PML oligomerization as well as ubiquitination, which lead to degradation of PML-RAR fusion protein [54]. It is highly plausible that As_2O_3 also binds to cysteine residues in the zinc finger domains in Gli1, thus causing Gli1 protein degradation. Furthermore, it has been reported that Gli1 appeared to regulate the stem cell transcription factors Sox2 and Oct4 [19, 55]. Thus, we speculate that As_2O_3 modulate Sox2 and Oct4 expression through Gli1 blockade, leading to the inhibitory effects on CSLCs. However, this mechanism should be further investigated.

Therefore, when all of the data presented here are taken in whole, it suggests that: (a) As_2O_3 could inhibit the proliferation and colony formation ability of lung cancer cells. (b) The formation of tumor spheres was decreased by As_2O_3 treatment. (c) As_2O_3 markedly reduced the expression of stem cell biomarker CD133 and stem cell transcription factors such as Sox2 and Oct4. (d) As_2O_3 decreased the expression levels of Gli1 and its downstream genes such as N-myc and GAS1. In a word, As_2O_3 is a promising new approach to inhibit CSLCs in lung cancer, and the underlying mechanism may involve Gli1 blockade. As an FDA-approved drug, As_2O_3

has been widely used to treat patients with APL, and the security of the drug at therapeutic doses has been confirmed in human body. Our founding will provide a foundation for the application of As_2O_3 in the clinical treatment of lung cancer.

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Disclosure of conflict of interest

None.

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