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# Curcumol Controls Choriocarcinoma Stem-Like Cells Self-Renewal via Repression of DNA Methyltransferase (DNMT)- and Histone Deacetylase (HDAC)-Mediated Epigenetic Regulation

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Data Interpretation D  
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**Background:** Cancer stem cells (CSCs), in choriocarcinoma and other carcinomas, possess the ability of self-renewal and multilineage differentiation potential. We previously isolated choriocarcinoma cancer stem-like cells (CSLCs), which hold the stemness characteristics of CSCs. Epigenetic modifications have emerged as drivers in tumorigenesis, but the mechanisms of CSCs are largely unknown, and new drug therapies are needed to break the persistence of CSCs.

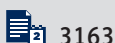
**Material/Methods:** Quantitative real-time PCR (qRT-PCR) and Western blot analysis were performed to detect the expression of DNMTs, HDACs, and stemness-genes. DNMTs and HDACs silencing and overexpressing lentivirus were transfected into JEG-3 cells to investigate the epigenetic functions in CSLCs. *In vivo* expression of curcumol effects on DNMTs and HDACs were analyzed by immunohistochemistry.

**Results:** Expression of DNMT1, DNMT3b, HDAC1, and HDAC3 were increased in choriocarcinoma CSLCs. Consistent with the inhibitory effect of 5-AzaC and TSA on CSLCs, DNMT/HDAC knockdown displayed significant repression of self-renewal in CSLCs. Curcumol inhibited the stemness ability of CSLCs *in vitro* and *in vivo*, and the inhibitory effect we observed was mediated in part through repressing activity of DNMTs and HDACs. Importantly, curcumol showed a better effect than DNMT and HDAC inhibitors combined in eliminating CSLCs.

**Conclusions:** These findings indicate that DNMT- and HDAC-mediated epigenetic regulation plays an important role in the biology of choriocarcinoma CSLCs, and curcumol has the potential to be a new drug to fight CSLCs, warranting further investigation of epigenetic-based therapies.

**MeSH Keywords:** **Choriocarcinoma • DNA Methylation • Histone Deacetylases • Neoplastic Stem Cells**

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## Background

Choriocarcinoma is a highly malignant trophoblastic tumor characterized by abnormal trophoblastic hyperplasia, and is a type of gestational trophoblastic neoplasia. Despite well-established chemotherapy, about 25% of choriocarcinoma patients showed incomplete response, or relapsed due to tumor remission [1,2]. Loss of fertility resulting in hysterectomy is the most concern outcome for patients, and is the final problem remaining to be solved. Recently, the cancer stem cells (CSCs) subpopulation, which has a high tumorigenic character, has been discussed as an explanation for tumor development, chemoresistance, and relapse after initial treatment [3–5]. CSCs have 2 important characteristics: self-renewal and multipotency. Our previous study isolated the choriocarcinoma stem-like cells (CSLCs) from the human choriocarcinoma JEG-3 cell line, showing the characteristics of CSCs [6]. The driver mutations affect a wide range of epigenetic regulators in different cancers, which provides direct evidence for the importance of epigenetic dysregulation in the formation of CSCs [7,8].

Epigenetic modifications have emerged as drivers in tumorigenesis [9,10]. Aberrant DNA methylation and histone deacetylation are 2 principal factors in epigenetic phenomena, and have been verified to be related to tumorigenesis in many studies. As the key enzymes, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) were recognized as antitumor drug targets in terms of epigenetic therapy [11,12]. DNMT1 maintains an established DNA methylation pattern, whereas DNMT3a and DNMT3b set up DNA methylation patterns in early development. Abnormal methylation by DNMTs overexpression induces tumor-suppressor genes silencing in human cancers. Given the reversibility of DNA methylation, DNMTs can be viable targets for the treatment of cancer. It was found that DNMT3a- and DNMT3b-mediated epigenetic regulation is pivotal for appropriate trophoblastic invasion [13]. Acetyl groups are removed on both histone and non-histone by HDACs, resulting in the alteration of protein functions [14]. HDACs have been shown to play crucial roles in the regulation of several proteins involved with cell cycle, proliferation, immunity, inflammation, and apoptosis [15,16]. Class I HDACs, especially HDAC1, HDAC2, and HDAC3, are found largely in the nucleus and account for the post-translational modification of histones into a deacetylated state, which cause transcription machinery alteration and alter nuclear signaling pathways relative to cell fate [17,18]. Especially in choriocarcinoma, HDAC1/2/3 inhibits the expression of multidrug resistance-associated protein 2 [19], and the HDAC inhibitor regulates the migration of JEG-3 cells [20]. Both DNMTs and HDACs induce tumor-genes silencing and cause the self-reinforcing nature of silencing mechanisms by interaction between them [21], and thus cause tumorigenesis by a bypass compensatory mechanism [22]. Interestingly,

breast CSCs were recently reported to be affected by combining DNMT and HDAC inhibition [23].

Curcumol is one of the major active components of *Curcuma zedoaria*, like curcumin [24,25], which is traditionally used for the treatment of gynecological tumors in China. Curcumol has been reported to show anti-cancer effects in several cervical cancer cells and ovarian cancer cells [26,27]. Choriocarcinoma treatment using Chinese herbs uses *Curcuma zedoaria* for adjuvant therapy with chemotherapy. However, to the best of our knowledge, the effect of the single component, curcumol, is still largely unknown in choriocarcinoma. In the present study, we tested the effect of curcumol on choriocarcinoma CSLCs via regulating epigenetic machinery.

## Material and Methods

### Drug and cell

Curcumol (purity  $\geq 96.7\%$ ), obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, CN), was dissolved in DMSO and then diluted in PBS with 0.1% DMSO. DNMT inhibitor 5-azacytidine (5-AzaC) and HDAC inhibitor Trichostatin A (TSA) were procured from Selleck (Houston, USA). The human choriocarcinoma JEG-3 cell line was obtained from the American Type Culture Collection (ATCC, USA) [28]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and antibiotics (50 U/ml penicillin, and 50  $\mu\text{g}/\text{mL}$  of streptomycin) at 37°C in a humidified incubator with 5%  $\text{CO}_2$  atmosphere.

### Sphere formation assay

JEG-3 CSLCs were isolated as described previously [6] for 7 days. The spheres were dissociated into single cells, then recultured for another 7 days. The first-generation spheres were treated with 5-azacytidine (75  $\mu\text{M}$ ) or TSA (100 nM) for 7 days.

### CD133+ cells isolation and flow cytometry analysis

The cells were labelled with a primary CD133 antibody (Miltenyi Biotec, GER), and the CD133+ and CD133- cells were subsequently magnetically isolated using the EasySep™ Human APC Positive Selection Kit (StemCell Technologies, CAN) following the manufacturer's instructions. Trypan blue staining was used to assess the sorted cell viability, and higher than 90% viability was considered acceptable for further downstream experiments. The dissociated single cells from spheres were stained with anti-CD133/APC and analyzed using a FACSCanto II Flow Cytometer instrument (BD Biosciences, USA). Acquired data were analyzed with FlowJo software.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Life Technologies, USA) following the manufacturer's protocol. The RNA was reverse transcribed to complementary DNA (cDNA) using a Transcriptor First Strand cDNA Synthesis Kit (Life Technologies, USA). qRT-PCR was performed using SYBR PremixEx Taq II method (Life Technologies, USA) on the ABI 7500 real-time PCR system (Thermo Fisher, USA). The expression level was calculated using the  $2^{-\Delta\Delta Ct}$  method. Experiments were repeated at least 3 times. The following primers obtained from Sangon Biotech (Shanghai, China) were used:

DNMT1 forward: 5'-CAGGAAGAACGGCCGAGCA-3',  
reverse: 5'-AGGCTTTGCCGGCTCCACG-3';  
DNMT3a forward: 5'-CAGTGCAGGTGACGAACATT-3',  
reverse: 5'-TGTTCCACCACCTGTTTTGA-3';  
DNMT3b forward: 5'-GGCAAGTTCTCCGAGGTCTCTG-3',  
reverse: 5'-TGGTACATGGCTTTTCGATAGGA-3';  
HDAC1 forward: 5'-GCCATCCTGGAAGTCTAAA-3',  
reverse: 5'-GGCTTGAAAATGGCCTCATA-3';  
HDAC2 forward: 5'-CCTGGAACAGGTGACATGTATGA-3',  
reverse: 5'-CGTAAGGGCACATTGAGACAATAG-3';  
NANOG forward: 5'-AGAACTCCAACATTCCTGAACCT-3',  
reverse: 5'-TGCCACCTCTAGATTTTCTCTCT-3';  
OCT4 forward: 5'-CTTGCTGCAGAAGTGGGTGGAGGAA-3',  
reverse: 5'-CTGCAGTGTGGGTTTCGGGCA-3';  
SOX2 forward: 5'-GGGAAATGGGAGGGGTGCAAAAAGA-3',  
reverse: 5'-TTGCGTGAGTGTGGATGGGATTGG-3';  
ABCG2 forward: 5'-GCAAGATGACTGCGGAAGA-3',  
reverse: 5'-CAGGTAGGCAATTGTGAGGAA-3'.

### Western blot analysis

The cells were harvested and lysed using ice-cold RIPA lysis buffer (Beyotime Biotechnology, CN). Following denaturation, equivalent amounts of protein from each sample (30–50  $\mu$ g) were separated on 10% SDS-PAGE. For immunodetection, resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Merck, Germany) in a semidry blotter (Bio-Rad) for 2 h using transfer buffer. The membranes were then blocked with TBST supplemented with 5% BSA for 1 h at room temperature, and then probed with indicated primary antibodies at 4°C overnight with gentle shaking. All the primary antibodies of DNMT1, DNMT3b, HDAC1, and HDAC3 were purchased from CST (Germany). The membranes were incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for 1 h. The bands of the target proteins were detected using enhanced chemiluminescence (ECL) reagent (Millipore, USA), and acquired by chemiluminescence system (Syngene, UK). The gray value of each band was measured by Image J software.

### Global DNA methylation quantification

The MethylFlash Methylated DNA Quantification kit (Epigentek Inc., NY) was used to measure the levels of the DNA methylation mark 5-methylcytosine (5-mC). The genomic DNA was isolated first, then the methylation of DNA was detected by the antibody, and the absorbance was read using a microplate spectrophotometer (Thermo Fisher, USA). The percentage of 5-mC in total DNA was calculated.

### HDAC activity analysis

The HDAC activity was analyzed using a colorimetric assay kit (Bio Vision, USA), according to the manufacturer's protocol. Briefly, nuclear cell lysates were extracted using the Nuclear Extract Kit (Active Motif, Belgium) and were incubated with the HDAC colorimetric substrate, the density was read at 405 nm on a Multimode Reader (Promega, USA).

### Cell transfection

Cells were seeded into 6-well plates before the day of transfection. At 30%–50% fusion, cells were transfected with different lentiviruses using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 12 h, the transfection medium was replaced with normal growth medium. The mRNA expression was assessed using qRT-PCR. The LV-RNAi of DNMT1 (CCGGCGACTACATCAAAGGCAGCAACTCGAGTTGCTG CCTTTGATGTAGTCGTTTTT), DNMT3b (CCGGCCTGCATTGTT TGATGGCATCTCGAGATGCCATCAAACAATGACAGGTTTTTG), HDAC1 (CCGGGCTGCTCAACTATGGTCTCTACTCGAGTAGAGAC CATAGTTGAGCAGCTTTTT) and HDAC3 (CCGGCCTGCATTA TGGTCTCTATAACTCGAGTTATA GAGACCATAATGCAGTTTTTG) and over-expression lentivirus (DNMT1 and HDAC1) were all provided by Genechem (Shanghai, China).

### Xenograft tumorigenicity assay

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Central South University (ID: 201603115).

BALB/c-nude mice (female, 4–5 weeks of age, body weight 18–20 g) were purchased from the Experimental Animal Center of Central South University. The animals were acclimated to their new environment for 1 week; the cells were inoculated subcutaneously into the left flank of mice. Tumor formation was monitored after inoculation. Tumors measuring at least 5 mm in diameter were considered to demonstrate successful model establishment. Tumor volume was calculated every 4 days using the equation  $(L*W^2)/2$ . After 3 weeks, mice were sacrificed after injection of cells, and the tumors were stripped

and weighed. The overall survival analysis was performed and recorded as the whole survival time per mouse.

### Immunohistochemistry analysis

Fresh tissues were immediately fixed with 4% paraformaldehyde and paraffin-embedded. All the tissue samples were sectioned into 4- $\mu$ m sections. Slides were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide. Antigen retrieval was performed by boiling the sections for 10 min in citrate buffer antigen retrieval solution (pH 6.0) at 100°C for 3 min. The sections were subsequently incubated overnight with primary antibody (DNMT1, 1: 200, DNMT3b, 1: 200, HDAC1, 1: 100, HDAC3, 1: 200), and then were washed with PBS and incubated with IgG anti-rabbit HRP-conjugated secondary antibodies IgG (CST, Darmstadt) for 30 min. The slides were stained with DAB (Solarbio, Beijing, China) and the stained cells were then analyzed. Five fields were randomly selected. The IHC scores were calculated according the method described previously [29].

### Statistical analysis

All data are expressed as means  $\pm$ SD. Differences were determined by one-way ANOVA test and chi-square test. Data not conforming to the homogeneity of variance were analyzed using the Kruskal-Wallis test. Survival curves were plotted by the Kaplan-Meier method and compared by log-rank test. A probability of  $p < 0.05$  was considered statistically significant. All statistical analyses were performed using SPSS 17.0 software.

## Results

### Increased expression of DNMTs and HDACs in choriocarcinoma CSLCs

The choriocarcinoma CSLCs were isolated using 2 different methodologies. The first one, using free-serum spheres formation, was as we described earlier. In another, using the CSC genetic marker, we separated CD133+ cells from CD133- cells by FACS sorting. To determine the DNA methylation levels in CSLCs and non-CSLCs, we measured the levels of the DNA methylation markers 5-mC. The results showed that the CSLC population exhibited significantly higher expression of 5-mC, irrespective of the method used for isolating CSLCs (Figure 1A, JEG-3 vs. Sphere,  $p < 0.01$ ; CD133- vs. CD133+,  $p < 0.001$ ). Further, significantly higher mRNA and protein expression of DNMT1 and DNMT3b were observed in the CSLCs, regardless of the isolation method used (Figure 1B, 1C;  $p < 0.05$ ). Moreover, the HDAC activity was higher in CSLCs than in non-CSLCs (Figure 1D;  $p < 0.05$ ). In both of the 2 different isolation methods, significantly higher mRNA (Figure 1E,  $p < 0.05$ ) and protein (Figure 1C,  $p < 0.001$ ) expression of HDAC1 and HDAC3 were found in all

CSLCs except for HDAC2. However, no significant difference was observed in the expression of HDAC2 mRNA in CD133+ cells as compared to CD133- cells. These findings indicate higher expression of DNMTs and HDACs in CSLCs.

### Inhibition of DNMTs and HDACs suppresses CSLCs self-renewal

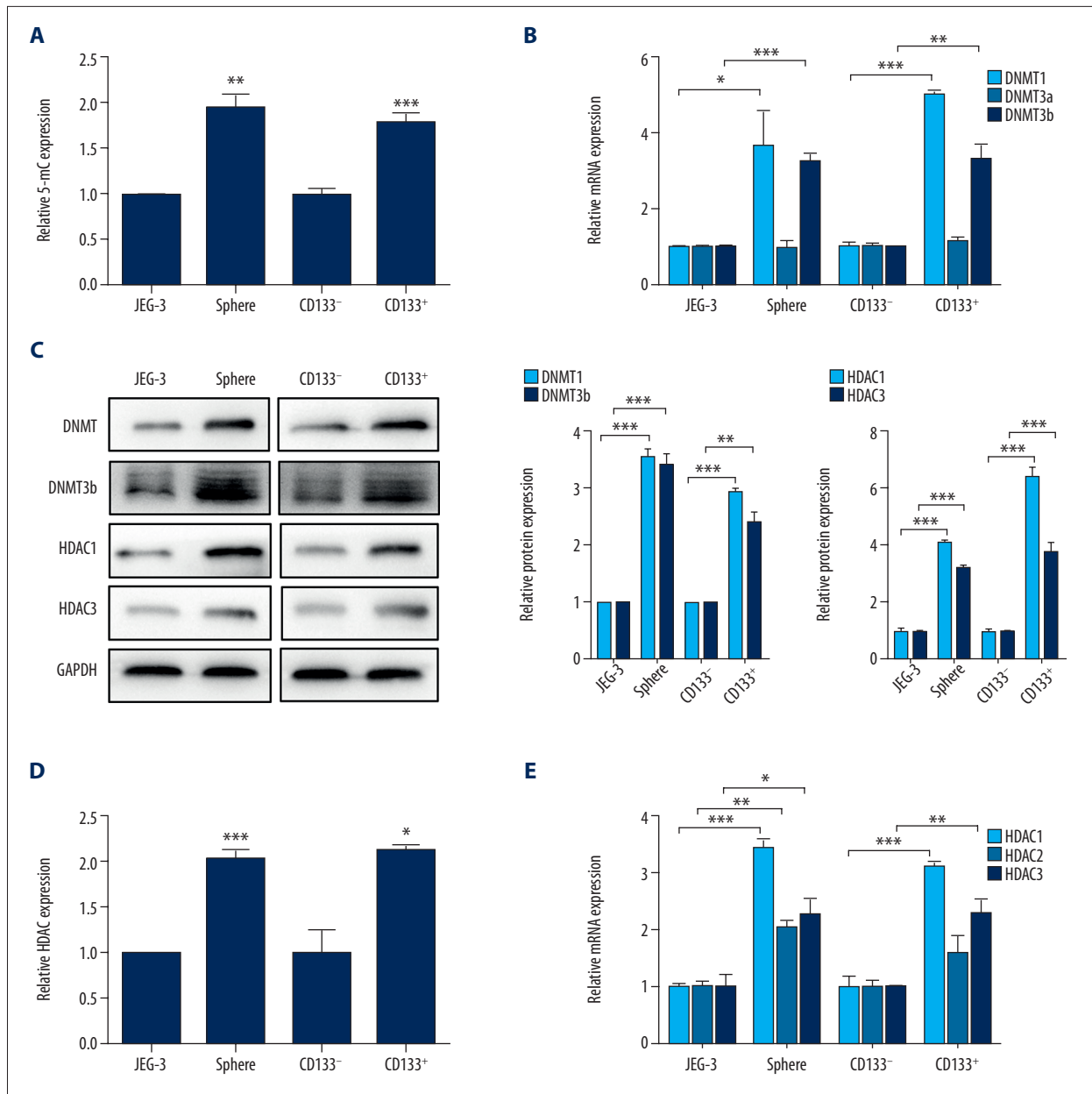
To further confirm the results above, DNMTs inhibitor 5-AzaC and HDACs inhibitor TSA were used to assess whether DNMT and HDAC inhibition could ablate choriocarcinoma CSLCs self-renewal. JEG-3 cells were treated with 5-AzaC or TSA in the process of sphere formation every other day for 7 days. DNMT1 and DNMT3b expression were reduced by 5-AzaC in spheres at both the mRNA and protein levels (Figure 2A, 2B,  $p < 0.01$ ). Similar suppression in the HDAC activity in spheres was observed with the treatment of TSA (Figure 2C,  $p < 0.05$ ); however, a significant decrease in HDAC1 and HDAC3 mRNA (Figure 2D,  $p < 0.05$ ) and protein (Figure 2A,  $p < 0.01$ ) expression was observed. For the self-renewal, 5-AzaC and TSA were significantly abrogated by CSLCs in both first- and second-generation (Figure 2E,  $p < 0.05$ ) and reduced the expression of stemness-associated genes, including NANOG, OCT4, SOX2, and ABCG2 (Figure 2F,  $p < 0.01$ ). The CD133 cell surface expression levels were significantly suppressed in spheres treated with 5-AzaC and TSA (Figure 2G,  $p < 0.05$ ). These data indicated that DNMTs and HDACs play significant roles in preserving the stemness state of CSLCs.

### Inhibition of CSLCs self-renewal with RNAi of DNMT1/3b and HDAC1/3

To further verify our hypothesis that DNMT and HDAC were indeed crucial for maintaining the stemness of CSLCs, we established transfected cells with LV-shRNA of DNMT1/3b and HDAC1/3 alone or combined. The expression of DNMT1/3b and HDAC1/3 protein decreased significantly in the transfected cells (Figure 3A, 3B,  $p < 0.001$ ). Further, the RNAi of DNMTs and HDACs inhibited the sphere formation (Figure 3C,  $p < 0.05$ ) and expression of stemness-associated genes (Figure 3D,  $p < 0.05$ ). Taken together, the results further support the vital role of DNMTs and HDACs in choriocarcinoma CSLCs.

### Curcumol suppresses CSLCs self-renewal and DNMT/HDAC activity *in vitro* and *in vivo*

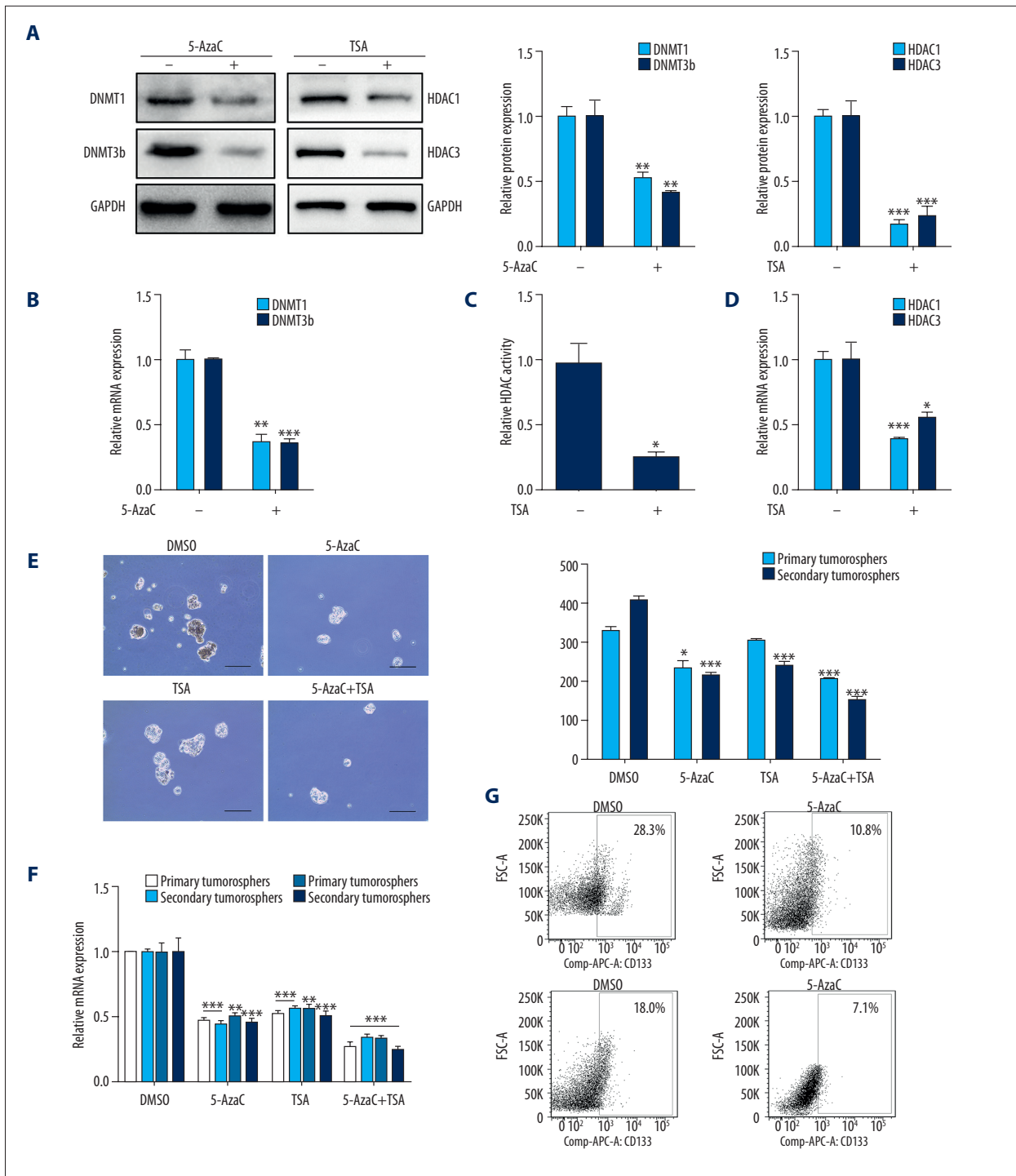
Next, to investigate the effect of curcumol (Figure 4A) on choriocarcinoma, 75  $\mu$ g/ml curcumol was used in JEG-3 cells, and the mean 50% inhibitory concentrations (IC50) of cell growth (Figure 4B) was calculated. Primary JEG-3 cells were treated with 75  $\mu$ g/ml curcumol every other day for 7 days for sphere formation. The treatment for functional level: (1) reduced the sphere formation rate (Figure 4C,  $p < 0.001$ ), (2) suppressed the



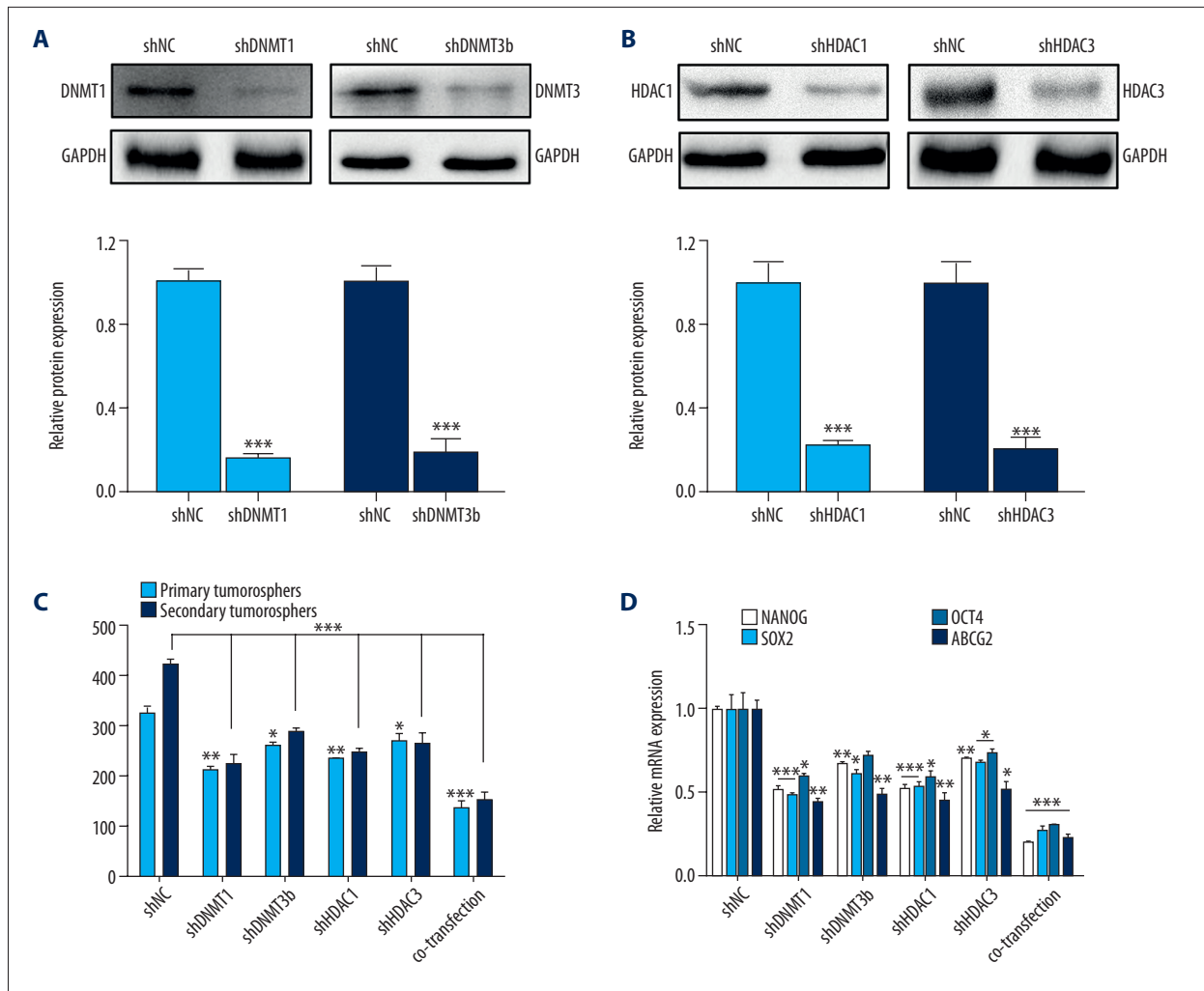
**Figure 1.** Higher DNMT and HDAC expression in choriocarcinoma CSLCs. **(A)** Quantification of 5-mC in non-CSLCs versus CSLCs (JEG-3 vs. sphere and CD133<sup>-</sup> vs. CD133<sup>+</sup>). Data are shown as relative expression compared with non-CSLCs (JEG-3 and CD133<sup>-</sup>). **(B)** RT-PCR analysis of DNMTs mRNA levels in non-CSLCs vs. CSLCs. **(C)** Western blot analysis of DNMT1, DNMT3b, HDAC1, and HDAC3 protein levels in non-CSLCs vs. CSLCs. GAPDH was used as the internal control (**left**). Respective relative changes in DNMTs (**middle**) and HDACs (**right**) protein levels. **(D)** Total HDAC activity of non-CSLCs vs. CSLCs. **(E)** RT-PCR analysis of HDACs mRNA levels in non-CSLCs vs. CSLCs. Data represent mean  $\pm$ SD from at least 3 independent experiments, \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

CD133 surface expression percent (Figure 4D,  $p < 0.01$ ), and (3) decreased the expression of stemness-associated genes (Figure 4E,  $p < 0.001$ ). In terms of epigenetic profile, a higher expression of 5-mC indicated DNA methylation in CSLCs was abrogated by curcumin treatment (Figure 4F,  $p < 0.001$ ). Furthermore, the treatment led to a significant reduction in

the expression of DNMT1 and DNMT3b (Figure 4G, 4H,  $p < 0.05$ ). Consistent with these findings, curcumin decreased HDAC activity in spheres (Figure 4I,  $p < 0.01$ ) and also significantly reduced the expression of the HDAC1/3 protein (Figure 4G,  $p < 0.05$ ) and mRNA (Figure 4H,  $p < 0.001$ ).



**Figure 2.** DNMT and HDAC inhibition decrease CSLCs self-renewal. **(A)** Western blot was used to analyze DNMT1/3b and HDAC1/3 protein levels following 5-AzaC or TSA treatment (**left**). Respective changes in DNMTs (**middle**) and HDACs (**right**) protein levels. **(B)** RT-PCR analysis of DNMT1 and DNMT3b mRNA levels with 5-AzaC treatment. **(C)** Total HDAC activity of CSLCs with TSA treatment. **(D)** RT-PCR analysis of HDAC1 and HDAC3 mRNA levels with TSA treatment. **(E)** Representative images of spheres (10x, **left**) and sphere counts (**right**) in first- and second-generation in cells with 5-AzaC or TSA treatment. **(F)** RT-PCR analysis of stemness-associated genes in sphere cells with 5-AzaC or TSA treatment (groups of 5-AzaC, TSA and 5-AzaC+TSA vs. DMSO group). Results were normalized to expression of GAPDH. **(G)** Flow cytometry analysis of CD133 cell surface expression in sphere cells with 5-AzaC or TSA treatment. Data represent mean  $\pm$ SD from at least 3 independent experiments, \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .



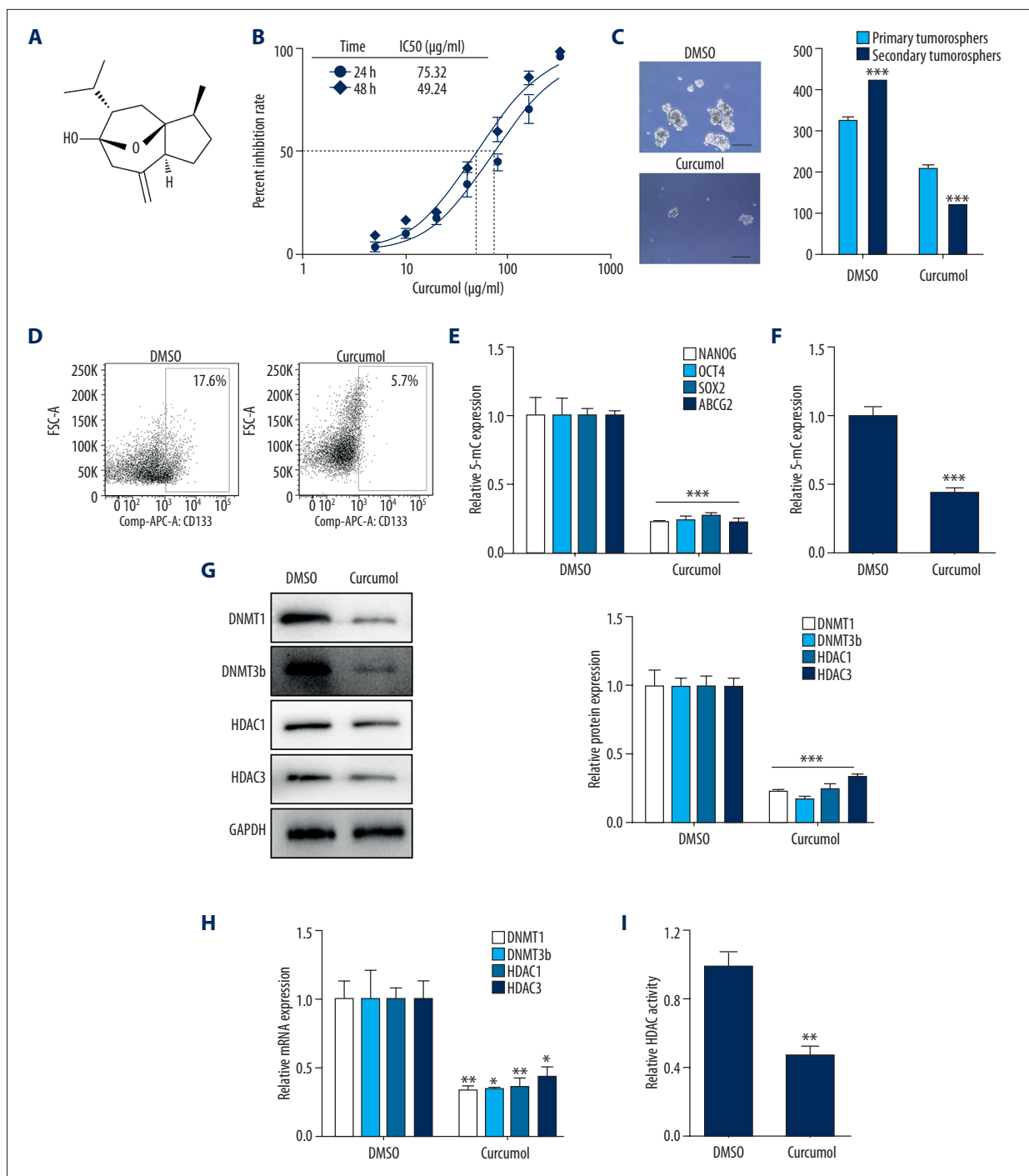
**Figure 3.** RNAi of DNMT1/3b and HDAC1/3 inhibit CSLCs self-renewal. **(A)** Western blot analysis of DNMT1 and DNMT3b protein expression with the shRNA interference (shDNMT1, shDNMT3b vs. shNC). **(B)** Western blot analysis of HDAC1 and HDAC3 protein expression with the shRNA interference (shHDAC1, shHDAC3 vs. shNC). **(C)** Sphere counts in first- and second-generation in cells with overexpressing transfection of DNMT1, DNMT3b, HDAC1, and HDAC3 (co-expression for 4 lentivirus transfections together; groups of shRNA vs. shNC group). **(D)** After the transfection, stemness-associate genes in the sphere cells were detected by RT-PCR (groups of shRNA vs. shNC group). Data represent mean  $\pm$ SD from at least 3 independent experiments, \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

Further, for the *in vivo* studies, choriocarcinoma CSLCs tumor-bearing nude mice were orally treated with curcumol (200 mg/kg) daily for 10 days when the tumor grew to 100 mm<sup>3</sup>. Two weeks after the termination of treatment, changes in the tumors in mice were observed (Figure 5A). A significant shrinkage both in tumor volume and weight was observed after treatment with curcumol (Figure 5B, 5C,  $p < 0.001$ ). Further, IHC analysis showed that the curcumol-treated xenografts exhibited elevated DNMT1, DNMT3b, HDAC1, and HDAC3 expression (Figure 5D). The Kaplan-Meier analysis revealed that mice treated with curcumol survived longer than in the DMSO group (Figure 5E,  $p = 0.0027$ ). These data demonstrate that

inhibition of curcumol overcomes the stemness potential of choriocarcinoma CSLCs.

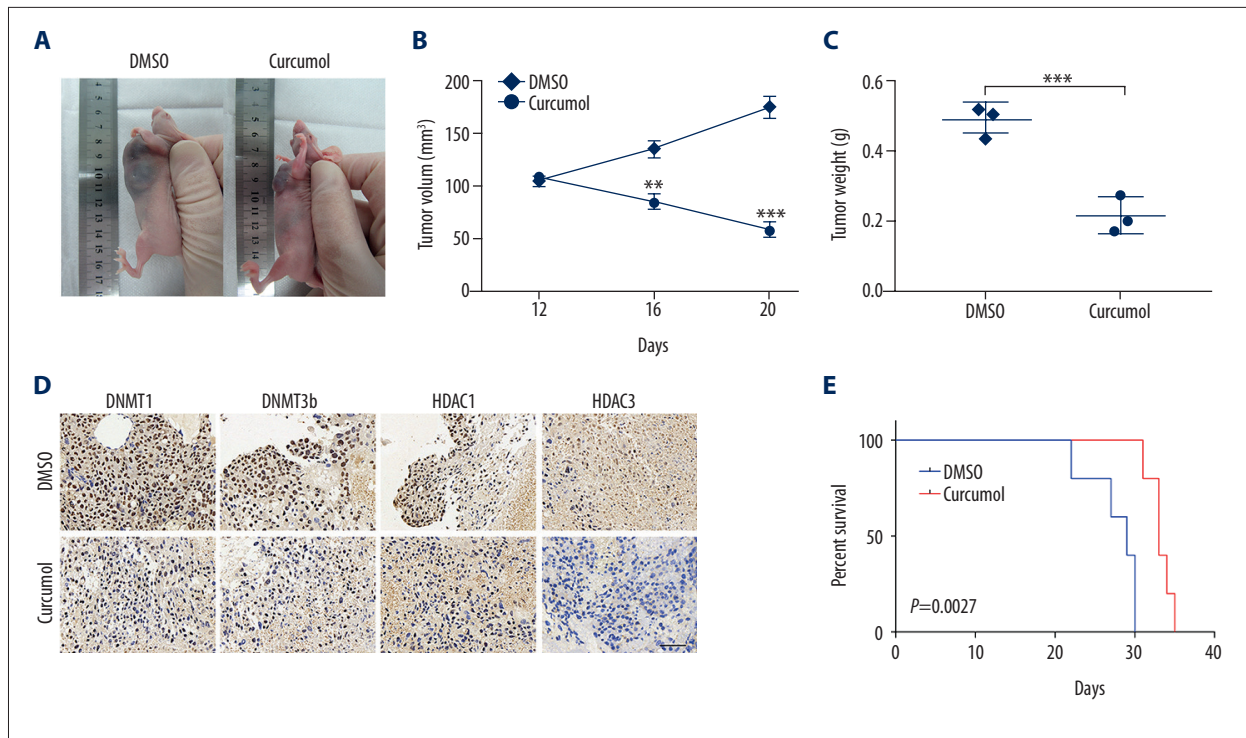
### Curcumol affects CSLCs through regulation of DNMTs and HDACs

Next, we assessed whether the inhibition of curcumol in choriocarcinoma CSLCs is through the regulation of DNMTs and HDACs. The primary cells treated with curcumol had significantly fewer sphere counts as compared to the combination of 5-AzaC and TSA in the secondary generation ( $p < 0.05$ ), and the NANOG, OCT4, SOX2, and ABCG2 expression levels ( $p < 0.05$ ). Further, the treatments of curcumol combined with 5-AzaC or



**Figure 4.** Curcumin decreases CSLCs self-renewal and DNMT/HDAC activity *in vitro*. **(A)** Chemical structure of curcumin. **(B)** IC50 dose response curves of JEG-3 cells with different doses curcumin at 24 h and 48 h. **(C)** Representative tumorspheres images (10×) of DMSO and treated 75 µg/ml curcumin for 7 days (**left**) and sphere counts in first- and second-generation in cells (**right**). **(D)** Flow cytometry analysis of CD133 cell surface expression in sphere cells with curcumin treatment. **(E)** RT-PCR analysis of NANOG, OCT4, SOX2, and ABCG2 mRNA expression levels in sphere cells with curcumin treatment. **(F)** Quantification of 5-mC in sphere cells with curcumin treatment. **(G)** Western blot analysis of DNMT1/3b and HDAC1/3 in sphere cells with RA treatment. GAPDH was used as the internal control (**left**). Respective changes in DNMTs and HDACs (**right**) protein levels. **(H)** The mRNA expression levels of DNMT1/3b and HDAC1/3 in cells with curcumin treatment. **(I)** After curcumin treatment, HDAC activity was detected. Data represent mean ±SD from at least 3 independent experiments, \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.





**Figure 5.** Curcumin decreases CSLCs self-renewal *in vivo*. (A) Tumor-bearing nude mice were treated with (200 mg/kg/day) orally for 10 days when the tumor grew to 100 mm<sup>3</sup>. Subcutaneous tumor in DMSO group vs. curcumin group of nude mice. (B) Tumor volumes were measured every 4 days for 3 weeks (DMSO vs. curcumin group). (C) Tumor weight in the indicated mice (DMSO vs. curcumin group). (D) IHC staining displaying the DNMT1, DNMT3b, HDAC1, and HDAC3 protein expression between DMSO and curcumin groups (IHC, 40×). (E) Kaplan-Meier curves of mice with curcumin treatment, compared with the DMSO group. Data represent mean ±SD from at least 3 independent experiments, \*\* p<0.01; \*\*\* p<0.001.

TSA were also evaluated. When curcumin was combined with 5-AzaC or TSA, both the combinations significantly enhanced the inhibition of CSLCs phenotypes, which was represented by decreased sphere formation rate and stemness-associated genes expression (Figure 6A, 6B, p<0.05). Nevertheless, given the exceptional role of DNMT1 and HDAC1 in CSLCs, we transfected their over-expression lentivirus into primary JEG-3 cells (Figure 6C, p<0.01). The curcumin-mediated reduction of CSLCs was reversed by DNMT1 and HDAC1 over-expression, including stemness-associated genes (Figure 6D, 6E, p<0.05). These results demonstrate that curcumin suppresses CSLCs through the significant regulation of DNMTs and HDACs in choriocarcinoma.

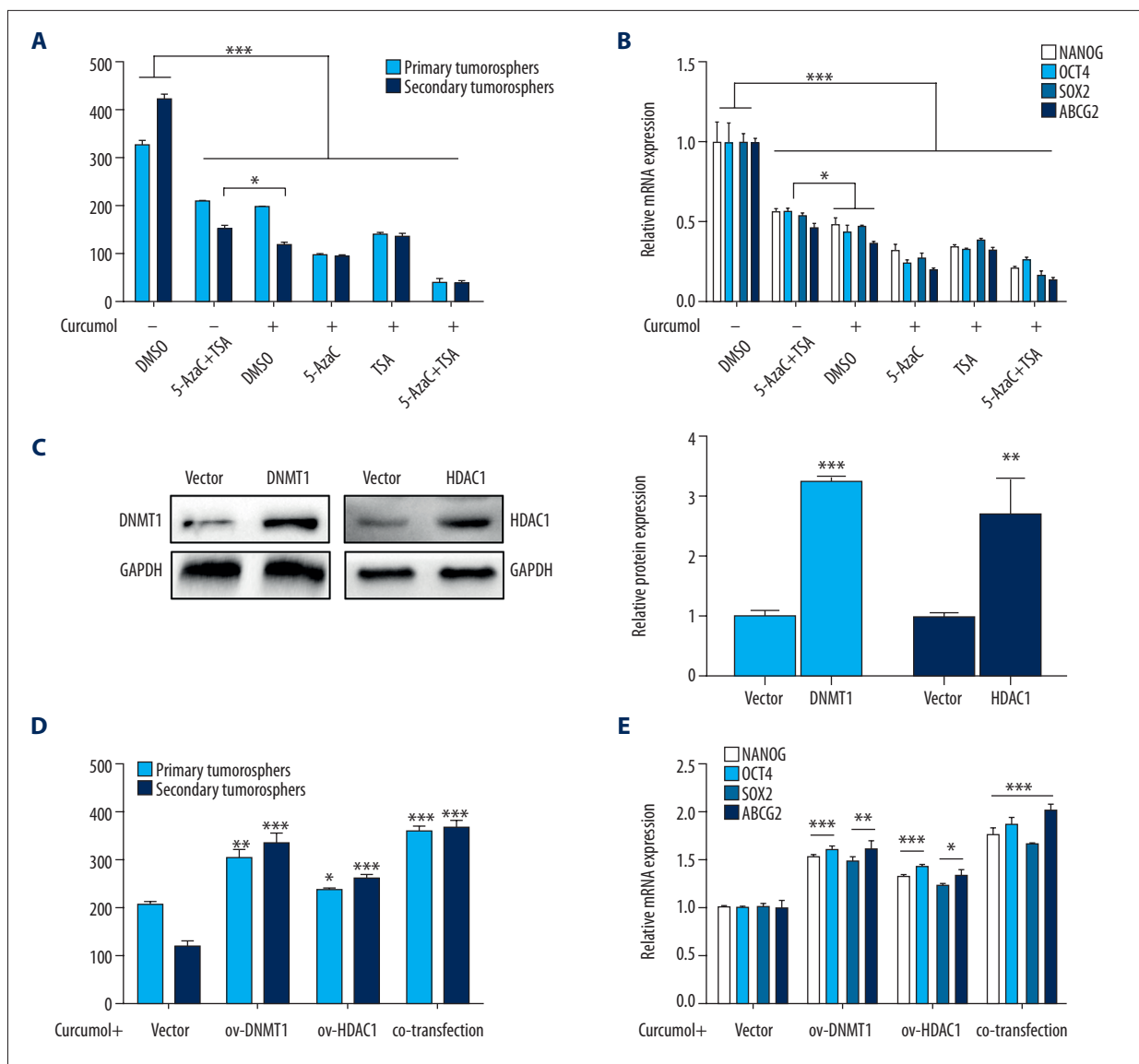
## Discussion

Because cancer stem cells play pivotal roles in occurrence, development, recurrence, and metastasis of cancer, it is imperative to elucidate the underlying molecular mechanisms of CSCs. Recently, the CSC epigenetics was shown to explain the significance of epigenetic-modifying agents in the stem-like characteristics of tumor cells [30]. The present study demonstrated

that curcumin could reduce the self-renewal of choriocarcinoma CSLCs with repression of the DNMT- and HDAC-mediated epigenetic regulation.

Epigenetic regulation is a heritable mechanism during stem cell fate specification [31], which involves intrinsic and extrinsic mechanisms deciding cell fate [32]. CSCs exhibit remarkable functional differences from their progenies that share the same genetic information, indicating the epigenetic changes they regulate. Thus, epigenetics must be the potential driver of the inherent heterogeneity [33]. Chemotherapy is the first-line therapy in choriocarcinoma patients; however, due to the childbearing age and the intrinsic chemotherapeutic sensitivity, there is a serious shortage of grouped clinical specimens. Despite this limitation, the present *in vitro* study supports that hypermethylation and activated histone deacetylation are consistent differential factors in CSCs as compared to non-CSCs.

Embryonic stem cells that lack DNA methyltransferases remain viable; however, they die when induced to differentiate [34]. Numerous studies reported that DNMTs are essential for cancer stem cell maintenance and tumorigenesis [35,36], including DNMTs themselves and DNMT-mediated epigenetics [37,38].



**Figure 6.** Curcumol affects CSLCs via regulation of DNMT and HDAC. With the treatment of curcumol combined with 5-AzaC or TSA, (A) the numbers of spheres in first- and second-generation cultures from JEG-3 cells (all groups vs. DMSO group; 0 μM curcumol+5-AzaC+TSA vs. 75 μg/ml curcumol+DMSO) and (B) stemness-associated genes mRNA expression levels. With the transfection of over-expression lentivirus of DNMT1 or HDAC1, (C) Western blot analysis of DNMT1 and HDAC1 protein levels (left) and respective changes (right), (D) sphere counts in first- and second-generation in cells (over-expression groups vs. Vector group) and (E) respective stemness-associated genes mRNA expression levels. Data represent mean ±SD from at least 3 independent experiments, \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

In another choriocarcinoma cell line, BeWo cells, both DNMT3a and DNMT3b, were found to affect migration and invasion [13]. Furthermore, the epigenetic mechanism has also been found to play a dynamic role in oncogenes. For instance, DNA methylation of E-cadherin promoter helps to recruit HDACs to the site, leading to histone deacetylation and transcriptional silencing [39]. The combination of DNMT and HDAC inhibitors, one of the most common combination therapies, have been extensively evaluated in clinical trials for the treatment of a

variety of cancers [40]. In the present study, DNMT1/3b and HDAC1/3 expression were consistently upregulated in CSLCs. The combination of 5-AzaC and TSA showed a noticeable effect on multipotential stemness, which indicated the critical role of DNMTs and HDACs in CSLCs.

Besides these known single-target compounds, many other multipotent-drugs are being developed and investigated in clinical trials. Thus, we sought to evaluate the effectiveness of the

traditional drugs with new and pleiotropic functions in cancer therapy. As an active component of a traditional Chinese medicine used in gynecological therapy, curcumin attracted our attention due to its low toxicity and its anti-cancer effects on many tumors. Therefore, in this study we investigated the anti-cancer activities of curcumin against choriocarcinoma CSLCs. Numerous studies have reported the anti-cancer mechanisms of curcumin. For instance, it repressed colorectal cancer cells via regulating IGF-R and p38 MAPK signaling pathways [41] and Akt/GSK3 $\beta$ /cyclin D1 pathways [42]. Some traditional Chinese medicines, such as curcumin, have been shown to affect epigenetic regulation in cancer cells [43,44]. However, there are no reports on its epigenetic-regulatory effect in cancer cells, particularly in CSCs. Notably, in the present study, the stemness repression in choriocarcinoma CSLCs was observed in both *in vitro* and *in vivo* studies with treatment of curcumin. Moreover, curcumin enhanced the overall survival rate of tumor-bearing mice. We further investigated the underlying regulatory mechanisms of curcumin. As hypothesized, DNMTs and HDACs expressions were significantly decreased in CSLCs by curcumin, and the stemness repression of curcumin can be

abrogated by DNMT/HDAC overexpression, remarkably. These findings indicated that DNMT/HDAC is the potential target of curcumin, which warrants further investigation. Moreover, we found that curcumin exhibited a significantly greater effect on stemness repression in CSLCs as compared to the combination of DNMT and HDAC inhibitors. Experimentation with various other types of inhibitors is required.

## Conclusions

In conclusion, our results underlined the significance of epigenetic mechanism in choriocarcinoma CSLCs through regulation of DNMTs and HDACs. Our results also demonstrated the effectiveness of curcumin against CSLCs, indicating its potential therapeutic role in epigenetic cancer therapies.

## Conflict of interest

None.

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