8-bromo-5-hydroxy-7-methoxychrysin targeting for inhibition of the properties of liver cancer stem cells by modulation of Twist signaling

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Abstract. Emerging evidence has suggested that cancer stem cells with expression of surface biomarkers including CD133 and CD44 have more aggressive biological behavior, including epithelial-mesenchymal transition (EMT), which are closely related to invasion. The upregulation and nuclear relocation of the EMT regulator Twist1 have been implicated in the tumor invasion and metastasis of human hepatocellular carcinoma (HCC). In this study, we aimed to isolate and characterize a small population of $CD133^+$ cells that existed in the HCC cell line SMMC-7721 by MACS and investigated the possible roles of 8-bromo-7-methoxychrysin (BrMC), a synthetic analogue of chrysin, in inhibiting the properties of $CD133^+$ sphere-forming cells (SFCs) derived from the HCC cell line SMMC-7721, namely liver cancer stem cells (LCSCs). Based on the data, BrMC inhibited the proliferation, self-renewal and invasion of LCSCs *in vitro* and *in vivo*, downregulated the expression of the LCSC biomarkers CD133 and CD44 and induced EMT by downregulating the expression of Twist and β-catenin in LCSCs. BrMC potentiated the inhibition of LCSCs self-renewal after reduction of twist protein levels, which was attenuated when twist was overexpressed. This study not only provides an important experimental and theoretical basis for investigation of BrMC in LCSCs, but also helps in the development of effective therapeutic medicine for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide and the third most common cause of cancer-related death (1), resulting in almost 700,000 deaths in 2008. Clinically, HCC is often diagnosed at the late stage and medical treatments including chemotherapy, chemoembolization, ablation and proton beam therapy remain disappointing. There is an urgent need for new therapies for this aggressive disease. Recently, HCC progression has been thought to be driven by a small subset of cells, namely liver cancer stem cells (LCSC), through their capacity for self-renewal, production of heterogeneous progeny, resistance to chemotherapy and limitless proliferation. Many research groups in leukemia and several solid tumors supported the existence of such a subpopulation, which was successfully isolated and manifested marked tumorigenic capacity assessed by NOD/SCID mouse xenograft assay (2-8).

CD133 (AC133) is a highly conserved antigen as the human homologue of mouse Prominin-1, which was originally identified as a 5-transmembrane cell surface glycoprotein expressed in a subpopulation of the CD34 hematopoietic stem and progenitor cells derived from human fetal liver and bone marrow (9,10). Notably, CD133 was expressed in most types of cancer stem cells within colon, breast, prostate, glioblastoma, medulloblastoma (MB) (11), and hepatocellular carcinoma (12,13). In the past few years, compelling evidence has emerged in support of the notion that CD133 is a surface marker for LCSCs in human liver cancer cell lines and clinical samples and that $CD133^+$ LCSCs are associated with a hypoxic marker in clinical HCC samples, suggesting that $CD133^+$ LCSCs have a critical role in tumor growth and resistance to anticancer therapy in liver cancers (14). CD44 is also regarded as an important marker for LCSC (15). In all HCC cell lines studied, CD133-positive cells showed higher cell migration activity and upregulated invasion- and EMT-associated genes including Twist (16).

The Twist1 gene encodes a transcription factor containing a basic helix-loop-helix (bHLH) domain (17) and an amino-acid motif present in a protein family involved in the regulation of organogenesis (18-20). Recently, a number of studies have revealed that Twist plays essential roles not only in the development of multiple organs and systems, but also in cancer metastasis (21-23). It has been reported that Twist1 overexpression correlates positively with HCC metastasis (24). Further study on different HCC cell lines revealed that

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HCC cells with higher levels of Twist1 have higher metastatic ability. This suggests that Twist1 induces EMT changes, which are partially responsible for the increased HCC cell invasiveness (24). β-catenin, encoded by the CTNNB1 gene, has multiple functions, including mediation of cell adhesion and signal transduction. It combines with a variety of proteins to regulate cell proliferation and differentiation, which is critical for embryonic development and tumorigenesis. In addition, β-catenin has been shown to be accumulated within 67% of HCC tissues and is closely related to the clinicopathological features of HCC (25-27).

8-bromo-7-methoxychrysin (BrMC) is a novel synthetic analogue of chrysin (5,7-dihydroxyflavone, ChR), which is a natural and biologically active flavone extracted from many plants, honey and bee propolis and has been shown to inhibit cell proliferation and induce apoptotic cell death in a variety of cancer cells (28-34). Because of the poor oral bioavailability, chrysin may not be successful when used as a dietary flavonoid for cancer chemotherapeutics (35). It has been reported that ChR halogenated derivatives had stronger bioactivities than the lead compound (36). Our previous study showed that the effect of BrMC on the inhibition of proliferation and induction of apoptosis in the colon cancer cell line HT-29 and the gastric cancer cell line SGC-7901, was stronger than that of ChR (37,38). BrMC also induces apoptosis of HCC cells by ROS generation and sustained JNK activation (39). Recently, our laboratory reported that BrMC affected the number of glioma stem-like cells (GSLCs) derived tumor spheres by MTT assay (40).

In this study, we investigated the possible functions of BrMC in inhibiting the characteristics of $CD133^+$ sphere-forming cells (SFC) derived from SMMC-7721 cell line *in vitro* and *in vivo* and explored the potential mechanisms.

Materials and methods

Cell culture and reagents. The hepatoma cell line SMMC-7721 and the immortalized embryo liver cell line L-02 were obtained from the Chinese Academy of Sciences (Shanghai, China). SMMC-7721 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies) in an incubator containing 5% CO_2 at 37˚C. BrMC was synthesized as described previously (37). Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Hyclone (Thermo Scientific, USA). Trypsin and dimethyl sulfoxide (DMSO) were from Amersco Co. (USA).

Immunomagnetic separation of CD133 hepatoma cells. Cells were suspended with PBE incubation solution (0.5% bovine serum albumin, 0.08% EDTA in PBS, pH 7.2) to a final concentration of $1x10^8$ cells in 0.5 ml, then incubated with anti-CD133 antibody (final concentration 20 μg/ml) at 4˚C for 30 min and incubated with antibody-coated superfine magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at 10˚C for 15 min and suspended in 20 times the total volume of PBE solution. The separation column was installed into a magnetic field and pretreated with 0.5 ml PBE which was naturally eluted due to gravity. The incubated cell suspension was added to the separation column and naturally eluted. The column was rinsed twice and then separated from the magnetic field and subsequently inserted into a new tube, followed by administration of 1-2 ml PBE along the needle core to remove the CD133-positive cells. Simultaneously, negative cells were collected and the two types of cells were rinsed with medium.

Flow cytometry (FCM). The freshly isolated cells from each fraction were prepared at a concentration of 10^5 cells/ml using William's E medium (containing 20% FBS) and incubated for 15-30 min at room temperature to block non-specific sites. These cells were then washed twice with PBS and re-suspended in 990 μl PBS. Subsequently, 10 μl of antibodies, including CD133 (PE-conjugated, Biolegend, USA) and isotype control IgG2b (PE-conjugated, Biolegend), were added to each cell suspension. After 30 min of incubation at 4˚C in the dark, the cells were washed twice with PBS, fixed in 0.1% formaldehyde and analyzed using the FACS Calibur™ system (BD Immunocytometry Systems, San Jose, CA, USA).

Spheroid formation and self-renewal assay. Parental cells were plated at a density of 2,000 cells/well in low adherence plates (6 wells) with serum-free stem cell conditional medium that containing DMEM/F12 (Gibco Invitrogen) plus 20 ng/ml EGF (Peprotech, NJ, USA), 10 ng/ml bFGF (Peprotech), 1X B27 (Invitrogen, CA, USA) and 0.4 μg/ml insulin (Peprotech). After 4 days of culture, sphere forming cells (SFCs) were visualized in a microscope and the number of cells after trypsin-EDTA digestion was counted.

To investigate self-renewal capacity of liver cancer sphere, single cell suspension prepared from the SFCs was diluted to 500 cells/ml. Single cell suspension (2 μl) was plated in 96-well ultra-low plates containing 150 μl serum-free medium per well. Wells containing about one or two cells were included and those with single cells were marked and monitored daily under a microscope (Nikon Eclipse TE2000-S) for 6 days. Then, the colonies were counted.

To analyze the effects of BrMC on self-renewal of SFCs, single cell suspension of SFCs was plated at a density of 2,000 cells/well in 6-well ultra-low plates. In addition, different concentrations of BrMC (0.1, 0.3 and 1.0 μM) were added to medium. After culturing for 6 days, the colonies were counted under a microscope.

Cell viability assay. Cell growth was measured by the MTT assay (Sigma). Briefly, SMMC-7721 cells were plated at a density of $5x10^3$ cells/well in 96-well plates and allowed to attach for 24 h for each conditions, resulting in log phase growth at the time of drug treatment. BrMC (0.1, 0.3, 1.0 and 3.0 μmol/l) was added to the wells for 48 h. After treatment, MTT reagent was added to each well at 5 mg/ml in a 20-μl volume and the reaction was incubated for another 4 h. The formazan crystals formed by viable cells were subsequently solubilized in DMSO. Absorbance was measured at 550 nm using an automated microplate reader (Bio-Rad 550). Cell viability was expressed as a percentage of the value for control cultures. The cytotoxic effects of BrMC on SMMC-7721 sorted or non-sorted cells were expressed as IC_{50} values (the

drug concentration that reduced the absorbance of treated cells by 50% compared to untreated cells), which was plotted using Graph Pad Prism 5 (GraphPad Software, San Diego, CA, USA). All experiments were carried out in triplicate.

Matrigel invasion assay. The invasion chamber has 24 cell culture inserts, each of which contains a polyethylene terephthalate membrane with 8-μm pores (Corning Inc., Lowell, MA, USA) coated with Matrigel. Serum-free DMEM (1 ml) was added to the apical side of an insert and then 1 ml of DMEM plus 10% fetal calf serum was added to the basal side of the insert as the chemoattractant. A total of 2,000 sorted or non-sorted SMMC-7721 cells were plated in the top chamber of the transwell and treated with BrMC (0.1, 0.3, 1.0 and 3.0 μmol/l) for 24 h. The cells that had not invaded through the pores of the insert were scraped off the apical side of the inserts with a sterile cotton swab and discarded. Cells invaded to the lower chamber were fixed with methanol, stained with crystal violet and counted.

Experimental studies by a xenograft model in nude mice. Eight-week-old BALB/c mice of either sex were purchased from Hunan Agricultural University [SCXK (Xiang 2002-003)], maintained in Hunan Research Center for Safety Evaluation of Drug (Experimental Animal Center of Hunan Province) and then bred under specific pathogen-free conditions. Mice were kept in ventilated and filtered cages, fed an irradiated diet and housed on irradiated bedding. Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with the guidelines of the Chinese government approved by the College BioResources Ethics Review Board. The area between the right leg and abdominal cavity in the nude mice was disinfected with iodine and a single cell suspension was injected into the mice subcutaneously using a 100-μl micro syringe. The needle was held in place for 1 min and then gradually withdrawn to prevent liquid return. After inoculation, mice were housed in a sterile barrier system at constant temperature (25±2˚C) and humidity (45-50%). Tumor formation and growth were observed daily. At the end of the experiment, the nude mice were sacrificed and the tumors were peered and fixed in fresh 4% paraformaldehyde, then embedded and H&E stained.

RNA interference and pcDNA3-Twist1 transfection. Twist1 siRNA (5'-GAU GGC AAG CUG CAG CUA UTT-3', 5'-AUA GCU GCA GCU UGC CAU CTT-3') and a non-silencing control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3', 5'-ACG UGA CAC GUU CGG AGA ATT-3') were synthesized by GenePharma (Shanghai, China). SiRNA transfections were performed according to the manufacturer's instructions. Briefly, Twist1 siRNA or negative control siRNA of 100 pmol was diluted in 250 μl of Opti-MEM I medium. Next, 5 μl Lipofectamine 2000 was diluted in 250 μl of Opti-MEM I Medium. After 5-min incubation, the diluted siRNA was mixed with diluted Lipofectamine 2000 gently and incubated for 20 min at room temperature. The oligomer-lipofectamine complexes were applied to the subconfluent cells which were seeded in a 6-well plate 24 h before the experiment. Forty-eight hours after transfection, the Twist1 protein levels were assessed by western blotting.

Table I. Comparison of sphere-forming ability between CD133$^+$ cells and parental cells.

Cell line	No. of spheres per 2,000 cells		Sphere volume (μm^3)	
	Parental cells	CD133$^+$ cells	Parental cells	CD133$^+$ cells
SMMC-7721	83±21	232±45[a]	314±34	986±52[a]

[a]P<0.05 vs parental cells.

pcDNA3-Twist1 was purchased from GenePharma. To generate Twist1-expressing stable transfectants, L-02 and SMMC-7721 cells and SFCs of SMMC-7721 cell line were transfected with pcDNA3-Twist1 and stable clones were selected with 1000 μg/ml of G418 (Calbiochem) for 4 weeks.

Western blotting. Cells were washed once in pre-cold PBS and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and protease inhibitors]. Sample proteins were separated by 10% SDS-PAGE gel, after electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Richmond, CA, USA) at 4˚C for 2 h at 100 mA. The membranes were detected by rabbit polyclonal antibodies against ZO-1 (Abcam), Twist (Cell Signaling) or mouse monoclonal antibodies against N-cadherin (Upstate), Vimentin Ab-2 (Neo Markers), E-cadherin (BD Transduction), β-catenin (Cell Signaling), β-actin (Sigma), respectively.

Statistical analysis. Data were presented as the mean ± SD. Comparisons of experimental values between BrMC-treated cells and untreated controls were conducted using analysis of variance or the Kruskal-Wallis rank test. Statistical significance was defined as P<0.05.

Results

LCSCs exist in hepatoma carcinoma cell line SMMC-7721 and in sorted CD133$^+$ cells. Human hepatoma carcinoma cell line SMMC-7721 was cultured *in vitro* as normal condition and the cells adhered to the culture slides (Fig. 1A, parental cells). In order to isolate LCSCs, SMMC-7721 cells were enzymatically dispersed into single-cell suspensions and subjected to immunostaining with an anti-CD133 antibody (Miltenyi Biotec Inc.) and FACS analysis. FACS results showed that the percentage of CD133$^+$ population was ~58.72% (Fig. 1C), which is much higher than that of CD133$^-$ population (1.07%) or non-sorted cells (3.53%), indicating our immunomagnetic separation system is efficient. On the other hand, a major characteristic of CSC cells is their capacity to form three-dimensional structures, or spheres. In the case of inoculation of 2,000 cells per well, there were many more spheres formed in the group of CD133$^+$ cells (Fig. 1A and Table I), while at the same time-point of sphere formation, the sphere volume of CD133$^+$ cells was much greater than that of parental cells both for the first generation and for the second generation (Fig. 1A).

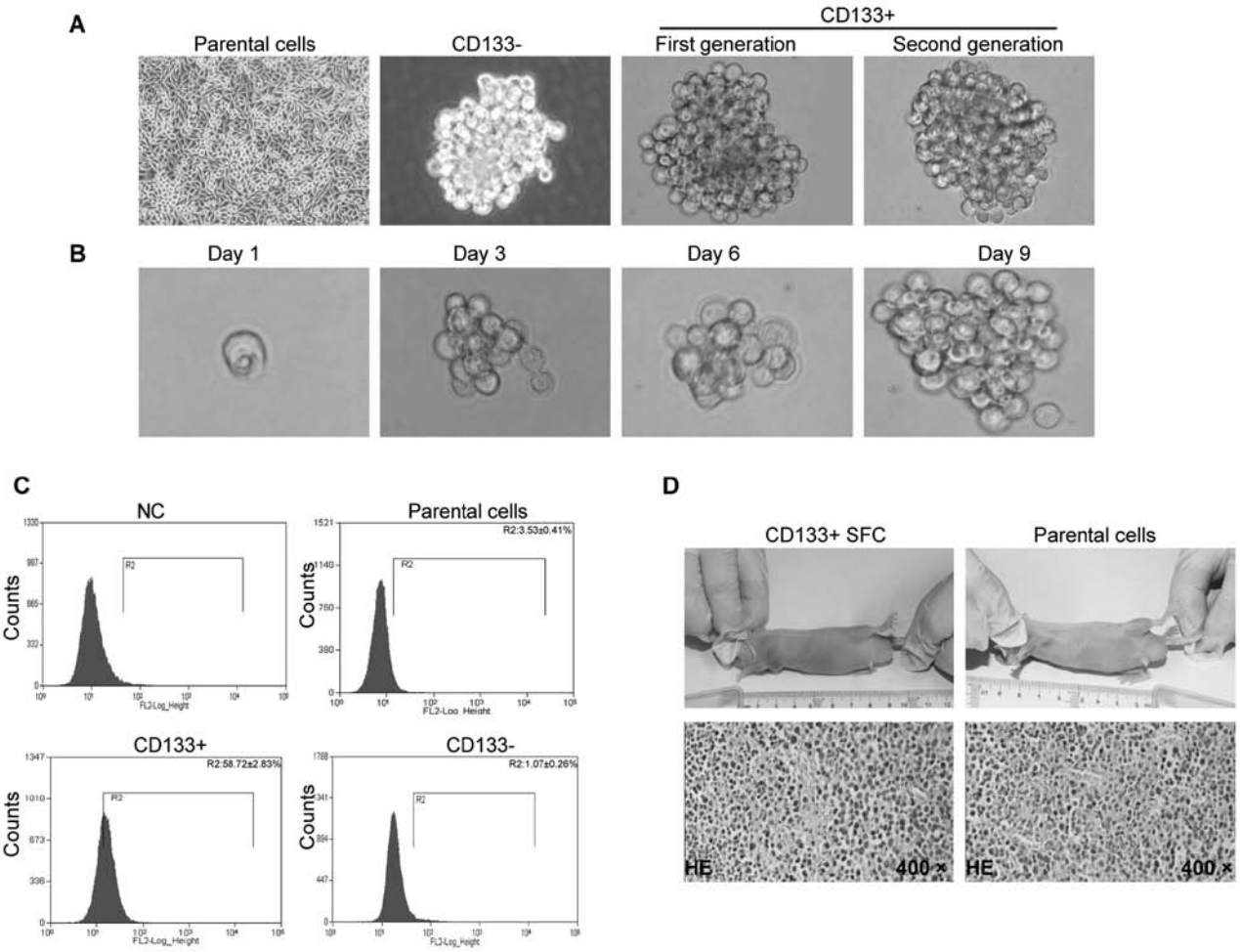

Figure 1. Identification of LCSCs in SMMC-7721 cell line. (A) Human hepatoma carcinoma cell line SMMC-7721 (parental cell) was cultured in DMEM medium. In conditional stem cell culture system, sphere-forming cells (SFCs) can be passaged and form spheres for the second generation and the volume and the size of CD133$^+$ SFCs were much larger than that of parental cells. (B) The process of a single CD133$^+$ SFCs of SMMC-7721 cell line to form a sphere was 1, 3, 6 and 9 days, respectively. (C) FACS analysis was performed to check the CD133 expression rates. (D) CD133$^+$ SFCs ($1x10^4$) derived from SMMC-7721 cell line, or $1x10^5$ parental cells were subcutaneous seeded into male Balb/c-nu mice. Six weeks later, there were visible tumors in CD133$^+$ SFCs xenotransplantation and no tumor seen in parental cells xenotransplantation. H&E staining revealed that the histological features of xenograft tumors in the CD133$^+$ SFCs were similar to those in parental SMMC-7721 cells.

One of the cancer stem cell characteristics is self-renewal. To test this, one SMMC-7721 cell per well was plated to a 96-well plate and the wells with one cell were visualized daily. Fig. 1B shows the process of single SMMC-7721 cell forming a sphere. The result showed that the SMMC-7721 derived CD133$^+$ cells have the ability to form spheres, even a single cell became a new hepatoma cancer sphere (Fig. 1B). These data indicate that the SMMC-7721 derived CD133$^+$ cell population has the capacity of self-renewal.

Xenotransplantation is the gold standard for evaluating tumorigenicity of tumor cells. We tested whether our sorted CD133$^+$ LCSCs have more tumor initiating capability. We injected different cell numbers of sphere-formed SMMC-7721 CD133$^+$ LCSCs, or parent cells into Balb/c-nu mice to get the minimum number of seeded cells *in vivo* tumorigenicity, respectively.

As shown in Table II and Fig. 1D, as few as 10,000 cells from the SMMC-7721 CD133$^+$ SFC were able to grow into tumors (4/4, 100%) when subcutaneously injected into Blab/c-nu mice, while $1x10^6$ parental cells were needed for tumor formation (4/4, 100%). There was also a great difference in the time needed for tumor formation, ~17 days for CD133$^+$ sphere

Table II. Xenotransplantation of human hepatoma carcinoma cell line SMMC-7721 and its LCSCs into Balb/c-nu immunodeficient mice.

Cell	Inoculum size	Tumor incidence[a]	Latency period (days)[b]
Parental cell	$1x10^4$	0/4	-
	$1x10^5$	1/4	47
	$1x10^6$	4/4	35
CD133$^+$ sphere cells	$2x10^3$	3/4	36
	$1x10^4$	4/4	17
	$1x10^5$	4/4	9

[a]The number of tumors detected/number of injections. [b]Approximate number of days from tumor cell injection to appearance of a tumor.

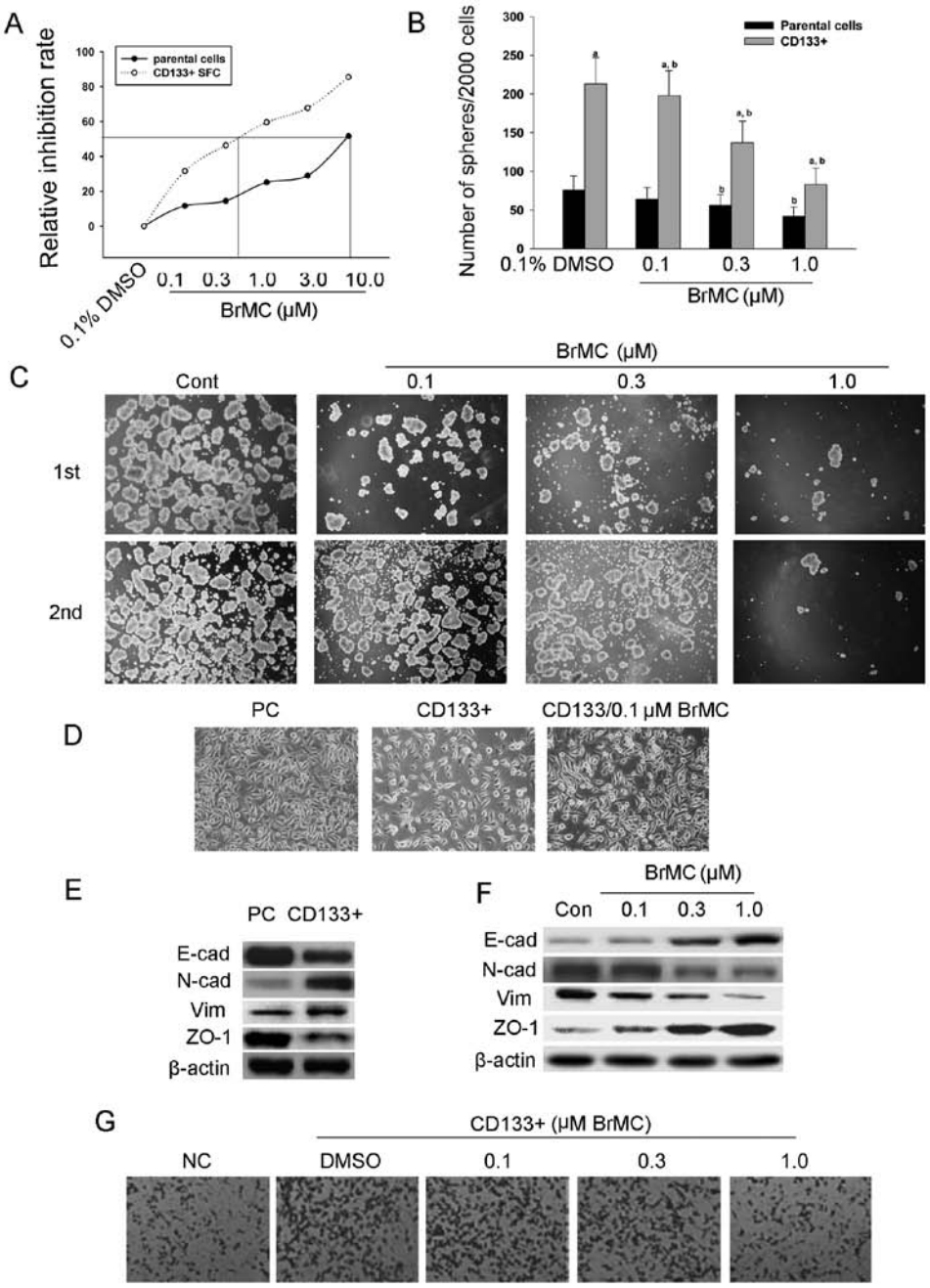

Figure 2. BrMC suppresses proliferation, self-renewal and invasion of LCSCs. (A) The relative inhibition rate (%) of BrMC on CD133+ SFCs or parent cells (PC) was determined by MTT assay. The IC_{50} of BrMC to CD133+ SFCs was 0.5 μmol/l, while the parental cells is 13.1 μmol/l. (B) BrMC reduced the numbers of spheroids in a dose-dependent manner. [a]P<0.05 vs parental cells treated by 0.1% DMSO. [b]P<0.05 vs CD133+ treated by 0.1% DMSO. (C) BrMC reduced the size and number of spheroids in a dose-dependent manner either for the first generation of LCSCs or for the second generation. (D) BrMC induced mesenchymal to epithelial transformation. The CD133+ SFCs were grown in plates, treated with 0.1 μM of BrMC, the cell morphology tended to be epithelial phenotype. (E) CD133+ SFCs highly express mesenchymal cell biomarker N-cadherin and Vimentin and express low levels of epithelial biomarkers E-cadherin and ZO-1. (F) BrMC decreased the expression of N-cadherin and Vimentin and increased the expression of E-cadherin and ZO-1 in CD133+ SFCs. (G) BrMC inhibited the invasion ability of CD133+ SFCs in a dose-dependent manner.

cells compared to ~35 days for the parental cells (Table II). These results demonstrate that CD133+ SFCs derived from SMMC-7721 cell line was highly tumorigenic and characteristic of CSC.

BrMC suppresses proliferation, self-renewal and invasion of LCSCs. Previously, our laboratory reported that the BrMC inhibits proliferation and induces apoptosis of HCC cells (39). Here, we performed MTT assay to analyze the influence of BrMC to CD133+ SFCs that derived from the SMMC-7721 cell line. As shown in Fig. 2A, as compared to negative control (0.1% DMSO), the IC_{50} of BrMC to CD133+ sphere cells is 0.5 μmol/l, which is much lower than that of parental cells (13.1 μmol/l), suggesting that BrMC preferentially inhibits the proliferation of CD133+ sphere cells.

In order to observe whether BrMC efficiently inhibits the self-renewal of our sorted SMMC-7721 CD133+ sphere cells, we treated the spheres seeded in 6-well low adherence plates

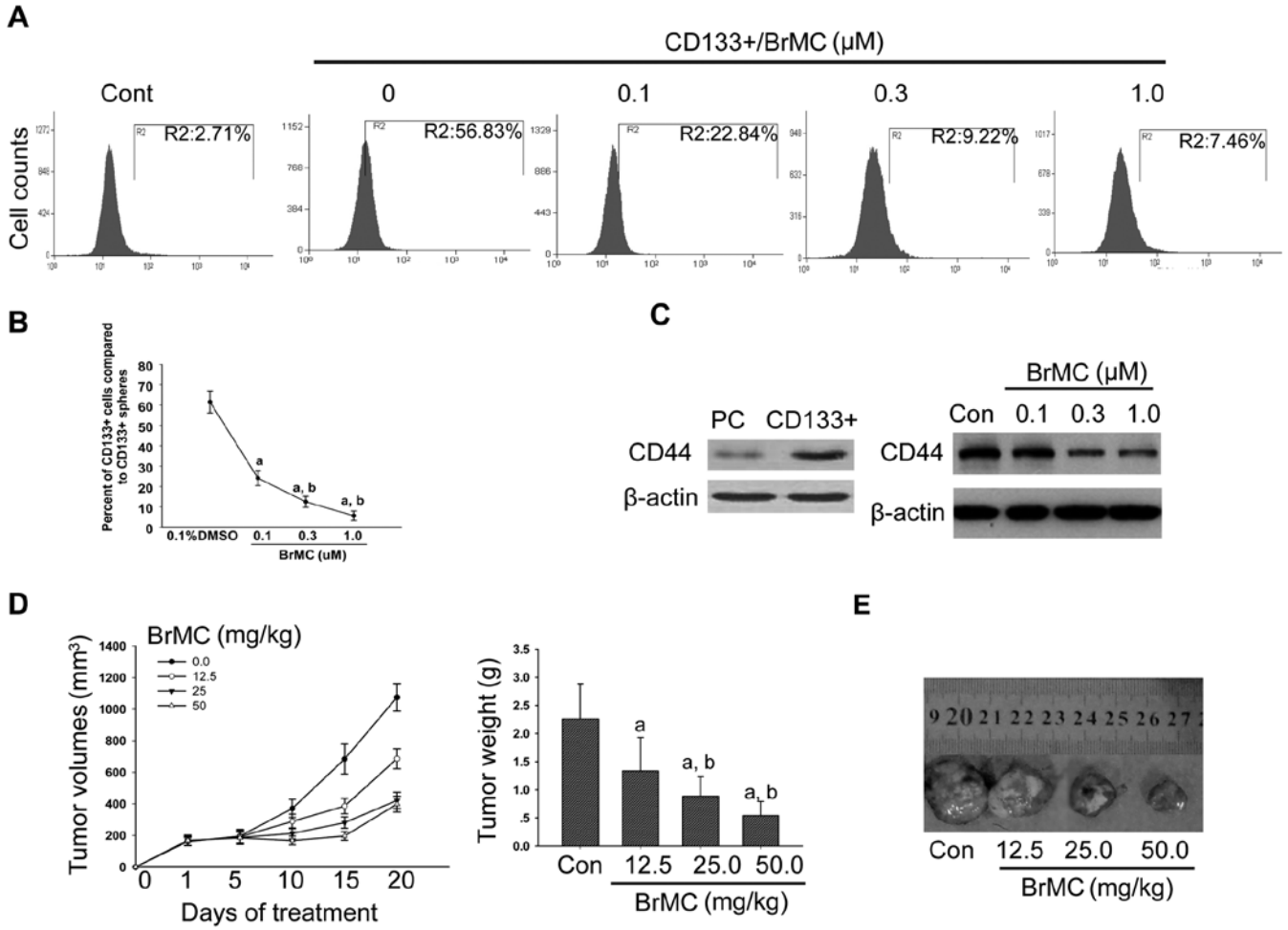

Figure 3. BrMC downregulates the expression of LCSC biomarkers CD133 and CD44. (A) FACS analysis results show the effects of BrMC on the expression of CD133 in CD133$^+$ SFCs of SMMC-7721 cell line. (B) Statistical analysis of (A), each experiments were performed in triplicate and representative examples are shown (^{a}P<0.01 vs groups treated by 0.1% DMSO; ^{b}P<0.05 vs groups treated with 0.1 μM of BrMC). (C) Western blot analysis show the effects of BrMC on expression of CD44. (D) BrMC suppressed tumor growth *in vivo*, while in CD133$^+$ SFC xenograft nude mouse models, different concentrations of BrMC (0, 12.5, 25 and 50 mg/kg) were daily lavaged for 20 days. (E) Representative images of tumors were taken.

with different concentrations of BrMC. Forty-eight hours after treatment, the spheres were passaged for second sphere formation without treatment, after 6-day culture, at which time-point, the second spheres were counted under a microscope. As depicted in Fig. 2B and C, BrMC inhibited both the size and the numbers of the first or second passaged spheres in a dose-dependent manner.

Epithelial-mesenchymal transition (EMT) is a critical process providing tumor cells with the ability to migrate and escape from the primary tumor and metastasize to distant sites. Recently, EMT has been shown to be associated with the cancer stem cell (CSC) phenotype in hepatoma cancer (2,41-43). Based on above mentioned results, we further examined whether BrMC affects the EMT process of CD133$^+$ SFCs derived from the SMMC-7721 cell line. As shown in Fig. 2D, the parental cells exhibited epithelial cell morphology. When the SMMC-7721 cell line derived CD133$^+$ sphere cells were cultured in 10% FBS and allowed to adhere to plates, the cells presented fusiform morphology, which is the mesenchymal cell phenotype. However, after treatment with 0.1 μM BrMC, the cell morphology tended to change to epithelial phenotype. The experiments showed that SMMC-7721-derived CD133$^+$ sphere cells possess mesenchymal cell morphology and BrMC induced mesenchymal to epithelial transformation.

We also performed western blot analysis to check the variety of EMT biomarkers in CD133$^+$ sphere cells, parental cells and the cell populations treated with the indicated concentration of BrMC (0.1, 0.3 and 1.0 μM). From Fig. 2C-F we can see that the CD133$^+$ sphere cells highly expressed mesenchymal cell biomarker N-cadherin and Vimentin and epithelial biomarkers E-cadherin and ZO-1 at low levels. While the treatment of BrMC leads to downregulation of N-cadherin and Vimentin and upregulation of E-cadherin and ZO-1 in CD133$^+$ sphere cells. Together, our data suggest that BrMC can effectively inhibit EMT in LCSCs.

We next performed a transwell assay to demonstrate whether BrMC affects invasion of LCSC. As described in Materials and methods, a total of 2,000 parental or CD133$^+$ cells derived from SMMC-7721 cells were treated with BrMC (0.1, 0.3, 1.0 and 3.0 μmol/l) for 24 h and then plated in the top chamber of the transwell, cells that invaded the lower chamber were counted. As shown in Fig. 2G, sorted CD133$^+$ sphere cells

Table III. Effects of BrMC on the secondary tumor formation ability in BALB/c nu mice for SMMC-7721 derived CD133+ SFCs.

	Secondary tumor incidence[a]		Secondary tumor volumes (mm^3)	
Days	Con	BrMC	Con	BrMC
1	0/12	0/12	-	-
3	0/12	0/12	-	-
6	3/12	0/12	19±2.7	-
12	8/12	0/12	92±27.6	-
15	12/12	1/12	258±89.1	14
18	12/12	1/12	599±152	18
21	8/8	1/8	394±64	29
24	8/8	1/8	638±168	37
27	4/4	0/4	227±82	-
30	4/4	0/4	493±91	-
33	4/4	0/4	686±187	-

[a]The number of tumors detected/number of injections.

were statistically significantly more invasive than parental SMMC-7721 cells. BrMC inhibited the invasion of CD133+ sphere cells in a dose-dependent manner.

BrMC downregulates the expression of LCSC biomarkers CD133 and CD44. In our study, we have shown that the CD133+ sphere cells derived from SMMC-7721 cell line had the property of self-renewal, EMT and were highly tumorigenic. To better understand the influence of BrMC on the biomarkers of LCSCs, we performed FACS and western blotting to analyze the expression of CD133 and CD44. The results provided evidence that in CD133+ sphere cells derived from SMMC-7721 cell line, BrMC downregulated the expression of CD133 (Fig. 3A and B) and CD44 in a dose-dependent manner (Fig. 3C).

BrMC suppresses tumor growth in vivo. To determine whether BrMC targeted the inhibition of growth of LCSCs *in vivo*, we transplanted human SMMC-7721-originated CD133+ spheres subcutaneously to Balb/c-nu mice for a xenograft nude mouse model. Two weeks after transplantation, mice were randomized to four groups and received daily gavage of indicated different dosage of BrMC (0, 12.5, 25 and 50 mg/kg). After treatment of 20 days, the volume of tumors that were treated by high doses for 25 and 50 mg/kg reduced to half size of that of model controls (Fig. 3D). To further confirm this result, we dissected these tumors in mice and re-seeded them subcutaneously to different nude mice. In order to avoid the possible changes caused by heterogeneity, we seeded 50,000 tumor cells from the model control in the side of forelimb, seeded another 50,000 tumor cells from xenograft nude mice that were treated by high dose of BrMC (50 mg/kg) into the other side of forelimb. Interestingly, we can see from Table III, the tumor cells from the control group grew very fast and the final volume reached 567-686 mm^3. However, the tumor cells originated from xenograft nude mice that were treated with BrMC did not grow until day 33 after transplantation. Among the 12 mice that received the BrMC treated tumor cells, only one mouse formed a small tumor (37 mm^3). In the control group, however, as early as the sixth day of tumor cell injection, the tumors emerged and all tumors appeared on the 15th day. These results suggest that BrMC is able to eliminate LCSCs in the initial transplanted tumors, thereby inhibiting tumor re-growth of the secondary inoculated mice, hinting that BrMC could eradicate LCSCs *in vivo*.

BrMC downregulates the expression of Twist and β-catenin in LCSCs. Transcription factor Twist and β-catenin were proved to be the critical epithelial-mesenchymal transitional molecules (44-47). In cancer cells, the nuclear translocation of CD44 causes stimulation of Twist transcription, which mediates the MSC-triggered epithelial-to-mesenchymal transition (EMT) of carcinoma cells (44). Based on the results that BrMC downregulates the expression of CD133 and CD44 and inhibits EMT in LCSCs, we further investigated the effects of BrMC on the expression of Twist and β-catenin, as reported by other groups (45,48,49), Twist and β-catenin were highly expressed in our sorted CD133+ sphere cells (Fig. 4A). Western blot analysis indicated that the protein levels were downregulated after these CD133+ SFCs were treated by the indicated concentration of BrMC (0.1, 0.3 and 1.0 μM) (Fig. 4A).

Synergistic inhibition of self-renewal of LCSCs by BrMC and Twist silencing. To further explore the biological functions of Twist in CD133+ SFCs and the maintenance of LCSC characteristics, we silenced the expression of Twist by RNA interference in CD133+ SFCs of the SMMC-7721 cells. The mRNA levels and protein expression of Twist decreased significantly after transfection with Twist siRNA (Fig. 4B). Interestingly, as the protein levels of Twist decreased, the β-catenin expression was reduced (Fig. 4B). Furthermore, the decreased expression of Twist also downregulated the sphere formation capacity of CD133+ SFCs of SMMC-7721 cell line (Fig. 4C and D). As BrMC inhibits proliferation and self-renewal of CD133+ sphere-forming cells derived from the SMMC-7721 cell line, to confirm the results, we treated CD133+ SFCs of the SMMC-7721 cells that were transfected with Twist siRNA with 0.1 μM of BrMC again. Fig. 4E shows the cells that were transfected with Twist siRNA, the addition of BrMC further decreased the protein levels of Twist and the sphere-forming capacity in this group also reduced more by the co-treatment with Twist siRNA and 0.1 μM of BrMC (Fig. 4F and G), indicating synergistic inhibition of self-renewal of LCSCs.

Overexpression of Twist attenuates inhibition of LCSC self-renewal by BrMC. To corroborate whether Twist rescued BrMC, we transfected the plasmid pcDNA-Twist and negative control pcDNA3.1 to the human embryonic liver cell line L-02, HCC cell line SMMC-7721 and CD133+ sphere cells derived from SMMC-7721 cells, respectively. As shown in Fig. 5A, compared to negative control vector pcDNA3.1, the three cell lines significantly overexpressed Twist. Tumor sphere forming experiment showed that the ectopic expression of Twist in these cell lines promoted the tumor sphere

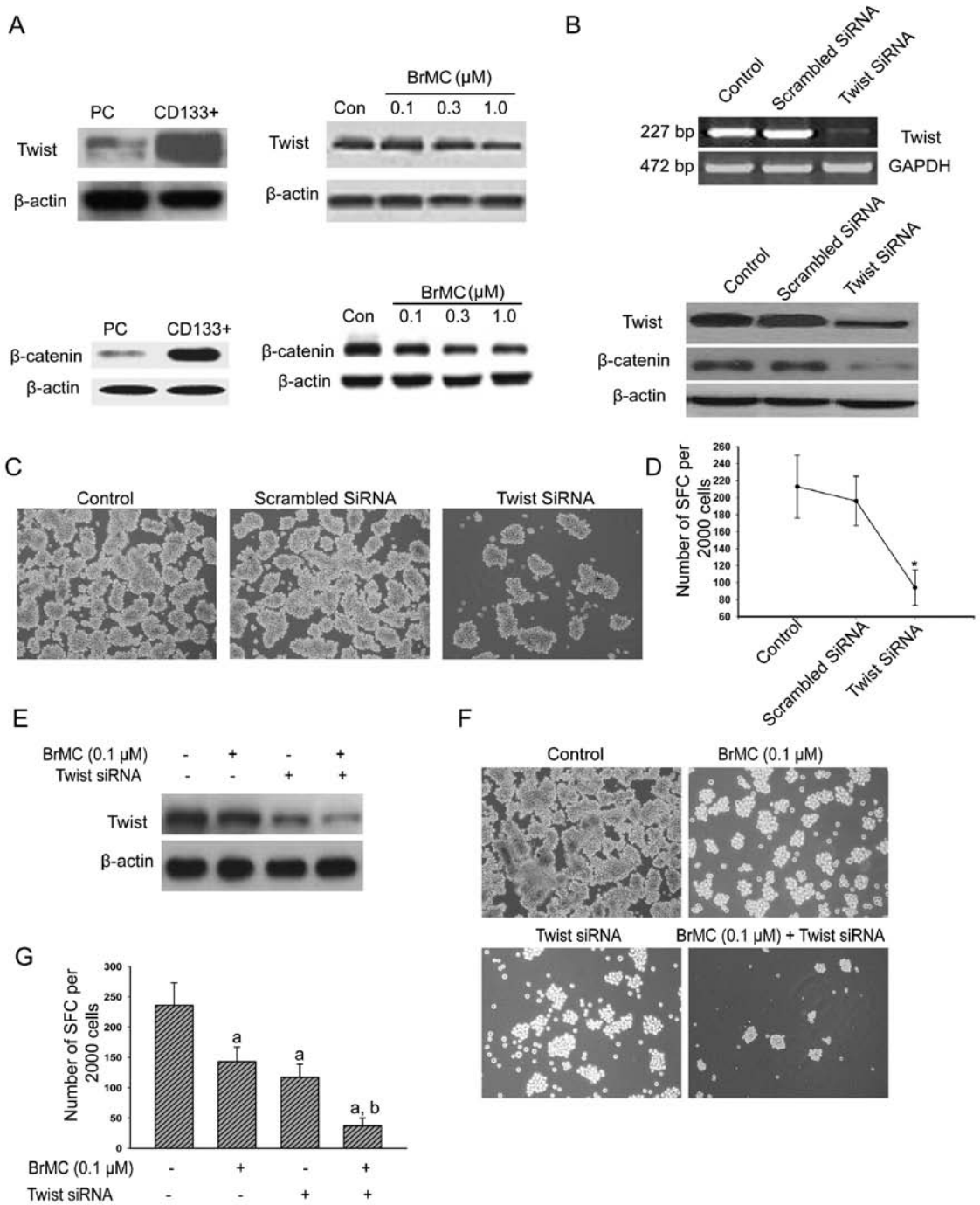

Figure 4. Synergistic inhibition of self-renewal of LCSCs by BrMC and Twist silencing. (A) Western blot analysis showed that Twist and β-catenin were highly expressed in CD133$^+$ SFCs of SMMC-7721 cell line, BrMC downregulated the expression of Twist and β-catenin in a dose-dependent manner. (B) The mRNA levels and protein levels of Twist decreased significantly after transfection with Twist siRNA. (C) The downregulation of Twist decreased the sphere forming ability of CD133$^+$ SFCs of SMMC-7721 cell line. (D) Statistical analysis of (C), each experiment was performed in triplicate and representative examples are shown. (E) Western blot analysis revealed that the addition of BrMC further decreased the protein levels of Twist. (F) Synergistic inhibition of the sphere forming ability of CD133$^+$ SFCs of SMMC-7721 cell line by BrMC and Twist silencing. (G) Statistical analysis of (F), each experiment was performed in triplicate and representative examples are shown.

formation (Fig. 5B and C). We also treated CD133$^+$ SFCs of SMMC-7721 cells that were transfected with pcDNA-Twist followed by treatment with 0.1 μM of BrMC. After addition of BrMC, the number of spheres formed in this group was relatively more than that of the control groups (Fig. 5D). These results suggest that Twist overexpression could partly reduce the inhibitory effect of BrMC on self-renewal of LCSCs.

Discussion

Cancer stem cell research is becoming a growing and exciting field. In fact, it appears that most cancer types contain populations of cells that exhibit stem-cell properties. CSCs have the ability to renew indefinitely, which can drive tumor development and metastatic invasion. As these cells are classically resistant to conventional chemotherapy and to

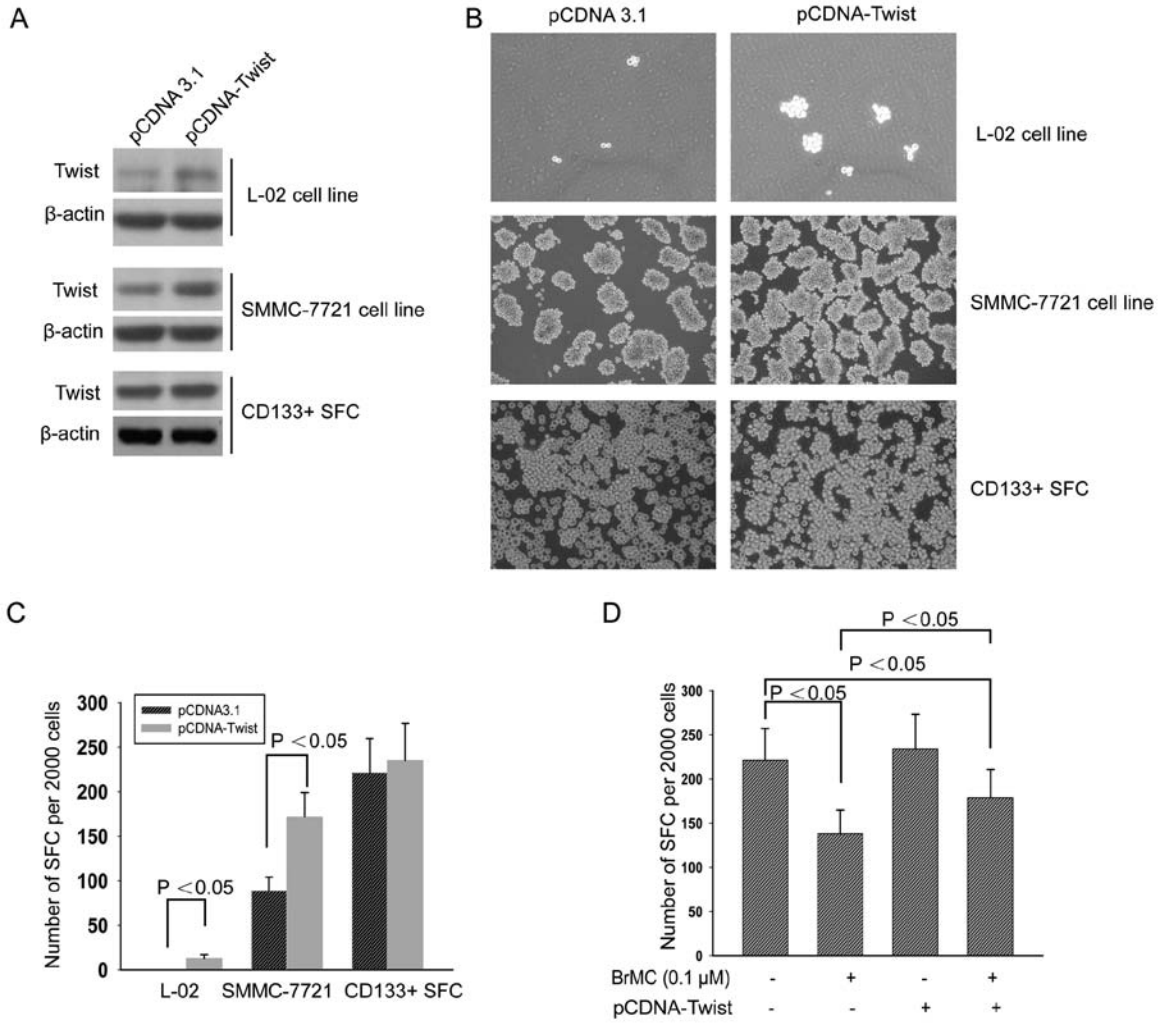

Figure 5. Overexpression of Twist attenuates the inhibitory effect of BrMC on LCSCs self-renewal. (A) Western blot analysis showed overexpression of Twist after transfection with plasmid pcDNA-Twist in L-02, SMMC-7721 cells and SFCs of SMMC-7721 cell line. (B) The ectopic expression of Twist in these cell lines promoted the tumor sphere formation. (C) Statistical analysis of (B), each experiment was performed in triplicate and representative examples are shown. (D) The sphere forming ability of $CD133^+$ SFCs derived from HCC SMMC-7721 cell line that was treated with BrMC was partly rescued after overexpression of Twist.

radiation therapy, they may contribute to treatment failure and relapse. Most cancer research experts focused on isolation and targeted killing of CSCs and the development of novel strategies for antitumor therapy relies on the use of biomarkers to identify, enrich and/or isolate the cell population(s) of interest. Magnetic activated cell sorting (MACS) is one of the specific methods to separate CSCs, which are based on cell surface markers. The proposed markers for liver CSCs include CD133, CD90, CD44, CD13, EpCAM and OV6, on the basis of the hypothesis that CSCs are originated from somatic stem cells and accordingly express the same surface markers (50,51). Prominin-1 (CD133) is generally regarded as one of the most important molecular markers for stem cells, cancer stem and stem-like cells in tumors originating from colon cancer (52), glioblastoma multiforme (GBM) cell line (53), HCC (12,54), pancreatic cancer (55), gastric cancer (56), and lung cancer (57) have been reported.

Although the cell surface expression of the human CD133 antigen, in particular of the AC133 epitope, is among those that have been most frequently studied in solid cancers, no mechanism has yet been proposed to link CD133 expression with the CSC phenotype (58). In our study, we quantified percentage of the isolated $CD133^+$ cells from the SMMC-7721 cell line by flow cytometry. There was no detectable $CD133^+$ cell in the $CD133^-$ population (1.07%), while the percentage of $CD133^+$ population was ~58.72%, indicating that CD133 is highly expressed in LCSCs derived from SMMC-7721, which was in agreement with the study by Yin *et al*, who also performed flow cytometry, for purity, before and after MACS sorting from SMMC-7721 cell line and the $CD133^+$ groups ranged from 60.2 to 91.2% compared to non-sorted SMMC-7721 cells (0.1-2%) (3,59).

Sphere formation experiment indicates high levels of CD133 were associated with increased spheroid forming capacity both for the first passage and for the second passage *in vitro*. Next, the ability of tumorigenicity was measured in BALB/c nu mice to verify $CD133^+$ sphere cells derived from SMMC-7721 is LCSCs. As expected, $CD133^+$ sphere-forming cell population exhibited stronger tumorigenicity than others, even 10,000 cells were enough to form tumors,

showing slight difference compared to results by De Hert *et al* (60), who used as few as 500 cells from the PLC/PRF/5 spheres to form a tumor when subcutaneously injected into NOD/SCID mice, while $2x10^5$ parental cells were needed.

During EMT, epithelial cells lose their characteristics and gain mesenchymal features. It has been suggested that transformed epithelial cells can activate embryonic programs of epithelial plasticity and switch from a sessile, epithelial phenotype to a motile, mesenchymal phenotype. Induction of EMT can, therefore, lead to invasion of surrounding stroma, intravasation, dissemination and colonization of distant sites. According to the cancer stem cell hypothesis, sustained metastatic growth requires the dissemination of a CSC from the primary tumor followed by its re-establishment in a secondary site. SNAI, ZEB and TWIST family members repress the CDH1 gene to induce EMT, but also regulate the transcription of other target genes. TWIST1 is upregulated in human breast cancer, gastric cancer, esophageal cancer and prostate cancer (61). The activation of Twist caused translocation of β-catenin into the nucleus and elevated Wnt/β-catenin signaling promotes EMT transition (62,63). In this study, the knockdown of Twist leads to reduced β-catenin expression (Fig. 4B), hinting that β-catenin is the downstream target gene of Twist, which is in agreement with a previous report that activation of β-catenin pathway by Twist is critical for the maintenance of EMT associated CSC-like characteristics (64).

BrMC is a novel ChR analogue synthesized by our laboratory. We previously showed that the effect of BrMC on the inhibition of proliferation and induction of apoptosis in the colon cancer cell line HT-29, breast cancer and in the gastric cancer cell line SGC-7901 was stronger than that of ChR (37,65,66). BrMC has been shown to induce apoptosis of HCC cell line in a dose-dependent manner, but have little effect on human embryo liver L-02 cells. In addition, BrMC also inhibits self-renewal of glioma stem-like cells (GSLCs) (39,40). In this study, we found that BrMC inhibited formation of primary and secondary spheroids in suspension and cell viability in those spheroids, inhibited self-renewal, EMT, cell invasion of $CD133^+$ sphere-forming cells from SMMC-7721 cell line *in vitro*. Furthermore, BrMC suppressed tumorigenicity in BALB/c nu mouse xenograft model. As the molecular mechanism, BrMC dose-dependently inhibited the expression of CD133 and CD44, which was related to LCSC characteristics and also reduced protein levels of EMT-associated crucial protein Twist and β-catenin.

In conclusion, we present supportive evidence for the first time that BrMC, a novel synthetic chrysin analogue, was able to target LCSCs both *in vitro* and *in vivo*. Furthermore, our study identified the blockage of Twist signaling pathway by BrMC as one of the possible mechanisms for this efficacy. This study supports the use of BrMC for HCC chemoprevention or chemotherapy.

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