CD133+ subpopulation of the HT1080 human fibrosarcoma cell line exhibits cancer stem-like characteristics

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Abstract. The cancer stem cell (CSC) theory holds that a minority population within tumors possesses stem cell properties of self-renewal and multilineage differentiation capacity and provides the initiating cells from which tumors are derived and sustained. However, verifying the existence of these CSCs has been a significant challenge. The CD133 antigen is a pentaspan membrane glycoprotein proposed to be a CSC marker for cancer-initiating subpopulations in the brain, colon and various other tissues. Here, CD133+ cells were obtained and characterized from the HT1080 cell line to determine the utility of this marker for isolating CSCs from human fibrosarcoma cells. In this study, CD133+ cells were separated from HT1080 cells using magnetic beads and characterized for their proliferation rate and resistance to chemotherapeutic drugs, cisplatin and doxorubicin, by MTS assay. Relative expression of tumor-associated genes *Sox2, Oct3/4, Nanog, c-Myc, Bmi-1* and *ABCG2* was measured by real-time polymerase chain reaction (PCR). Clonal sphere formation and the ability of CD133+ cells to initiate tumors in BALB/c nude mice was also evaluated. We found that CD133+ cells showed a high proliferation rate, increased resistance to chemotherapy drugs and overexpression of tumor-associated genes compared with these features in CD133- cells. Additionally, CD133+ cells were able to form spherical clusters in serum-free medium with high clonogenic efficiency, indicating a significantly greater tumor-initiating potential when compared with CD133- cells. These findings indicate that CD133+ cells identified within the HT1080 human fibrosarcoma cell line possess many CSC properties and may facilitate the development of improved therapies for fibrosarcoma.

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Introduction

Human fibrosarcoma is an aggressive type of tumor composed of fibroblasts with variable collagen production and, in classical cases, a herringbone architecture. This tumor is relatively uncommon, accounting for only 1 to 3% of adult sarcomas (1,2). It can develop in any fibrous tissue of the body and strike at any age, but most commonly in middle-aged and elderly adults. Generally, the incidence of fibrosarcoma is equal among the genders.

Despite advances in chemotherapies and surgical treatments, human fibrosarcoma has a higher recurrence rate compared with these of other soft tissue sarcomas. The probability of local recurrence relates to the completeness of excision, with recurrence rates of 12-79% (3-5). Fibrosarcomas metastasize to lungs and bone, especially the axial skeleton, but rarely to lymph nodes. Metastasis is time- and grade-dependent and occurs in 9-63% of patients. The 5-year survival remains only 39-54% (3,5). Many patients relapse and their tumors become drug-resistant after a period of treatment, thus long-term patient survival rates have remained poor (6).

It is well known that solid tumors consist of heterogeneous cell populations that include cells with stem-like properties, such as a high proliferation rate and invasive growth potential (7,8). The cancer stem cell (CSC) theory states that these cells exist as a minority population within the tumor, possess stem cell properties of self-renewal and multilineage differentiation and are the root cells from which tumors are derived and sustained (9-11). CSCs were initially identified and isolated from leukemia patients (12). The discovery of leukemic stem cells prompted further research into other types of cancer, leading to the identification of CSCs in numerous solid tumors, including cancers of the breast (13), brain (14), prostate (15) and other different types of sarcomas (16-18). CSCs are postulated to be resistant to standard cytotoxic chemotherapeutic agents both *in vitro* and *in vivo*, and this resistance may be the major cause of treatment failure, as the surviving reservoir of stem cells can populate the tumor and lead to relapse. New therapies targeting these cells, which are fundamental for tumor progression, can significantly improve the clinical management of cancer (19). Therefore, identifying within tumors subpopulations of cells exhibiting significant

alterations in proliferation, stem cell marker expression and behavior are essential to understanding cancer pathology.

The cell surface glycoprotein CD133 is a pentaspan transmembrane protein that was originally identified in hematopoietic and neural stem cells (20). Interest in CD133 as a CSC marker has grown dramatically since it was associated with a cancer-initiating subpopulation in the brain (14) and prostate (21). Moreover, $CD133^+$ cells have also been found in various sarcomas, including Ewing's sarcoma and osteosarcoma (22-26). However, until now, it has not been determined whether $CD133^+$ cells with tumorigenic potential can be prospectively isolated from human fibrosarcomas. In this study, a population of HT1080 human fibrosarcoma cells expressing CD133 was identified and fulfilled the criteria of being characterized as CSCs both *in vivo* and *in vitro*. These cells may be highly valuable targets for the therapy of recurrent and chemoresistant disease.

Materials and methods

Cell culture. The HT1080 fibrosarcoma cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Quality Biological, Gaithersburg, MD, USA), and maintained at 37˚C (humidified, in 5% CO_2). The medium was replaced every 2-3 days, and the monolayer cultures were trypsinized and recultured when reaching 70-80% confluency. Single-cell suspensions for flow cytometry and cell sorting were generated by trypsinization with a solution of 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA).

Magnetic-activated cell sorting (MACS) and flow cytometric analysis. Cultured cells were trypsinized and resuspended in MACS buffer [PBS without Ca^{2+} and Mg^{2+}, supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA] at a concentration of $1x10^8$ cells/300 μl. A minimum of 10^6 events was collected and acquired using CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). For MACS, single-cell suspensions were then incubated with FcR blocking reagent (1 μl per million cells) and CD133 microbeads (1 μl per million cells) for 30 min at 4˚C. After washings, $CD133^+$ cells were separated according to the manufacturer's recommendations by positive selection on LS columns, and the $CD133^-$ cells were collected in the flow-through. Prior to and following separation, samples were analyzed by fluorescence-activated cell sorting (FACS) using CD133/2 phycoerythrin (293C) antibody and isotype control mouse IgG2b phycoerythrin (1/10) in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The purity of $CD133^+$ and $CD133^-$ cell populations was evaluated by standard flow cytometric analysis. All reagents were purchased from Miltenyi Biotech (Bergisch Bladbach, Germany).

Hoechst 33342 exclusion assay. HT1080 fibrosarcoma cells were resuspended at $1.0x10^6$ cells/ml in pre-warmed DMEM culture medium and divided into 2 portions. One portion was treated with 50 mM verapamil (Sigma, St. Louis, MO, USA) as an inhibitor of the ABC transporter, and the other was left untreated. Both portions were incubated in DMEM culture medium with 5 μg/ml Hoechst 33342 (Sigma) for 90 min at 37˚C. Cells were washed in PBS after the incubation, kept on ice for 5 min and analyzed for Hoechst 33342 efflux using a BD FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA). The Hoechst 33342 dye was excited at 357 nm, and the fluorescence was analyzed at dual wavelengths (blue, 402-446 nm; red, 650-670 nm).

Sphere formation assay. Cells expressing CD133 were enriched using CD133 microbeads and a MACS device. To further improve the purity of the $CD133^-$ fraction, a second sorting step was performed by adding fresh CD133 microbeads to the $CD133^-$ cell fraction and running a depletion protocol on the MACS. Single $CD133^+$ or $CD133^-$ cells were seeded into low attachment 96-well plates (Corning Incorporated, Corning, NY, USA). Visual inspection was performed the day after the initial plating to confirm that each well contained a single cell. After 3-4 weeks, spheres containing >50 cells were counted. Cells were inoculated into N2/1% methylcellulose medium without serum. The N2 medium consisted of 2X DMEM/F12 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with progesterone (20 nM), putrescine (100 μM), sodium selenite (30 nM), transferrin (25 μg/ml), insulin (20 μg/ml), human recombinant epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml), mixed with an equal volume of 2% methylcellulose. All reagents were purchased from Sigma. Fresh aliquots of EGF and bFGF were added every other day. After 3-4 weeks of culture, spherical colonies were quantified using an Olympus CK2 inverted phase contrast microscope (Olympus, Japan). The capacity for secondary sphere formation was also investigated by repeated dissociation and re-culturing in anchorage-independent, serum-starved conditions on ultra low attachment plates.

Efficacy of chemotherapy drugs on $CD133^+$ and $CD133^-$ cells. The $CD133^+$ and $CD133^-$ cells were dissociated, seeded into 96-well microtiter plates (Corning Incorporated) at a density of 2,000 cells/100 μl/well and allowed to attach in DMEM/10% FBS for 8 h at 37˚C. Cells were then exposed to 10 μl of cisplatin (CDDP) or doxorubicin (DXR) (both from Sigma) at different concentrations for 48 h. Viabilities of both drug-treated and non-treated cells were measured by the MTS colorimetric assay using the CellTiter 96® AQueous One Solution Cell Proliferation assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Drug resistance in $CD133^+$ spheroids and adherent cells was compared for a more detailed characterization of the stemness properties of the $CD133^+$ cell population. The $CD133^+$ cells were seeded into 96-well ultra low attachment microplates (Corning Incorporated) in N2/1% methylcellulose media at a density of 5,000 cells/100 μl/well for 2 weeks to allow sphere formation. $CD133^+$ spheroids and adherent cells were then treated with 10 μl of CDDP or DXR at different concentrations for 48 h, and cell viability was assessed by the MTS assay.

Protein isolation and western blot analysis. Sorted cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl 1 mM EDTA, 1% NP-40, 0.1% SDS,

Table I. Primers for *Sox2, Oct3/4, Nanog, c-Myc, Bmi-1* and *ABCG2*.

Gene	Forward (5'-3')	Reverse (5'-3')
c-Myc	GCATACATCCTGTCCGTCCA	CAAGAGTTCCGTAGCTGTTCAAG
Sox2	ATCACCCACAGCAAATGACA	CAAAGCTCCTACCGTACCACTA
Oct3/4	TATTCAGCCAAACGACCATCT	TCAGCTTCCTCCACCCACTT
Bmi-1	CTCCACCTCTTCTTGTTTGC	GATGACCCATTTACTGATGATTT
Nanog	AGGCAAACAACCCACTTCT	'TCACACCATTGCTATTCTTCG
ABCG2	GATAAAGTGGCAGACTCCAAGGT	CCAATAAGGTGAGGCTATCAAACA
GAPDH	GGGAAACTGTGGCGTGAT	GAGTGGGTGTCGCTGTTGA

1% Na-deoxycholate, 1 mM Na-vanadate, and protease inhibitors (5 mg/ml pepstatin, 1 mM PMSF, 10 mg/ml leupeptin and 1 mM NaF) (Sigma) for 1 h in ice. After centrifugation at 13,000 x g for 10 min at 4˚C, the protein concentrations of the supernatants were measured by the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Lysates were mixed (1:1) with Laemmli buffer (Sigma). Fifty micrograms of protein was electrophoresed per lane in 10% SDS polyacrylamide gels and transferred onto PVDF membranes (Sigma). Membranes were blocked with nonfat milk for 1 h at room temperature and incubated overnight at 4˚C with the corresponding antibodies in Tris-buffered saline (TBS) with 5% BSA (Sigma) and 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, CA, USA). After being washed 3 times in TBS with 0.1% Tween-20, blots were incubated with Ig-specific, peroxidase-conjugated secondary antibodies for the appropriate species. Immunoreactive bands were detected with the ECLPlus SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) for 60 sec. Protein levels were normalized with respect to the band density of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Primary antibodies against *Nanog, Oct3/4, Sox2, ABCG2, c-Myc* and *Bmi-1* (all diluted 1:1,000) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The GAPDH-specific antibody (1:5,000) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Real-time PCR. cDNA was obtained using Moloney murine leukemia virus reverse transcriptase and RNase H minus (Promega Corporation). Typically, 250 ng of template total RNA and 250 ng of random hexamers were used per reaction. Real-time PCR amplification was performed using the TaqMan Universal PCR Master Mix and Assays-On-Demand gene expression products or Power SYBR-Green Master Mix and specific PCR primers in an ABI Prism 7900 instrument (Applied Biosystems, Carlsbad, CA, USA). Relative quantification of each target, normalized to an endogenous control (cyclophilin A), was performed using the comparative Ct method (Applied Biosystems). Probes were used to detect *c-Myc, Sox2, Oct3/4, Bmi-1, Nanog* and *ABCG2*. The primer sequences used for amplification of the above genes are listed in Table I. The GAPDH gene was used as an internal control for normalizing template amounts.

In vivo tumorigenicity studies. Groups of 6 female nude athymic mice (6- to 8-weeks old) (BALB/c nu/nu; Vital River Laboratory Animal Center, Beijing, China) were injected with either sorted or unsorted cells at different concentrations subcutaneously into the right flank in a 1:1 mixture of Matrigel and Hank's balanced salt solution (HBSS) in a final volume of 300 μl. Engrafted mice were inspected biweekly for tumor development by visual observation and palpation. Mice were sacrificed by cervical dislocation when the tumor diameter reached 15 mm or at 4 months post-transplantation. Xenograft tumors were excised using aseptic techniques and processed for hematoxylin and eosin (H&E) staining and anti-CD133 immunohistochemical analysis. In addition, tumor samples were digested using collagenase II (Sigma), and the $CD133^+$ cells were isolated and immediately re-injected into mice to generate second-round tumors. All animal procedures were approved by the Institutional Animal Care and Use Committee in Teaching and Research of Harbin Medical University.

Histopathological and immunohistochemical analysis. All samples of the mouse transplants were placed in 10% formalin immediately, processed with routine histological procedures and embedded in paraffin. Serial sections (5-μm) were cut and either stained with H&E for routine histological observation under a light microscope or used for immunohistochemical detection of CD133. After deparaffinization (hydration), sections were treated sequentially with normal goat serum, anti-CD133/2 antibody (Miltenyi; clone 293C3), biotin-labeled goat anti-mouse IgG and avidin-biotin-peroxidase complex (ABC). Peroxidase binding sites were detected by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. The number and area of CD133-positive foci >0.2 mm in diameter and the total area of the examined sections were measured using an Olympus C5050Z digital camera. Images were acquired digitally using MagnaFire Software (Optronics) and processed in Adobe Photoshop.

Statistical analysis. All assays were repeated at least 3 times. Results were reported as means ± standard deviation (SD) after analysis by using SPSS 19.00 software. Differences with P-values <0.05 were considered significant.

Results

Separation of $CD133^+$ and $CD133^-$ fractions from the HT1080 fibrosarcoma cell line. CD133 is a phylogenetically conserved cell surface molecule that was first described in

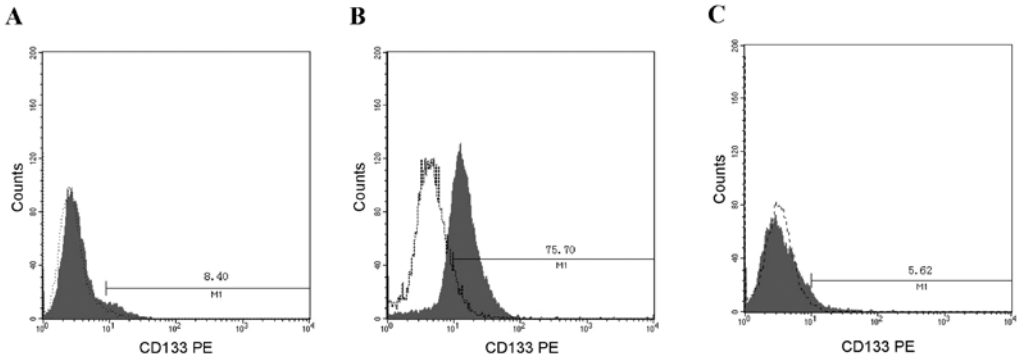

Figure 1. Flow cytometric analysis of the expression ratio of CD133 in the HT1080 cell line. (A) The ratio of CD133 expressed in HT1080 was 8.40%. After CD133 magnetic bead purification, the ratio of (B) CD133+ cells was 75.70% and the ratio of (C) CD133- cells was 94.38%. Black bars and percentage indicate the ratio of the CD133+ cell population. Black broken lines indicate isotype matched control. Representative data of 3 independent experiments are shown.

neuro-endothelial progenitors and recently has been proposed to be a selective marker for CSCs in many cancer types. We first assessed the presence and size of the cell populations expressing CD133 on the surface of HT1080 fibrosarcoma cells by standard flow cytometry. Analysis of this fibrosarcoma model cell line revealed that it consistently contained a CD133+ subpopulation at a frequency of 8.40% in HT1080 cells. Subsequently, the CD133+ and CD133- fractions were separated by MACS sorting. As shown in Fig. 1, a CD133+ enriched population (75.70%) and a CD133- cell population (94.38%) were obtained (Fig. 1).

Side population. A very small subset (0.30%) of the HT1080 cell displayed the characteristic profile of a side population. The side population phenotype is considered the most significant attribute of CSCs. The percentage of side population cells was markedly diminished by treatment with verapamil, an inhibitor of the pumps responsible for the exclusion of the Hoechst 33342 dye, indicating that this population truly represented side population cells. Thus, this result showed the detection of a side population in a fibrosarcoma cell line (Fig. 2).

Functional characterization of CD133+ cells. Although no *in vitro* assay can specifically identify CSCs, some growth properties, such as spheroid formation, can be assessed in culture as a potential characteristic of CSCs. Therefore, the ability of freshly isolated CD133+ and CD133- cells to form spheres in serum-free culture conditions was tested. Because of the high likelihood of this assay containing potential CD133+ contaminant cells in the negative fraction, a second round of depletion was performed on the CD133- cells. Under serum-free conditions, the CD133+ population proliferated as floating spheres (Fig. 3A), whereas most of the CD133- population did not survive. By re-culturing in anchorage-independent, serum-starved conditions, cells dissociated from the spheres were shown to be capable of repeatedly forming spheres (Fig. 3B). When floating spheres of CD133+ cells were seeded into serum-containing medium, the cells migrated from the spheres within a few hours and adhered to the bottom of the flasks, showing spindle-like morphology similar to that of the original cell culture (Fig. 3C). Single CD133+ or CD133- cells were individually seeded into ultra low adhesion 96-well plates that favor the proliferation of undifferentiated cells. The number of spheres was scored after 3-4 weeks in culture. Importantly, the CD133+ fraction gave rise to an ~5-6-fold greater number of spheres than the CD133- fraction, and this increase was statistically significant (Table II).

Table II. Shere-forming efficiency of individual CD133+ and CD133- cells plated in N2 medium for 3-4 weeks.

	CD133+	CD133-
Wells with spheres	64/384	11/384
Sphere-forming efficiency	16.7%	2.86%

Spheres of >50 cells were counted, and the results are the sum from 2 independent experiments using 192 wells/experiment.

CD133+ cells have increased resistance to chemotherapeutic drugs. CSCs have been reported to be more resistant to chemotherapy than other tumor cells. Therefore, viability was examined for the sorted CD133+ and CD133- cells that were exposed to increasing doses of cisplatin (CDDP) or doxorubicin (DXR), drugs which are currently in use in the clinical setting for the treatment of fibrosarcoma. As shown in Fig. 4, both CD133+ and CD133- cells displayed a dose-dependent sensitivity to these chemotherapeutic agents. However, the CD133+ population was significantly resistant to both CDDP and DXR in comparison with the CD133- population. To better understand how the stem cell properties of the CD133+ cell population could confer resistance to therapy, differences between CD133+ spheroids in serum-free media and CD133+ adherent cells grown in serum-containing media were examined. The spheroids were found to be more resistant to both chemotherapeutics in comparison with the adherent cells.

CD133+ cells are enriched in expression of 'stem cell' genes. Real-time PCR and western blotting were used to examine whether the CD133+ cells were enriched in the expression of genes that have been postulated to play key roles in stem cell biology (Fig. 5). Real-time PCR analysis revealed that the expression levels of genes involved in the maintenance of

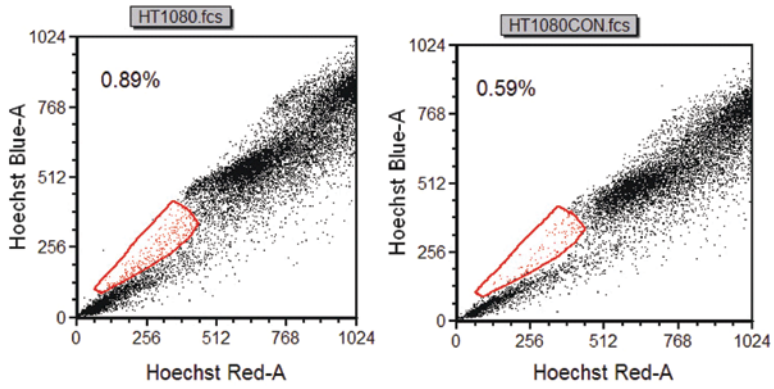

Figure 2. Cytometric analysis of the side population (SP) in the HT1080 cell line. The populations of SP cells, in the presence or absence of verapamil, are shown. SP cells are marked by red dotted lines to show the proportion of SP cells among the total living cells. HT1080 cells treated with verapamil as a control (right panel).

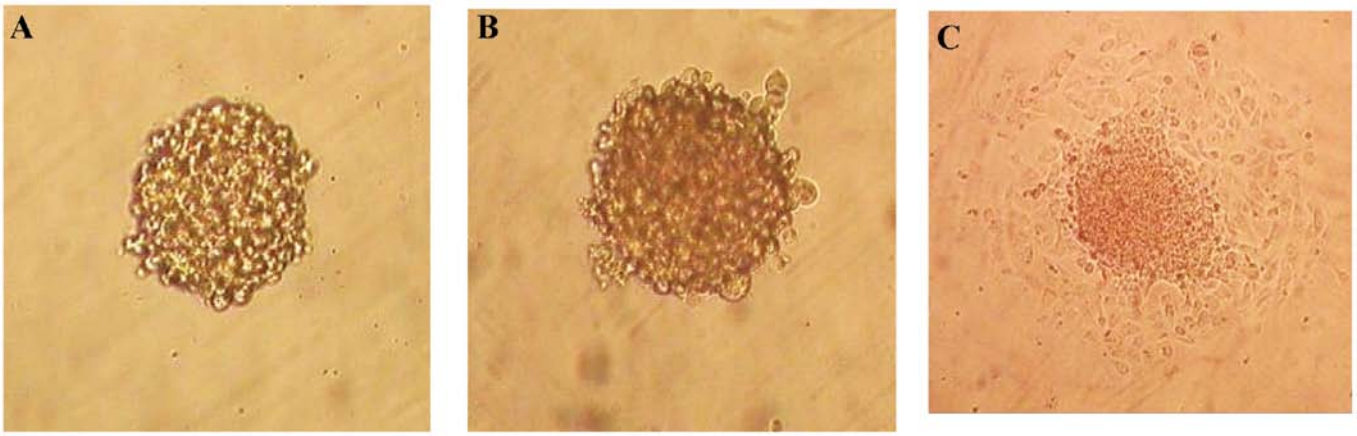

Figure 3. *In vitro* characterization of CD133$^+$ cells. (A) Primary sphere formation of HT1080 cells in anchorage-independent, serum-free conditions. (B) Dissociated HT1080 primary sphere cells showed a capacity for secondary sphere formation. (C) When these spheres were seeded in serum-containing medium, they exhibited a spindle-shaped structure replicating the primary cell culture.

stemness, namely *Sox2* and *Nanog*, were significantly higher in the CD133$^+$ population in comparison with the CD133$^-$ population. Moreover, expression levels of the *Oct3/4, c-Myc*, *Sox2* and *Bmi-1* genes were consistently increased in the CD133$^+$ population compared with their CD133$^-$ counterparts. A 6.3-fold increase in *Nanog* mRNA and an 8.8-fold increase in *Sox2* mRNA in CD133$^+$ cells compared to CD133$^-$ cells were detected by real-time PCR. The level of *c-Myc* was also upregulated in CD133$^+$ cells in comparison with CD133$^-$ cells, while *Bmi-1* expression was nearly the same in the 2 populations according to the western blotting results. In addition, both gene and protein expression levels of the *ABCG2* drug transporter were significantly increased in the CD133$^+$ cells when compared with the levels in the CD133$^-$ cells.

CD133$^+$ cells exhibit tumor-initiating activity in vivo. The definition of cancer-initiating cells relies mainly on their functional properties such as self renewal. The other defining characteristic of CSCs is recapitulating the cellular heterogeneity of the original tumor. Therefore, the tumorigenic capacity of HT1080-derived CD133$^+$ and CD133$^-$ cell populations was investigated. Cells were MACS-sorted into CD133$^+$ and CD133$^-$ fractions and expanded in culture as independent populations for 3 to 5 passages. Subsequently, a range of CD133$^+$ or CD133$^-$ cells (200-10^6) were injected subcutaneously into the right flank of individual BALB/c nude mice. Unsorted cells were injected as a control. After 6-10 weeks, tumors were detected in the flank region with an average size of 15 mm. Tumor formation was noted after injection of as few as 10^3 CD133$^+$ cells (Table III). By contrast, only 4 of 6 of the mice injected with 10^6 CD133$^-$ cells and none of the mice injected with 10^5 or fewer CD133$^-$ cells developed a tumor, suggesting that this population was relatively depleted of tumor-initiating cells. Injections of unsorted cells also reliably resulted in tumor formation, and 3 out of 6 mice injected with 10^5 unsorted cells developed a tumor. Importantly, tumors that arose from CD133$^+$ cells could be serially transplanted into secondary recipients. Gross tumor appearance and histology were similar for tumors arising from the unsorted cells, CD133$^+$ cells and CD133$^-$ cells. Immunohistochemistry of these xenografts demonstrated that only a small minority of tumor cells expressed a high level of CD133, even in tumors that arose from implantation of purified CD133$^+$ cells (Fig. 6), further supporting the hypothesis that they represent true CSCs able to generate serially transplant-

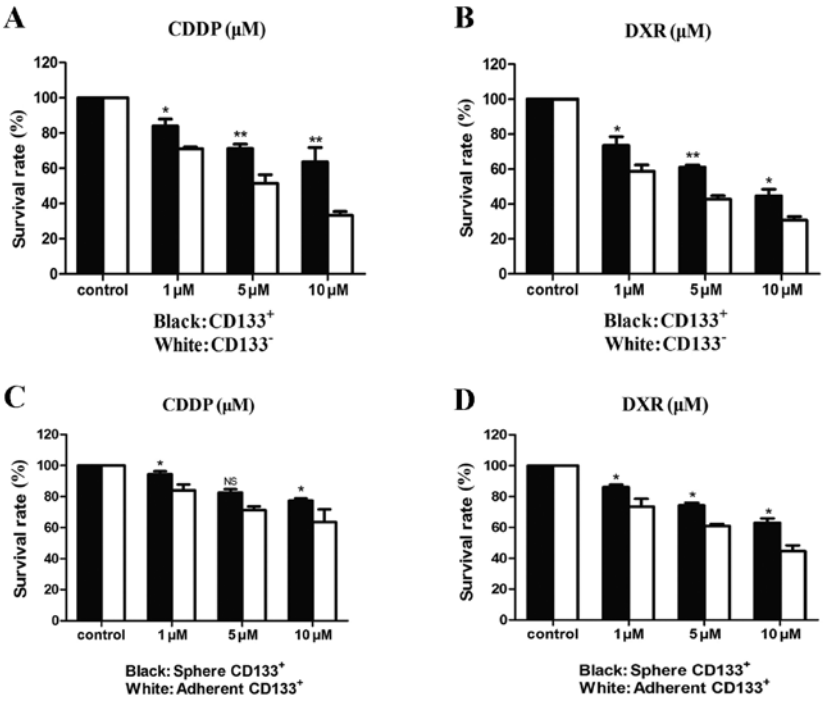

Figure 4. Drug resistance of CD133+ and CD133- cells. The CD133+ population was highly resistant to (A) CDDP and (B) DXR in comparison with the CD133- population. The spheroids were significantly resistant to (C) CDDP and (D) DXR in comparison with the adherent CD133+ cells (± SD; *P<0.05; **P<0.01). NS, not significant; CDDP, cisplatin; DXR, doxorubicin.

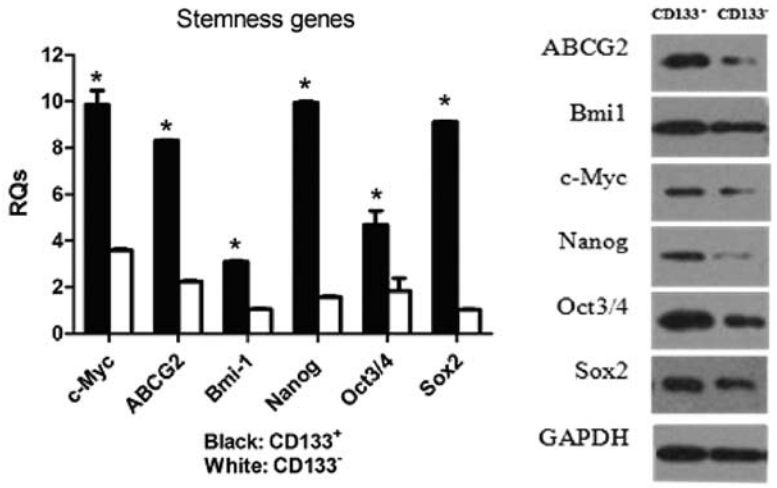

Figure 5. Gene expression profile of CD133+ cells. (A) Real-time polymerase chain reaction (PCR) analysis of stemness-related gene expression in the CD133+ and CD133- cells. (B) Western blot analysis of the stemness-related genes. GAPDH was used as a loading control (± SD; *P<0.05; **P<0.01). RQ, relative quantity.

able tumors containing mostly CD133- non-stem cells. Together these results provide preliminary *in vivo* evidence that the tumor-initiating potential of CD133+ cells is greater than that of CD133- cells and that CD133+ tumor-initiating cells undergo asymmetric cell division during tumor growth to generate both CD133+ and CD133- progeny.

Discussion

Fibrosarcoma, a malignant tumor which prevailingly affects adults, is associated with higher recurrence and metastasis rates in comparison with other soft tissue sarcomas. The concept that a small fraction of cancerous cells residing within each tumor with stem cell properties, which are responsible for tumor initiation, invasive growth and metastasis, is generally accepted. CSCs are hypothesized to have a phenotype defined by their cell of origin and retain the stem cell properties of self-renewal, capacity to differentiate and drug resistance. Therefore, new therapies targeting these cells may significantly improve the clinical outcome of cancer patients. However, obtaining conclusive evidence for the existence of CSCs has been a significant challenge in cancer research. Recently, various

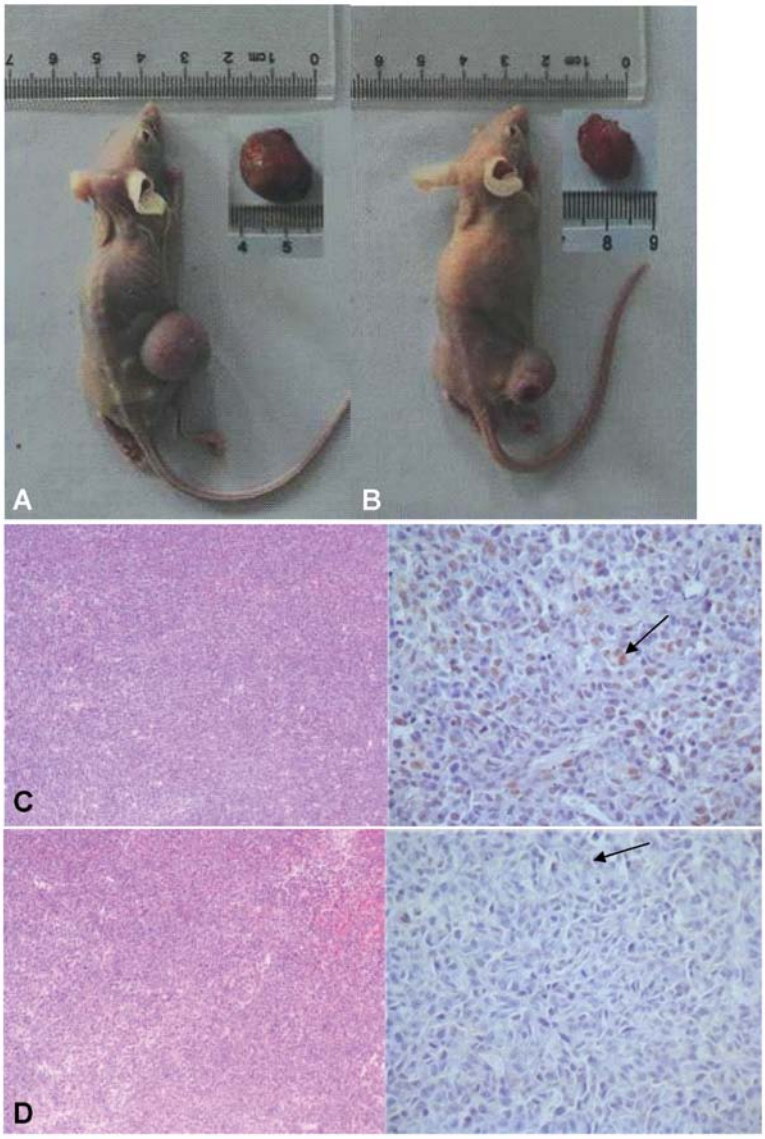

Figure 6. Analysis of tumors grown from CD133$^+$ and CD133$^-$ subpopulations. Mice displaying tumor growth and the gross appearance of resected tumors with (A) CD133$^+$ cells and (B) CD133$^-$ cells. Histopathological (left) and immunohistochemical analysis (right) of (C) CD133$^+$ cell-derived xenografts and (D) CD133$^-$ cell-derived xenografts. (CD133$^+$ cells were small round brown cells and are marked with arrows).

Table III. Tumor initiating capacity of various cell populations.

	Cell number injected				
	200	10^3	10^4	10^5	10^6
Unsorted		0/6	0/6	3/6	5/6
CD133$^+$	0/6	3/6	5/6	6/6	
CD133$^-$		0/6	0/6	0/6	4/6

Data values are the number of mice bearing tumors/total mice/group.

studies using phenotypical analyses have reported stem-like cell populations in numerous types of cancers (8,10,12). The presence of a CD133$^+$ subpopulation with stem-like features within human solid tumors has been documented both *in vitro* and *in vivo* by many reports (14,21-25). In the present study, the CD133 antigen was used to demonstrate that the subpopulation expressing this marker within the stabilized fibrosarcoma cell line HT1080 carries some characteristics of stem cells, including the capacity to generate a heterogeneous population, *in vitro* clonogenic activity and *in vivo* tumorigenic activity. The CD133$^+$ subset was found to be 8.40% of the total fibrosarcoma cells, consistent with the assumption that CSCs comprise only a very small proportion of tumor cells. In addition, a side population also was detected in the HT1080 cell line for the first time in this study. The ability of cancer cells to exclude small lipophilic molecules such as Hoechst 33342 depends on the presence of ABC (ATP-binding cassette) transporters actively pumping out these molecules, and this phenomenon has been exploited for the identification of putative stem cells in a variety of human cancers (27-29). The side population was found to comprise a much smaller fraction (0.30%) of the total cells in this study, Therefore, this may be considered to be an effective sorting approach for CSCs.

CD133+ cells in this study showed many differences with respect to their negative counterparts both *in vitro* and *in vivo*. In assays generally utilized to assess indices of self-renewal, tumorigenicity and clonogenicity, CD133+ cells displayed a stronger ability to form spherical colonies in anchorage-independent, serum-starved culture conditions. This finding was also supported by use of tumor xenografts *in vivo*. Only 1,000 CD133+ cells were needed to form fibrosarcomas, and these cells remained tumorigenic in continuous passages, while up to 100,000 injected CD133- cells could not form tumors in nude mice. The results confirmed that the tumor-initiating potential of CD133+ cells was much greater than that of the CD133- cells.

More recent evidence has indicated that there is a mutual genomic fingerprint between embryonic stem (ES) cells, adult tissue stem cells and CSCs. Thus, relative expression levels of genes that play an important role in the maintenance and promotion of stem cell pluripotency and nuclear reprogramming, include *Sox2, Oct3/4, Nanog, c-Myc* and *Bmi-1* in CD133+ and CD133- cells. The *Sox* family of transcription factors plays a critical role in cell differentiation and development. *Sox2* was originally thought to be the only *Sox* protein expressed in ES cells (30,31). The *Oct3/4* transcription factor and *Nanog* are critically involved in self-renewal and the maintenance of pluripotency in undifferentiated ES cells (32,33). *c-Myc* is recognized as a dominant-acting oncogene that encodes a transcription factor thought to regulate the G0-G1 cell cycle transition. *Bmi-1* is a member of the mammalian polycomb group (Pc-G) gene family and has recently emerged as a Myc-cooperating oncogene, which contributes to the proliferative capacity and self-renewal of both normal and malignant stem cells (34-36). All genes above were shown by real-time PCR in this study to be distinctly overexpressed in the CD133+ fraction. These data provide compelling evidence that a CD133+ subpopulation with stem-like properties exists in HT1080 cells.

To further investigate the CSC properties of CD133+ cells, the sensitivity of cells to chemotherapy drugs was examined. The results showed that CD133+ cells were more resistant to standard cytotoxic chemotherapy in comparison with the CD133- population. Advanced cancer is generally initially responsive to standard chemotherapies, but that response is almost inevitably followed by development of a drug-resistant phenotype. One increasingly accepted hypothesis of chemoresistance posits that standard therapies fail to target tumor progenitors, which are believed to express normal stem cell phenotypes, such as a low mitotic index, enhanced DNA repair and expression of membrane efflux transporters. The ABC superfamily of proteins contribute to multidrug resistance by pumping chemotherapy drugs out of the cell (37,38). *ABCG2*, a member of this family and a membrane transporter, is associated with the side population phenotype. Thus, chemosensitizers are being developed for the reversal of these transporters which play a prominent role in the multidrug resistance mechanism of tumors. The expression of *ABCG2* was recently reported in many types of cancers, and it was interpreted as a conserved feature of stem cells (39-41). Consistent with that hypothesis, CD133+ cells were shown here to express high levels of *ABCG2*, suggesting a significant role for these cells in fibrosarcoma chemoresistance. However, targeted therapy with an *ABCG2* antagonist can only partially inhibit the growth of CD133+ cells, perhaps due to expression by CSCs of other drug-resistant proteins such as *ABCB1* and *ABCC1* (38,42,43). Moreover, the CD133+ cell-derived spheres showed greater resistance than the CD133+-adherent cells. These results suggest that although CD133 is present in stem-like populations, using it as a single marker to isolate these cells would be insufficient. Further study is required to determine additional definitive surface markers for fibrosarcoma stem cells, such as *ABCG2*, that may be used in concert with CD133 to sort CSCs.

The above results revealed that CD133 may be used as a marker for isolation of fibrosarcoma CSCs from the human fibrosarcoma cell line HT1080. The isolated CD133+ cell subpopulation was demonstrated to have CSC characteristics. This finding prompts further study to more precisely define sarcoma stem cells and their mechanisms of drug resistance. A better understanding of fibrosarcoma CSC biology will facilitate the determination of prognosis and treatments. In particular, expression studies of fibrosarcoma CSCs will help to identify more specific diagnostic markers and therapeutic targets.

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