Sphere-forming tumor cells possess stem-like properties in human fibrosarcoma primary tumors and cell lines

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Abstract. Fibrosarcoma is a malignant soft tissue tumor of mesenchymal origin. Despite advances in medical and surgical treatment, patient survival rates have remained poor. According to the cancer stem cell hypothesis, tumors are comprised of heterogeneous cell populations that have different roles in tumor formation and growth. Cancer stem cells are a small cell subpopulation that exhibits stem-like properties to gain aggressiveness and recurrence. These cells have been identified in a variety of cancerous tumors, but not in human fibrosarcoma. In this study, we observed that HT1080 cells and primary fibrosarcoma cells formed spheres and showed higher self-renewal capacity, invasiveness and drug resistance compared with their adherent counterparts. Moreover, we demonstrated that the cells showed higher expression of the embryonic stem cell-related genes Nanog, Oct3/4, Sox2, Sox10 and their encoding proteins, as well as greater tumorigenic capacity in nude mice. In conclusion, our data suggest the presence of a stem-like cell population in human fibrosarcoma tumors, which provides more evidence for the cancer stem cell hypothesis and assistance in designing new therapeutic strategies against human fibrosarcoma.

Introduction

Fibrosarcoma is a malignant neoplasm derived from fibrous connective tissue and is characterized by immature or undifferentiated anaplastic spindle cells (1). Fibrosarcoma may occur in soft tissues such as muscles, connective tissue, blood vessels, fat and even in bones. Generally, fibrosarcoma develop equally among males and females. According to data compiled by the NCI in the SEER database between 2000 and 2004, the age-adjusted incidence of all bone and joint sarcoma was 0.9 per 100,000 individuals per year (2).

Although the incidence is low, long-term patient survival rates have remained poor (3). The reason for this may be the resistance of fibrosarcoma to radiation therapy and chemotherapy. Fibrosarcoma mainly metastasizes to the lung and unless metastases are completely resected, almost all patients with metastatic disease succumb to the disease. In addition, although fibrosarcomas have been studied for decades, their biological characteristics and cellular origins have not been well elucidated.

Current opinion is that cells in a tumor are hierarchically organized with respect to their capacity to initiate and sustain tumor growth. Cancer stem cells (CSCs) are a rare subpopulation of cancer cells that possess stem-like characteristics. The CSC hypothesis proposes that CSCs are responsible for forming the bulk of the tumor (4-6). CSCs are similar to stem cells and are capable of renewal and differentiation into all types of cells within a tumor. Furthermore, it is also believed that CSCs may play a key role in chemotherapeutic resistance, metastasis and recurrence (7).

Recent studies have identified CSCs in certain epithelial tumors and sarcomas, including leukemia, breast cancer, brain tumors, melanoma and Ewing's sarcoma, but not fibrosarcoma (8-12). Therefore, we aimed to detect the possible presence of cells possessing stem cell-like properties in human fibrosarcomas using the sphere-forming assay, which has been previously used to isolate cells that acquire a colony-forming capacity. The sarcospheres were then compared with adherent cells in terms of their stem cell-like properties using cell self-renewal assays, invasion assays, drug resistance assessments, western blot analysis, real-time quantitative PCR and *in vivo* tumor transplantation assay.

In the present study we show, for the first time, that sarcospheres are observed in primary fibrosarcoma tumor cells. Moreover, we demonstrated that these sphere-forming cells display higher self-renewal capacity, invasiveness and drug resistance compared with adherent cells. In addition, the sphere-forming cells showed greater expression of the embryonic stem cell-related genes and proteins. Taken together, our data suggest that stem-like cells may be found in human

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fibrosarcoma. These data may be of paramount importance in understanding the biology of stem cell-like cells as well as for designing novel therapies for human fibrosarcoma.

Materials and methods

Ethics statement. The patient in this study provided written informed consent for the publication of his case details. The protocol of the study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of Harbin Medical University, Harbin, China. The animal experimentation was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Use of Live Animals in Teaching and Research of the Harbin Medical University, Harbin, China (SYSK 2011-009).

Primary tumor cells and HT1080 cell line culture. A tumor sample from a 42-year-old male patient who had been diagnosed with fibrosarcoma in the left thigh muscle was obtained directly after surgical removal. The tumor sample was mechanically dissociated, digested in collagenase II (Sigma, Beijing, China) and incubated in a shaking water bath for 2 h at 37˚C. Pre-separation filters (Miltenyi Biotec, Beijing, China) were used to remove clumps and erythrolysis was performed in hypotonic solution (0.2% NaCl followed by 1.2% NaCl to stop lysis). The sample was purified with a dead cell removal kit (Miltenyi Biotec) and prepared as a cell suspension.

The HT1080 fibrosarcoma cell line was purchased from the American Type Culture Collection (Rockville, MA, USA). HT1080 cells and purified primary fibrosarcoma cells were maintained in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS; Invitrogen, Beijing, China) at 37˚C in a 5.0% CO_2 atmosphere.

Sphere formation assay. At ~80% confluence in DMEM/10% FBS medium, monolayer cells were dissociated with trypsin-EDTA into single-cell suspensions. The cells were then inoculated into N2-supplemented DMEM/F12/1% methylcellulose medium without serum at a density of $1x10^5$ cells/well in ultra-low-attachment six-well plates (Corning, Inc., Corning, NY, USA). Fresh aliquots of human recombinant epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml) were added every other day. Following 10-14 days in culture, colonies that contained >10 cells were quantitated by inverted phase contrast microscopy (Olympus CK2; Tokyo, Japan).

Single-cell suspension assay. Fibrosarcospheres were mechanically dissociated and adherent cells were digested into single-cell suspensions. The cells were then reintroduced into 96-well ultra low-attachment plates (Corning, Inc.) at a density of 1 cell/well in anchorage-independent methylcellulose medium to investigate their ability to self-renew through secondary sphere formation.

Assessment of drug resistance to doxorubicin

Cell Counting Kit-8 assay. Fibrosarcospheres were mechanically dissociated, and adherent cells were digested into single-cell suspensions. The fibrosarcosphere cells ($4x10^3$/well) and adherent cells ($4x10^3$/well) were then split into 96-well plates and incubated overnight to allow the cells to adhere. The cells were then were exposed to gradient doses of doxorubicin for 48 h. The cells were then incubated with WST-8 solution at 37˚C for 1 h and the absorbance at 450 nm was measured on a microplate reader (MPR-A4i, Tosoh Corporation, Tokyo, Japan). The cell viability index was calculated according to the following formula: experimental OD value/control OD value x 100%.

Crystal violet assay. Fibrosarcospheres and adherent cells ($5x10^4$/well) were seeded into 6-well plates and cultured overnight. The medium was then replaced with complete culture medium containing doxorubicin (10 μmol/l) for an additional 48 h. The cells were then washed twice with pre-warmed PBS, and the remaining cells were stained for 1 h with a crystal violet solution (0.1% crystal violet, 20% methanol). Images were captured using a camera.

Matrigel invasion assay. The Matrigel invasion assay was performed according to the manufacturer's instructions. Briefly, $1x10^5$ PFT or HT1080 sphere-forming cells were plated onto the Matrigel-coated membrane in the top chamber (24-well insert; pore size, 8 μm; Corning, Inc.). The adherent cells were processed in the same way as the control. All cells were added to the transwell inserts suspended in 0.5 μl medium containing 1% FBS and the inserts were placed in 750 μl complete medium. Following 48 h of incubation, cells that had migrated through the Matrigel were stained with hematoxylin. Cells in five representative microscopic fields were then counted and photographed.

Quantitative real-time PCR analysis. Quantitative real-time PCR was performed as previously described (13). Briefly, total RNA was extracted using a Qiagen RNeasy kit (Qiagen, Hilden, Germany) and then converted to cDNA with the Omniscript First-Strand synthesis system (Qiagen) using random primers (Qiagen). RT-PCRs were carried out using ABI Power SYBR Green mix (ABI, Applied Biosystems, Inc., Foster City, CA, USA) on a BioRad Chromo 4 instrument (BioRad, Richmond, CA, USA). Reactions were carried out in triplicate with RT controls; the gene for the ribosomal protein HL32 was used as a reference gene (14). The data were analyzed using the modified $\Delta\Delta$Ct method.

Western blotting. Protein lysates of PFT and HT1080 cells were prepared and separated onto SDS-polyacrylamide gels as previously described (15). Blots were stained with anti-β-actin antibody as an internal control for the amounts of target proteins. Anti-STAT3, -Oct 3/4, -Nanog, -Sox 2, -Sox 10 and -MDR1 primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used.

Nude mice xenografts. Five-week-old athymic nude mice (BALB/c nu/nu; Vital River Laboratory Animal Center, Beijing, China) were divided into two groups. Trypsinized fibrosarcospheres and adherent cells ($5x10^2$-$1x10^5$ unfractionated cells) were subcutaneously injected into the left and right flank, respectively. The mice were then inspected daily for 12 weeks. Tumor size was measured with a caliper and tumor volume was calculated using the formula (axb^2)/2, with a being the longest diameter and b the shortest diameter of the tumor.

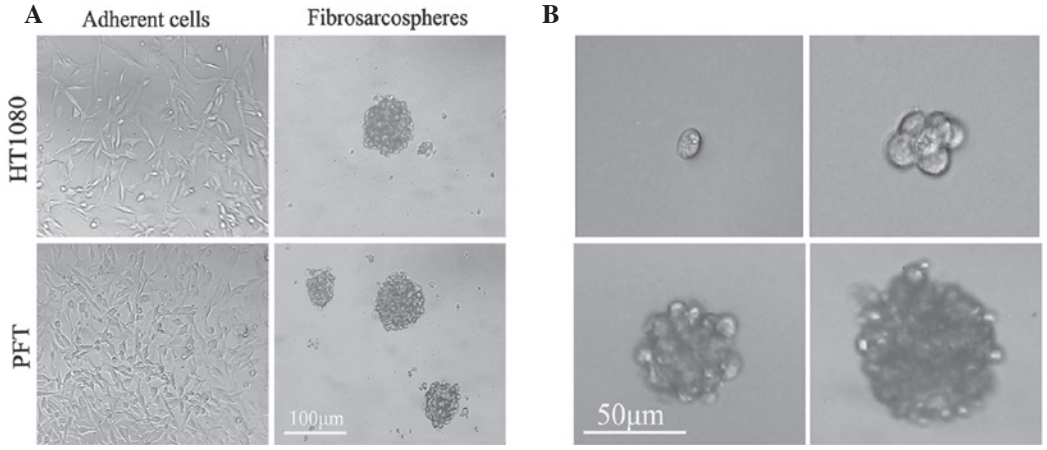

Figure 1. Human fibrosarcoma cells formed anchorage-independent, self-renewing fibrosarcospheres. (A) Phase-contrast images of adherent HT1080 (top left) and PFT (bottom left) cells cultured in serum-containing medium (DMEM with 10% FBS). Fibrosarcospheres formed by HT1080 (top right) and PFT cells (bottom right) under anchorage-independent, serum-free culture conditions for 14 days. PFT cells formed fibrosarcospheres at a higher frequency. Scale bar, 100 μm. (B) Phase images of a single fibrosarcosphere-derived cell cultured in a 96-well ultra-low-attachment plate under anchorage-independent, serum-free conditions. The propagation of a single cell was recorded at day 1 (top left), day 5 (top right), day 10 (bottom left) and day 14 (bottom right). Scale bar, 50 μm.

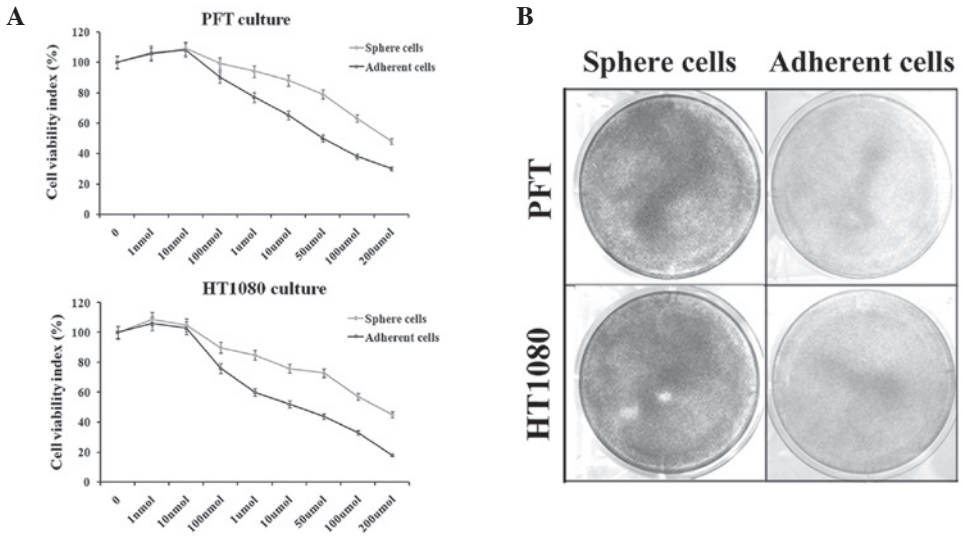

Figure 2. Fibrosarcosphere cells show a stronger drug resistance capacity to the chemotherapeutic agent doxorubicin. (A) The viability of cells was assessed by the Cell Counting Kit-8 assay. *P<0.05. (B) The proliferation of cells was measured using the crystal violet assay. Selected figures are representative of three independent experiments.

Statistical analysis. The results are expressed as the mean ± standard deviation and the Student's t test was used to evaluate statistical significance. P<0.05 was considered to indicate a statistically significant result.

Results

Fibrosarcosphere formation and self-renewal from primary tumors and the HT1080 cell line. When cultured in serum-containing medium (DMEM with 10% FBS), primary fibrosarcoma culture (PFT) and HT1080 cells showed an adherent growth pattern with a spindle-shaped morphology (Fig. 1A).

The cells were then trypsinized and replated in serum-free medium supplemented with bFGF and EGF in six-well ultra-low-attachment plates, and their growth characteristics and morphology were monitored. Within 48 h of replating, the single cells began to form loose clumps that continued to increase in density. At day 4, the loose clumps aggregated. At day 8, spheroids began to take shape. By day 12, spheroids had completely formed and became well-rounded structures composed of numerous, compacted cells (Fig. 1A). The PFT and HT1080 tumor cells eventually formed spheroids at a frequency of ~1/100 (928.25±30.25 colonies/1x10^6 cells) and 1/130 (769.75±69 colonies/1x10^6 cells).

To further investigate the self-renewal capacity of spherical cells, the single fibrosarcosphere cells were replated into 96-well ultra-low-attachment plates (1 cell/well) under serum-starved conditions. Both sphere-forming cells demonstrated self-renewal through the formation of secondary spheres at a frequency of approximately 1/100 (Fig. 1B). These data suggest that human fibrosarcomas possess the ability to generate suspended spherical clones and contain a population of self-renewing primitive cells as well as populations of differentiating cells.

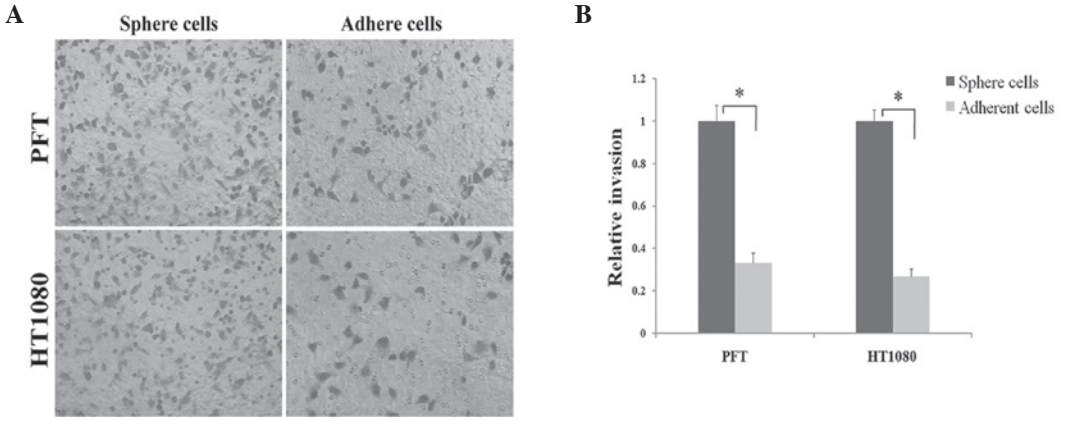

Figure 3. Fibrosarcosphere cells possess stronger invasive characteristics than adherent cells in Matrigel assays. (A) Photomicrographs of cells that passed through Matrigel. Sphere cells from PFT and HT1080 cultures showed significantly higher invasive capacity than their corresponding adherent cells (original magnification, x100). (B) Quantification of cells that passed through Matrigel. *P<0.05.

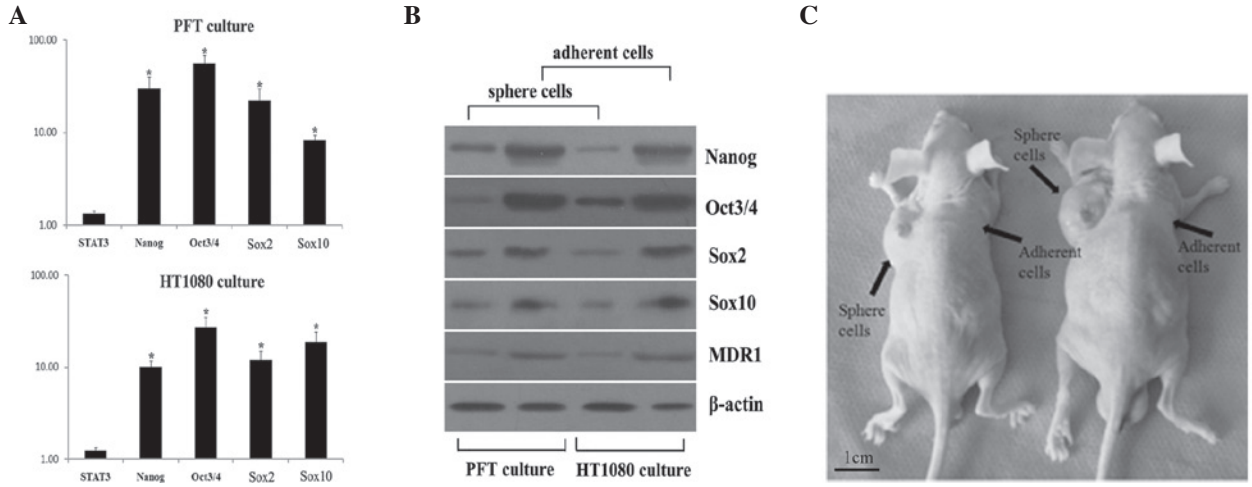

Figure 4. Fibrosarcospheres showed higher expression of stemness-related genes and proteins as well as exhibiting high tumorigenicity *in vivo*. (A) Quantitative RT-PCR analysis of mRNA expression of stemness-related genes. The ratio of the expression of the stemness-related genes STAT3, Oct3/4, Nanog, Sox2 and Sox10 in fibrosarcospheres compared with that in adherent cells is shown. *P<0.05. All mRNA levels except STAT3 were markedly increased in fibrosarcospheres from HT1080 and PFT cultures. (B) Western blotting analysis showed similar results and demonstrated a higher expression of MDR1 in fibrosarcospheres compared with adherent cells. (C) A total of $5x10^3$ sphere cells from HT1080 cultures were injected into nude mice and formed xenograft tumors (~1.3 cm^3, left mice), and $5x10^3$ injected fibrosarcospheres from PFT cultures formed larger tumors (~2.2 cm^3, right mice) at 8 weeks. Visible xenograft tumors from adherent cells from both cultures were not detected. Scale bar, 1 cm.

Fibrosarcosphere cells are more resistant to the chemotherapeutic agent doxorubicin. In this study, the Cell Counting Kit-8 assay was used to assess the cell viability rate. When exposed to gradient doses of doxorubicin for 48 h, the growth of PFT and HT1080 cultures was inhibited in a dose-dependent manner. The difference in growth inhibition rate between spherical and adherent cells was statistically significant. For example, 10 μM doxorubicin inhibited PFT and HT1080 cell growth by up to 38 and 48% in adherent cultures and 11 and 25% in spherical cultures, respectively (Fig. 2A). These results were further confirmed by the crystal violet assay (Fig. 2B).

Fibrosarcosphere cells possess stronger invasive characteristics than adherent cells in Matrigel assays. Certain studies have shown that CSCs in tumors are more invasive compared with their adherent counterparts, which is defined as an inherent characteristic of CSCs (7,12). As shown in Fig. 3, after 48 h of incubation PTF and HT1080 spherical cells readily migrated through the Matrigel chamber in relatively high numbers, whereas their adherent counterparts exhibited a marked reduction in invasion.

Fibrosarcospheres express markers of pluripotent embryonic stem cells. In this study, as shown in Fig. 4A, PFT and HT1080 fibrosarcospheres grown in the serum-free, anchorage-dependent system showed a significantly greater (P<0.05) expression of Oct3/4, Nanog, Sox2 and Sox10 than cells in adherent culture. Notably, both fibrosarcosphere and adherent cultures showed similar levels of STAT3 mRNA.

Consistent with the real-time quantitative PCR results, sphere cells from PFT and HT1080 cultures showed a higher protein expression of Oct3/4, Nanog, Sox2 and Sox10 than adherent cells (Fig. 4B). Moreover, MDR1, a protein related to chemotherapy resistance in tumors, was detected in both cultures. MDR1 expression in sphere cells was higher than

Table I. Tumorigenicity of fibrosarcosphere cells and adherent cells of PFT and HT1080 cultures in nude mice.

Cell type	Cell numbers injected	Tumor incidence[a]	Latency (days)[b]
Fibrosarcosphere cells of PFT culture	$1x10^2$	0/3	-
	$5x10^2$	1/3	45
	$1x10^3$	1/3	38
	$5x10^3$	2/3	32
	$5x10^4$	3/3	21
	$1x10^5$	2/2	13
Adherent cells of PFT culture	$5x10^2$	0/3	-
	$5x10^3$	0/3	-
	$5x10^4$	1/3	25
	$1x10^5$	2/2	18
Fibrosarcosphere cells of HT1080 culture	$1x10^2$	0/3	-
	$5x10^2$	0/3	-
	$5x10^3$	1/3	35
	$5x10^4$	2/3	23
	$1x10^5$	2/2	14
Adherent cells of HT1080 culture	$5x10^2$	0/3	-
	$5x10^3$	0/3	-
	$5x10^4$	1/3	28
	$1x10^5$	1/2	19

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

that in adherent cells. This result was consistent with the result from the drug resistance assessment assay.

Spherical cells show higher tumorigenic potential in xenografts. To study the tumor-initiating capability of the spherical cells, we generated xenografts using nude mice. As shown in Table I, spherical cells formed visible xenograft tumors in certain mice 2 weeks after injection, whereas their adherent counterparts did not form any visible tumors. Notably, despite the largest xenograft tumors initiated by spherical cells having reached 2.5±0.4 cm in diameter at week 12, few tumors were observed with the adherent cells. Moreover, as little as 500 PFT spherical cells were capable of initiating a tumor, whereas no tumor was found with $>5x10^3$ PFT adherent cells (Fig. 4C).

Discussion

Previous studies have suggested that the characteristics of normal stem cells, including self-renewal, the ability to differentiate and the activation of anti-apoptotic pathways, may be shared by tumor cells. In a tumor, CSCs comprise a relatively small subpopulation of cells and possess primitive phenotypes, the capability of initiating tumor formation, resistance to chemotherapy and invasion into other tissues (16). Therefore, CSCs may survive therapy and begin to differentiate and reform a bulk tumor. Hence, CSCs are proposed to be responsible for chemoresistance, recurrence and progression in a number of tumors (5,16).

In the present study, we demonstrated that HT1080 and PFT cells have the ability to form fibrosarcospheres and to self-renew in a culture system previously developed to isolate stem cells from brain and breast tumors (10,17). This anchorage-independent, serum-free culture system yielded clonogenic stem-like cells that possessed many attributes common to normal stem cells. Furthermore, we found that fibrosarcosphere cells presented the characteristics of tumor stem cells previously described for CSCs present in other tumor types, including osteosarcoma, melanoma, breast and colon tumors.

By RT-PCR analysis, the fibrosarcosphere cells were found to highly express the stemness-related genes compared with adherent cells. This result was confirmed by western blot detection of their gene products. Oct3/4 is a POU family homeoprotein initially expressed in the inner cell mass of embryos and is essential for the maintenance of pluripotency. After maturity, Oct3/4 is only observed in certain early progenitor cells, but not in somatic cells (18). Nanog is a divergent homeoprotein that is capable of maintaining self-renewal in ES cells. Overexpression of Nanog is associated with an increased self-renewal capacity of ES cells (19,20). Together, our data suggest that fibrosarcospheres contain cells that possess pluripotent and self-renewal capacity.

In the Cell Counting Kit-8 and crystal violet assays, we demonstrated that fibrosarcosphere cells showed higher resistance potential to the chemotherapeutic agent doxorubicin. Although the mechanisms of drug resistance remain to be elucidated, several studies have revealed a possible involvement of the ATP-binding cassette (ABC) drug transporters (21). In this study, spherical cells highly expressed multidrug resistance transporter (MDR1). Goodell *et al* demonstrated that ABC transporters may be involved in the efflux capacity of CSCs (22). This transporter protein has been found to contribute to Hoechst dye efflux and produce a cancer stem cell phenotype in a wide variety of tissues (21). The expression of ABC transporters has been analyzed in various malignancies in relation to the drug resistance of CSCs (23). As such, the higher expression of MDR1 may be a mechanism of the resistance of fibrosarcosphere cells to the chemotherapeutic agents that was observed in our study.

Furthermore, we found that certain cells derived from fibrosarcospheres were able to reform spherical colonies with a similar or greater frequency in the cell renewal assay *in vitro*, indicating that the fibrosarcospheres contain self-renewing daughter cells and differentiating cells through asymmetric cell divisions. We also found that as few as 500 fibrosarcosphere cells were able to initiate tumor formation in nude mice. Collectively, these results suggest that both PFT and HT1080 cell populations contain enriched stem-like cells that retain self-renewal, chemotherapeutic resistance, invasive and strong tumor-initiating abilities. Thus, these cells have a number of characteristics indicative of 'stem-like cancer cells'.

Although the cell of origin has still not been clearly determined, specific molecular markers for cancer stem cell populations in certain epithelium-derived tumors have been identified, such as CD133 (prominin-1), which was used initially as a marker for neuroepithelial stem cells and

subsequently as a maker for many CSCs, including those of the brain and colon (24-28). To develop CSC-targeted therapy, it is important to specifically isolate the CSCs. Although we have demonstrated the existence of a cell population in human fibrosarcomas that has stem cell characteristics, no specific molecular markers of human fibrosarcoma CSCs were determined in this study. Therefore, future studies will employ immunochemistry and fluorescence-activated cell sorting (FACS) to investigate specific molecular markers for the isolation of the CSCs in human fibrosarcomas.

In conclusion, our study results suggest that both human fibrosarcoma primary tumors and cell lines contain a subpopulation of stem cell-like cells that possess a self-renewal capacity, strong tumor-initiating ability, higher resistance to chemotherapy and greater invasiveness, as well as primitive phenotypes of embryonic stem cells. These data may lead to a considerable increase in our understanding of the biology of human fibrosarcoma, raise the possibility that human fibrosarcoma is a stem cell malignancy and provide key insight into improved drug design and therapy in the future.

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