Stem-Like Cells in Bone Sarcomas: Implications for Tumorigenesis[1]

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Abstract

Bone sarcomas are a clinically and molecularly heterogeneous group of malignancies characterized by varying degrees of mesenchymal differentiation. Despite advances in medical and surgical management, survival rates for high-grade tumors have remained static at 50% to 70%. Tumor stem cells have been recently implicated in the pathogenesis of other heterogeneous, highly malignant tumors. We demonstrate here the existence of a small subpopulation of self-renewing bone sarcoma cells that are capable of forming suspended spherical, clonal colonies, also called "sarcospheres," in anchorage-independent, serum-starved conditions. These bone sarcoma cells as well as tissue specimens express activated *STAT3* and the marker genes of pluripotent embryonic stem (ES) cells, *Oct 3/4* and *Nanog*. Expression levels of *Oct 3/4* and *Nanog* are greater in sarcospheres than in adherent cultures. A subset of bone sarcoma cells displays several surface markers of mesenchymal stem cells (Stro-1, CD105, and CD44) as well as attributes of mesodermal, ectodermal, and endodermal differentiation. Although previously documented in brain and breast tumors, our results support the extension of the cancer stem cell hypothesis to include tumors of mesenchymal lineage. Furthermore, they suggest the participation of ES cell homeobox proteins in non–germ cell tumorigenesis.

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Introduction

Bone sarcomas, including osteosarcoma and chondrosarcoma, are among a group of mesenchymal malignancies that exhibit clinical, histologic, and molecular heterogeneity [1,2]. Osteosarcoma is the most common primary bone malignancy of childhood and adolescence, comprising almost 60% of the common histologic subtypes of bone sarcomas in childhood [3]. Despite advances in surgery and multiagent chemotherapy, long-term survival rates have stag-

nated at approximately 65% [4]. Chondrosarcoma, however, is a disease of adults for which chemotherapy has proven ineffective. Both tumors metastasize to the lung, and unless lung metastases can be resected completely, almost all patients with metastatic bone sarcoma succumb to their disease.

The biology of sarcomagenesis is understood poorly. Recent studies have implicated stem-like cells in the pathogenesis of leukemia, brain tumors, and breast tumors [5–8]. The cancer stem cell theory holds that there is a small subpopulation of cells within a tumor, which, like normal stem cells, has the ability to self-renew. These few cells can divide asymmetrically, producing an identical daughter cell and a more differentiated cell, which, on subsequent divisions, generates the vast majority of tumor bulk. This rare stem-like cell is responsible for initiating and maintaining the growth of the tumor and, if not completely eradicated by surgical extirpation or chemotherapy, might be responsible also for local and distant recurrence. For this study, we hypothesized that the heterogeneity and relative resistance of bone sarcomas to chemotherapy might be associated with the presence of tumor stem-like cells. Demonstration of stem-like cells in these tumors would extend support for the stem cell theory of carcinogenesis from hematologic and ectodermal tumors to mesenchymal tumors [5–9].

Stem cells have the potential to self-renew and generate a developmental hierarchy of differentiating progeny. The original culture methodology employed by Reynolds et al. [10] and Reynolds and Weiss [11] to show that the adult mammalian brain contains cells that give rise to neurosphere clones has

been used to isolate and characterize cells suspected of possessing attributes of stem and progenitor cells. Stressful growth conditions of this system (serum starvation and anchorage independence) may allow dedifferentiation of certain cells, or may select for the most primitive cells by eliminating the differentiated cells that are unable to survive. Similarly, suspending dissociated normal and cancerous tissues in semisolid media without serum selects for primitive clonogenic cells that can be expanded and can give rise to different classes of cells. We have shown previously that isolated cells from malignant human brain tumors exhibit a stem cell–like phenotype in a neurosphere culture system that exploits anchorage independence, serum starvation, and the pleiotropic growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [7]. Others have further validated this model based on generally accepted cell surface marker expression, fluorescence-activated cell sorter isolation and cloning, and reestablishment of tumorigenicity following xenografting [12,13]. Similar sphere culture systems have been used to identify tumor stem cells that are capable of self-renewal and tumorigenicity in mouse models [6,8,9,13] for both brain and breast malignancies. Despite prospective identification of tumor stem cells by these approaches, molecular mechanisms controlling their self-renewal and differentiation are poorly understood. Here, we report the identification of a subpopulation of stem-like cells in bone sarcomas using a neurosphere culture system, and we demonstrate that these cells express the key molecular machinery of embryonic stem (ES) cells.

Materials and Methods

Cell Culture

Osteosarcoma and chondrosarcoma cultures were established from patient biopsies. Specimens were minced in DMEM/F12 medium, digested with 5 mg/ml Collagenase Type II (Gibco BRL Invitrogen Corporation, Grand Island, NY) for 3 to 12 hours at 37°C, and passed through a 70-μm Cell Strainer (Becton Dickinson Lab Ware, Franklin Lakes, NJ) to prepare a single-cell suspension, as described previously [7]. Samples were obtained with consent, using protocols approved by the Institutional Review Board of the University of Florida College of Medicine. The age range for osteosarcoma patients was 11 to 34 years (median age, 12 years), whereas that for chondrosarcoma patients was 55 to 67 years (median age, 57.5 years). Cell cultures 99-1 and 99-2 were supplied by the University of Colorado Health Sciences Center. The human osteosarcoma cell line MG 63 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM/F12 medium supplemented with 10% FBS (HyClone, Logan, UT) at 37°C and 7.0% CO_2.

Neurosphere/Sarcosphere System Assays

Cells were plated at a density of 60,000 cells/well in six-well ultralow attachment plates (Corning Inc., Corning, NY) in N2 medium with 1% methylcellulose. For this, 2× DMEM/F12 with progesterone (20 nM), putrescine (100 μM), sodium selenite (30 nM), transferrin (25 μg/ml), insulin (20 μg/ml; Sigma Biochemicals, St. Louis, MO), and the growth factors human EGF (10 ng/ml) and human bFGF (10 ng/ml; Pepro Tech, Rocky Hill, NJ) were mixed with an equal volume of 2% methylcellulose (Sigma Biochemicals). In addition, fresh aliquots of EGF and bFGF were added every other day. After culture for 7 to 12 days, colonies containing more than 50 cells were quantitated by inverted phase contrast microscopy (Nikon Eclipse TS100).

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit and was treated with RNAase-Free DNase (Qiagen Sciences, Valencia, CA) according to the manufacturer's instructions. RNA was quantitated by spectrophotometry at OD_{260}. First-strand cDNA was made using Oligo(dT)$_{12-18}$ (Invitrogen Life Technologies, Carlsbad, CA) primers, 1.5 μg of total RNA, and SuperScript II RNase H Reverse Transcriptase (Invitrogen Life Technologies), according to the manufacturer's recommendations. The target cDNA was amplified using Platinum Taq DNA Polymerase (Invitrogen Life Technologies) for 35 to 37 cycles at 90°C for 1 minute, at 55 or 60°C for 30 seconds, and at 72°C for 1 minute. The primers are provided in Table 1. Aliquots of 8 μl of the amplification products were separated by electrophoresis in 1.2% agarose gels

Table 1. Primer Pairs Used in RT-PCR.

Gene	Forward Primer	Reverse Primer	Product (bp)
Gata-4	GCCCAAGAACCTGAATAAATCTAAG	AGACATCGCACTGACTGAGAACGTC	208
Gata-6	TTCCCCCACAACACAACCTACAG	GTAGAGCCCATCTTGACCCGAATAC	118
AFP	GGTGTAGCGCTGCAAACGATG	AATTTAAACTCCCAAAGCAGCACGA	210
STAT3	GGGTGGAGAAGGACATCAGCGGTAA	GCCGACAATACTTTCCGAATGC	198
RUNX1	CTCAGGTTTGTCGGTCGAAGTGGAA	CCGCAGCTGCTCCAGTTCAC	216
RUNX2	CTCCCTGAACTCTGCACCAAGTCCT	GGGGTGGTAGAGTGGATGGACG	156
RUNX3	CCGAGCCATCAAGGTGACCGTGGAC	GGGCTGGCTGCTGAAGTGGCTTGT	187
Osteocalcin	CCCTCACACTCCTCGCCCTATT	AAGCCGATGTGGTCAGCCAACTCGT	259
ALP	CACTGCGGACCATTCCCACGTCTT	GCGCCTGGTAGTTGTTGTGAGCATA	206
IBSP	GGGCAGTAGTGACTCATCCGAAGAA	CTCTCCATAGCCCAGTGTTGTAGCA	166
Nanog	GCTGAGATGCCTCACACGGAG	TCTGTTTCTTGACTGGGACCTTGTC	163
Oct 3/4	TGGAGAAGGAGAAGCTGGAGCAAAA	GGCAGATGGTCGTTTGGCTGAATA	186
β-III Tubulin	CTGCTCGCAGCTGGAGTGAG	CATAAATACTGCAGGAGGGC	141
β-Actin	GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG	229

and visualized by ethidium bromide staining. Only RNA samples that gave completely negative results in PCR without reverse transcriptase were used for analyses.

Western Blot Analysis

Cells were lysed in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1% Na-deoxycholate, 1 mM Na-vanadate, and protease inhibitors 5 μg/ml pepstatin, 1 mM PMSF, 10 μg/ml leupeptin, and 1 mM NaF (Sigma Biochemicals) for 1 hour in ice. After centrifugation at 12,000g for 10 minutes at 4°C, the protein concentration of the supernatants was measured by the BCA Protein Assay kit (Pierce, Rockford, IL). Lysates were mixed (1:1) with Laemmli buffer (Sigma Biochemicals). Fifteen micrograms of protein per lane was electrophoresed in 8% to 16% or in 10% to 20% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Sigma Biochemicals). Membranes were blocked with nonfat dry milk for 1 hour at room temperature and incubated overnight at 4°C with the corresponding antibodies in 5% bovine albumin (Sigma Biochemicals), Tris-buffered saline (TBS), and 0.1% Tween 20 (Bio-Rad Laboratories, Hercules, CA). After being washed six times in TBS with 0.1% Tween 20, blots were incubated with appropriate species: Ig-specific, peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA; Jackson Immuno Research Laboratories, West Grove, PA). Immunoreactive bands were detected by ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ) for 60 seconds. We used primary antibodies anti-*STAT3* (R&D Systems, Minneapolis, MN), phospho-*STAT3* (Tyr705; Cell Signaling Technology), β-*III tubulin* (BAbCO, Berkeley, CA), anti–β-*actin* (Sigma Biochemicals), alpha fetoprotein (*AFP*; C-19), and *Oct 3/4* (N-19; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunocytochemistry

Cells were grown for 3 to 5 days on glass coverslips coated with polyornithine (10 μg/ml)/laminin (5 μg/ml) in DMEM/F12 medium with 10% FBS in 12-well plates. Cells were fixed in freshly prepared cold 4% paraformaldehyde (Sigma Biochemicals) for 15 minutes at room temperature and made permeable with ice-cold 0.5% Triton X-100 and 2% sucrose in Dulbecco's phosphate-buffered saline (DPBS) for 5 minutes. After blocking for 20 minutes with 25% goat serum (Sigma Biochemicals) in DPBS, cells were incubated for 30 minutes at room temperature in 25% goat serum in DPBS with primary antibodies β-*III tubulin* (BAbCO) and Stro-1 (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA) and visualized by indirect immunofluorescence microscopy with secondary antimouse antibody. All proteins were labeled using Alexa Fluor 594–conjugated antibody (Molecular Probes, Eugene, OR). For F-actin visualization, cells were labeled with fluorescein phalloidin.

Histologic Analyses and Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections (5 μm) were deparaffinized sequentially, rehydrated, and blocked for endogenous peroxidase activity. Following a 95°C, 25-minute antigen retrieval in Trilogy unmasking solution (Cell Marque, Hot Springs, AR), slides were biotin-blocked, serum-blocked, and immunostained using a goat ABC Elite Kit (Vector Laboratories, Burlingame, CA). Antibodies to *Oct 3/4* and *Nanog* (R&D Systems) were applied at 1:50 for 1 hour at room temperature. Positive staining was detected with 3,3′-diaminobenzidene, and light green SF yellowish (Sigma Biochemicals) was used as counterstain.

Differentiation Staining

Cells derived from bone sarcoma spheres were grown on an adhesive substrate in adipogenic and osteogenic media, as previously described [14], for 14 days. Lipid-induced cells were fixed with 10% formalin, washed, and stained with Oil Red O for 7 minutes. Hematoxylin was used as the counterstain. Cells in osteogenic medium were fixed in ethanol, followed by von Kossa stain and exposure to sunlight for 20 minutes. Cells were washed three times in distilled water and then counterstained with Nuclear Fast Red.

Statistical Analyses

Statistical analysis was completed using Student's *t* tests to determine the RNA expression difference between adherent and sarcosphere culture conditions for each tumor. Pearson's correlation coefficient was performed to compare the correlation of *Oct 3/4* and *Nanog* coexpression between sphere and adherent cultures (Statistica; StatSoft, Tulsa, OK).

Results

Sarcosphere Formation and Self-Renewal from Bone Sarcoma Culture

To determine whether bone sarcomas might contain stem-like cells, we first established adherent cultures from biopsies of untreated chondrosarcoma and osteosarcoma, as well as from the commercially available osteosarcoma cell line MG 63. We evaluated each for its ability to generate spherical clones and to self-renew in our neurosphere culture system. At ~80% confluence, we dissociated these monolayer cultures into single-cell suspensions and inoculated them into a methylcellulose medium without serum, at a clonogenic density of 60,000 cells/well in antiadhesive six-well plates. It should be noted that these conditions have been optimized to preclude reaggregation of single cells.

After 10 to 14 days, all nine initial bone sarcoma cultures and the MG 63 cell line formed spherical colonies ("sarcospheres") at an epigenetic frequency of 10^{-2} to 10^{-3} (Figure 1, *A* and *B*). This frequency of sphere formation is similar to that reported by others for brain and breast malignancies [6,7,9]. Sarcospheres were also generated at a similar frequency from fresh tumor dissociates produced at the time of biopsy (data not shown). This high frequency suggests an epigenetic mechanism, rather than a mutational mechanism, underlying these cells' ability to form clonal colonies under our culture conditions.
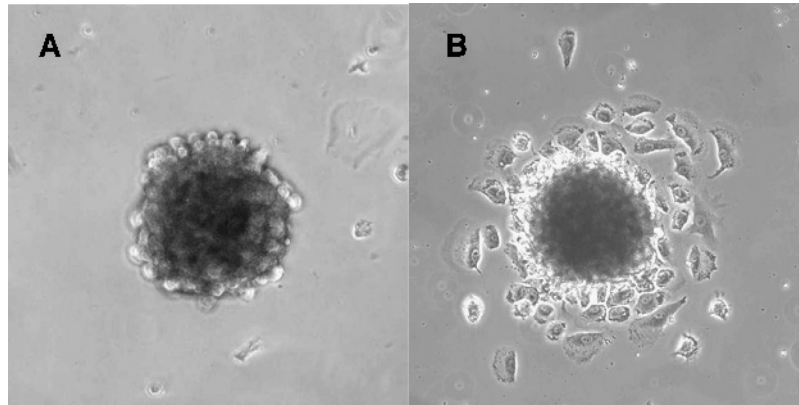
Figure 1. *Phase contrast images of monoclonal sarcospheres formed from self-renewing cells from bone sarcoma. Monolayer cultures of cells isolated from bone sarcoma were seeded at clonogenic density into N2 medium with 1% methylcellulose (see Materials and Methods section) and cultured for 10 to 14 days. Under these conditions, spheres form at a frequency of 1/100 to 1/1000 cells. (A) Representative image of a sarcosphere in the suspension culture. Cells within the sphere show a compact undifferentiated morphology. (B) Sarcosphere removed from the suspension culture and allowed to attach to a substratum. Adherent cells can be seen expanding from the sphere.*

We then asked whether clone-forming cells could self-renew in the sarcosphere system. Cultured sarcospheres from five representative cultures (osteosarcomas OS 521, OS 01-187, and OS 99-1, chondrosarcoma CS 828, and osteosarcoma MG 63) were dissociated to single cells and allowed to grow in monolayers. At near-confluence, the cells were harvested and reseeded as single cells into a suspension culture, as described above. All five cultures demonstrated self-renewal through the formation of secondary spheres at a similar or an increased frequency of approximately 10^{-2}. Three of these were recloned serially for four passages and continued to generate spheres at approximately the same frequency. These data show that bone sarcomas share with ectodermal tumors [7,15] the ability to generate suspended spherical clones under growth-constraining conditions, and they suggest that they contain a small subpopulation of self-renewing primitive cells.

Expression of Oct 3/4*,* Nanog*, and* STAT3 *in Bone Sarcoma Cells*

Pluripotency in embryonic settings is associated with the ability to maintain the expression of genes specific for multiple cellular lineages and then to differentiate along one of those lineages while restricting the others [16]. Current evidence from murine studies suggests that self-renewal and pluripotency of undifferentiated ES cells are maintained by a crosstalk involving three transcription factors: the POU family member Oct 3/4, the recently identified homeoprotein Nanog, and activated STAT3 [17,18]. To determine whether the genes for these proteins are expressed in bone sarcoma cultures in both sphere and adherent conditions, we first performed semiquantitative RT-PCR analyses.

As shown in Figure 2A, detectable levels of mRNA were present in each culture type for all three transcription factors. Both spheres and adherent cultures demonstrated similar robust levels of *STAT3* mRNA. Strikingly, sarcospheres grown in the serum-starved, anchorage-independent system showed significantly greater ($P < .05$) expression of

both *Oct 3/4* and *Nanog* than cells grown in adherent culture supplemented with serum (Figure 2, A and B). To determine if these genes were expressed at the protein level, a series of Western blot and immunohistochemical analyses was performed. Unfortunately, despite numerous attempts, we were able only to isolate usable protein from the adherent cultures and not from spheres in methylcellulose culture. However, as shown in Figure 2C, in the adherent cultures, we were able to detect specific immunoreactive species that corresponded in size with Oct 3/4. We also detected STAT3 and its activated form, represented by phosphorylation at Tyr705, in each of the cultures examined (Figure 2C). Immunohistochemical staining of sarcospheres from paraffin sections, however, enabled the detection of both Nanog and Oct 3/4 in similar patterns (Figure 2D). Interestingly, the smaller immature spheres showed a greater proportion of cells that stained positive for Oct 3/4 and Nanog than did the larger spheres. Typical of that shown in the figure, the cells in the periphery of the spheres showed a more intense staining than the internal cells, giving a ring-like appearance.

Following these observations, we addressed whether Oct 3/4 and Nanog are expressed in actual tumor tissues. For this, paraffin sections from eight bone sarcoma patients were evaluated using immunohistochemistry. Nanog and Oct 3/4 nuclear staining was observed in seven of eight tumors studied. In each case, as determined by histologic criteria, the stained nuclei were from malignant cells and not from infiltrating normal cells (Figure 3). Between tumor specimens, the number of Oct 3/4− and Nanog-positive cells varied considerably. Positive Oct 3/4 staining ranged from a few percentages of the cells in some tumors up to 25% in others. Nanog staining was also quite variable, ranging from ~1% of the cells to nearly 50% in certain samples. Cumulatively, previous data demonstrate the expression of ES cell−associated gene products in adherent cultures, sarcospheres, and tissue sections from bone sarcomas, and they support the hypothesis that cells in these tumors possess attributes of stem cells.

Expression of Genes from Multiple Lineages in Bone Sarcoma Cells

Based on previous findings, we reasoned that if bone sarcomas express some of the molecular machineries of ES cells, they might, in addition to mesodermal genes, express genes from endodermal and ectodermal lineages. RT-PCR analyses of mRNA from adherent and sarcosphere cultures of the eight tumors examined previously, plus an additional chondrosarcoma culture (CS 828 LM), revealed the expression of *Gata-4*, *Gata-6*, and *AFP*, which are indicative of endodermal differentiation. Expression of β-*III tubulin* RNA, which is believed to be a marker of neural ectoderm but has been demonstrated in some poorly differentiated malignancies [19], was seen as well. Using Western blot analyses, expression of AFP and β-III tubulin at the protein level was demonstrated in each of the seven cultures tested (Figure 4*B*). β-III Tubulin was also detected in paraffin sections of tumors using immunohistochemistry (Figure 4, *C* and *D*) and in cell culture samples using immunocytochemistry (Figure 4, *E* and *F*).

Bone Sarcoma Cells Have Attributes of Mesenchymal Stem Cells

Although the bone sarcomas studied here express transcription factors associated with pluripotent stem cells, their histologic phenotype is, by definition, one of arrested mesenchymal differentiation. Therefore, we examined the respective cultures for cells with characteristic bone marrow stromal cells, also termed mesenchymal stem cells. These multipotent cells differentiate along mesenchymal lineages, such as bone, cartilage, fat, and muscle [14,20–22]. Using immunocytochemistry, we first screened for the expression of Stro-1, CD44, and CD105, which are cell surface proteins associated with mesenchymal stem cells [22,23]. Stro-1 has been shown previously to be expressed in the permanent osteosarcoma cell line MG 63 [24].

These assays revealed that 2% to 10% of adherent cells in each of the chondrosarcoma and osteosarcoma cultures were positive for the Stro-1 surface protein (Figure 5*A*). Additionally, CD105 expression was demonstrated in 30% to 50%—and CD44 expression was demonstrated in 75%
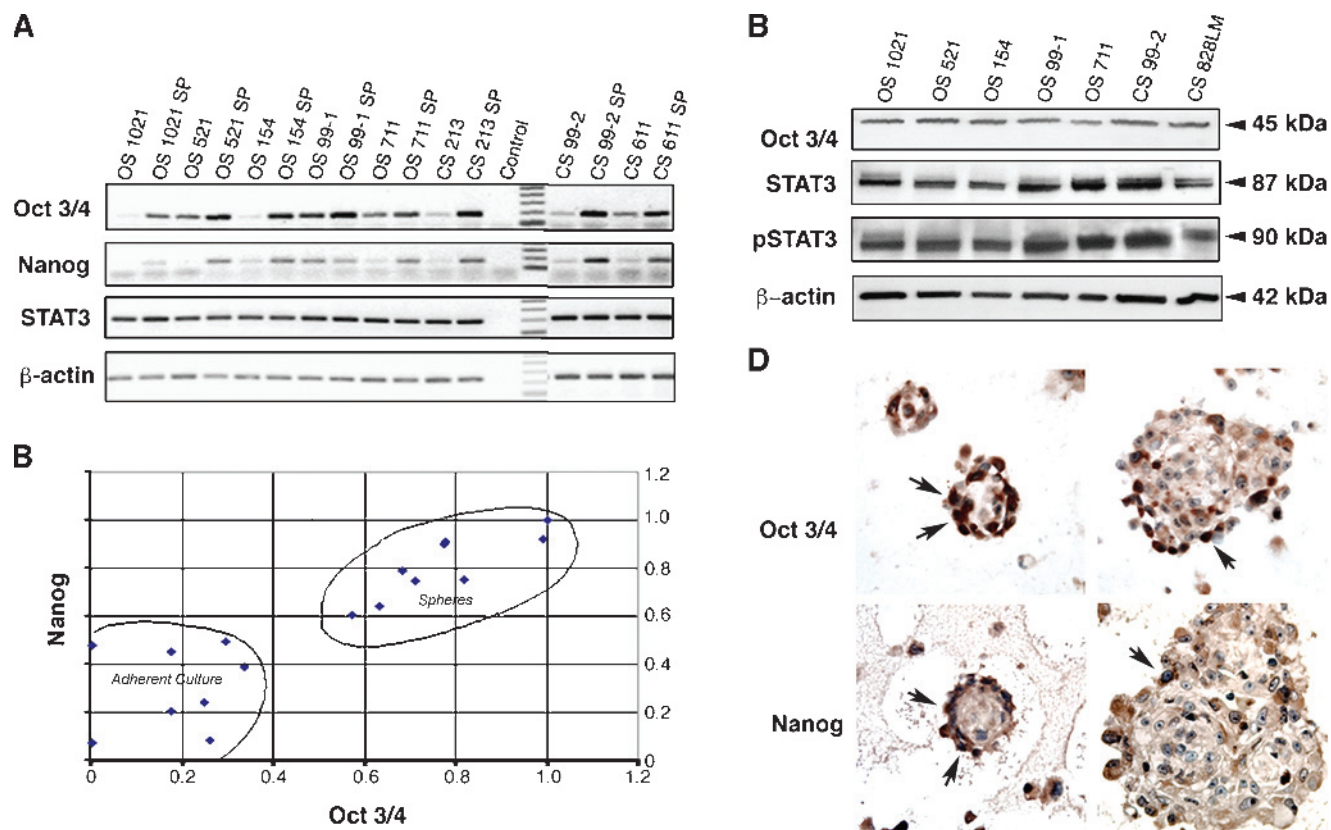

Figure 2. *Genes specific to ES cells show increased expression in sphere cultures derived from bone sarcomas. (A) Monolayer and sarcosphere (SP) cultures initiated from five osteosarcoma (OS) and three chondrosarcoma (CS) biopsies were analyzed for the expression of* Oct 3/4, Nanog, *and* STAT3 *using semiquantitative RT-PCR.* β-Actin *expression was used as a positive control. Sphere cultures demonstrate increased transcription of both* Oct 3/4 *and* Nanog *over adherent cultures.* STAT3 *expression was uniform between both culture types. (B) Relative band intensities for* Oct 3/4 *and* Nanog *for each culture from (A) were quantitated by densitometry, normalized relative to* β-actin, *and plotted on the graph (*Oct 3/4, x-axis; Nanog, y-axis*). As indicated by the grouping, the sphere cultures of each sarcoma showed a significantly greater expression of both* Oct 3/4 *and* Nanog *than adherent cultures (P < .05, Pearson's correlation coefficient). (C) Western blot analysis of lysates from representative bone sarcoma cell cultures for the protein expression of* Oct 3/4, STAT3, *and activated (phosphorylated, p)* STAT3. β-Actin *was used as a positive control for loading, membrane transfer, and immunoblotting. All cultures showed positive staining of protein bands of appropriate sizes, as indicated. (D) Small (left) and large (right) sarcospheres were embedded in paraffin and stained using immunohistochemistry for* Oct 3/4 *and* Nanog, *as indicated. Small spheres show an intense staining of cells in the periphery (arrows). Large spheres show similar numbers of darkly staining cells (arrows), with dramatically increased numbers of poorly staining cells in the interior of the sphere.*
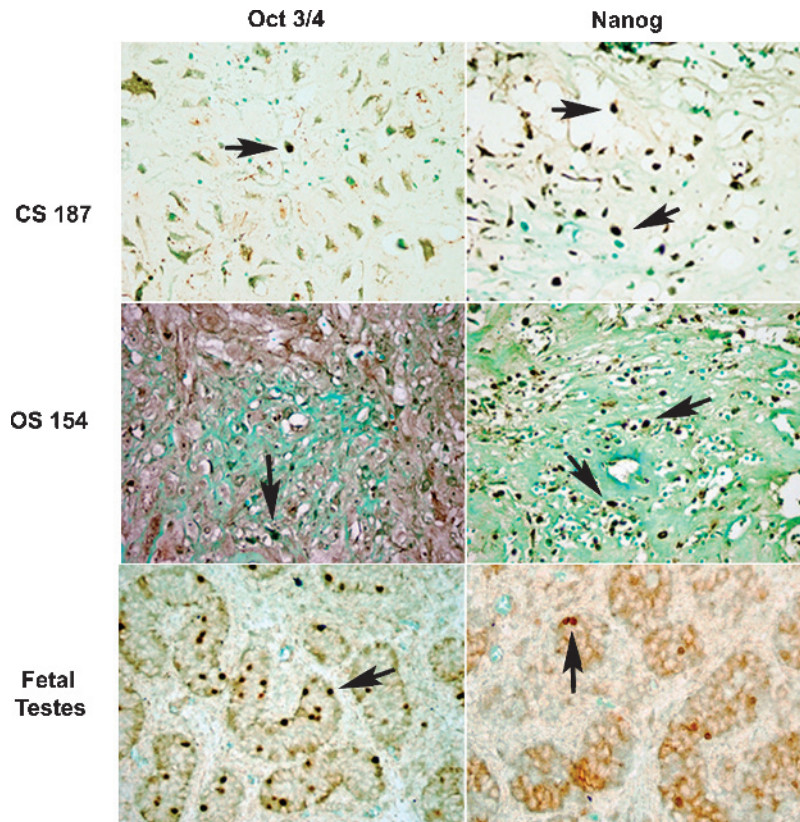
Figure 3. *Immunohistochemical staining for* Oct 3/4 *and* Nanog *in sections from tumor biopsies of chondrosarcoma and osteosarcoma. One representative osteosarcoma (OS 154) and chondrosarcoma (CS 187) and a positive control, human fetal testes, are shown, as indicated. CS 187 shows a single nucleus positive (brown) for* Oct 3/4 *and multiple nuclei positive (brown) for* Nanog *in lung metastasis from a chondrosarcoma. OS 154 sections demonstrate scattered* Oct 3/4 *nuclear staining and near-complete nuclear* Nanog *staining in a primary fibular osteosarcoma. Twenty-six-week fetal testes with scattered* Oct 3/4 *and* Nanog *nuclear staining are shown as positive controls.*

to 100%—of the culture cells tested (data not shown). There were no obvious morphologic differences between the positive- or negative-staining cells for these three antigens. Next, to determine if cells within the cultures were indeed multipotent, we attempted to induce adherent cultures of osteosarcoma and chondrosarcoma cells to differentiate along two distinct mesenchymal lineages by culture in osteogenic and adipogenic media. Similar to that shown in Figure 5*A*, within each adherent culture tested, we observed discrete foci of mineralization in cells grown in osteogenic medium and also observed fields of lipid-laden cells in those grown in adipogenic medium. Finally, when RNA from the respective cultures was examined using RT-PCR, multiple genes indicative of mesenchymal lineage were expressed in both adherent cells and sarcospheres (Figure 5*B*). Although differences in expression levels were noted within and among culture types other than alkaline phosphatase (*ALP*), which was expressed more highly in monolayer cells than in sarcospheres, no discernable pattern was observed.

Discussion

The goal of this study was to determine if bone sarcomas contain cells with stem-like properties. To this end, we first demonstrated that a subset of bone sarcoma cells has the capacity to form sarcospheres and to self-renew in a culture system previously developed to isolate stem cells from brain and breast tumors [7,9,11]. This system yielded clonogenic stem-like cells that have many attributes in common with both normal stem cells and glioblastoma stem cells. Furthermore, we found cells derived from these tumors that bear mesenchymal stem cell markers: Stro-1, CD44, and CD105 can be induced to differentiate along at least two mesenchymal lineages. In addition, cultures of cells from bone sarcomas express genes associated with all three germ layers mesoderm, ectoderm, and endoderm. We also have shown that sarcospheres preferentially express key marker genes of ES cell pluripotency, *Oct 3/4* and *Nanog*. These proteins were also seen in paraffin-embedded sarcoma tumor specimens, extending the relevance of these data beyond *in vitro* systems. Altogether, these data provide compelling evidence of the existence of a subpopulation of stem-like cells in bone sarcomas.

The expression profiling of our sarcoma cultures identified differences between cells grown as adherent cultures supplemented with serum and those grown as sarcospheres. The most striking difference between the two was the increased expression of *Oct 3/4* and *Nanog* in sarcospheres. These proteins were also detected in tumor tissues by immunohistochemistry. Interestingly, *STAT3*, which is considered to be central to the maintenance of the ES cell phenotype in mouse cells [16] but not in human cells [25], did not

differ in expression level between sarcosphere and adherent cultures. *STAT3* has, however, been shown to be highly expressed in several human malignancies, promoting metastasis, angiogenesis, and immune evasion [26,27], and, thus, may play a role in bone sarcomas not directly related to "stemness." To our knowledge, this is the first evidence that constitutively activated STAT3 is present in bone sarcomas [28].

In-depth evaluation of the sarcospheres revealed that, following seeding as a single-cell suspension, the early small spheres that arose primarily comprised cells that were positive for Oct 3/4 and Nanog. As the spheres grew to a larger size with increasing cell number, immunohistochemical staining displayed increasingly greater heterogeneity of the cells, with a lesser percentage expressing these ES cell markers. These observations suggest a situation where relatively primitive stem-like cells undergo a transition from symmetric to asymmetric division and thereupon produce daughter cells of various states of differentiation. Along these
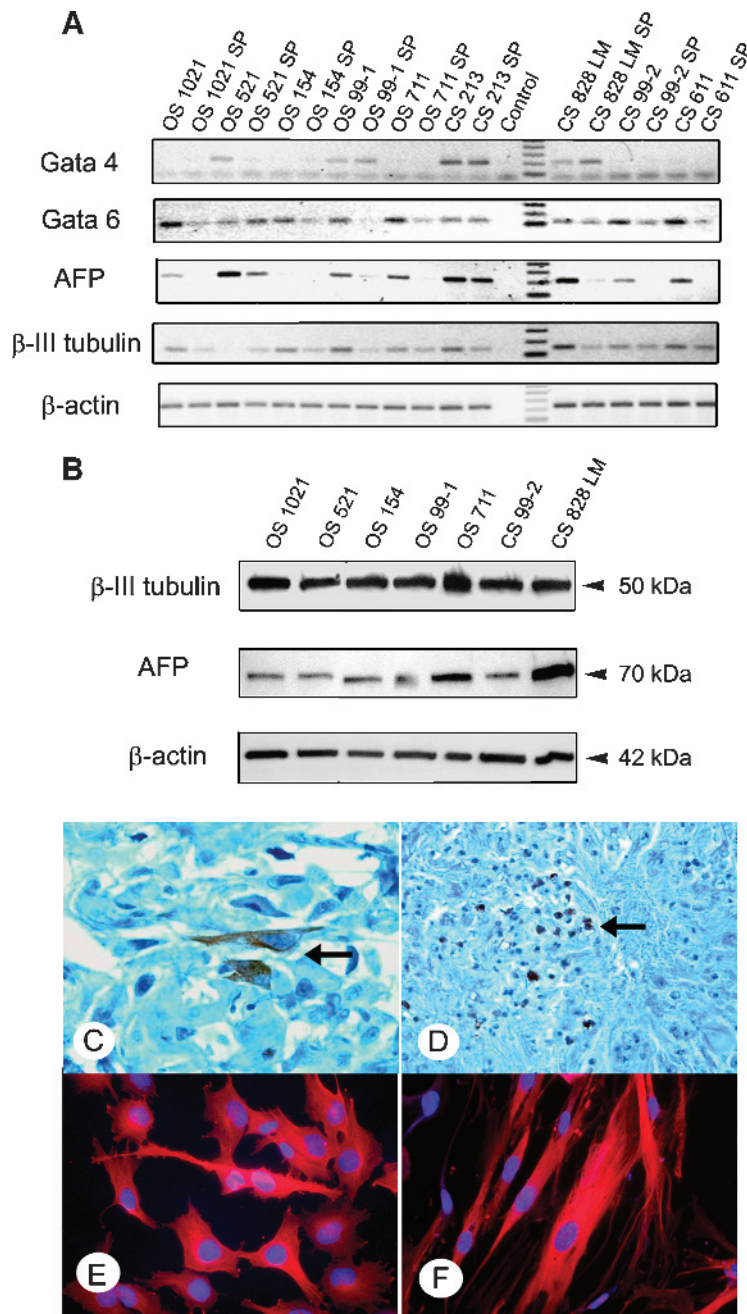

Figure 4. *Analyses of bone sarcoma cultures for the expression of genes of endodermal and ectodermal lineages. (A) RT-PCR analyses of adherent and sarcosphere cultures as described in Figure 1 for the transcription of endoderm-associated genes (*Gata-4*, *Gata-6*, and *AFP*) and the neuroectoderm marker *β-III tubulin. Primers for *β-actin were used as positive reaction controls, as indicated. The control lane represents parallel RT-PCR reactions performed without reverse transcriptase. (B) Western blot analyses for the expression of *β-III tubulin and *AFP* from lysates of adherent cultures are shown in panel A, demonstrating protein expressions of endoderm and neuroectoderm-associated genes. (C and D) Expression of *β-III tubulin in tissue specimens from bone sarcomas as detected by immunohistochemistry, and in adherent cultures (E and F) as demonstrated by immunocytochemistry. In panels C and D, arrows indicate regions of positive staining. In panels E and F, areas of *β-III tubulin *staining are seen in red. Nuclei were counterstained blue using Hoechst's stain.*
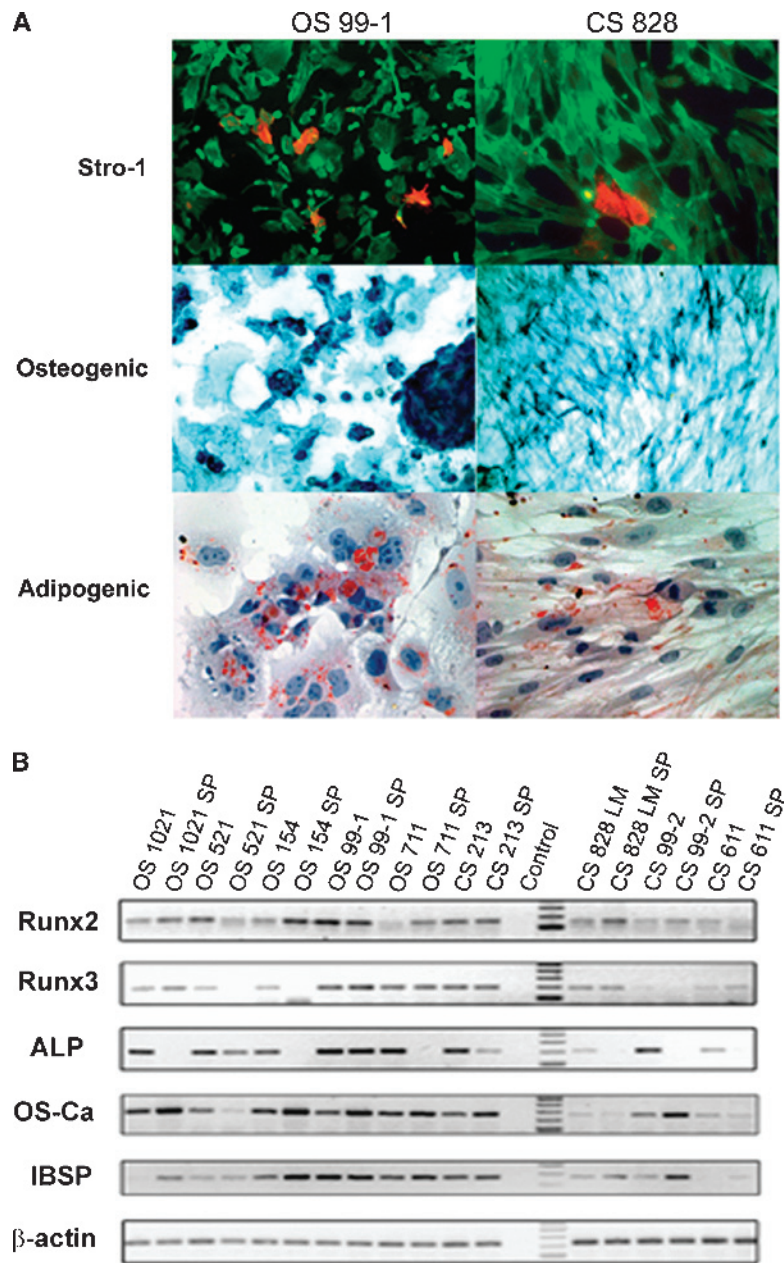
Figure 5. *Multipotent cells in bone sarcoma and the expression of mesenchymal lineage genes. (A) Immunocytochemical staining for Stro-1, a cell surface marker of mesenchymal stem cells, in cultures of osteosarcoma (OS 99-1) and chondrosarcoma (CS 828) cells. Positive staining is shown in orange. Counterstaining for F-actin is seen in green. Following incubation in osteogenic or adipogenic media to induce differentiation along mesenchymal lineages, the respective cultures were analyzed for mineralization by von Kossa stain or for lipid vacuoles by Oil Red O stain. As shown, both cultures showed focal staining for osteogenic and adipogenic differentiation. (B) Semiquantitative RT-PCR analyses for the expression of mesenchymal lineage genes in adherent and sarcosphere (SP) cultures from osteosarcoma (OS) and chondrosarcoma (CS). Reaction products for the respective cultures using primer pairs specific for* RUNX2, RUNX3, ALP, osteocalcin (OS Ca), *and bone sialoprotein (*IBSP) *are shown, as indicated. Reactions using primer pairs for β-actin were used as positive controls and also to normalize band intensities between samples. With the exception of* ALP, *which was preferentially expressed in the adherent cultures, no significant differences were seen between the different culture types. All genes assayed were expressed at detectable levels in both conditions.*

lines, it is interesting to note that bone sarcomas, which are considered to be mesenchymal malignancies, were found to express genes from endoderm and ectoderm lineages, in addition to mesodermal genes. At this point, we do not know the position(s) that a bone sarcoma stem cell might occupy in the stem cell hierarchy. It is possible that the expression of genes, such as *Gata-4* and *Gata-6*, is a product of dysregulated gene expression in the tumor cells. An alternative explanation is that these genes are indicative of aberrant pluripotent differentiation of cancer stem cells.

Curiously, despite the expression of endoderm and ectoderm genes in cells in culture, the phenotype of the bone sarcoma *in vivo* is clearly mesenchymal. Consistent with this, numerous cells in the tumor cultures that displayed proteins characteristic of mesenchymal stem cells were identified. Despite the fact that the respective cultures originated from

tumors of distinct mesenchymal phenotypes (osteoblastic and chondrocytic), it was possible to induce the differentiation of the cells along alternate mesenchymal lineages. This suggests that bone sarcoma stem cells might arise from cells at least as primitive as mesenchymal stem cells.

In our studies, we examined cells from distinct bone sarcomas. Chondrosarcoma is a tumor derived from cartilage cells or cartilage precursors and occurs primarily in middle-aged to older adults. The samples in this study were obtained from patients with ages ranging from 55 to 67 years. Osteosarcoma is a tumor comprising bone-forming or osteoblastic cells and primarily occurs in young adults. Those used in this study were from patients with ages ranging from 11 to 34 years. Despite the divergent origins of these cancers, the expression profiles and stem-like properties of the respective cell types were indistinguishable. This suggests that the cellular machinery responsible for the maintenance of the cancer stem cell phenotype may likewise be very similar in these tumors.

Oct 3/4 is a Pou family homeoprotein expressed initially in the inner cell mass of embryos and is essential for the maintenance of pluripotency. It is downregulated as development progresses and, following implantation of the embryo, is restricted to primordial germ cells [16]. After maturity, it is seen only in type A spermatogonia. Oct 3/4 has also been observed in testicular germ cell tumors [29], teratocarcinomas, and some early progenitor cells [30]. It is not seen in somatic cells. Recently, Gidekel et al. [29] have demonstrated that aberrant Oct 3/4 expression contributes to the neoplastic process in germ cells. In addition, these authors were able to demonstrate that nonneoplastic cells engineered to overexpress Oct 3/4 became tumorigenic. Nanog is a recently identified divergent homeoprotein that can maintain self-renewal in ES cells independent of leukemia-inhibitory factor. Overexpression of *Nanog* is associated with increased self-renewal capacity [17,18], and its expression is apparently restricted to ES cells [16], teratocarcinomas, and germ cell tumors [29]. Similar to *Oct 3/4*, expression of *Nanog* has not been demonstrated in somatic cells. Although we have not demonstrated a causal relationship between these proteins and the genesis of bone sarcomas, given what we know of the role of Oct 3/4 and Nanog in maintaining stemness in ES cells, they emerge as potential players in sarcoma stem cell biology. Together, Oct 3/4, Nanog, and STAT3 are responsible for the maintenance of the pluripotent state and for ability for self-renewal in murine ES cells [16]. If they perform similar functions in cancer stem cells, they could be important molecular targets of directed therapy.

This study lends further support to the cancer stem cell hypothesis that a small number of cells within the bulk of a solid tumor are responsible for the initiation and growth of malignancy and, furthermore, suggests a possible mechanism for this stem cell–like behavior. Assuming that the cancer stem cell theory [7,13,31] is correct, cytotoxic chemotherapeutic agents, developed in part by assessing the gross response of the tumor mass, may not address this small proportion of tumorigenic cells. The cancer stem cell theory could explain the relative resistance of osteosarcoma to chemotherapy (in which survival of patients treated with chemotherapy alone is only 20%) [32] and the near-complete resistance of chondrosarcoma to standard drug therapy. The novel findings presented here suggest that the transcription factors Oct 3/4 and Nanog may be part of a mechanism by which these cells maintain stemness. Exploring the roles these genes play, in conjunction with Stro-1 and other surface markers associated with mesenchymal precursors, may allow us to prospectively identify the tumorigenic cells in bone sarcomas. Perhaps more importantly, together, they may serve as potential targets for selective noncytotoxic therapy in bone sarcoma patients, as these tumors are rather resistant to current therapeutic protocols.

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