Published in final edited form as:

Cancer Res. 2010 June 1; 70(11): 4602–4612. doi:10.1158/0008-5472.CAN-09-3463.

CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance

Amit S. Adhikari^{1,4}, Neeraj Agarwal^{1,4}, Byron M. Wood^{1,4}, Constance Porretta², Bernardo Ruiz³, Radhika R. Pochampally⁵, and Tomoo Iwakuma^{1,4}

¹ Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

² Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

³ Department of Pathology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

⁴ Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA

⁵ Department of Pharmacology, Tulane University Health Sciences Center, New Orleans, LA 70112, USA

Abstract

Emerging evidence indicates the presence of tumor-initiating cells (TIC) or cancer stem cells (CSCs) in osteosarcoma. However, no study has demonstrated specific markers to identify osteosarcoma TICs with *in vivo* tumor formation ability. Additionally, there has been a lack of investigations gauging the contribution of osteosarcoma TICs to metastatic and drug-resistant properties. In this study, we have identified mouse and human osteosarcoma TICs using mesenchymal stem cell (MSC) markers CD117 and Stro-1. These markers were preferentially expressed in spheres and doxorubicin-resistant cells. Both mouse and human cells expressing these markers were sorted and analyzed for their abilities of tumor formation with as few as 200 cells, self-renewability, multipotency, drug resistance, metastatic potential, and enrichment of a metastasis-associated marker CXCR4 and a drug-resistance marker ABCG2. CD117⁺Stro-1⁺ cells efficiently formed serially transplantable tumors, whereas CD117⁺Stro-1[−] cells rarely initiated tumors. Upon orthotopic injections, CD117⁺Stro-1⁺ cell-derived tumors metastasized at a high frequency. Further, CD117⁺Stro-1⁺ cells showed high invasive and drug-resistant properties and were efficiently enriched for CXCR4 (20–90%) and ABCG2 (60–90%). These results suggest possible mechanisms for the high metastatic and drug-resistant properties of osteosarcoma TICs. In summary, CD117 and Stro-1 identify osteosarcoma TICs associated with the most lethal characteristics of the disease - metastasis and drug resistance - and these markers offer candidates for TIC-targeted drug delivery aimed at eradicating osteosarcoma.

Keywords

osteosarcoma; tumor-initiating cells; cancer stem cells; CXCR4; ABCG2

Introduction

Osteosarcoma, the most common bone cancer, is the second highest cause of cancer-related death in children and adolescents. Approximately 90% of cases show micro-metastasis at diagnosis, making systematic chemotherapy the first choice of treatment (1). Despite intensive chemotherapy, the survival rate for high-grade osteosarcomas remains only 50–80% (2). The inadequacy of current treatments may result from the inability to effectively target tumor-initiating cells (TICs) or cancer stem cells (CSCs) of osteosarcoma (3, 4). TICs represent a small fraction of a tumor's cellular population, having the ability to generate new tumors identical in cellular composition to the tumor of origin. Given that normal adult stem cells are capable of becoming TICs through the acquisition of mutations in several types of cancer, markers for stem cells have been widely used to identify TICs (4–7). However, the origin of the TICs remains controversial (8).

TICs have been proposed to be responsible for cancer metastasis, since cancer cells use the same molecular machinery for invasion and metastasis as normal stem cells do for homing or mobilization (9). The chemokine SDF1/CXCL12 and its receptor CXCR4 are involved in hematopoietic stem cell (HSC) mobilization and homing as well as breast cancer cell migration and metastasis (10). CXCR4 is also shown to be enriched in CD133⁺ glioblastoma TICs (11). Recently, CXCR4⁺ pancreatic TICs showed a markedly increased metastatic potential compared to CXCR4[−] TICs (12). Thus, increasing evidence suggests a crucial role of CXCR4 in the metastatic property of TICs.

Another important property of TICs is resistance to anti-cancer drugs (13). This phenotype is mainly mediated by ATP-binding cassette (ABC) transporters such as MDR1/ABCB1 and BCRP1/ABCG2. These transporters also allow cells to efflux Hoechst 33342 dye, a property used to identify a subpopulation of cells, called the Side Population (SP) (14). The SP cells are shown to be enriched with tumorigenic stem-like cancer cells (15). Recently, ABCB5, another ABC member, has been reported to identify melanoma stem cells (16). These findings indicate the significant involvement of ABC transporters in the drug-resistant property of TICs.

Despite numerous efforts to identify osteosarcoma TICs, no report has yet successfully demonstrated markers to identify them, specifically regarding *in vivo* tumor formation (17–20). To discover novel and effective therapies for eradicating osteosarcoma, we have isolated a subpopulation of cells with TIC properties from mouse and human osteosarcoma cell lines using mesenchymal stem cell (MSC) markers because of its mesenchymal origin. As few as 200 cells expressing CD117 and Stro-1 efficiently initiated tumors in immunocompromised mice. Additionally, CD117⁺Stro-1⁺ cells showed high metastatic and drug-resistant properties with enrichment of cells positive for CXCR4 and ABCG2.

Materials and Methods

Cell lines

Mouse K7M2, human KHOS/NP (R-970-5), and MNNG/HOS osteosarcoma cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) (21). Primary mouse osteosarcoma cell lines 318-1 and P932 were previously established (22, 23). BCOS is a primary cell line established by Dr. Pochampally's laboratory from an untreated osteosarcoma-bearing 25-year-old male patient undergoing biopsy in accordance with the protocol approved by Tulane University Health Sciences Center Institutional Review Board. The tumor was located in the left radius and the histological subtype was high-grade osteoblastic osteosarcoma.

Sphere culture

For sphere formation studies in Fig. 1A, five cells per well were plated on 96-well ultra-low attachment plates under serum-free sphere-specific conditions described by Gibbs et al. (18). These spheres were processed to form the next generation of spheres every 14 days. Third generation spheres were used for all the following experiments.

Differentiation studies

Cells (15,000) were plated on 96-well plates. When cells reached confluency, they were cultured in adipogenic differentiation medium for 5 days. Cells were fixed with 10% buffered formalin and stained with Oil Red O (Sigma Biochemicals) (24). For osteogenic differentiation, cells were cultured in osteogenic differentiation media for 3 weeks and stained using the Von Kossa Staining kit (Polysciences, Warrington, PA) (25).

Tumor sample preparation for flow cytometry, cells sorting, and serial transplantability

Tumors were minced and incubated with 3 mg/ml collagenase/dispase solution (Roche Applied Science, Indianapolis, IN). Isolated cells were cultured for 1 day in complete media to ensure the removal of dead and non-adherent cells. To eliminate non-specific binding of antibodies and dead cells for flow cytometry and cell sorting, mouse FC Block and 7-amino-actinomycin D staining (BD Biosciences) were used, respectively. Flow cytometric acquisition was performed using FACSCalibur (BD Biosciences) and analyzed using BD CellQuest™ Pro. Isotype controls were used for all experiments. Cell sorting was performed using FACSARIA (BD Biosciences) at the LSUHSC Alcohol Research Center.

Tumor formation and metastasis studies

Cultured cells including spheres were dissociated into single cell suspensions using non-enzymatic cell dissociation solution (Sigma Biochemicals, St. Louis, MO). Live cells were counted by trypan blue staining, suspended in 4.5 mg/ml of Matrigel (BD Biosciences) in Hanks Balanced Salt Solution, and subcutaneously injected into NIH-III nude mice. For orthotopic injections, cells were injected into femoral bone marrow space of anesthetized NOD/SCID mice. The injection entry point was sealed using bone wax (World Precision Instrument, Sarasota, FL). Mice were monitored daily until 4 months after injection or when tumors reached 1 cm in diameter. Data were accumulated from at least three independent experiments. Tumor nodules more than 1 mm in diameter were taken for pathological examination. When tumor nodules were identified at secondary sites apart from a primary injection site and diagnosed as osteosarcoma, the nodules were determined as metastases.

Invasion assay

Invasion assays were performed for monolayer (Mo), CD117⁻Stro-1⁻ (double negative, DN), and CD117⁺Stro-1⁺ (double positive, DP) cells using Growth Factor-Reduced Matrigel-coated 24-well transwell chambers (BD Biosciences). In order to recover cells from sorting stress, DN and DP cells were cultured for 48 h in complete medium before assays. Cells (3,000 cells/well) were plated in culture media containing 0.2% fetal bovine serum (FBS) in duplicates. Culture media containing 10% FBS was used as a chemo-attractant. Invading cells were fixed after 19 h and stained with Diff-Quik Stain Set (Dade Behring, Newark, DE, USA). Cells on the entire filter were counted to determine relative invasive potential.

Drug sensitivity assessment

318-1 and K7M2 (Mo, DN, and DP) cells were cultured for two days in complete media to recover cells from sorting stress. Cells (5,000 cells/well) were cultured in 96-well plates for one day and then treated with increasing concentrations of doxorubicin (0 to 8.0 μ M) for 24

h. Cell viability was measured by methyl-thiazol-tetrazolium (MTT) assay (26) to determine the mean 50% inhibitory concentration (IC50) values.

Immunohistochemistry

Immunohistochemistry was performed as previously described (27). Paraffin-embedded tissues sections (5 μ m) were deparaffinized, rehydrated, and processed for antigen retrieval using citrate buffer solution. Following hydrogen peroxide treatment, samples were incubated with appropriate antibodies. Signal detection was performed using the Vectastain Elite ABC kit (Vector laboratories) followed by counterstaining with haematoxylin. Stro-1 immunohistochemistry was performed using the BenchMark XT (Ventana medical systems, Inc, Tucson, Arizona, USA). For signal detection, the ultraView universal DAB detection kit (Ventana medical systems) was used, followed by counterstaining with haematoxylin.

Statistical analyses

Each experiment was performed independently at least three times. Values were expressed as means $\pm$ standard deviations (SD). Statistical significance ($p < 0.05$) was determined using Student's *t*-test.

Antibodies, mice, Hoechst 33342 dye-excluding side population (SP) analysis, Pathology, X-ray imaging

See Supplementary Materials and Methods.

Results

Murine osteosarcoma sphere cells efficiently initiate tumors and are enriched for stem cell markers CXCR4, ABCG2, CD117, and Stro-1

Tumor-initiating cells (TICs) are capable of forming spheres when cultured without serum and in an anchorage-independent manner (28). We first tested if mouse osteosarcoma cell lines contained a cellular population possessing this property using an established cell line K7M2, and two primary cell lines 318-1 and P932. After 14 days of culture in sphere-specific conditions, cells from all three cell lines formed spheres, but with different numbers and sizes (Fig. 1A). This suggests that each cell line contained a different number of TICs or, alternatively, TICs with different growth properties. Next, we examined the tumor forming ability of sphere cells. Single cell suspensions from third generation spheres (Sp) and monolayer cultures (Mo) from 318-1 and K7M2 cell lines were subcutaneously injected into NIH-III nude mice. As few as 200 cells from spheres successfully initiated tumors (2 out of 5 in 318-1 and 2 out of 4 in K7M2 cells), and over 60% of mice injected with 500 and 2,000 sphere-derived cells developed tumors in both cell lines (Fig. 1B and Table 1A). In contrast, 200 and 500 monolayer cells did not initiate tumors, and only a few mice injected with 2,000 monolayer cells developed tumors (1 out of 5 in 318-1 and 2 out of 4 in K7M2 cells, Table 1A).

CXCR4 and ABCG2 are linked to metastatic and drug-resistant properties of TICs in diverse tumor types (10, 29). Thus, we wanted to examine if spheres were enriched with cells expressing these markers. Less than 10% and 1% of monolayer cultures for all three cell lines expressed CXCR4 and ABCG2, respectively (Fig. 1C). Consistent with the rare existence of ABCG2⁺ cells in monolayer cultures, Hoechst 33342 dye-excluding side population (SP) cells were present at only at $1.7 \pm 0.5\%$ in P932 cells (Fig. S1A). In contrast to monolayer cultures, spheres derived from all three cell lines contained significantly more CXCR4⁺ cells compared to monolayer cultures ($p < 0.005$). ABCG2⁺ cells were minimally enriched only in 318-1 and P932 sphere cells ($p < 0.05$, Fig. 1C and Fig. S1B). Cells from spheres also showed the ability to differentiate into both osteogenic and adipogenic lineages

(Fig. S1C). These results suggest that spheres from murine osteosarcoma cell lines are enriched with cells having TIC properties.

Given that osteosarcoma TICs are enriched in spheres and share certain properties of mesenchymal stem cells (MSC), MSC markers that are predominantly expressed in spheres could be used to identify potential osteosarcoma TICs. Our flow cytometric analyses revealed that spheres contained higher percentages of cells expressing CD117/c-kit and Stro-1, but not CD44, CD105, and CD49b, as compared to monolayer cultures (Fig. 1D and Fig. 1SD). Cells double positive for CD117 and Stro-1 were present at 1–3% of monolayer cultures and 6–14% of sphere cells in all three cell lines, suggesting the possibility of using these markers as a strategy to identify osteosarcoma TICs.

Cells expressing CD117 and Stro-1 show higher resistance to doxorubicin

TICs from certain tumors show chemotherapy resistance (16, 30), hence we additionally examined which subpopulations of MSC marker-expressing cells could survive when monolayer cultures were exposed to doxorubicin, a commonly used chemotherapy drug for the treatment of osteosarcoma. Cells surviving doxorubicin treatment contained higher percentages of cells expressing CD117 and Stro-1 compared to control DMSO-treated cells (Fig. 2A and Fig. S2A). Since two independent strategies to enrich TICs resulted in essentially the same consequence, we hypothesized that CD117 and Stro-1 can be used to isolate subpopulation of osteosarcoma cells possessing TIC properties. To investigate the drug-resistant property of cells expressing these markers, we first sorted monolayer cultures (Mo) for CD117⁺Stro-1⁺ (double-positive, DP) and CD117[−]Stro-1[−] (double-negative, DN) populations. Purities for DP and DN populations were constantly over 85% and 90%, respectively (Fig. S2B). Mo, DN, and DP cells from 318-1 and K7M2 cell lines were exposed to increasing concentrations of doxorubicin in order to determine the mean 50% inhibitory concentration (IC₅₀, Fig. 2B). The IC₅₀ value for DP cells was 2.2 μM compared to 1.5 μM in Mo and DN cells in 318 cells, while that in K7M2 DP cells was 2.6 μM compared to 1.5 μM in Mo and DN cells. Thus, in both cell lines, DP cells showed a higher resistance to doxorubicin than Mo and DN cells.

CD117⁺Stro-1⁺ cells efficiently initiate tumors with a high frequency of metastasis

The most significant property of TICs is their ability to initiate tumors. We, therefore, subcutaneously injected DP and DN cells in immunocompromised mice. In each cell line, cells were independently sorted more than three times. In 318-1 and K7M2 cells, the majority of mice injected with as few as 200 DP cells formed tumors, whereas only one mouse injected with 2,000 318-1 DN cells showed tumor formation (Fig. 3A and Table 1B). This DN-derived tumor was considerably smaller than those with 2,000 DP cells (Fig. S3A). For P932 cells, 2,000 DP cells were required for tumor formation, but 2,000 and 6,000 DN cells failed to form any tumors (Fig. 3A and Table 1B). When we re-isolated DP cells from three different 318-1 DP-derived tumors, we observed tumor formation in 67% of mice (n=9) injected in triplicate with 500 DP cells. These results suggest the serial transplantability of DP cells.

Next, we tested the ability of DP cells to initiate osteosarcomas in their own niche, by performing orthotopic injections into NOD/SCID mice (Fig. 3B and Table 1C). Similar to subcutaneous injections, all four mice injected with as few as 200 318-1 DP cells and seven out of eight mice with 2,000 DP injection formed tumors. These tumors were confirmed as osteosarcomas that on pathology showed pleomorphic tumor cells with osteoid formation (Fig. 3B). 200 DN cells failed to form tumors, and only one out of twelve mice injected with 2,000 DN cells formed a tumor (Table 1C).

To investigate the metastatic potential of DN and DP cells, we counted the number of metastatic nodules in tumor-bearing mice. Since 2,000 DN cells were insufficient to initiate tumors, we increased the number of DN cells injected to 20,000 and 200,000. This higher number of DN cells efficiently generated tumors (5 out of 7 and 4 out of 4 mice with 20,000 and 200,000 cells, respectively, Table 1C and Table S1). Notably, to generate tumors of approximately 1 cm in diameter, a three-month period was required for 20,000 DN cells, compared to the two-month period for 2,000 DP cells (Fig. S3B). Slow-growing tumor formation using higher number of DN cells may indicate the possibility of either contamination of DP cells in DN population during cell sorting procedures or the presence of other populations with tumor-initiating potential. Nonetheless, the average number of lung nodules from 200 and 2,000 DP cell-derived tumors was 29 per tumor-bearing mouse (n=11), whereas that of 2,000 to 200,000 DN cell-derived tumors was only 6 (n=10, Table 1C and Table S1). To further confirm the high metastatic potential of DP cells, we performed *in vitro* invasion assays using 318-1 monolayer (Mo), DN, and DP cells (Fig. 3C). DP cells showed a two-fold higher invasive potential compared to that of Mo, whereas DN cells showed a similar invasive potential to that of Mo, supporting our *in vivo* observation of DP cells possessing high metastatic potential.

We further wanted to compare the expression patterns of stem cell markers CD117, Stro-1, ABCG2, and CXCR4 in primary tumors with those in lung metastases. We performed immunohistochemistry of primary tumors and their lung metastases from five mice with intrafemoral injections of 318-1 DP cells (Fig. 3D and Fig. S3C). Although we detected cells positive for all four markers in both primary and metastatic tumors, the intensely stained cells were more frequent at the metastatic sites compared with those in their primary sites (Fig. 3D and Fig. S3C). These markers did not show any specific intratumoral localization patterns at both primary and metastatic sites (Fig. 3D, and Fig. S3C). These results support previous findings that high expression of TIC markers positively correlates with cancer metastasis (31–33).

CD117⁺Stro-1⁺ population is multipotent and enriched for CXCR4 and ABCG2

TICs are capable of self-renewal and differentiation into multiple cell populations (3). Thus, we examined for the MSC marker profile of 318-1 DP-derived tumors and compared it to that of monolayer cultures. The percentages of CD117⁺Stro-1⁺ cells in 318-1 DP-derived tumors were similar to those of monolayer cultures (Fig. 4A), indicating that DP cells can generate other cellular populations in tumors. Percentages of other MSC markers including CD44, CD105, and CD49b were also similar between monolayer cultures and DP-derived tumors (Fig. S4). We further confirmed successful differentiation of DP cells into both osteogenic and adipogenic lineages, suggesting the multipotent nature of these cells (Fig. 4B).

Given that DP cells showed a high metastatic potential, we investigated percentages of CXCR4⁺ cells in this population using flow cytometric analyses. Strikingly, over 70% of DP cells from all three lines expressed CXCR4, whereas only a few CXCR4⁺ cells were present in monolayer cultures (Mo) and DN cells (Fig. 4C). Single-positive CD117⁺Stro-1[−] (CD117) cells also contained 30–75% of CXCR4⁺ cells, however enrichment of CXCR4⁺ cells in CD117[−]Stro-1⁺ (Stro-1) cells was observed only in 318-1 cells.

Since DP cells showed resistance to doxorubicin, we next examined for the percentage of ABCG2⁺ cells. Similar to the results of CXCR4, over 60% of DP cells from all three lines expressed ABCG2, compared to less than 2% of Mo and DN cells. Approximately 30–75% of single positive CD117⁺Stro-1[−] (CD117) cells expressed ABCG2, whereas only 0–25% were ABCG2⁺ in CD117[−]Stro-1⁺ (Stro-1) cells (Fig. 4D). In summary, mouse osteosarcoma

DP cells showed TIC properties as indicated by their efficient tumor-initiating ability, self-renewability, multipotency, high metastatic potential, and drug resistance.

Human osteosarcoma CD117⁺Stro-1⁺ cells also show TIC properties

The major objective in establishing a mouse system is its translation into human research. We therefore investigated the TIC properties of DP cells isolated from the established human osteosarcoma cell lines KHOS, MNNG/HOS, and a primary cell line BCOS that we derived from an osteosarcoma patient. Isolated DP and DN populations were approximately 85% and 95% in purities, respectively (Fig. S5A). Upon subcutaneous injections of these populations into NIH-III nude mice, seven out of eight mice with 500 KHOS DP cells, seven out of eight mice with 1,000 BCOS DP cells, and three out of four mice with 1,000 MNNG/HOS DP cells initiated tumors (Fig. 5A and Table 1D). In contrast, only one out of eight mice with 500 KHOS DN cells, one out of eight mice with 1,000 BCOS DN cells, and no mice injected with 1,000 MNNG/HOS DN cells formed tumors (Table 1D). Moreover, the KHOS DN-derived tumor was significantly smaller in size compared to those from DP cells (Fig. S5B). Serial transplantability of DP cells was tested by injecting 500 DP cells in duplicate after re-isolating them from three different tumors induced by 500 KHOS DP cells. We found tumor formation in four out of six injections. Additionally, DP cells were capable of differentiating into adipogenic lineage (Fig. S5C). These results suggest that human osteosarcoma DP cells also represent TICs. We then examined expression profiles of CXCR4 and ABCG2 in the DP population of KHOS and BCOS cells. While only a few CXCR4⁺ cells were present in monolayer and DN cells, approximately 25% of DP cells from both cell lines contained CXCR4⁺ cells (Fig. 5B). Unlike the mouse system, only CD117⁺Stro-1⁺ (Stro-1) cells contained high percentages of CXCR4⁺ cells similar to that of DP cells, whereas CXCR4⁺ cells were not enriched in CD117⁺Stro-1⁻ (CD117) cells. Regarding ABCG2 expression, approximately 90% of DP cells from both cell lines contained ABCG2⁺ cells, whereas only a few monolayer and DN cells were positive for ABCG2. Contrary to the mouse system, enrichment of ABCG2⁺ cells in the CD117⁺Stro-1⁺ (Stro-1) population was more efficient (70–80%) than that in the CD117⁺Stro-1⁻ (CD117) population (30–50%) (Fig. 5C). Although enrichment profiles of CXCR4 and ABCG2 were different between mouse and human, our results indicate that human osteosarcoma DP cells also demonstrate TIC properties with high enrichment of metastasis-and drug resistance-associated markers.

Discussion

Markers for the identification of osteosarcoma TICs demonstrating *in vivo* tumor formation have not yet been reported. We have selected MSC markers CD117 and Stro-1 due to their preferential expression in spheres and in cells surviving doxorubicin treatment. Our data strongly suggest that CD117⁺Stro-1⁺ (DP) cells from both mouse and human osteosarcoma cell lines are enriched with TICs. CD117/c-kit is a 145 kDa transmembrane glycoprotein and is expressed in both HSCs and MSCs. CD117 has been suggested as a prognostic marker for osteosarcoma, since its higher expression, mainly due to gene-amplification rather than mutations, is associated with a poorer outcome in patients, metastasis, and recurrence of the local disease (34–37). Considering that CD117 can be a marker for osteosarcoma TICs associated with metastasis and drug resistance, overexpression of CD117 in patients with worse prognosis observed in these reports may indicate that these osteosarcomas contain a high population of TICs. Stro-1, originally identified as an antigen expressed by stromal elements in human bone marrow, is exclusively present in MSCs (38). However, the clinical relevance of Stro-1 in cancer remains to be explored. Given that TICs are considered to be derived from their normal adult stem cells, we believe that osteosarcoma TICs also carry over the properties of MSCs including expression of cell

surface markers. Nonetheless, it remains unknown if CD117 or Stro-1 plays an active role in the properties of osteosarcoma TIC, in addition to solely serving as its marker. Further studies are required to clarify this issue.

We demonstrate that CD117⁺Stro-1⁺ (DP) cells showed high metastatic potential compared to CD117⁺Stro-1⁻ (DN) cells by both intrafemoral injection studies and *in vitro* invasion assays. We also demonstrated that DP cells were enriched with cells expressing a metastasis-associated stem cell marker CXCR4. This observation is in agreement with the previous studies suggesting that CXCR4 plays a crucial role in the metastatic property of TICs (12). Since CXCR4 is also linked to poor prognosis or metastasis of osteosarcoma (39, 40), our findings suggest that targeting DP cells may help prevent metastasis. In addition to the high metastatic property, DP cells exhibited a higher IC-50 value for doxorubicin treatment than DN cells, demonstrating their drug-resistant property. We also observed that DP cells were enriched with ABCG2⁺ cells at over 60% of the population in all cell lines examined. ABCG2 is a major contributor of the SP phenotype, which is well correlated with the drug-resistant property of cells (19, 41, 42), and its expression is associated with a poor clinical outcome or resistance to therapy in several types of cancer (43, 44). Therefore, our observation of enrichment of ABCG2⁺ cells in DP cells may suggest the contribution of ABCG2 to the drug-resistant property of osteosarcoma TICs. Thus, significant enrichment of cells positive for both CXCR4 and ABCG2 in DP population suggests possible mechanisms for the high metastatic and drug-resistant properties of osteosarcoma TICs.

Our flow cytometric analyses using triple staining for CD117, Stro-1, and CXCR4 or ABCG2 revealed that expression profiles of CXCR4 and ABCG2 for single marker CD117⁺Stro-1⁻ (CD117) or CD117⁺Stro-1⁺ (Stro-1) cells were not identical between mouse and human osteosarcoma cell lines. Since CD117⁺Stro-1⁻ (CD117) cells, but not CD117⁺Stro-1⁺ (Stro-1) cells, displayed similar expression profiles of CXCR4 and ABCG2 to those of DP cells in mouse osteosarcoma cell lines, CD117 may play a more critical role in TIC properties than Stro-1 in mouse osteosarcoma. On the other hand, in human cell lines, CD117⁺Stro-1⁺ (Stro-1) cells showed similar expression profiles of CXCR4 and ABCG2 to those of DP cells. Thus, in human osteosarcoma, Stro-1 may contribute to TIC properties more than CD117. These results suggest that each marker, CD117 and Stro-1, has a differential influence on TIC properties between the two organisms. Further investigation is required to decipher the role of the individual stem cell markers.

Although our study delineates CD117 and Stro-1 as markers for isolating osteosarcoma TICs, we do not exclude the possibility that other markers also identify them. Our intrafemoral injection studies demonstrate the slow-growing tumor formation using a higher number of DN cells. This observation may suggest the presence of other populations with tumor-initiating potential, but we can not exclude the possibility of contamination of DP cells in the DN population during cell sorting procedures. Nonetheless, our study is the first to demonstrate isolation of an osteosarcoma subpopulation with TIC properties including efficient tumor-initiating ability, self-renewability, multipotency, high metastatic potential, and drug resistance. Further, this study identifies CD117 and Stro-1 as potential candidates for TIC-targeted drug delivery, which will accelerate the development of new therapies that are associated with the most lethal characteristics of osteosarcoma—metastasis and chemotherapy resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This work was supported by grants from NIH P20 RR020152-02 (P.D.), ACS RSG-09-169-01-CSM (T.I.), LCRC Start-up (T.I.) and Seed funds (T.I. and R.R.P.).

We thank Drs. Deborah Sullivan, Jay K. Kolls, and Wu Lingtao for helpful discussion and Dr. Ping Zhang, Dr. Beatriz Finkel-Jimenez, Mr. Luis Marrero, Mr. Kevon Hekmatdoost, and Ms. Alana M. Whitehead for technical assistance. We also thank Mr. Shuo Cui and Dr. Britt Bromberg for editing the manuscript.

References

1. Himelstein BP, Asada N, Carlton MR, Collins MH. Matrix metalloproteinase-9 (MMP-9) expression in childhood osseous osteosarcoma. *Med Pediatr Oncol*. 1998; 31:471–4. [PubMed: 9835898]
2. Meyers PA. Muramyl tripeptide (mifamurtide) for the treatment of osteosarcoma. *Expert Rev Anticancer Ther*. 2009; 9:1035–49. [PubMed: 19671023]
3. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–11. [PubMed: 11689955]
4. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene*. 2004; 23:7274–82. [PubMed: 15378087]
5. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997; 3:730–7. [PubMed: 9212098]
6. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature*. 2004; 432:396–401. [PubMed: 15549107]
7. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2007; 445:106–10. [PubMed: 17122772]
8. Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer*. 2005; 5:899–904. [PubMed: 16327766]
9. Dewan MZ, Ahmed S, Iwasaki Y, Ohba K, Toi M, Yamamoto N. Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer. *Biomed Pharmacother*. 2006
10. Gelmini S, Mangoni M, Serio M, Romagnani P, Lazzeri E. The critical role of SDF-1/CXCR4 axis in cancer and cancer stem cells metastasis. *J Endocrinol Invest*. 2008; 31:809–19. [PubMed: 18997494]
11. Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer*. 2006; 5:67. [PubMed: 17140455]
12. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell*. 2007; 1:313–23. [PubMed: 18371365]
13. Borst P, Jonkers J, Rottenberg S. What makes tumors multidrug resistant? *Cell Cycle*. 2007; 6:2782–7. [PubMed: 17998803]
14. Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct “side population” of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A*. 2004; 101:14228–33. [PubMed: 15381773]
15. Hadnagy A, Gaboury L, Beaulieu R, Balicki D. SP analysis may be used to identify cancer stem cell populations. *Exp Cell Res*. 2006; 312:3701–10. [PubMed: 17046749]
16. Schatton T, Murphy GF, Frank NY, et al. Identification of cells initiating human melanomas. *Nature*. 2008; 451:345–9. [PubMed: 18202660]
17. Tirino V, Desiderio V, d'Aquino R, et al. Detection and characterization of CD133+ cancer stem cells in human solid tumours. *PLoS ONE*. 2008; 3:e3469. [PubMed: 18941626]
18. Gibbs CP, Kukekov VG, Reith JD, et al. Stem-like cells in bone sarcomas: implications for tumorigenesis. *Neoplasia*. 2005; 7:967–76. [PubMed: 16331882]

19. Di Fiore R, Santulli A, Ferrante RD, et al. Identification and expansion of human osteosarcoma-cancer-stem cells by long-term 3-aminobenzamide treatment. *J Cell Physiol*. 2009; 219:301–13. [PubMed: 19160414]
20. Fujii H, Honoki K, Tsujiuchi T, Kido A, Yoshitani K, Takakura Y. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. *Int J Oncol*. 2009; 34:1381–6. [PubMed: 19360350]
21. Khanna C, Prehn J, Yeung C, Caylor J, Tsokos M, Helman L. An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. *Clin Exp Metastasis*. 2000; 18:261–71. [PubMed: 11315100]
22. Lang GA, Iwakuma T, Suh YA, et al. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell*. 2004; 119:861–72. [PubMed: 15607981]
23. Iwakuma T, Tochigi Y, Van Pelt CS, et al. Mtbp haploinsufficiency in mice increases tumor metastasis. *Oncogene*. 2008; 27:1813–20. [PubMed: 17906694]
24. Wan C, He Q, McCaigue M, Marsh D, Li G. Nonadherent cell population of human marrow culture is a complementary source of mesenchymal stem cells (MSCs). *J Orthop Res*. 2006; 24:21–8. [PubMed: 16419965]
25. Maxson S, Burg KJ. Conditioned media cause increases in select osteogenic and adipogenic differentiation markers in mesenchymal stem cell cultures. *J Tissue Eng Regen Med*. 2008; 2:147–54. [PubMed: 18383557]
26. Mossman BT. In vitro approaches for determining mechanisms of toxicity and carcinogenicity by asbestos in the gastrointestinal and respiratory tracts. *Environ Health Perspect*. 1983; 53:155–61. [PubMed: 6363051]
27. Evans SC, Viswanathan M, Grier JD, Narayana M, El-Naggar AK, Lozano G. An alternatively spliced HDM2 product increases p53 activity by inhibiting HDM2. *Oncogene*. 2001; 20:4041–9. [PubMed: 11494132]
28. Bleau AM, Howard BM, Taylor LA, et al. New strategy for the analysis of phenotypic marker antigens in brain tumor-derived neurospheres in mice and humans. *Neurosurg Focus*. 2008; 24:E28. [PubMed: 18341405]
29. Lou H, Dean M. Targeted therapy for cancer stem cells: the patched pathway and ABC transporters. *Oncogene*. 2007; 26:1357–60. [PubMed: 17322922]
30. Drewa T, Styczynski J, Szczepanek J. Is the cancer stem cell population “a player” in multi-drug resistance? *Acta Pol Pharm*. 2008; 65:493–500. [PubMed: 19051593]
31. Balic M, Lin H, Young L, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res*. 2006; 12:5615–21. [PubMed: 17020963]
32. Maeda S, Shinchi H, Kurahara H, et al. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. *Br J Cancer*. 2008; 98:1389–97. [PubMed: 18349830]
33. Charafe-Jauffret E, Ginestier C, Iovino F, et al. Aldehyde Dehydrogenase 1-Positive Cancer Stem Cells Mediate Metastasis and Poor Clinical Outcome in Inflammatory Breast Cancer. *Clin Cancer Res*. 2009
34. Wei H, Zhao MQ, Dong W, Yang Y, Li JS. Expression of c-kit protein and mutational status of the c-kit gene in osteosarcoma and their clinicopathological significance. *J Int Med Res*. 2008; 36:1008–14. [PubMed: 18831895]
35. Entz-Werle N, Gaub MP, Lavaux T, et al. KIT gene in pediatric osteosarcomas: could it be a new therapeutic target? *Int J Cancer*. 2007; 120:2510–6. [PubMed: 17294449]
36. Entz-Werle N, Marcellin L, Gaub MP, et al. Prognostic significance of allelic imbalance at the c-kit gene locus and c-kit overexpression by immunohistochemistry in pediatric osteosarcomas. *J Clin Oncol*. 2005; 23:2248–55. [PubMed: 15800315]
37. Sulzbacher I, Birner P, Toma C, Wick N, Mazal PR. Expression of c-kit in human osteosarcoma and its relevance as a prognostic marker. *J Clin Pathol*. 2007; 60:804–7. [PubMed: 17018686]
38. Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood*. 1994; 84:4164–73. [PubMed: 7994030]

39. Oda Y, Yamamoto H, Tamiya S, et al. CXCR4 and VEGF expression in the primary site and the metastatic site of human osteosarcoma: analysis within a group of patients, all of whom developed lung metastasis. *Mod Pathol.* 2006; 19:738–45. [PubMed: 16528367]
40. Laverdiere C, Hoang BH, Yang R, et al. Messenger RNA expression levels of CXCR4 correlate with metastatic behavior and outcome in patients with osteosarcoma. *Clin Cancer Res.* 2005; 11:2561–7. [PubMed: 15814634]
41. Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res.* 2007; 67:4827–33. [PubMed: 17510412]
42. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood.* 2002; 99:507–12. [PubMed: 11781231]
43. Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. *Leukemia.* 2002; 16:1443–7. [PubMed: 12145683]
44. Yoh K, Ishii G, Yokose T, et al. Breast cancer resistance protein impacts clinical outcome in platinum-based chemotherapy for advanced non-small cell lung cancer. *Clin Cancer Res.* 2004; 10:1691–7. [PubMed: 15014021]

Figure 1.

Sphere cells from mouse osteosarcoma cell lines efficiently initiate tumors and express stem cell-associated markers. **A**, Representative phase-contrast micrographs of spheres from 318-1, P932, and K7M2 cell lines (10x magnification). The graph shows percentages of first generation spheres derived from monolayer cultures ($n=1,000$, means $\pm$ SD). **B**, Sphere cells (Sp) and monolayer cultures (Mo) were subcutaneously injected into NIH-III nude mice. Representative pictures show injected sites (broken circles), cell numbers, and observation periods. **C**, Flow cytometric analyses for CXCR4 and ABCG2 using Mo (grey) and Sp (black). Graphs illustrate percentages of CXCR4⁺ or ABCG2⁺ cells ($n=3$, means $\pm$ SD, Student's *t*-test, * $p<0.05$, ** $p<0.005$, NS: not significant). **D**, Distribution of MSC markers in Mo (grey) and Sp (black). Graphs illustrate percentages of cells expressing markers indicated ($n=3$, means $\pm$ SD).

Figure 2.

Cells expressing CD117 and Stro-1 show resistance to doxorubicin. **A**, MSC marker analysis following doxorubicin treatment. Cells were treated with 1 μ M doxorubicin for 48 h and surviving cells were stained for indicated MSC markers. Graphs summarize the percentage of each MSC marker-expressing cells in control DMSO- (grey) and doxorubicin-treated (black) cells ($n=3$, means $\pm$ SD, Student's t -test, * $p<0.05$, ** $p<0.005$, NS: not significant). **B**, Determination of IC50 values for doxorubicin. Monolayer (Mo, triangle), CD117⁻Stro-1⁻ (DN, circle), and CD117⁺Stro-1⁺ (DP, square) cells from 318-1 (left) and K7M2 (right) cell lines were treated with increasing concentration of doxorubicin for 24 h. Percentages of surviving cells are shown along with the IC50 values.

Figure 3.

Mouse osteosarcoma CD117⁺Stro-1⁺ (DP) cells efficiently initiate tumors with a high frequency of metastasis. **A**, DP and DN (CD117⁻Stro-1⁻) cells were subcutaneously injected into NIH-III nude mice. Representative pictures demonstrate tumor formation of DP cells along with injected sites, cell numbers, and observation periods. **B**, An x-ray image of orthotopically injected mouse with 2,000 318-1 DP cells and an enlarged region show a primary tumor (broken circle) and multiple metastases (arrows), respectively. Gross pictures of lungs and liver with haematoxylin- and eosin-stained tissues indicate osteosarcoma metastasis. **C**, Invasive potential of 318-1 Mo, DN, and DP cells. Relative percent invasion compared to the number of invading Mo cells is shown (n=3, means $\pm$ SD, Student's *t*-test, *p<0.05). **D**, Representative immunohistochemistry (20X) comparing the expression of CD117, Stro-1, CXCR4, and ABCG2 between primary tumors (top) and their lung metastases (bottom).

Figure 4.

Characterization of mouse osteosarcoma cells expressing CD117 and Stro-1. **A**, Flow cytometric analyses of 318-1 cells comparing percentages of DP cells among monolayer cultures, DP-sorted cells, and DP-derived tumors. Representative results and a summarized graph indicate the multipotent nature of DP cells ($n=3$, means $\pm$ SD). **B**, Results of Von Kossa and Oil red O stainings show osteogenic and adipogenic differentiation of DP cells, respectively (20x magnification). **C** and **D**, Flow cytometric analyses showing percentages of CXCR4⁺ (**C**) or ABCG2⁺ (**D**) cells in monolayer culture (Mo), DN, DP, CD117⁺Stro-1⁻ (CD117), and CD117⁺Stro-1⁺ (Stro-1) populations. Representative results (**C**, 318-1 and **D**, P932) and the summarized graphs for all three cell lines show efficient enrichment of CXCR4⁺ and ABCG2⁺ cells in the DP population ($n=4$, means $\pm$ SD).

Figure 5.

Human osteosarcoma double-positive (DP, CD117⁺Stro-1⁺) cells show TIC-like properties. A, DP and DN (CD117⁻Stro-1⁻) cells from KHOS, BCOS, and MNNG/HOS cell lines were subcutaneously injected into NIH-III nude mice. Representative pictures indicate tumor formation of DP cells along with injected sites, cell numbers, and observation periods. B and C, Flow cytometric analyses showing percentages of CXCR4⁺ (B) or ABCG2⁺ (C) cells in each population. Representative results (B, KHOS and C, BCOS) and the summarized graphs for KHOS and BCOS cell lines show efficient enrichment of CXCR4⁺ and ABCG2⁺ cells in the DP population (n=3, means $\pm$ SD).

Table 1

Tumor formation studies of mouse and human osteosarcoma cells

A. Comparison between monolayer cultures and spheres via s.c injections						
Cell line	318-1		K7M2			
Cell number	200	500	2,000	200	500	2,000
Monolayer	0/5	0/5	1/5	0/4	0/4	2/4
Sphere	2/5	3/5	3/5	2/4	3/4	4/4

B. Comparison between mouse DN and DP cells via s.c. injections						
Cell line	318-1		K7M2		P932	
Cell number	200	500	2,000	200	500	2,000
DN	0/7 (4)	0/7 (5)	1/7 (5)	0/6 (4)	0/5 (4)	0/5 (3)
DP	7/7 (4)	6/7 (5)	7/7 (5)	4/6 (4)	4/5 (4)	3/5 (3)

C. Tumor formation and metastasis of 318-1 cells following orthotopic injections					
Injected cell number	200	2,000	20,000	200,000	Average # of lung nodules in tumor-bearing mice
DN	0/4 (3)	1a/12(7)	5 ^b /7(4)	4 ^c /4(3)	6/10
DP	4 ^d /4 (3)	7 ^c /8 (5)			29/11

D. Comparison between human DN and DP cells via s.c. injections				
Cell lines	KHOS	BCOS	MNNG/HOS	
Cell number	500	1,000	1,000	
DN	1/8 (5)	1/8 (4)	0/4 (3)	
DP	7/8 (5)	7/8(4)	3/4(3)	

Osteosarcoma cells were subcutaneously (s.c) injected into NIH-III nude mice. Numbers indicate tumor-bearing mice/injected mice.

Numbers indicate tumor-bearing mice/injected mice. Parentheses indicate number of independent sorting procedures. DN and DP: double-negative and double-positive for CD117 and Stro-1, respectively.

Cells were orthotopically injected into NOD/SCID mice. Numbers at the left indicate tumor-bearing as mice/injected mice. Numbers at the right indicate average number of lung nodules in tumor-bearing mice. Parentheses indicate number of independent sorting procedures.

^aNo metastasis was found.
^bFive mice had metastasis.

^cTwo mice had metastases.

^dTwo mice had metastases.

^eSeven mice had metastases. Supplementary Table SI for detailed information.

Numbers indicate tumor-bearing mice/injected mice. Parentheses indicate number of independent sorting procedures.