Tumor and Stem Cell Biology

CD117 and Stro-1 Identify Osteosarcoma Tumor-Initiating Cells Associated with Metastasis and Drug Resistance

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Abstract

Emerging evidence indicates the presence of tumor-initiating cells (TIC) or cancer stem cells in osteosarcoma. However, no study has shown 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 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. On 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. Cancer Res; 70(11): 4602–12. ©2010 AACR.

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 micrometastasis at diagnosis, making systematic chemotherapy the first choice of treatment (1). Despite intensive chemotherapy, the survival rate for high-grade osteosarcomas remains only 50% to 80% (2). The inadequacy of current treatments may result from the inability to effectively target tumor-initiating cells (TIC) or cancer stem cells of osteosarcoma (3, 4). TICs represent a small fraction of the cellular population of a tumor, 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 because 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 with 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 anticancer 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; ref. 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 shown markers to identify them, specifically about 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 immuno-compromised 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 the American Type Culture Collection (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 histologic subtype was high-grade osteoblastic osteosarcoma.

Sphere culture
For sphere formation studies in Fig. 1A, five cells per well were plated on 96-well ultralow attachment plates under serum-free-sphere-specific conditions described by Gibbs and colleagues (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; ref. 24). For osteogenic differentiation, cells were cultured in osteogenic differentiation media for 3 weeks and stained using the Von Kossa Staining kit (Polysciences; ref. 25).

Tumor sample preparation for flow cytometry, cell sorting, and serial transplantability
Tumors were minced and incubated with 3 mg/mL collagenase/dispease solution (Roche Applied Science). Isolated cells were cultured for 1 day in complete media to ensure the removal of dead and nonadherent cells. To eliminate nonspecific 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 FACS Calibur (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 Louisiana State University Health Sciences Center Alcohol Research Center.

Tumor formation and metastasis studies
Cultured cells including spheres were dissociated into single-cell suspensions using nonenzymatic cell dissociation solution (Sigma Biochemicals). Live cells were counted by trypan blue staining, suspended in 4.5 mg/mL Matrigel (BD Biosciences) in HBSS, and s.c. injected into NIH-III nude mice. For orthotopic injections, cells were injected into femoral bone marrow space of anesthetized nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. The injection entry point was sealed using bone wax (World Precision Instrument). 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 >1 mm in diameter were taken for pathologic 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). To recover cells from sorting stress, DN and DP cells were cultured for 48 hours in complete medium before performing assays. Cells (3,000 per well) were plated in culture media containing 0.2% fetal bovine serum (FBS) in duplicates. Culture media containing 10% FBS were used as a chemoattractant. Invading cells were fixed after 19 hours and stained with Diff-Quik Stain Set (Dade Behring). 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 2 days in complete media to recover cells from sorting stress. Cells (5,000 per well) were cultured in 96-well plates for 1 day and then treated with increasing concentrations of doxorubicin (0–8.0 μmol/L) for 24 hours. Cell viability was measured by MTT assay (26) to determine the mean IC50 values.

Immunohistochemistry
Immunohistochemistry was performed as previously described (27). Paraffin-embedded tissues sections (5 μm) 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 hematoxylin. Stro-1 immunostaining was performed using the BenchMark XT (Ventana Medical Systems, Inc.). For signal detection, the ultraView Universal DAB Detection kit (Ventana Medical Systems) was used, followed by counterstaining with hematoxylin.

Statistical analyses
Each experiment was performed independently at least thrice. Values were expressed as mean ± SD. Statistical significance (P < 0.05) was determined using Student’s t test.
Antibodies, mice, Hoechst 33342 dye-excluding SP analysis, pathology, and 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

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 and Mo from 318-1 and K7M2 cell lines were s.c. injected into NIH-III nude mice. As few as 200 cells from spheres successfully initiated tumors (two of five in 318-1 and two...
of four in K7M2 cells), and >60% of mice injected with 500 and 2,000 sphere-derived cells developed tumors in both cell lines (Fig. 1B; Table 1A). In contrast, 200 and 500 Mo cells did not initiate tumors, and only a few mice injected with 2,000 Mo cells developed tumors (one of five in 318-1 and two of four 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 Mo for all three cell lines expressed CXCR4 and ABCG2, respectively (Fig. 1C; Supplementary Fig. S1C). These results suggest that spheres from murine osteosarcoma cell lines are enriched with cells having TIC properties.

Table 1. Tumor formation studies of mouse and human osteosarcoma cells

<table>
<thead>
<tr>
<th>A. Comparison between Mo and spheres via s.c. injections*</th>
<th>318-1</th>
<th>K7M2</th>
</tr>
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<tbody>
<tr>
<td>Cell line</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>Mo</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Sphere</td>
<td>2/5</td>
<td>3/5</td>
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<tr>
<th>B. Comparison between mouse DN and DP cells via s.c. injections†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
</tr>
<tr>
<td>Cell number</td>
</tr>
<tr>
<td>DN</td>
</tr>
<tr>
<td>DP</td>
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<table>
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<tr>
<th>C. Tumor formation and metastasis of 318-1 cells following orthotopic injections‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injected cell number</td>
</tr>
<tr>
<td>DN</td>
</tr>
<tr>
<td>DP</td>
</tr>
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*Osteosarcoma cells were 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.
‡Cells were orthotopically injected into NOD/SCID mice. Numbers at the left indicate tumor-bearing mice/injected mice. Numbers at the right indicate average number of lung nodules/tumor-bearing mice. Parentheses indicate number of independent sorting procedures. See Supplementary Table S1 for detailed information.
§No metastasis was found.
||Five mice had metastases.
|†Two mice had metastases.
|**Two mice had metastases.
|††Seven mice had metastases.
‡‡Numbers indicate tumor-bearing mice/injected mice. Parentheses indicate number of independent sorting procedures.
expressing CD117/c-kit and Stro-1, but not markers such as CD44, CD105, and CD49b, compared with Mo (Fig. 1D; Supplementary Fig. S1D). Cells DP for CD117 and Stro-1 were present at 1% to 3% of Mo and at 6% to 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 Mo cells 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 with control DMSO-treated cells (Fig. 2A; Supplementary Fig. S2A). Because 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 Mo for CD117⁺Stro-1⁺ (DP) and CD117⁻Stro-1⁻ (DN) populations. Purities for DP and DN populations were constantly more than 85% and 90%, respectively (Supplementary Fig. S2B). Mo, DN, and DP cells from 318-1 and K7M2 cell lines were exposed to increasing concentrations of doxorubicin to determine the mean IC₅₀ (Fig. 2B). The IC₅₀ value for DP cells was 2.2 μmol/L compared with 1.5 μmol/L in Mo and DN cells in 318 cells, whereas that in K7M2 DP cells was 2.6 μmol/L compared with 1.5 μmol/L 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 s.c. injected DP and DN cells in immunocompromised mice. In each cell line, cells were independently sorted more than thrice. 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; Table 1B). This DN-derived tumor was considerably smaller than those with 2,000 DP cells (Supplementary Fig. S3A). For P932 cells, 2,000 DP cells were required for tumor initiation.
Figure 3. Mouse osteosarcoma CD117+Stre-1+ (DP) cells efficiently initiate tumors with a high frequency of metastasis. A, DP and DN (CD117−Stre-1−) cells were s.c. injected into NIH-III nude mice. Representative pictures show 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 H&E-stained tissues indicate osteosarcoma metastasis. C, invasive potential of 318-1 Mo, DN, and DP cells. Relative percent invasion compared with the number of invading Mo cells is shown (n = 3). Columns, mean; bars, SD. *, P < 0.05, Student’s t test. D, representative immunohistochemistry comparing the expression of CD117, Stre-1, CXCR4, and ABCG2 between primary tumors (top) and their lung metastases (bottom). Magnification, ×20.
formation, but 2,000 and 6,000 DN cells failed to form any tumors (Fig. 3A; Table 1B). When we reisolated 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; Table 1C). Similar to s.c. injections, all four mice injected with as few as 200 318-1 DP cells and seven 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). Two hundred DN cells failed to form tumors, and only 1 of 12 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. Because 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 (five of seven and four of four mice with 20,000 and 200,000 cells, respectively; Table 1C; Supplementary Table S1). Notably, to generate tumors of ~1 cm in diameter, a 3-month period was required for 20,000 DN cells compared with the 2-month period for 2,000 DP cells (Supplementary 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; Supplementary Table S1). To further confirm the high metastatic potential of DP cells, we performed in vitro invasion assays using 318-1 Mo, DN, and DP cells (Fig. 3C). DP cells showed a 2-fold higher invasive potential compared with 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; Supplementary 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; Supplementary Fig. S3C). These markers did not show any specific intratumoral localization patterns at both primary and metastatic sites (Fig 3D; Supplementary 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 Mo. The percentages of CD117−Strom-1− cells in 318-1 DP-derived tumors were similar to those of Mo (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 Mo and DP-derived tumors (Supplementary 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, >70% of DP cells from all three lines expressed CXCR4, whereas only a few CXCR4+ cells were present in Mo and DN cells (Fig. 4C). Single-positive CD117−Strom-1− (CD117) cells also contained 30% to 75% of CXCR4+ cells; however, enrichment of CXCR4+ cells in CD117−Strom-1− (Strom-1) cells was observed only in 318-1 cells.

Because DP cells showed resistance to doxorubicin, we next examined for the percentage of ABCG2+ cells. Similar to the results of CXCR4, >60% of DP cells from all three lines expressed ABCG2 compared with <2% of Mo and DN cells. Approximately 30% to 75% of single-positive CD117−Strom-1− (CD117) cells expressed ABCG2, whereas only 0% to 25% were ABCG2+ in CD117−Strom-1− (Strom-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−Strom-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 and 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 (Supplementary Fig S5A). On s.c. injections of these populations into NIH-III nude mice, seven of eight mice with 500 KHOS DP cells, seven of eight mice with 1,000 BCOS DP cells, and three of four mice with 1,000 MNNG/HOS DP cells initiated tumors (Fig. 5A; Table 1D). In contrast, only one of eight mice with 500 KHOS DN cells, one 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 with those from DP cells (Supplementary Fig S5B). Serial transplantability of DP cells was tested by injecting 500 DP cells in duplicate after reisolating them from three different tumors induced by 500 KHOS DP cells. We found tumor formation in four of six injections. Additionally, DP cells were capable of differentiating into adipogenic lineage (Supplementary 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. Whereas only a few CXCR4+ cells were present in Mo and DN cells, ~25% of DP cells from both cell lines contained CXCR4+ cells (Fig. 5B). Unlike the mouse system, only CD117−Strom-1− cells contained high percentages of CXCR4+ cells similar to that of DP cells, whereas CXCR4+ cells were not enriched in CD117−Strom-1− (CD117) cells. About ABCG2 expression, ~90% of DP cells from both cell lines contained ABCG2+ cells, whereas only a few Mo and DN cells were positive for ABCG2. Contrary to the mouse system, enrichment of ABCG2+ cells in the CD117−Strom-1− (Strom-1) population was more efficient (70–80%) than that in the CD117−Strom-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 show TIC properties.
with high enrichment of metastasis-associated and drug resistance–associated markers.

Discussion

Markers for the identification of osteosarcoma TICs showing 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 because 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 TICs, in addition to solely serving as its marker. Further studies are required to clarify this issue.

We show that CD117+Stro-1+ (DP) cells showed a high metastatic potential compared with CD117 Stro-1− (DN) cells by both intrafemoral injection studies and in vitro invasion assays. We also showed that DP cells were enriched with cells expressing a metastasis-associated stem cell marker (CXCR4). This observation agrees with the previous studies, suggesting that CXCR4 plays a crucial role in the metastatic property of TICs (12). Because 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, showing their drug-resistant property. We also observed that DP cells were enriched with ABCG2+ cells at >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. Because 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 show 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 cannot exclude the possibility of contamination of DP cells in the DN population during cell sorting procedures. Nonetheless, our study is the first to show 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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