Identification of chemoradiation-resistant osteosarcoma stem cells using an imaging system for proteasome activity

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Abstract. Osteosarcoma is the most common primary bone malignancy in pediatric and adolescent populations. Recurrence and metastatic potential can be due to a subpopulation of cells with stem cell-like characteristics, such as tumor-initiating cells (TICs), which maintain the capacity to regenerate entire tumors. Targeting the TICs in osteosarcoma is a promising avenue for the development of new therapies for this devastating disease. TICs are usually quiescent with a low protein turnover, decreased metabolism, and downregulation of proteasome activity. Recently, cancer cells with low proteasome activity have been identified as TICs in several types of cancer. We stably infected two osteosarcoma cell lines, MG-63 and U2-OS, with an expression vector for a fusion protein between the green fluorescent protein, ZsGreen, and the C-terminal degron of the murine ornithine decarboxylase to monitor the 26S proteasome activity in living cells. We separated the osteosarcoma cells with low proteasome activity using fluorescence-activated cell sorting (FACS) and verified whether these ZsGreen$^+$ cells had TIC-like properties. The ZsGreen$^+$ cells showed enhanced sphere formation capacity and underwent asymmetric divisions into ZsGreen$^+$ and ZsGreen$^-$ cells, whereas ZsGreen$^-$ cells underwent only symmetric divisions into ZsGreen$^-$ cells. Moreover, the ZsGreen$^+$ cells were more chemo- and radioresistant. Thus, the present study demonstrated that chemoradiation-resistant TICs can be visualized by this system and suggested the rationale for further study of osteosarcoma stem cells.

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Introduction

Osteosarcoma is the most common primary bone malignancy in children and young adults. Osteosarcoma occurs in the long bones of the limbs, particularly in the distal femur and proximal tibia. Osteosarcoma is a locally aggressive tumor and tends to produce early distant metastases, particularly to the lung. Before 1970, amputation was the only treatment for osteosarcoma patients and 80% patients died of metastatic disease (1). Since the 1970s, the combination of limb-sparing surgery and conventional chemotherapy agents, including methotrexate (MTX), cisplatin (CDDP), and doxorubicin, has been used to treat osteosarcoma. However, the 5-year patient survival has plateaued at ~60-70% (2).

Tumors are organized into a hierarchy of heterogeneous cell populations. Recurrence and metastatic potential may be due to a subpopulation of cells with stem cell-like characteristics, such as cancer stem cells (CSCs) or tumor-initiating cells (TICs), which maintain the capacity to regenerate entire tumors (3). Targeting the TICs in osteosarcoma may be a promising avenue to explore for the development of new therapies for this devastating disease.

Increasing evidence of the existence of TICs in patients with osteosarcoma has been reported. Identification of osteosarcoma TICs has been performed using CD133 (4,5), side populations (6,7), PKH26 (8), ALDH1 (9,10), and the promoter reporter assays of hTERT (11) and Oct3/4 (12). TIC-enriched osteosarcoma cell populations exhibit capacity for self-renewal, multilineage differentiation, tumorigenicity,

and chemo- and radioresistance. Furthermore, TICs are usually quiescent, with a low protein turnover, decreased metabolism, and downregulation of proteasome activity. The ubiquitin-proteasome system is the major non-lysosomal system for the degradation of intracellular proteins. Recently, cancer cells with low proteasome activity have been identified as TICs in patients with breast cancer, glioma (13), pancreatic cancer (14), and esophageal cancer (15) using a fluorescence marker system for the level of proteasome activity. However, no study has reported the identification of TICs in human osteosarcoma cell lines on the basis of low proteasome activity.

Here we showed that a small subpopulation of osteosarcoma cells with low proteasome activity had TIC-like properties. Human osteosarcoma cell lines were transfected with a retroviral vector that monitored proteasome activity using a fluorescent protein (ZsGreen). We isolated a fraction of cells with low proteasome activity from these human osteosarcoma cell lines and identified these cells to have tumor-initiating capacity.

Materials and methods

Cell culture. Human osteosarcoma cell lines U2-OS and MG-63 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), penicillin, and streptomycin (Sigma). All cells were grown in a humidified incubator at 37˚C with 5% CO_2.

Generation of stable cell lines expressing ZsGreen-cODC fusion proteins using retroviral transduction. The retroviral expression vector pQCXIN-ZsGreen-cODC, containing green fluorescence ZsGreen-labeled degron ODC, was kindly provided by Dr Shinji Tanaka. The retroviral vector was transfected into platinum A (Plat-A) to generate a retrovirus. The vector was transfected into Plat-A retroviral packaging cells using FuGENE 6 (Promega); the virus collected from the supernatant of Plat-A was used to infect osteosarcoma cells. The stable transfectants were selected with Geneticin (Invitrogen) and the accumulation of ZsGreen-labeled degron ODC protein was monitored by fluorescence microscopy and flow cytometry. To determine if this was a stable transfection, the cells were exposed to proteasome inhibitor MG-132 (Wako, Japan) for 12 h.

Time-lapse analysis. After FACS, ZsGreen$^+$ cells were separately plated at a density of 10^4 cells in 35-mm dishes in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and Pen/Strep (Sigma). After incubation in 5% CO_2 at 37˚C overnight, cell attachment was confirmed. Image analysis was performed using a FV1200 confocal microscope (Olympus).

Flow cytometry. We used a FACS Aria II (BD Biosciences, San Jose, CA, USA) for cell sorting. Osteosarcoma cells were washed with phosphate-buffered saline (PBS) and enzymatically dissociated with 0.05% trypsin-EDTA (Invitrogen). Cells were gently triturated and filtered through cell strainer caps (35-μm mesh) to obtain a single cell suspension (~1x10^7 cells/ml). The presence of ZsGreen allows the selection of cells with low proteasome activity using flow cytometry with standard fluorescein isothiocyanate (FITC) for cell sorting.

Measurement of proteasome activity. Using Proteasome-Glo™ cell-based assays (Promega), the chymotrypsin-like, trypsin-like, and caspase-like activities were measured according to the manufacturer's protocol. Proteasome-Glo™ buffer was mixed with luciferin detection reagent, and the substrate was added to the mixture and incubated at room temperature for 1 h. An equal volume of proteasome-Glo™ reagent was added to the samples and further incubated for 15 min. The luminogenic substrate containing the Suc-LLVY sequence was recognized by the proteasome. Following the proteasome cleavage, the substrate for luciferase (aminoluciferin) was released, allowing the luciferase reaction to proceed and produce light. The luminescence was measured using a luminometer (Thermo Fisher Scientific, Waltham, MA, USA).

Sphere formation assay. ZsGreen$^+$ and ZsGreen$^-$ cells were sorted by FACS, plated separately at a density of 10,000 cells on low-attachment 6-well plates (Costar, Corning, NY, USA), and incubated in serum-free Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with b-FGF, EGF (Sigma), and N2 (Wako). After 14 days, spheres with diameters >100 μm were counted.

Tumorigenicity assay. Our animal studies were approved by the Animal Experiments Committee of Osaka University (Suita, Japan). Following FACS, the portions containing 1x10^3, 1x10^4 and 1x10^5 cells were mixed with BD Matrigel (Becton-Dickinson, Franklin Lakes, NJ, USA) at a 1:1 ratio and were subcutaneously injected into 4- to 5-week-old female NOD/SCID mice (Charles River, Japan). The animals were anesthetized with isofluorane and maintained under sterile airflow conditions during the experiments.

Cell proliferation assay. Proliferation assays for the U2-OS and MG-63 cells treated with MTX and CDDP (both purchased from Wako, Japan) were performed to test chemosensitivity. Briefly, a total of 2x10^3 of both ZsGreen$^+$ and ZsGreen$^-$ cells were sorted, individually seeded into 96-well plates (Corning), and treated with varying concentrations of the drugs for 72 h. The assay was performed using a commercially available cell counting kit-8 (Dojindo, Japan). The remaining viable cell count was determined by measuring the absorbance at 450 nm using an Enspire plate reader (Perkin-Elmer).

Clonogenic survival assay. The appropriate number of ZsGreen$^+$ and ZsGreen$^-$ cells were sorted, individually seeded in 6-cm dishes, and exposed to radiation at 0, 2, 4, 6, and 8 Gy by Cs-137 gamma irradiation generated using a Gammacell 40 Exactor (MDS Nordion, Ottawa, Canada). After incubation for 14 days, the colonies were fixed and stained with crystal violet. Colonies containing >50 cells were counted as survivors. At least three parallel samples were scored in three to five repetitions conducted for each irradiation condition.

Statistical analysis. Data are expressed as means ± SDs. Statistically significant differences were determined by

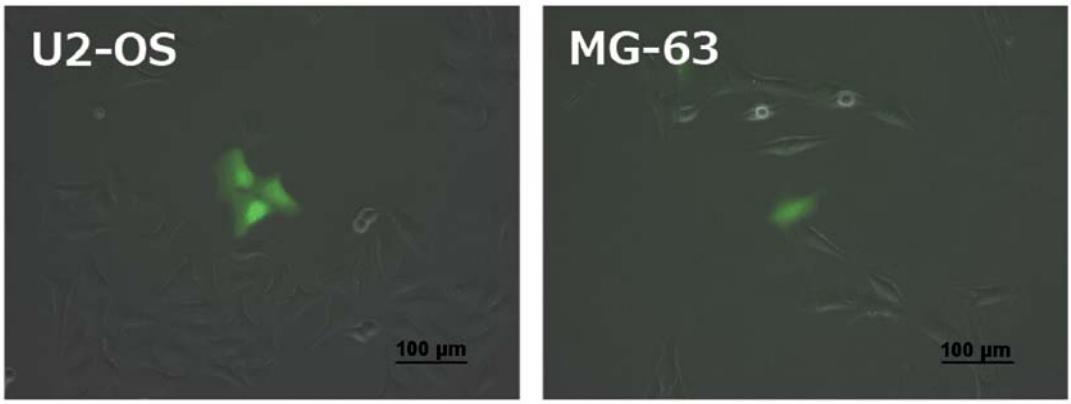

Figure 1. Establishment of osteosarcoma cell lines transfected with a proteasome sensor vector. The frequency of cells with accumulation of ZsGreen-ODC protein in human osteosarcoma cultures (bar, 100 μm).

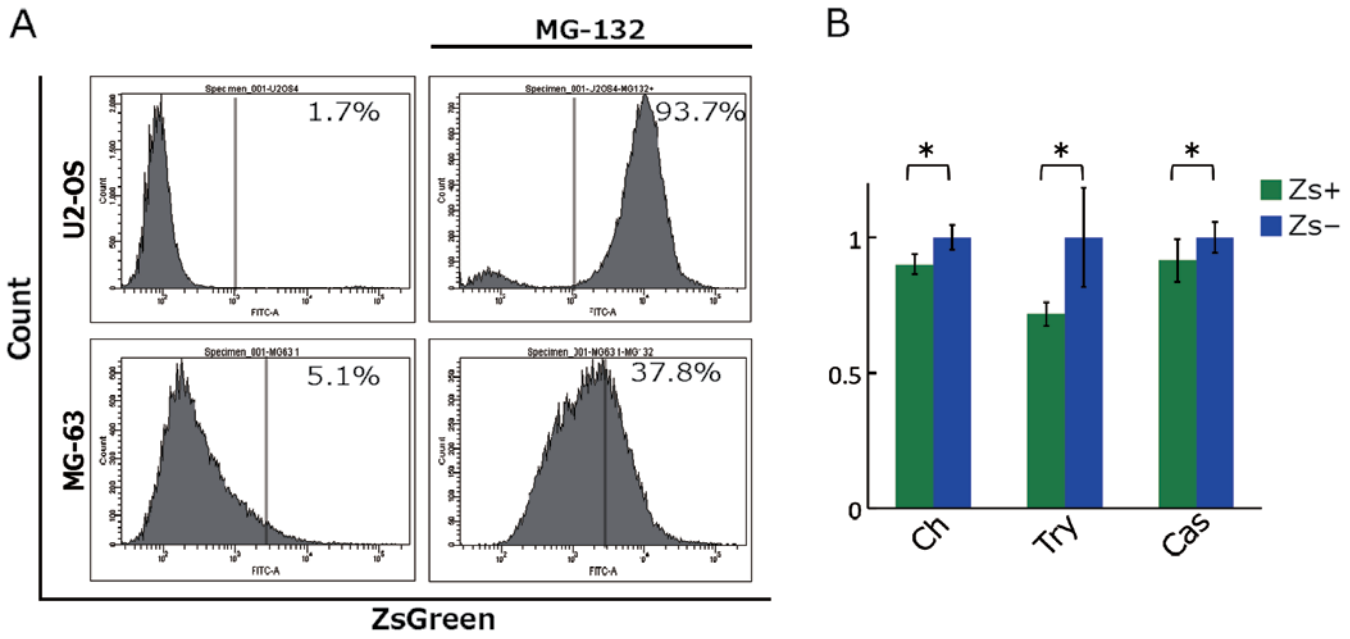

Figure 2. ZsGreen$^+$ cells showing low proteasome activity. (A) Fractions of ZsGreen$^+$ cells were measured by fluorescence-activated cell sorting (FACS) (left). When two cell lines are incubated with the proteasome inhibitor MG-132 overnight all cells accumulate the fusion protein. (B) ZsGreen$^+$ cells of MG-63 showing low proteasome activity. The value is standardized with ZsGreen$^-$ cells. Ch, chymotrypsin-like activity. Try, trypsin-like activity. Cas, caspase-like activity. $^*p<0.05$. Error bars indicate means ± SD.

two-way ANOVA analysis and Student's t-test, where appropriate, and were defined as $p<0.05$.

Results

Establishment of osteosarcoma cell lines transfected with proteasome sensor vector. To monitor proteasome activity, two osteosarcoma cell lines, U2-OS and MG-63, were stably transfected with retroviral vector pQCXIN-ZsGreen-cODC. After geneticin selection and single cell cloning, we established two osteosarcoma cell lines transfected with a proteasome sensor vector. We divided these cells into ZsGreen$^+$ and ZsGreen$^-$ groups according to their fluorescence intensity. We could easily identify ZsGreen$^+$ cells (Fig. 1). The FACS analysis showed that the fractions of ZsGreen$^+$ cells were 1.7% in U2-OS and 5.1% in MG-63 (Fig. 2A). To validate the proteasome activity monitoring, we performed proteasome inhibition of these established cell lines with MG-132. Fig. 2A shows that proteasome inhibition dramatically increased the fraction of ZsGreen$^+$ cells from 1.7 to 93.7% in U2-OS and from 5.1 to 37.8% in MG-63. Furthermore, we directly measured proteasome activity, such as trypsin-like, chymotrypsin-like, and caspase-like activities, using Proteasome Glo; this showed that ZsGreen$^+$ cells had lower proteasome activity compared with ZsGreen$^-$ cells in MG-63 (Fig. 2B), but not in U2-OS (data not shown).

ZsGreen$^+$ cells regenerate ZsGreen$^+$ and ZsGreen$^-$ cells. TICs have self-renewal and multilineage differentiation capacity. To test this, we separately cultured ZsGreen$^+$ and ZsGreen$^-$ cells after cell sorting for U2-OS. Time-lapse imaging showed asymmetric divisions of the ZsGreen$^+$ cells into ZsGreen$^+$

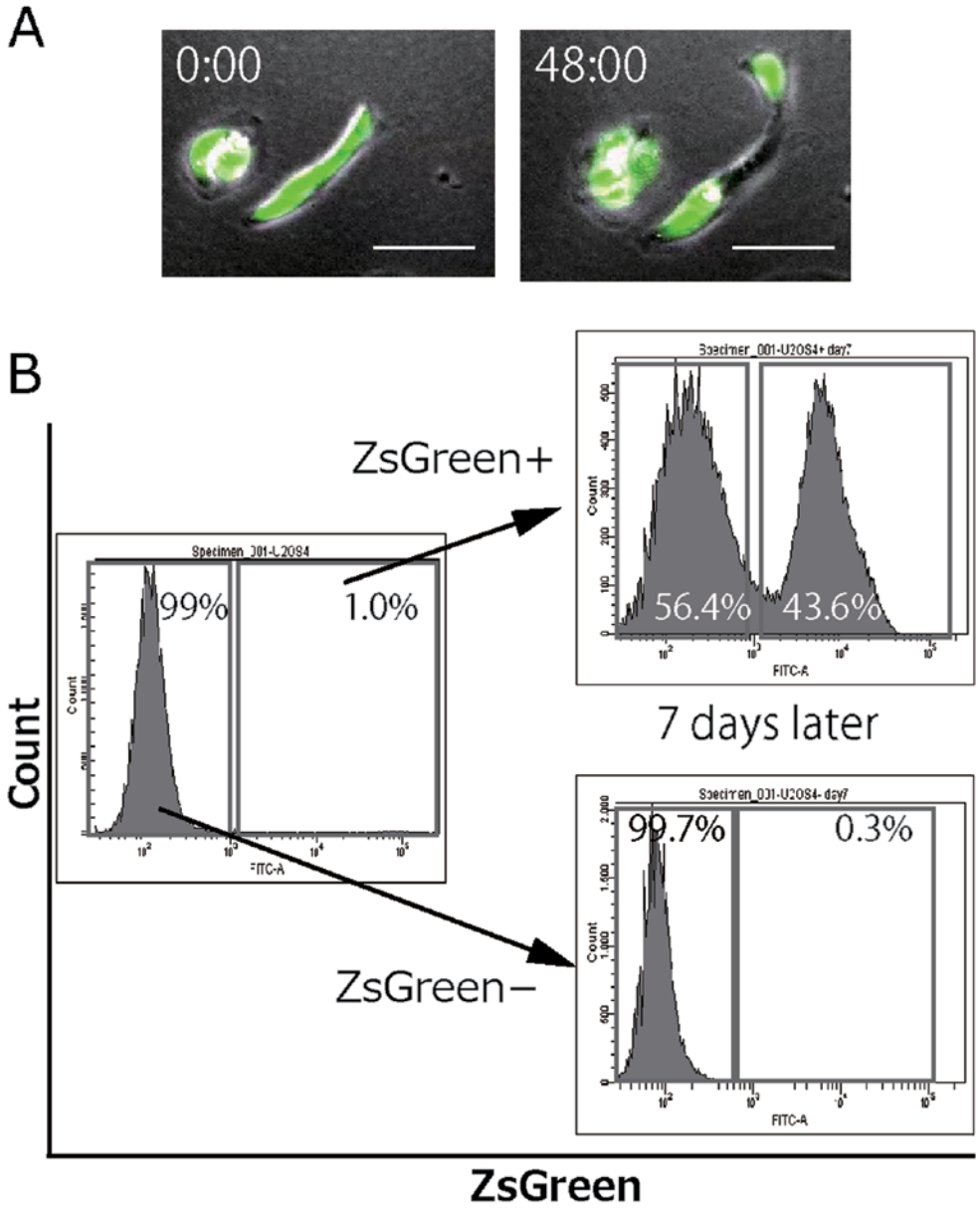

Figure 3. ZsGreen$^+$ cells divided into ZsGreen$^+$ and ZsGreen$^-$ cells. (A) Time-lapse imaging shows the asymmetric division of ZsGreen$^+$ cells from U2-OS. (B) Immediately after sorting, ZsGreen$^+$ and ZsGreen$^-$ cells of U2-OS were separately cultured. After 7 days, fluorescence-activated cell sorting (FACS) analysis revealed that ZsGreen$^+$ cells divide into ZsGreen$^+$ and ZsGreen$^-$ cells, whereas ZsGreen$^-$ cells divide into only ZsGreen$^-$ cells.

and ZsGreen$^-$ cells (Fig. 3A), whereas the ZsGreen$^-$ cells did not divide into ZsGreen$^+$ cells. Similarly, the FACS analysis showed that the ZsGreen$^+$ cells of U2-OS regenerated into ZsGreen$^+$ and ZsGreen$^-$ cells, whereas the ZsGreen$^-$ cells divided into only ZsGreen$^-$ cells (Fig. 3B). These findings indicate that the ZsGreen$^+$ population had the capacity for self-renewal and multilineage differentiation. However, the ZsGreen$^+$ and ZsGreen$^-$ cells of MG-63 divided into both ZsGreen$^+$ and ZsGreen$^-$ cells, which indicated that both the populations had the capacity for differentiation.

Sphere-forming capacity. To evaluate self-renewal, we evaluated the sphere-forming capacity of ZsGreen$^+$ and ZsGreen$^-$ cells. A total of 10,000 ZsGreen$^+$ and ZsGreen$^-$ cells were sorted and cultured in serum-free conditions. The frequencies of sphere formation for ZsGreen$^+$ and ZsGreen$^-$ were 0.16 and 0.013% ($p<0.001$) in U2-OS and 0.23 and 0.097% ($p=0.027$) in MG-63 cells (Fig. 4A and B), respectively. The ZsGreen$^+$ cells showed a high frequency of sphere formation compared with the ZsGreen$^-$ cells.

Tumorigenicity assay. TICs are primarily characterized by the ability to form tumors in NOD/SCID mice. To determine whether ZsGreen$^+$ cells were more tumorigenic than ZsGreen$^-$ cells *in vivo*, we injected both the populations separately into NOD/SCID mice. After 3 months, no tumors were observed (Table I).

Treatment resistance. To test chemoresistance, ZsGreen$^+$ and ZsGreen$^-$ cells from two osteosarcoma cell lines were exposed to MTX and CDDP. The difference in chemoresistance between the ZsGreen$^+$ and ZsGreen$^-$ cells was assessed using

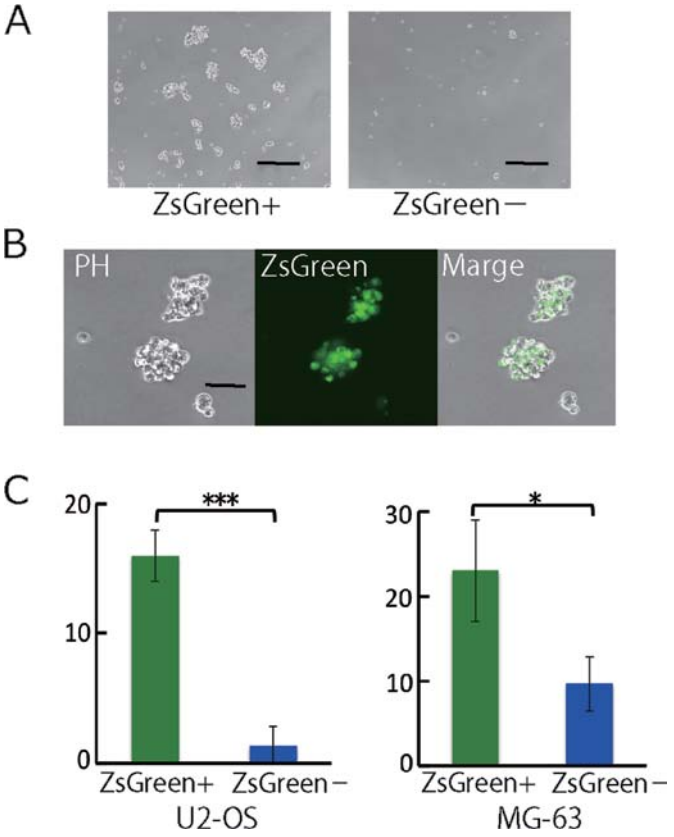

Figure 4. Sphere-forming assay. Immediately after sorting, a total of 10,000 ZsGreen$^+$ and ZsGreen$^-$ cells are separately cultured in serum-free medium supplemented with b-FGF, b-EGF, and N2. After 14 days, a strong sphere-forming capacity is observed in ZsGreen$^+$ cells. (A) Microscopic images in the low-power field (bar, 300 μm). (B) Microscopic images in the high-power field show that ZsGreen$^+$ cells primarily comprise spheres (bar, 100 μm). (C) Quantitative analysis of the number of spheres. Data are presented as means ± SD (t-test: $^*p<0.05$, $^{***}p<0.001$).

Table I. Tumorigenesis of ZsGreen$^+$ and ZsGreen$^-$ cells of U2-OS in NOD/SCID mice.

	No. of cells per injection		
	$1x10^3$	$1x10^4$	$1x10^5$
ZsGreen$^+$ cells	0/6	0/6	0/6
ZsGreen$^-$ cells	0/6	0/6	0/6

ZsGreen$^+$ and ZsGreen$^-$ cells were isolated separately and injected into the subcutaneous space of NOD/SCID mice. Tumor formation was observed for 12 weeks after injection.

the cell proliferation assay. Of the two cell lines, the ZsGreen$^+$ cells exhibited significant resistance to MTX, but not to CDDP (Fig. 5A).

Next, to evaluate radioresistance, we performed clonogenic survival assays for ZsGreen$^+$ and ZsGreen$^-$ cells of the two cell lines. As shown in Fig. 5B, ZsGreen$^+$ cells were more radio-resistant than ZsGreen$^-$ cells in both cell lines. These results indicated that the ZsGreen$^+$ cells were resistant to chemo-therapy and radiotherapy.

Discussion

Our findings indicate that low proteasome activity can serve as a marker to identify a subpopulation of TIC cells in osteosarcoma. The ZsGreen$^+$ cells displayed several features that are typically observed in TICs, including self-renewal, generation of differentiated progeny, and treatment resistance. We observed that the fractions of ZsGreen$^+$ cells were 1.7 and

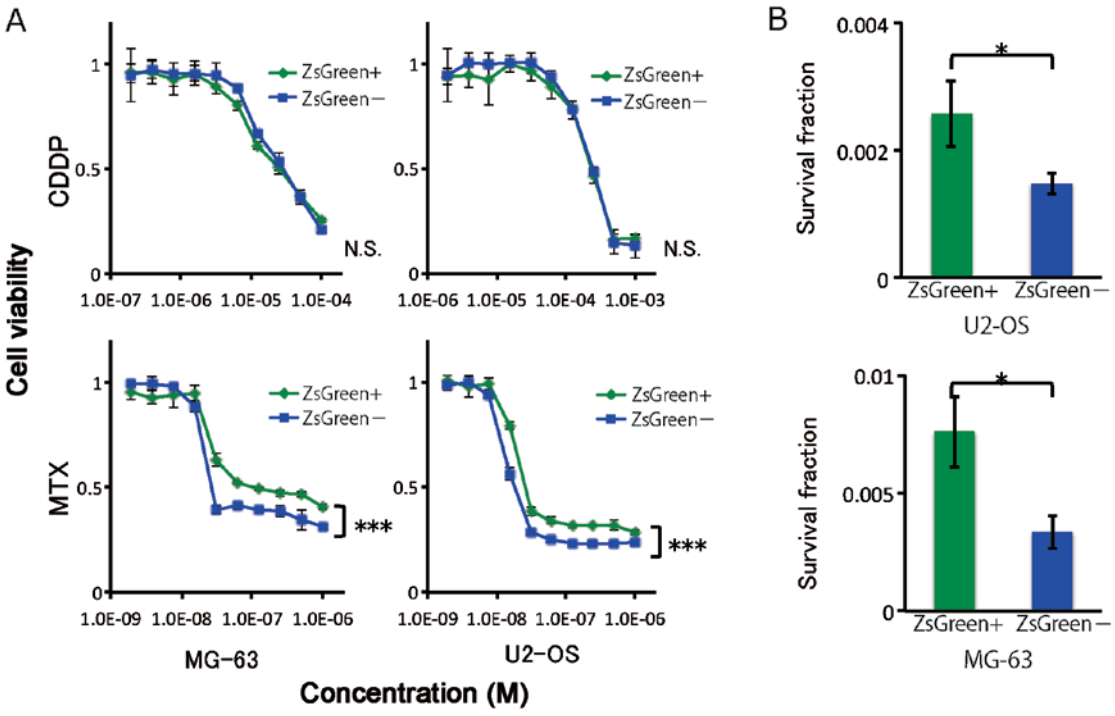

Figure 5. Treatment resistance. (A) A cell proliferation assay reveals that compared with ZsGreen$^-$ cells, ZsGreen$^+$ cells are more chemoresistant to MTX, but not to CDDP. Data are presented as means ± SDs (two-way ANOVA, $^{***}p<0.001$). (B) A clonogenic survival assay reveals that ZsGreen$^+$ cells are more radioresistant than ZsGreen$^-$ cells after 8 Gy of irradiation. Data are presented as means ± SD (t-test: $^{**}p<0.01$).

5.1% in the U2-OS and MG-63 cells, respectively. Previous reports have shown that the fractions of $ZsGreen^+$ cells were <4% in U87MG (13) and 0.5% in Panc-1 (14). The proteasome activity of $ZsGreen^+$ cells are lower than $ZsGreen^-$ cells in MG-63, whereas no difference was found in U2-OS; this is likely because this proteasome activity assay can detect partial proteasome activity. The sphere-forming capacity in established osteosarcoma cell lines has been previously demonstrated (4,8,9,11,12). Interestingly, Honoki *et al* showed that the frequency of sphere formation was 0.25% in ALDH1-positive MG-63 cells (9). Moreover, primary cultures of osteosarcoma tended to have enhanced sphere-forming capacities (5,8,16).

Although sphere-forming capacity may be a good *in vitro* assay to study osteosarcoma TICs, the gold standard to identify TICs is the formation of tumors after limiting dilution transplantation in immunodeficient mice. There was no tumor formation when we performed limiting dilution transplantation in NOD/SCID mice. Wang *et al* also showed that the TIC-like populations (high ALDH activity) of the osteosarcoma cell lines did not form tumors, but they did show enhanced tumor formation in populations with high ALDH activity obtained from a xenograft (10). Similar to the observation in this study, the population with low proteasome activity in osteosarcoma cells *in vivo* may be more tumorigenic. We could not inoculate a larger number of cells, such as 10^6 cells, because of the extensive long time required for the sorting process.

We studied treatment resistance of the $ZsGreen^+$ cells and found that they were chemo- and radioresistant. The mechanism by which treatment resistance occurs in TICs has not been elucidated. Di Fiore *et al* manufactured novel TIC-enriched cell lines such as 3AB-OS that were irreversibly selected from MG-63 cells after long exposure with 3-aminobenzamide (3AB) and expressed higher levels of the drug resistance marker ABCG2 (17). Furthermore, Honoki *et al* showed that the high ALDH1 population of MG-63 enhanced the resistance to CDDP and doxorubicin (9). Fujiawa *et al* recently found that miR-133a expression was correlated with chemo-resistance (18).

In conclusion, osteosarcoma cells with low proteasome activity had TIC properties, including sphere formation and chemo- and radioresistance. This result may lead to new approaches for the development of a more specific therapy and for an improvement in prognosis.

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