# Expression of an Exogenous Human Oct-4 Promoter Identifies Tumor-Initiating Cells in Osteosarcoma

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## Abstract

**We explored the nature of the tumor-initiating cell in osteosarcoma, a bone malignancy that predominately occurs in children. Previously, we observed expression of Oct-4, an embryonal transcriptional regulator, in osteosarcoma cell cultures and tissues. To examine the relationship between Oct-4 and tumorigenesis, cells from an osteosarcoma biopsy (OS521) were stably transfected with a plasmid containing the human Oct-4 promoter driving a green fluorescent protein (GFP) reporter to generate the transgenic line OS521Oct-4*p*. In culture, only ~24% of the OS521Oct-4*p* cells were capable of activating the transgenic Oct-4 promoter; yet, xenograft tumors generated in NOD/SCID mice contained ~67% GFP$^+$ cells, which selectively expressed the mesenchymal stem cell–associated surface antigens CD105 and ICAM-1. Comparison of the tumor-forming capacity of GFP-enriched and GFP-depleted cell fractions revealed that the GFP-enriched fractions were at least 100-fold more tumorigenic, capable of forming tumors at doses of <300 cells, and formed metastases in the lung. Clonal populations derived from a single Oct-4/GFP$^+$ cell were capable of forming tumors heterogeneous for Oct-4/GFP expression. These data are consistent with the cancer stem cell model of tumorigenesis in osteosarcoma and implicate a functional link between the capacity to activate an exogenous Oct-4 promoter and tumor formation. This osteosarcoma tumor-initiating cell appears highly prolific and constitutes a majority of the cell population in a primary xenograft tumor, which may provide a biological basis for the particular virulence of this type of cancer.** [Cancer Res 2009;69(14):5648–55]

## Introduction

Osteosarcoma is a malignant mesenchymal tumor in which the cancerous cells produce osteoid, the organic extracellular matrix of bone. It is the most common primary, nonhematologic malignancy in children, occurring most frequently in patients between ages 10 and 25 years (1). Before multiagent chemotherapy, amputation provided a long-term survival rate of only ~20%. Currently, the use of chemotherapeutics in combination with aggressive surgery has improved the long-term survival in these patients to ~60% (2, 3). Despite intensive efforts to improve both surgical and medical management, this survival rate has not improved over the last 30 years, and fully 40% of osteosarcoma patients die of their disease (4).

Despite the supposed clonal origin of cancer (5), the constituent cells of a tumor can show significant heterogeneity with respect to surface antigens, proliferation kinetics, colony-forming activity, and tumorigenic and metastatic potential (6, 7). The biological basis for this heterogeneity remains unclear and has potential therapeutic implications, as many antineoplastic agents were developed under the assumption that cancer cells are functionally homogeneous.

Two models have been proposed to explain intratumoral heterogeneity (6, 8). The stochastic model predicts that all cells in a tumor are homogeneous for tumorigenic and metastatic potential and that heterogeneity arises from intrinsic and extrinsic factors that affect cell behavior in a random fashion. In contrast, cancer stem cell model proposes that heterogeneity occurs as a result of a hierarchal organization reminiscent of normal stem cell-driven organogenesis. In this model, tumor-initiating cells constitute a distinct subpopulation and share important properties with normal tissue stem cells, including self-renewal and differentiation (9).

In considering the types of cancer likely to arise from stem/progenitor cells, osteosarcoma seems a favorable candidate. Bone is a rich reservoir of growth factors and adult stem and progenitor cells. It is one of the few human organs with the capacity for regeneration, and children, more so than adults, are capable of regenerating large segments of bone lost to trauma or surgery. Osteosarcoma occurs most commonly near active growth plates in long bones during adolescence. During this phase of postnatal bone development, stem and progenitor cells are highly active in expansion and differentiation (10, 11). If stem/progenitor cells are indeed vulnerable to oncogenic disruption, this time and site of vigorous organogenesis would seem opportune for the development of a malignant stem-like cell.

Previously, we found that cultures derived from osteosarcoma biopsies contained a subpopulation of cells capable of self-renewal as spherical clones ("sarcospheres") under anchorage-independent, serum-starved culture conditions (1). We also detected expression of the embryonic stem cell–specific transcription factors *Oct-4* and Nanog in monolayer culture, which was markedly enhanced in sarcospheres. These cells also expressed genes associated with multiple lineages and could be differentiated toward several mesenchymal phenotypes. Our results suggested that subpopulations of cells in bone sarcomas possessed stem-like properties.

Here, we worked to explore the nature of the tumor-initiating cell in osteosarcoma, hypothesizing that tumorigenesis is driven by a defined subset of cells that use regulatory networks of embryonic stem cells. To test this, we stably transfected a tumorigenic osteosarcoma cell line (OS521) with a plasmid in which the human

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*Oct-4* promoter drives expression of green fluorescent protein (GFP). Fractionation of cells from tumors based on the activity of the fluorescent reporter identified a functionally distinct, stem-like tumor-initiating cell population.

## Materials and Methods

### Cell Culture

Osteosarcoma cultures were established from patient biopsies as described (12). Samples were obtained with consent using protocols approved by the institutional review board of the University of Florida College of Medicine. Human mesenchymal stem cells were obtained from the Tulane University Center for Gene Therapy. Human fetal osteoblasts obtained from the American Type Culture Collection (CRL-11372 ATCC). Cultures were maintained in complete culture medium (DMEM/F-12, 1% penicillin/streptomycin, and 10% fetal bovine serum; Life Technologies), with the exception of human fetal osteoblasts and OS521Oct-4*p* (complete culture medium with 0.3 mg/mL G418; Mediatech).

### Generation of Oct-4/GFP Transgenic Osteosarcoma Cell Lines

OS521Oct-4*p* cell line was generated by transfection of OS521 with phOct-4/GFP (a gift from Dr. Wei Cui, Rosyln Institute; ref. 13) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Selection was done using standard culture medium with 1.0 to 0.5 mg/mL G418 (Mediatech).

### Xenotransplantation/Tumorigenicity Assays

Animal experiments were approved by the University of Florida Institutional Animal Use and Care Committee. Tumors were grown in 6-week-old female NOD/SCID mice (The Jackson Laboratory) by subcutaneous inoculation with $3 \times 10^2$ to $3 \times 10^6$ cells suspended in Opti-MEM. Tumor onset was set at 0.5 cm diameter, and tumors were harvested at a diameter of 0.8 to 1.0 cm. Recovered cells were cultured overnight in complete medium. For fluorescence-activated cell sorting, cultures were trypsinized and resuspended in PBS/2.0% bovine serum albumin. Fractionated cell populations were resuspended in Opti-MEM for subsequent injection into NOD/SCID mice.

### Isolation/Expansion of OS521Oct-4*p* GFP$^+$ Clones

Tumors from transplant of unsorted OS521Oct-4*p* cells were harvested and dissociated as above. Cultures were fractionated into GFP-enriched and GFP-depleted populations resuspended at $1 \times 10^2$ cells/mL, and 0.01 mL of the cell suspension was then seeded to each well of a flat-bottomed 96-well plate. Wells verified to contain a single cell were monitored for growth of colonies. Selected clones were expanded and characterized for GFP, surface antigens, and tumorigenicity.

### Immunohistochemistry

Tumor specimens were fixed in 4°C in 4% paraformaldehyde, incubated overnight in 20% sucrose/PBS at 4°C, and embedded in OCT (Sakura Finetek). Sections (5 μm) were immunostained using rabbit anti-GFP (ab290; Abcam). Slides were heat retrieved in 10 mmol/L citrate buffer (pH 6.0), blocked with normal serum, and stained overnight at 4°C. Signal was detected with anti-rabbit Alexa Fluor 488 (Molecular Probes).

### Microscopy

Expression of Oct-4/GFP and CMV/GFP was visualized using a Leica DMIL inverted fluorescence microscope (Leica Microsystems). Images were captured using the Retiga 1300R camera (Q Imaging) and analyzed using the manufacturers' software. Histologic images were captured using 3.3 MPX Camera (Imaging Planet) mounted on a Zeiss Axioskop 40 microscope (Carl Zeiss Microimaging) and analyzed using Image Planet Capture software (Imaging Planet).

### Flow Cytometry

**Monolayer.** Cells were trypsinized, resuspended in PBS/0.05% bovine serum albumin at $5 \times 10^6$/mL, and blocked with human IgG (1 μg/$10^5$ cells) before incubation with the specified antibodies and isotype controls (1:10 dilution). Analyses were done using a LSRII flow cytometer (BD Biosciences).

**Tumors.** Tumors were removed at 0.8 to 1.0 cm and dissociated as indicated for fluorescence-activated cell sorting enrichment and resuspended in PBS/0.05% bovine serum albumin ($5 \times 10^6$ cells/mL). Blocking and antibody/isotype hybridization were done as above.

**Antibodies.** Anti-CD44, CD106, and HLA-A/B/C (MHC class I) were purchased from BD Pharmingen; anti-CD133/2 from Miltenyi Biotech; anti-CD56 (FAB240P), CD90 (FAB10971P), CD105 (FAB2067P), CXCR-4 (FAB170P), SSEA-4 (FAB1435P), and EpCAM (FAB9601A) from R&D Systems; anti-CD29 from eBioscience; and anti-ICAM1 from Abcam.

## Results

**Development of an assay for tumorigenesis.** Cell lines established from human osteosarcoma biopsies were evaluated for the capacity to generate tumors following subcutaneous transplantation into NOD/SCID mice. One cell line, OS521, which originated from a high-grade distal femoral osteosarcoma, was capable of reliable, robust tumor formation. Resulting tumors showed evidence of osteoid arising from malignant spindle cells, recapitulating the histologic phenotype of the patient's osteosarcoma (Fig. 1).

In later experiments, we found that delivery of $3 \times 10^6$ and $3 \times 10^5$ OS521 cells produced tumors of >1.0 cm diameter with 100% efficiency. At a dose of $3 \times 10^4$ cells, the efficiency of tumor formation was reduced to ~75%. We selected this as the starting cell dose for subsequent tumorigenicity assays because it represented the highest dose at which differences in tumorigenic potential could be detected.

**Phenotypic characterization of OS521 cells *in vitro*.** To attempt to identify phenotypically distinct subpopulations within the OS521 line for comparison in tumorigenicity assays, we characterized these cells for expression of a variety of surface antigens associated with normal and malignant stem cells in other tissues (14, 15). Monolayer cultures of OS521 were composed of a largely homogenous population (Fig. 2*A*); they uniformly expressed MHC class I and the mesenchymal stem cell–associated antigens CD29, CD90, CD105, and CD44, implicated as a marker for breast and colon cancer stem cells (14, 16–18). They also expressed adhesion molecules, ICAM-1 and CD56. OS521 cells were negative for the embryonic stem cell–specific surface antigen SSEA-4 and the chemokine receptor CXCR4. They also were negative for the tumor-associated adhesion molecule VCAM-1 and the colon cancer stem cell marker EpCAM as well as the neural and brain tumor stem cell–associated marker CD133 (16, 19–21). This apparent lack of heterogeneity prohibited fractionation of OS521 cells based on antigen phenotype.

**A subset of OS521 cells activates the exogenous *Oct-4* promoter.** We devised an alternative approach to identify distinct subpopulations based on the capacity to activate an exogenous human *Oct-4* promoter element. We obtained the plasmid phOct-4/GFP, containing the entire 4 kb human *Oct-4* promoter sequence positioned upstream of a GFP reporter (13). This plasmid also contains an independent SV40 promoter-driven, neomycin resistance cassette, which allows for positive selection of cells that acquire the plasmid irrespective of the capacity to activate the Oct-4 promoter.

phOct-4/GFP was transfected into three cell cultures: OS521, human mesenchymal stem cells, and a human fetal osteoblast line. As positive controls, parallel cultures were similarly transfected with

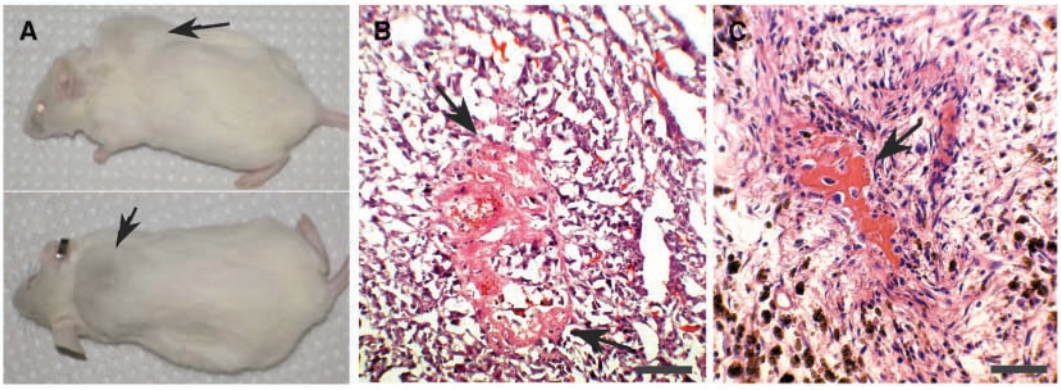


**Figure 1.** OS521 cells are tumorigenic and regenerate the histologic phenotype of the primary tumor in NOD/SCID xenografts. *A,* NOD/SCID mouse with a tumor (*arrows*) from subcutaneous injection of $3 \times 10^6$ OS521 cells. *B,* xenograft OS521 tumor stained with H&E. *C,* H&E-stained section of the primary OS521 osteosarcoma. *B* and *C, arrows,* osteoid. Magnification, ×200. *Bar,* 100 μm.

a plasmid, pEGFP-N1 containing a CMV promoter-driven GFP reporter. Despite the homogeneity of the cells in culture with regard to surface proteins, following stable transfection of the OS521 line (hereafter termed OS521Oct-4*p*), only 24% of the G418-resistant population expressed Oct-4/GFP (Fig. 3*A* and *B*). Despite expression of the CMV/GFP reporter in >95% of the cells in all three cultures, Oct-4/GFP expression was observed only in OS521 (Fig. 3*B*).

**Oct-4/GFP$^+$ cells from tumors selectively express CD105 and ICAM-1.** To explore the role of the Oct-4/GFP$^+$ cells in tumor initiation, we injected $3 \times 10^4$ OS521Oct-4*p* cells (both GFP$^+$ and GFP$^-$ cells) as well as nontransfected OS521 cells into separate groups of six NOD/SCID mice. At ~5 weeks, tumors >0.5 cm diameter had formed in 5 of 6 and 4 of 6 animals of the respective groups, indicating that integration of the phOct-4/GFP plasmid did not influence the tumor-initiating potential of the OS521 cells. Histologic examination showed the tumors were heterogeneous for Oct-4/GFP expression, exhibiting discrete regions of GFP$^+$ and GFP$^-$ cells (Fig. 3*C*). Flow cytometry showed the proportion of GFP$^+$ cells to be ~67%, increasing ~3-fold over the 24% observed in monolayer culture (Fig. 3*B*), suggesting a selective amplification of cells that are able to activate the Oct-4 promoter during tumorigenesis.

Interestingly, analysis of surface antigen expression of the cells recovered from tumors produced results similar to those from monolayer cells shown in Fig. 2*A*, with the notable exceptions of CD105 and ICAM-1. Expression of these antigens corresponded closely with that of Oct-4/GFP, indicating that, in cells isolated directly from tumors, the capacity to transcriptionally activate the exogenous *Oct-4* promoter is linked with a distinct cellular phenotype.

**Oct-4/GFP$^+$ cell fractions exhibit heightened tumorigenicity.** To test if the GFP$^+$ OS521Oct-4*p* cells showed enhanced tumorigenesis, cells recovered from OS521Oct-4*p* xenograft tumors were pooled and fractionated by fluorescence-activated cell sorting into GFP-enriched and GFP-depleted populations. Secondary analyses showed that the enriched fraction was composed of ~92% GFP$^+$ cells, whereas, in the GFP-depleted fraction, GFP$^+$ cells numbered ~3% (Fig. 4*A*). Starting from a dose of $3 \times 10^4$ cells, we injected serial 10-fold dilutions of the respective fractions, as well as equivalent numbers of unfractionated OS521Oct-4*p* cells, into groups of 8 NOD/SCID mice and examined the rate of tumor formation over a period of 90 days.

Cells in the GFP-enriched fraction proved to be >100-fold more tumorigenic than those in the GFP-depleted fraction (Table 1). At doses as low as $3 \times 10^2$ cells, the GFP-enriched fraction formed tumors in all of the mice inoculated. For the GFP-depleted group, at $3 \times 10^3$ cells, only 1 of 8 mice developed a tumor, and at $3 \times 10^2$ cells, none of the mice developed tumors. At the lowest dose, the GFP-enriched fraction was also significantly more tumorigenic than the unsorted population as only 3 of 8 animals formed tumors. Curiously, all tumors formed from the GFP-enriched and GFP-depleted fractions were highly GFP$^+$ (Fig. 4*A* and *B*). In both cases, similar to that shown in Fig. 2*B*, expression of CD105 and ICAM-1 was restricted to the GFP$^+$ cells. Altogether, these results indicated that tumorigenesis in OS521 is functionally linked with the capacity to activate the exogenous Oct-4 promoter.

We passaged the OS521Oct-4*p* GFP$^+$ cells three additional times in mice, whereby the cells were injected, harvested from tumors, fractionated, and reinjected at $3 \times 10^2$ cells. We found that the tumors appeared to increase in virulence with passage, producing tumors with shorter time to onset and more rapid growth rate. By the third passage, we noted the formation of multiple tumor nodules following a single injection. Analysis of the lungs of these mice showed clear evidence of metastases, with clusters of GFP$^+$ cells readily identified throughout (Fig. 4*C*). The formation of multiple tumor nodules or lung metastases had not been observed in any of the prior experiments, suggesting that these changes were the result of selection by serial passage *in vivo*.

**OS521Oct-4*p* GFP$^+$ clones generate heterogeneous tumors.** To determine if the heterogeneity in Oct-4/GFP expression in tumors reflected differences in growth rates of preexisting GFP$^+$ and GFP$^-$ cell populations in the inocula, or signaled asymmetric division by GFP$^+$ OS521Oct-4*p* tumor-initiating cells, cells from OS521Oct-4*p* xenograft tumors were sorted by fluorescence-activated cell sorting and seeded at single-cell density into individual wells of 96-well plates. Oct-4/GFP$^+$ clones arose with ~90% efficiency, and three were selected for expansion and characterization. We were unable to isolate GFP$^-$ cells capable of growth at low density, suggesting that these cells were nonclonogenic *in vitro* (22–25). The disparate *in vitro* clonogenic potential of the GFP$^+$ and GFP$^-$ populations was consistent with our *in vivo* results regarding tumorigenicity.

Each of the three Oct-4/GFP$^+$ clonal populations was highly uniform for GFP expression *in vitro* (Fig. 5*A*), which was

maintained with passage. Following delivery of $3 \times 10^4$ cells of the respective clones into NOD/SCID mice, tumors readily formed within 2 to 3 weeks. Analysis by flow cytometry showed that the tumors had reestablished heterogeneity with respect to Oct-4/GFP expression as well as CD105 and ICAM-1. On first passage, $GFP^+$ clones generated tumors composed of cells whose fluorescence intensity ranged over 3 logs, showing that $GFP^-$ cell populations could arise from $GFP^+$ cells *in vivo.* Interestingly, by the third serial passage of unfractionated cells, the total cell population had resolved into discrete $GFP^+$ and $GFP^-$ populations of approximately equal proportions.

To assay for differences in tumorigenic capacity of the $GFP^+$ and $GFP^-$ cells, we harvested third passage tumors from one of the clones (S1), fractionated the cells based on fluorescence, and transplanted them at decreasing doses, similar to that described earlier. Consistent with our previous results, the GFP-enriched cell fraction was significantly more tumorigenic than the GFP-depleted fraction (Table 1). Analysis of the resulting tumor cell populations for Oct-4/GFP expression showed that the GFP-enriched fractions formed tumors composed of ~90% $GFP^+$ cells (Fig. 5*B*). Tumors derived from transplantation of GFP-depleted fractions contained ~45% $GFP^+$ cells. Altogether, these findings support the idea that OS521Oct-4*p* $GFP^+$ cells are capable of tumor initiation and self-renewal and can give rise to functionally disparate cell populations in xenografts.

## Discussion

**Characteristics of osteosarcoma tumor-initiating cells.** Among cells from a primary tumor biopsy, the subpopulation capable of activating an Oct-4 promoter/GFP reporter construct showed significantly enhanced tumorigenic activity. Following

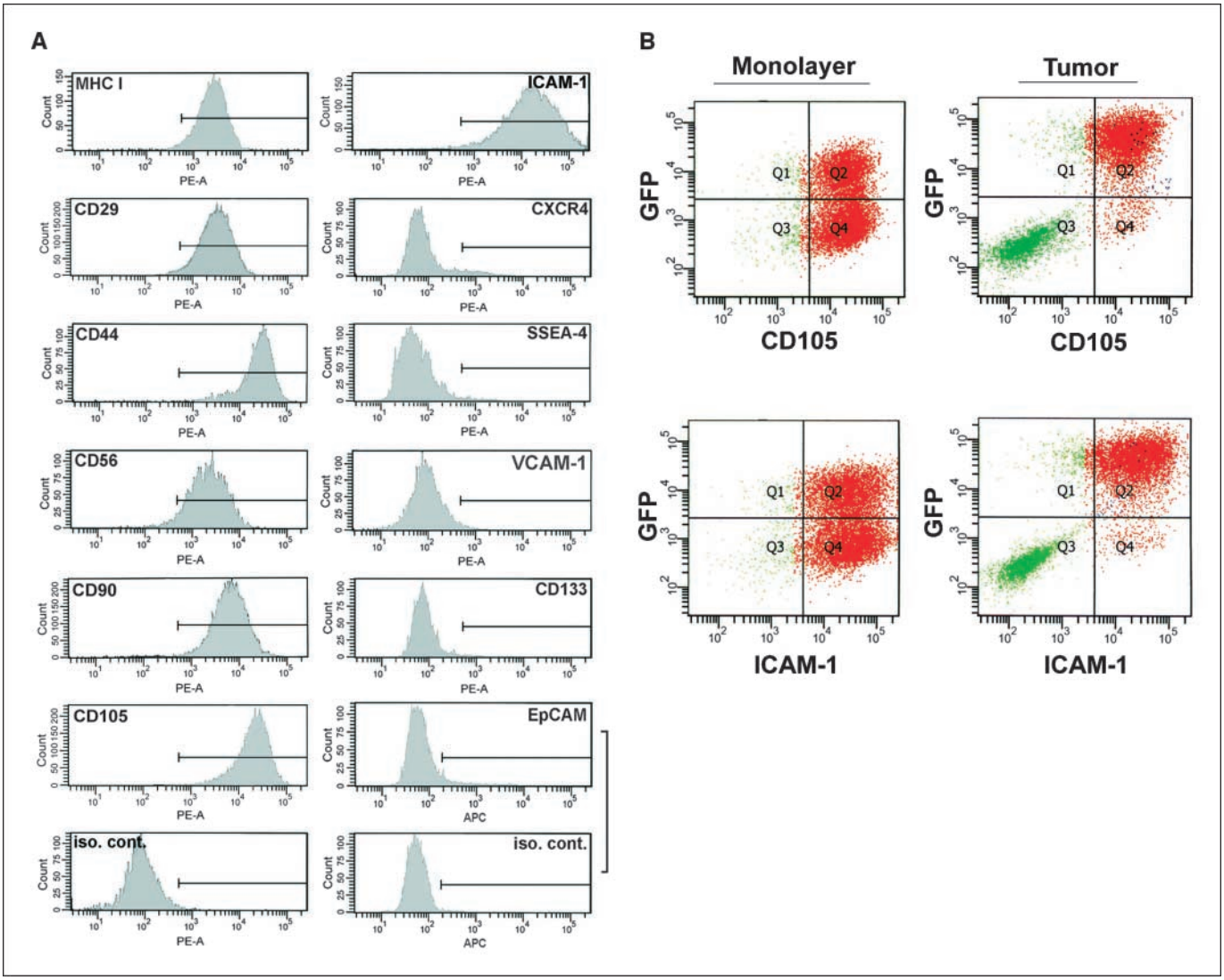


**Figure 2.** Analysis of OS521 cells for expression of stem cell–associated surface antigens. *A,* cultures of OS521 incubated with specific antibodies or isotype controls (*iso. cont.*) conjugated with either phycoerythrin (*PE-A*) or allophycocyanin (*APC*) were analyzed for surface antigen expression by flow cytometry. OS521 was uniformly positive for expression of MHC class I, CD29, CD44, CD56, CD90, CD105, and ICAM-1 but negative for others tested. *Vertical axes,* cell number; *horizontal axes,* relative levels of fluorescence; *horizontal bar,* gating indicating fluorescence exceeding 95% of isotype controls. *B,* OS521Oct-4*p* cells analyzed by flow cytometry for coexpression of GFP with CD105 or ICAM-1 in monolayer or xenograft tumor. Oct-4/GFP activation in tumors was closely associated with cells expressing CD105 and ICAM-1. *Vertical axis,* GFP fluorescence; *horizontal axis,* CD105 or ICAM-1.

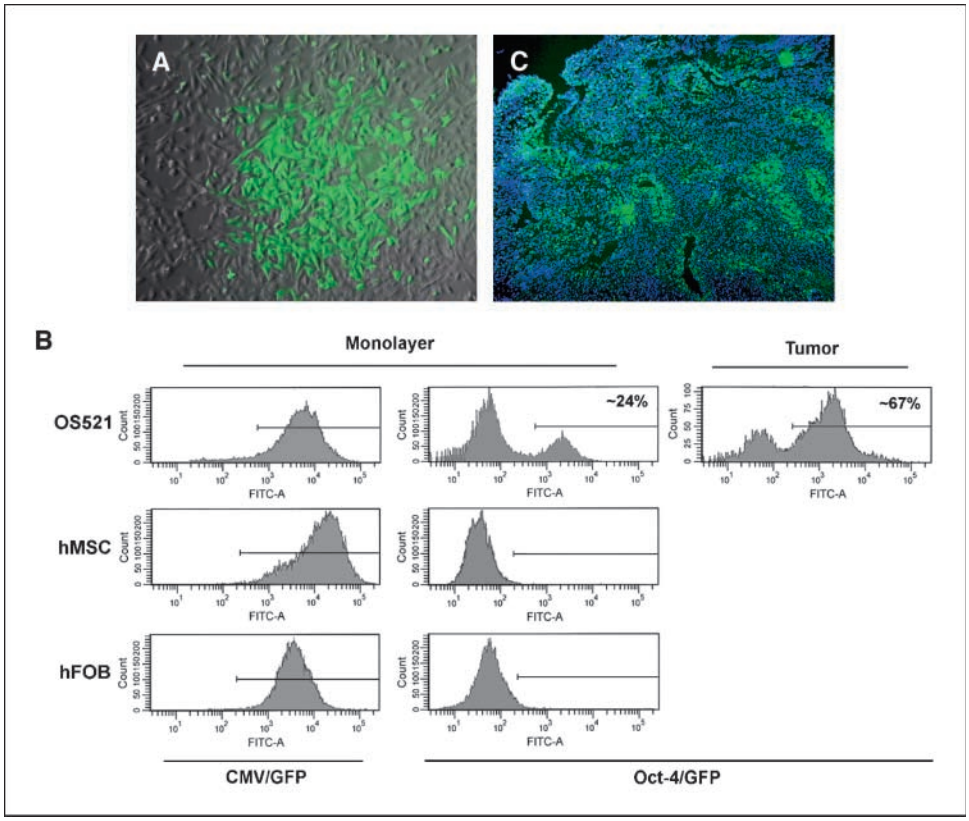


**Figure 3.** OS521 is heterogeneous for expression of the Oct-4/GFP reporter. Cultures of OS521 cells, human mesenchymal stem cells, and human fetal osteoblast (*hFOB*) cells were stably transfected with plasmids containing a GFP reporter driven by the CMV promoter/enhancer or the human Oct-4 promoter (CMV/GFP and Oct-4/GFP, respectively). *A,* fluorescence microscopy of OS521 cells stably transfected with phOct-4/GFP (OS521Oct-4*p* cells) shows variable GFP expression. *B,* fluorescence of the respective transfected cultures (*Monolayer*) using flow cytometry and cells isolated from xenograft tumors in NOD/SCID mice generated by subcutaneous injection of $3 \times 10^4$ unfractionated OS521Oct-4*p* cells (*Tumor*). In monolayer, ~24% of the OS521Oct-4*p* cells expressed the GFP reporter, whereas in tumors ~67% were GFP$^+$. *C,* immunohistochemical detection of GFP expression in frozen sections of a xenograft tumor generated from unfractionated OS521Oct-4*p* cells. Discrete foci of cells expressing the Oct-4 promoter reporter (GFP$^+$) are seen throughout the tumor.

ectopic transplantation, these cells reliably formed tumors at doses of 300 cells or less, representing >100-fold enrichment of tumorigenic capacity. Phenotypic characterization showed that the tumor-initiating cells selectively expressed surface antigens CD105 and ICAM-1.

This tumorigenic osteosarcoma cell appears both highly prolific and plastic, such that it not only comprises a majority of the cells in a tumor but also gives rise to phenotypically divergent progeny (CD105- and ICAM-1–negative) that are incapable of activating the *Oct-4* promoter or efficiently forming tumors in the xenograft model. Despite the finding that this cell activates an embryonic stem cell-specific promoter element, we did not detect expression of embryonic stem cell surface markers SSEA-4 and CXCR4. Instead, tumor-initiating cells remained intrinsically mesenchymal, expressing surface antigens commonly associated with mesenchymal stem cells (CD29, CD44, CD56, CD90, and CD105). Moreover, the tumors formed displayed pleiomorphic, malignant, spindle cells that secrete osteoid, the characteristic osteosarcoma phenotype (26). We found no link between tumorigenicity and expression of CD133, EpCAM, or CD44, markers associated with tumor-initiating cells in brain, breast, and colon cancer (14, 17, 18, 21).

**Generation of Oct-4/GFP$^+$ tumors from GFP-depleted cell populations.** We routinely observed tumors that were highly GFP$^+$ arising from transplantation of GFP-depleted cell fractions. This is likely attributable to one of two scenarios. The first is that GFP$^-$

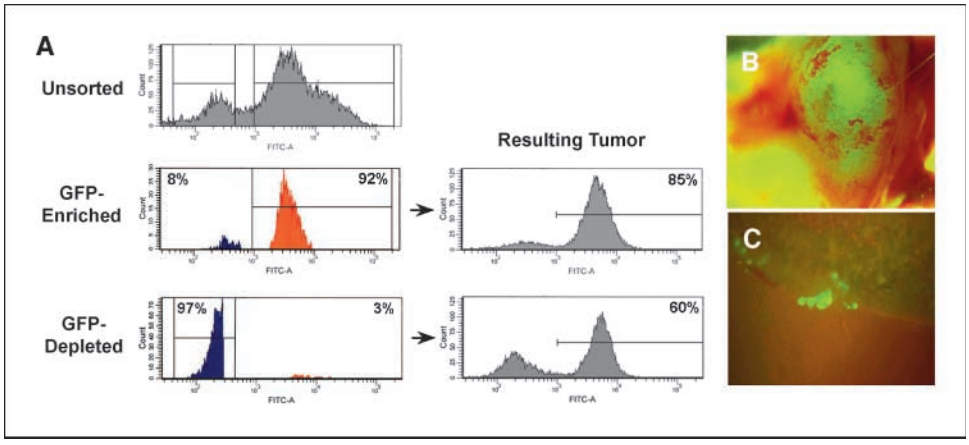


**Figure 4.** GFP$^+$ OS521Oct-4*p* cells show heightened tumorigenicity and metastases. *A,* fluorescence of OS521Oct-4*p* cells prior (*Unsorted*) and following fractionation. *Brackets,* gates used to define GFP$^+$ and GFP$^-$ cells. GFP-enriched fraction contained ~92% GFP$^+$ cells (*orange*); the depleted fraction was composed of ~97% GFP$^-$ cells (*blue*) and ~3% GFP$^+$ cells. *B,* regardless of dose, resulting tumors from both fractions were highly fluorescent. Fluorescent image of a tumor formed from $3 \times 10^4$ cells from the GFP-depleted fraction. Resulting tumors following delivery of $3 \times 10^4$ GFP-enriched or GFP-depleted cells were composed of 85% and 60% GFP$^+$ cells, respectively. *C,* clusters of fluorescent metastatic cells in the lungs of mice following serial passage of GFP-enriched cells.

**Table 1.** Tumor incidence and time to onset following xenotransplantation of fractionated OS521Oct-4*p* cells or an OS521Oct-4*p* GFP$^+$ clone

OS521Oct-4*p*

| Cell dose | GFP-enriched | | Unsorted | | *P** | GFP-depleted | | *P*† |
|---|---|---|---|---|---|---|---|---|
| | Incidence | Onset (d) | Incidence | Onset (d) | | Incidence | Onset (d) | |
| $3 \times 10^4$ | 8/8 | 22 | 6/8 | 26 | 0.233 | 5/8 | 47 | 0.10 |
| $3 \times 10^3$ | 8/8 | 34 | 6/8 | 44 | 0.233 | 1/8 | 51 | <0.001 |
| $3 \times 10^2$ | 8/8 | 45 | 3/8 | 60 | 0.012 | 0/8 | 90 | <0.001 |

OS521Oct-4*p* GFP$^+$ clone

| Cell dose | GFP-enriched | | GFP-depleted | | *P*† |
|---|---|---|---|---|---|
| | Incidence | Onset (d) | Incidence | Onset (d) | |
| $3 \times 10^4$ | 4/4 | 23 | 2/4 | 47 | 0.2143 |
| $3 \times 10^3$ | 4/4 | 36 | 1/4 | 50 | 0.0714 |
| $3 \times 10^2$ | 4/4 | 44 | 0/4 | 90 | 0.0142 |

NOTE: Fisher-Irwin exact test was used to determine if the probability of tumor formation for the GFP-enriched fraction was greater than the GFP-depleted or unsorted (OS521Oct-4*p* only) fractions. Tumor incidence = number of mice with tumors / mice injected.
*$P$ values for enriched versus unsorted populations.
†$P$ values for enriched versus depleted populations.

cells within the Oct-4/GFP-depleted fractions acquired the capacity to reactivate the Oct-4 promoter/reporter, resulting in a high percentage of GFP$^+$ cells in the tumor. We feel a more plausible explanation is that the ~3% GFP$^+$ cells contaminating the GFP-depleted fractions were sufficient to initiate tumor formation and thereby generated tumors that were largely GFP$^+$. Because as few as 300 cells from the GFP-enriched fractions readily formed tumors in mice, it seems reasonable then that the ~900 contaminating GFP$^+$ cells in the $3 \times 10^4$ cell dose (3% of $3 \times 10^4$) of the GFP-depleted fraction would likewise be capable of tumorigenesis (see Table 1). If the contaminating GFP$^+$ cells are indeed responsible for generating the tumors in the GFP-depleted fractions, then our approximation of 100-fold increased tumorigenic activity in these cells is a vast underestimate.

**Oct-4/GFP expression and tumorigenesis in osteosarcoma.** The expression of the Oct-4/GFP reporter appears directly linked to tumorigenesis in our xenograft model. Although we have not yet elucidated the exact nature of this relationship, it suggests the

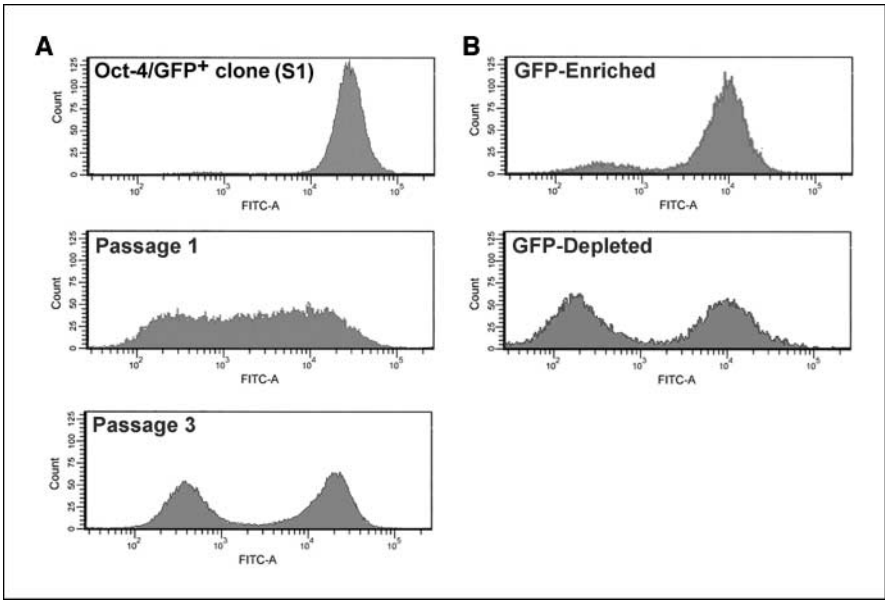


**Figure 5.** OS521Oct-4*p* GFP$^+$ clones are capable of self-renewal and generate heterogeneous tumors following xenotransplantation. Cells from OS521Oct-4*p* GFP-enriched fractions were seeded at single-cell density into multiwell plates. *A,* analysis by flow cytometry of a representative clone expanded in culture (*S1*) shows that, in monolayer, cells uniformly expressed the exogenous Oct-4/GFP reporter. Xenotransplantation of S1 cells into NOD/SCID mice generated tumors composed of cells that heterogeneously express the Oct-4/GFP reporter (*passage 1*). Following the third passage, *in vivo* tumors are composed of distinct populations of GFP$^+$ and GFP$^-$ cells, which were fractionated into GFP-enriched and GFP-depleted populations and tested for relative tumor-forming capacity. GFP-enriched fraction was significantly more tumorigenic (see Table 1). *B,* tumors formed from both GFP-enriched and GFP-depleted fractions contained a significant proportion of GFP$^+$ cells (~90% and 45%, respectively).

molecular machinery of embryonic stem cells is active in osteosarcoma and is critical to the phenotype of the tumor-initiating cell.

The Oct-4/GFP reporter construct used in these studies contains the elements critical for *Oct-4* tissue-specific gene expression and includes both distal and proximal enhancers. Gerrard and colleagues (13) showed that, in human embryonic stem cells stably transfected with this construct, GFP expression faithfully represented expression of endogenous Oct-4 protein in undifferentiated embryonic stem cells and its subsequent loss during neural differentiation. Similarly, in our studies, the loss of Oct-4/GFP expression was associated with an apparent differentiation event, evidenced by a reduction in tumorigenic potential and a change in cell surface phenotype. Furthermore, the promoter/reporter was only active in our tumor-initiating cells and not in the normal mesenchymal stem cells or the more differentiated osteoblast cell line.

We have attempted to characterize *Oct-4* expression in OS521Oct-4*p* cells using several methods, including reverse transcription-PCR, Western blot, and immune staining of cells and tumor sections; however, discrepancies were noted in the results among these assays. Indeed, definitive detection of *Oct-4* expression remains an area of active controversy in the cancer literature (27, 28). The identification of numerous *Oct-4* pseudogenes and splice variants as well as reports questioning the specificity of the commercially available antibodies used to detect this protein cloud the issue surrounding *Oct-4* expression (27, 28).

Regardless of our inability to conclusively correlate endogenous Oct-4 protein with the activation of our reporter, other groups using similar Oct-4 promoter-driven constructs have shown that activation of the exogenous promoter is restricted to embryonic stem and primordial germ cells *in vitro* (29, 30). Moreover, in transgenic mice, expression of the exogenous Oct-4 promoter is specifically limited to germ-line cells in embryos and adult mice (30, 31). Additionally, Oct-4/GFP expression is used routinely as an indicator of cellular reprogramming following transplant of somatic nuclei into ooplasms and during fusion of somatic and embryonic stem cells (32–37). Therefore, whether or not Oct-4 specifically contributes to oncogenesis in osteosarcoma, activation of the Oct-4 promoter/reporter in the osteosarcoma initiating cells suggests that these cells have likewise undergone cellular reprogramming and possess a transcriptional profile related to that of embryonic cells (38, 39). This implies that the regulatory networks controlling stem cell function are active in osteosarcoma and are functionally linked to tumorigenesis.

Although the data shown here are derived from a single osteosarcoma, we have since established three additional primary osteosarcoma cultures capable of forming tumors in the NOD/SCID mouse. These tumor lines have been stably transfected with the Oct-4/GFP construct, and ongoing studies show heterogeneous GFP expression, suggesting that the activation of exogenous Oct-4 promoter may be a generalized phenomenon in this type of cancer. We are currently evaluating the relative tumorigenicity of the respective GFP-enriched and GFP-depleted cell fractions for these lines.

**An osteosarcoma stem cell?** Tumorigenesis in osteosarcoma appears most consistent with the cancer stem cell model as defined by the prospective selection of a discrete subpopulation of cells within a tumor with enhanced tumorigenic capacity (8). In addition to activating the *Oct-4* promoter and bearing surface antigens frequently associated with mesenchymal stem cells, the tumor-initiating cells we identified possess several stem-like properties. Clonal populations generate antigenically distinct progeny and give rise to heterogeneous tumors composed of tumorigenic and nontumorigenic cell populations. These tumor-initiating cells also self-renew, as shown through serial transplantation in NOD/SCID mice, and spontaneously metastasize.

In contrast to other malignancies in which cancer stem cells are described as rare and slowly dividing (16), osteosarcoma tumor-initiating cells appear highly proliferative and comprise much of the tumor cell population. This may be a manifestation of their enhanced capacity for self-renewal and a more plastic cellular phenotype, enabling the initiating cells to adapt to the stringent environment of the xenograft. This is supported by the observation that *in vitro* the percentage of cells expressing Oct-4/GFP remains stable; yet, significant changes in the proportion of $GFP^+$ and $GFP^-$ cells are observed following a single passage *in vivo*. Further, following serial transplantation, we observed an increase in virulence and the acquisition of metastatic capability, suggesting a selective adaptation analogous to the process of tumor progression (5, 40, 41).

The preponderance of $GFP^+$ cells in our xenografts may therefore represent selection and early expansion of the tumor-initiating population by symmetric division. Once a critical mass has been achieved and a suitable microenvironment formed, the cells then begin to differentiate, producing the $GFP^-$ cells. This is supported by the image shown in Fig. 3, displaying dense zones of Oct-4/$GFP^+$ cells distributed throughout a xenograft tumor. Using this interpretation, it is possible that the xenograft tumors exhibit a disproportionately high percentage of tumor-initiating cells because of their small size at the time of harvest, which may not have permitted sufficient time for both expansion and generation of a mature cellular hierarchy.

Alternatively, it may mean that osteosarcomas in general are composed of a large percentage of highly proliferative, tumor-initiating cells. This could explain the extreme virulence of this form of cancer and of this cell line in particular. The aggressiveness of a specific sarcoma is directly related to its histologic grade, determined predominantly by the degree of differentiation. Grade has been shown to be the single most predictive variable related to survival in patients not already having metastases (42). Interestingly, OS521 was derived from a poorly differentiated osteosarcoma that exhibited scant osteoid production. The clinical course of this patient was one of rapid progression to metastases and death despite chemotherapy, reflecting the aggressive appearance of the histology.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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