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## Human osteosarcoma CD49f<sup>-</sup>CD133<sup>+</sup> cells: impaired in osteogenic fate while gain of tumorigenicity

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### Abstract

The biological relationships among self-renewal, tumorigenicity, and lineage differentiation of human osteosarcoma-initiating cells (OSIC) remain elusive, making it difficult to identify and distinguish OSIC from osteosarcoma-forming cells (OSFC) for developing OSIC-targeted therapies. Using a new inverse lineage tracking strategy coupled with serial human-to-mouse xenotransplantation, we identified a subpopulation of osteosarcoma cells with OSIC-like properties and sought to distinguish them from their progeny, OSFC. We found that serial transplantation of cells from different osteosarcoma cell lines and primary osteosarcoma tissues progressively increased the CD49f<sup>+</sup> subpopulation composing the bulk of the osteosarcoma mass. These CD49f<sup>+</sup> cells displayed characteristics of OSFC: limited *in vivo* tumorigenicity, weak lineage differentiation, more differentiated osteogenic feature, and greater chemo-sensitivity. By contrast, their parental CD49f<sup>-</sup>CD133<sup>+</sup> cells had an inhibited osteogenic fate, together with OSIC-like properties of self-renewal, strong tumorigenicity, and differentiation to CD49f<sup>+</sup> progeny. Hence, the CD49f<sup>-</sup>CD133<sup>+</sup> phenotype appears to identify OSIC-like cells that possess strong

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Conflict of interest

The authors declare no conflict of interest.

**Data deposition.** The microarray data from the manuscript have been submitted to the GEO database (<http://www.ncbi.nlm.nih.gov/geo>). Series: GSE30807; 3 samples: GSM764199, GSM764200, and GSM764201. Title: Gene expression of normal human mesenchymal stem cells, osteosarcoma U2OS cells, and U2OS cell-derived UT2 cells by serial transplantation. File format, GEOarchive; user name, Lingtao Wu. Confidential access for peer-reviewers: user ID, USC007; password, usc007.

**Supplemental Information:** Supporting information includes: a) Supplemental Materials and Methods; b) Supplemental Tables (4), and c) Supplemental Figures (3).

tumorigenicity correlated with an impaired osteogenic fate and the ability to initiate tumor growth through generation of CD49f<sup>+</sup> progeny. These findings advance our understanding of OSIC-like properties and, for the first time, provide a much-needed distinction between OSIC and OSFC in this cancer.

### Keywords

Osteosarcoma-initiating cells; self-renewal; tumorigenicity; lineage differentiation; osteosarcoma-forming cells; osteogenic differentiation

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### Introduction

Osteosarcoma, generally considered a disease of cell differentiation (1, 2), is the most common malignant bone tumor (1, 3). Recent findings suggest that non-targeted, cytotoxic chemotherapy can effectively kill the vast majority of osteosarcoma cells; but a small fraction of transformed cells, OSIC, survive and thus instigate osteosarcoma re-initiation, drug resistance, and clinical relapse (2). Hence, identifying OSIC would be crucial steps toward devising OSIC-targeted therapies, thereby avoiding treatment resistance and the severe toxicity associated with current, non-selective forms of osteosarcoma chemotherapy. Investigators focusing on the identification of OSIC have reported that some subpopulations of osteosarcoma cells express prospective cancer stem cell markers, including CD117<sup>+</sup>, Stro-1<sup>+</sup> (4), or CD133<sup>+</sup> (5-7). Moreover, by activating an exogenous *Oct-4* promoter in primary osteosarcoma cells, Oct-4/GFP<sup>+</sup> cells display properties of cancer-initiating cells (8); whereas an osteosarcoma subpopulation with high ALDH activity also exhibits OSIC features (9). To date, the relationships among OSIC self-renewal, tumorigenicity, and lineage differentiation remain unclear, making it difficult to identify and distinguish OSIC from their OSFC for developing OSIC-targeted therapies.

The cancer stem cell hypothesis predicts that only a small fraction of transformed cells are capable of reconstituting all of the diverse cell types within a particular tumor (10, 11), as demonstrated in hematologic malignancies (12-14), central nervous system tumors (15), breast tumors (16), and colon cancer (17). An increasing number of studies show that some tumor cell subpopulations isolated by prospective stem cell markers possess the properties of cancer stem cells; however, these results are often confounded by the ability of marker-negative counterpart subpopulations to induce tumors. This often led to common controversy (11, 18-20) that is currently observed in identifying cancer stem cells (15, 21-23). For example, although the role of CD133 as a marker of cancer stem cells (15, 21) is well documented (11, 19, 20), some studies demonstrate that both CD133<sup>+</sup> and CD133<sup>-</sup> cells can initiate tumor formation (22, 23). Other studies suggest that a combination of CD133<sup>+</sup> with other markers or ALDH activity is needed in identifying cancer stem cells (23-26). These controversies reflect the complexity of cancer stem cells. Here, we used an inverse lineage tracking strategy coupled with serial transplantation to identify OSIC properties. Our studies show that the gain of strong tumorigenicity seen with OSIC-like CD49f<sup>-</sup>CD133<sup>+</sup> cells correlates to diminished osteogenic fate, which distinguish them from

their CD49f<sup>+</sup> progeny that possess limited tumorigenicity in association with more differentiated osteogenic features.

## Results

### Serial xenotransplantation enriches self-renewal and tumorigenicity of osteosarcoma cells

To identify osteosarcoma cells with OSIC properties, we used serial xenotransplantation as a means to enhance OSIC self-renewal and promote lineage differentiation. We first screened tumorigenicity of four human osteosarcoma cell lines and six primary osteosarcoma samples by engrafting them into nude mice with subcutaneous injection. We found that KHOS/NP cells formed tumors at 2 weeks post-injection, U2OS cells at 2 months, and TTC444 cells at 2-3 months (Supplemental Tables 1, 2). Since KHOS/NP cells are virus-transformed cells, whereas well-established U2OS cells afford the best window of time for amplifying the OSIC-like property by serial transplantation, we used these cells to derive different generations of tumor xenografts for *in vivo* analysis of self-renewal and tumorigenicity. The results showed that the self-renewal and tumorigenicity of U2OS cells from primary tumor xenograft to sequential progeny xenografts, designated UT1, UT2, and UT3 cells (Figure 1A), were progressively enhanced. Indeed, a reduction in cell number for transplantation (from  $1 \times 10^7$  to  $1 \times 10^5$  cells) was associated with a reduced time to tumor formation (from 60 days to 7 days). HE staining confirmed that the tumor mass derived from UT2 engraftment retained the same properties as the parental U2OS osteosarcoma xenograft (Figure 1B). These results show that OSIC activity is progressively enhanced by serial xenotransplantation, leading to an enhanced production of OSFC that forming the osteosarcoma mass.

We next examined, using orthotopic transplantation, whether the phenotype of osteosarcoma cells is maintained after serial engraftments. Because UT2 cells offered a better experimental window-time and have a similar tumorigenicity compared to UT3 cells, we intrafemorally injected  $2 \times 10^5$  UT2 cells into mice. Consistent with enhanced self-renewal and tumorigenicity ascribed to UT2 cells (Figure 1A, third engraft), tumor formation in bone was taken 2 weeks (Figure 1C, panel ii). HE staining showed tumor growth in the distal femurs (Figure 1D). These results demonstrate that the enhanced OSIC activity remains capable of producing OSFC progeny able to form phenotypically recognizable osteosarcoma in bone.

### Enriched OSIC activities correlate with accelerated sphere formation and expression of stem/progenitor cell-associated genes

Cancer stem cells often possess the ability to produce unattached spheres in the absence of serum (27, 28). To test the enriched OSIC activities for this property, we cultured cells isolated from different xenograft tissues with basal medium containing methylcellulose. We found that after 12 days of culture, both the size and number of the floating spheres were progressively increased, from U2OS to UT1 to UT2 cells. More than 20 spheres with a 70 to 80- $\mu$ m diameter were observed among each  $10^3$  of the initially seeded UT1 or UT2 cells, in contrast to spheres of <22- $\mu$ m diameter formed by U2OS cells (Figure 2A). This greater stem-like sphere formation suggests that OSIC activities are enriched in UT2 cells.

Because analyses of global gene expression pattern between normal and malignant stem cell populations can provide critical insights into cancer stem cell properties (29), we determined whether enhanced self-renewal and tumorigenicity in UT2 cells correlate with increased expression of stem/progenitor cell-associated genes, using microarray analysis as described (30). Genes whose expression differed from 2 to 1,000-fold in UT2 vs. U2OS cells were identified, with more than 3,000 genes classified as up-regulated in UT2 cells (Supplemental Table 3). Among them, 21 stem or progenitor cell-associated genes were up-regulated (Figure 2B). Moreover, molecules involved in regulation of self-renewal signaling pathways in cancer stem cells (11, 18) were up-regulated in UT2 cells compared to U2OS and normal human mesenchymal stem cells (MSC), including those in the Notch, Wnt, and TGF $\beta$  pathways (Figure 2C-H). These data indicate a genetic basis for the enhanced self-renewal and tumorigenicity in UT2 cells.

### **Enhanced OSIC self-renewal and tumorigenicity induce a progressive expansion of CD49f<sup>+</sup> cells forming the osteosarcoma mass**

Given that cancer stem cells arise from normal stem cells (10, 11), we predict that OSIC retain some normal human MSC properties and intrinsic mesenchymal origin, expressing surface antigens commonly associated with MSC. Thus, enhanced OSIC self-renewal by serial transplantation should increase OSIC propensity to differentiate to OSFC progeny that express stem or progenitor markers and form the osteosarcoma mass. To test this idea, we analyzed the change in expression of different potential phenotypic markers of mesenchymal origin in U2OS, UT2, and UT3 cells by FACS analysis, using anti-human CD28, CD43, CD49f, CD59, CD117, CD133 and STRO-1 antibodies. We found that, while many of these markers showed no or only minor up-regulation in those cells (Figure 3A), the size of the CD49f<sup>+</sup> subpopulation was significantly increased from U2OS to UT2 to UT3 cells (Figure 3B, 3C). Because this increase was associated with enhanced formation of the osteosarcoma mass from U2OS to UT2 to UT3 cells (Figure 1), we further investigated the expression levels of CD49f in primary osteosarcoma cells (TTC444, TTC606, TTC712, TTC595, TTC445, TTC618, TTC814, and CHLA99), MSC, and virus-transformed osteosarcoma KHOS/NP cells. Interestingly, TTC444, KHOS/NP, UT2, and UT3 cells, which show high levels of CD49f expression (Figure 3B-D; Supplemental Figure 1), formed osteosarcoma xenografts within 6-90 days (Figure 1; Supplemental Tables 1, 2). Moreover, higher levels of CD49f<sup>+</sup> cells were inversely related to the time required to form a tumor (Figure 3E, 3F). Since CD49f is a marker of human MSC progenitors (31, 32), our studies suggest that it identifies OSFC with the capacity to proliferate actively to constitute the bulk of the osteosarcoma mass.

### **CD49f<sup>-</sup> cells with greater tumorigenicity and drug resistance generate CD49f<sup>+</sup> subpopulation**

Identification of CD49f<sup>+</sup> cells (Figure 3) forming the osteosarcoma mass raises a question: What subpopulation of U2OS cells possesses OSIC-like properties to give rise to CD49f<sup>-</sup> progeny? Because CD49f<sup>+</sup> cells account for about 70% of the UT2 cells, we first tested *in vitro* whether these cells were derived from the CD49f<sup>-</sup> fraction. We cultured CD49f<sup>+</sup> and CD49f<sup>-</sup> subpopulations of UT2 cells for 2 weeks, and then determined the changes in genetic lineage. FACS analyses showed that the CD49f<sup>-</sup> subpopulation generated about

10% of the CD49f<sup>+</sup> cells, whereas CD49f<sup>+</sup> cells (99.6%) remained no change (Figure 4A, left panel). We then tested the ability of CD49f<sup>-</sup> cells to generate CD49f<sup>+</sup> subpopulation *in vivo*, by subcutaneously transplanting purified CD49f<sup>-</sup> and CD49f<sup>+</sup> subpopulations into NOD/SCID mice. FACS analysis of tumor xenografts showed that the CD49f<sup>+</sup> marker was retained on 99.0% of the xenograft cells transplanted with CD49f<sup>+</sup> subset, whereas in xenografts generated by the CD49f<sup>-</sup> subpopulation, more than 89% of the cells became CD49f<sup>+</sup> positive (Figure 4A, right panel). These data demonstrate that CD49f<sup>-</sup> cells can differentiate to CD49f<sup>+</sup> progeny and that certain factors from *in vivo* vicinity promote tumorigenicity (33) of CD49f<sup>-</sup> cells. Together, the above results suggest that the enriched OSIC-like self-renewal and tumorigenicity in CD49f<sup>-</sup> fraction gives rise to a large number of lineage-committed CD49f<sup>+</sup> cells (Figures 3, 4A) that ultimately form the osteosarcoma mass.

It has been proposed that daughter cells generated from cancer stem cells can differentiate to the various cell types seen in the bulk tumor (11, 34, 35). We therefore examined osteogenic and adipogenic potential of both parent CD49f<sup>-</sup> and daughter CD49f<sup>+</sup> cells. Purified CD49f<sup>-</sup> or CD49f<sup>+</sup> cells, grown to 70~80% confluence in regular medium, were further cultured with BDM or FDM for 21 days for inducing osteogenic and adipogenic differentiation. We found that by using ARS and ORO staining, both CD49f<sup>-</sup> and CD49f<sup>+</sup> cells displayed osteoblast and adipocyte fates, whether they were isolated from UT2 or KHOS/NP cells (Figure 4B). These data suggest that CD49f<sup>+</sup> progeny arising from the CD49f<sup>-</sup> fraction retain essentially the same differentiation potential as their parental cells.

Because CD49f<sup>-</sup> cells differentiate to CD49f<sup>+</sup> cells (Figure 4A), we sought to test the tumorigenicity of both subpopulations in NOD/SCID mice. With subcutaneous injections of 10,000 down to 1,000 cells (3 mice per group), we observed that CD49f<sup>-</sup> cells showed higher tumorigenicity (33%) than did CD49f<sup>+</sup> cells (17%) for up to 32 days (data not shown). Further tests with subcutaneous injection of 500 cells showed that CD49f<sup>-</sup> cells had consistently higher tumorigenic potential (Figure 4C). Since the increase in proportion of tumor formation was not striking (56% vs. 44%), there may be other subpopulations within the CD49f<sup>-</sup> fraction that possess property of OSIC-like tumorigenicity.

Cancer stem cells show greater resistance to drugs and toxins than their more differentiated progeny. We thus assessed the response of both CD49f<sup>-</sup> and CD49f<sup>+</sup> cells to cisplatin or doxorubicin, drugs currently used to treat osteosarcoma (36). The results indicated that the CD49f<sup>+</sup> subpopulation was drug sensitive compared to controls (Figure 4D; Supplemental Figure 2). We further determined the inhibition concentration of drug for 50% of cells (IC<sub>50</sub>) in CD49f<sup>-</sup>, CD49f<sup>+</sup>, and non-sorted (mock) cells using the XTT proliferation assay. The results showed an IC<sub>50</sub> value of 18.9 μM for cisplatin in UT2-CD49f<sup>-</sup> cells, compared to 4.49 μM and 2.88 μM in UT2-CD49f<sup>+</sup> and mock cells (Figure 4E). Similarly, IC<sub>50</sub> value of >30 μM was observed in TTC606-CD49f<sup>-</sup> cells, compared to 13.9 μM and 17.2 μM in TTC606-CD49f<sup>+</sup> and mock cells (Figure 4F). The CD49f<sup>-</sup> cells also showed greater resistance to idarubicin and taxol than did CD49f<sup>+</sup> cells, with IC<sub>50</sub> values that were as much as two times higher than those in CD49f<sup>+</sup> cells (Figure 4G). Because the CD49f<sup>-</sup> subpopulation gives rise to CD49f<sup>+</sup> cells, possesses greater tumorigenicity, and has lower sensitivity to drug treatment than its CD49f<sup>+</sup> daughter cells (Figure 4A, 4C-G), we conclude

that CD49f<sup>-</sup> subpopulation exhibits OSIC-like properties: self-renewal capacity, strong tumorigenicity, differentiation to tumor-forming CD49f<sup>+</sup> progeny, and increased drug resistance.

### CD49f<sup>-</sup> cells generate the CD133<sup>+</sup> subpopulation

Because the intact CD49f<sup>-</sup> population does not show prominent tumorigenicity, the OSIC-like properties likely reside in one or more fractions of these cells. To address this issue, we analyzed a group of markers that have been implicated in cancer stem-like cell behavior. CD133 (alias prominin-1) is a transmembrane protein, and has been classified as a potential marker of osteosarcoma cells with stem-like cell properties (5-7) and of neural and colon cancer cells (15, 17, 37). Moreover, CD117<sup>+</sup> and Stro-1<sup>+</sup> subpopulations of osteosarcoma cells possess the OSIC-like properties of tumorigenicity and drug resistance (4). We thus tested whether CD133, CD117, and/or Stro-1 are potential markers of OSIC-like cells within the CD49f<sup>-</sup> fraction. FACS analysis confirmed that among UT2 cells, about two thirds (63.63% + 5.52%) are CD49f<sup>+</sup> and 30% CD49f<sup>-</sup>; whereas 0.97% CD133<sup>+</sup> cells were in the CD49f<sup>-</sup> subpopulation compared to 5.52% CD133<sup>+</sup> cells in the CD49f<sup>+</sup> subpopulation (Figure 5A). These results indicate that the majority of cells in the CD49f<sup>-</sup> subpopulation lack the CD133<sup>+</sup> marker, and that the CD49f<sup>-</sup>CD133<sup>+</sup> subpopulation accounts for only about 1% of UT2 cells. Interestingly, after culturing of CD49f<sup>-</sup> and CD49f<sup>+</sup> subpopulations for 2 weeks, the proportions of CD133<sup>+</sup> cells increased to 27.8% among CD49f<sup>-</sup> cells and to 8.4% among CD49f<sup>+</sup> cells (Figure 5B, sections 2 vs. 3 and 4). Because CD49f<sup>-</sup> cells differentiate to both CD49f<sup>+</sup> and CD133<sup>+</sup> cells, we suggest that the increased smaller proportion of CD133<sup>+</sup> cells in the CD49f<sup>+</sup> subpopulation (Figure 5B, sections 2 vs. 3) originates from CD49f<sup>-</sup> cells. By contrast, the CD117<sup>+</sup> subpopulation remained essentially unchanged in both the CD49f<sup>+</sup> and CD49f<sup>-</sup> fractions of UT2 cells after 14 days of culture (Figure 5C), whereas Stro-1<sup>+</sup> cells showed a slight increase in the CD49f<sup>-</sup> fraction compared with the CD49f<sup>+</sup> fraction (Figure 5D). This implies that CD133<sup>+</sup> cells within the CD49f<sup>-</sup> fraction are more promising candidate population with OSIC-like properties than CD117<sup>+</sup> or Stro-1<sup>+</sup> cells.

Further to test whether candidate partner of CD49f<sup>-</sup> cells possesses OSIC properties, we examined the tumorigenicity of CD133<sup>+</sup>, CD133<sup>-</sup>, CD117<sup>+</sup>, and Stro-1<sup>+</sup> cells by xenotransplantation of these cells into NOD/SCID mice. The results showed that CD117<sup>+</sup> cells did not initiate tumor formation, whereas Stro-1<sup>+</sup> cells showed low tumorigenicity (Figure 5E). However, while both CD133<sup>+</sup> and CD133<sup>-</sup> populations initiated tumor formation, CD133<sup>+</sup> cells showed stronger tumorigenicity than CD133<sup>-</sup> cells (Figure 5F). Given that CD49f<sup>-</sup> cells can differentiate to both CD49f<sup>+</sup> and CD133<sup>+</sup> cells (Figures 4A; 5B), and that both the CD49f<sup>-</sup> and CD133<sup>+</sup> subpopulations show greater tumorigenicity than either CD49f<sup>+</sup> or CD133<sup>-</sup> cells (Figures 4C; 5F), we suggest that the CD49f<sup>-</sup>CD133<sup>+</sup> phenotype serves as a marker for cells with OSIC-like properties.

### Strong tumorigenicity of CD49f<sup>-</sup>CD133<sup>+</sup> cells correlates with inhibited osteogenic capacity

To explore OSIC-like CD49f<sup>-</sup>CD133<sup>+</sup> properties, we first asked whether the CD133<sup>-</sup> and CD133<sup>+</sup> cells share similar traits of multipotency. After culturing CD133<sup>+</sup> or CD133<sup>-</sup> cells with BDM and FDM for 21 days, we performed ARS and ORO staining for analyzing

osteogenic and adipogenic differentiation. We found that similar to CD49<sup>-</sup> or CD49<sup>+</sup> cells, both osteogenic and adipogenic differentiation were observed in CD133<sup>+</sup> and CD133<sup>-</sup> cells isolated from either UT2 or KHOS/NP cells (Figure 6A). Because CD49<sup>-</sup> cells generate both CD49<sup>+</sup> and CD133<sup>+</sup> subpopulations whereas both CD49<sup>-</sup> and CD133<sup>+</sup> cells are more tumorigenic than CD49<sup>+</sup> and CD133<sup>-</sup> cells, these results suggest that the ability of multipotent differentiation is preserved in both OSIC-like CD49<sup>-</sup>CD133<sup>+</sup> cells and their CD49<sup>+</sup> and CD133<sup>-</sup> progeny.

To test the OSIC-like properties of CD49<sup>-</sup>CD133<sup>+</sup> cells, we compared the tumorigenicity of CD49<sup>-</sup>CD133<sup>+</sup> versus CD49<sup>-</sup>CD133<sup>-</sup> subpopulations of UT2 cells in NOD/SCID mice. The results showed that with transplantation of 500 cells, tumors were induced at day 17 in 3 of 4 mice given the CD49<sup>-</sup>CD133<sup>+</sup> cells, whereas tumors were not induced (0/4) in any of the mice given the CD49<sup>-</sup>CD133<sup>-</sup> cells for up to 6 months (Figure 6B, left panel). HE staining confirmed that the tumor mass derived from CD49<sup>-</sup>CD133<sup>+</sup> xenografts (Figure 6B, middle panel) retained the same properties of the parental U2OS osteosarcoma (Figure 1B). Importantly, analysis of phenotypic changes in CD49<sup>-</sup>CD133<sup>+</sup> xenografts showed that more than 95% of the engrafted cells gained the CD49<sup>+</sup> marker (Figure 6B, right panel), the hallmark of cells constituting the bulk of osteosarcoma (Figures 1,3,4). Hence, CD49<sup>-</sup>CD133<sup>+</sup> cells appear to identify the OSIC-like properties: self-renewal, osteosarcoma initiation, and differentiation to CD49<sup>+</sup> progeny that form the osteosarcoma mass. Because the tumorigenicity of transplanted CD49<sup>-</sup>CD133<sup>+</sup> cells remained less than 100% (Figure 6B, left panel), we conclude that the OSIC-like CD49<sup>-</sup>CD133<sup>+</sup> subset of osteosarcoma cells likely coexists with an additional tumorigenic subpopulation(s) within the CD49<sup>-</sup> fraction.

Since multipotency is an accepted classical feature of cancer stem cells (11), we investigated the potential for bone and fat differentiation by CD49<sup>-</sup>CD133<sup>+</sup> and CD49<sup>-</sup>CD133<sup>-</sup> cells, using ARS and ORO staining analyses. Interestingly, while the two fractions had similar adipogenic capacities (Figure 6C, section 3 vs. 4), the CD49<sup>-</sup>CD133<sup>+</sup> cells showed reduced osteogenic differentiation compared to both CD49<sup>-</sup>CD133<sup>-</sup> cells and MSC in the presence of BDM (Figure 6C, sections 1 vs. 2 and 6). Because this reduction of osteogenic fate in CD49<sup>-</sup>CD133<sup>+</sup> cells correlated with an increased rate of tumor production (Figures 6B, left panel), we investigated osteogenic and adipogenic gene expressions during the transition from U2OS through the UT2 cells, using microarray analysis. We found that in contrast to non-inhibition of adipogenic gene expression, osteogenic gene expression was essentially blocked in UT2 cells compared to U2OS cells (Figure 6D). We further analyzed changes in the expression of osteogenic and adipogenic target genes in both CD49<sup>-</sup> and CD49<sup>+</sup> subpopulations during the transition from U2OS to UT2 cells. qRT-PCR analyses of osteoblastic differentiation genes, osteopontin (OPN) and osteocalcin (OC), showed that in contrast to the adipogenic genes PPAR- $\gamma$  and adipophilin that had only a modest change in CD49<sup>-</sup> and CD49<sup>+</sup> subpopulations, the expression of OPN and OC decreased strikingly over the same transition (Figure 6E vs. 6F). Hence, these findings suggest that the self-renewal and tumorigenicity of OSIC properties exhibited by OSIC-like CD49<sup>-</sup>CD133<sup>+</sup> subpopulation are associated with an inhibited osteogenic potential, in contrast to their CD49<sup>+</sup> progeny that display limited tumorigenicity with more differentiated osteogenic feature.

## The CD49<sup>f</sup>-CD133<sup>+</sup> phenotype identifies a small fraction of transformed cells in patient samples of either primary or metastatic osteosarcoma

Previous studies have suggested that cancer stem cells constitute a small fraction of transformed cells. They are capable of extensive self-renewal and can repeatedly generate copious numbers of progeny that ultimately form the bulk of the tumor mass, while the initial proportion of cancer stem cells remains essentially the same (11). We find that during the transition from the low tumorigenicity of U2OS cells to the high tumorigenicity of UT2 and UT3 cells, OSIC-like CD49<sup>f</sup>-CD133<sup>+</sup> cells consistently represent a much smaller proportion of osteosarcoma cells than do their CD49<sup>f</sup><sup>+</sup> progeny (Figures 4-6). To investigate whether this phenomenon exists in human osteosarcoma patient samples, we first examined CD49<sup>f</sup>- versus CD49<sup>f</sup><sup>+</sup> versus CD49<sup>f</sup>-CD133<sup>+</sup> populations in TTC444 cells, which were derived from the primary bone tumor and their earlier passage was proven to have the tumorigenicity *in vivo* (Figure 3F; Supplemental Table 2). FACS analysis showed that in contrast to the 84.5% of cells expressing CD49<sup>f</sup>, 9.7% and 2.1% expressed CD49<sup>f</sup>- and CD49<sup>f</sup>-CD133<sup>+</sup>, respectively (Figure 7A). Further to determine such populations in osteosarcoma tissues, the paired samples from both primary and concordant metastatic pulmonary sites in 3 osteosarcoma patients were examined with HE staining and immunohistochemical analysis. The results showed that in contrast to scant expression of CD133 in both the primary and metastatic sites, a massive CD49<sup>f</sup> expression was detected in these tissues (Figure 7B-D, left panels). Because osteosarcoma most commonly metastasizes to the lungs (3) whereas cancer stem cells are thought to be responsible for establishing metastasis (38, 39), we examined in these patient tissues whether CD133<sup>+</sup> subpopulation generated by CD49<sup>f</sup>- cells (Figure 5) co-existed with a subpopulation expressing CXCR4, a marker identified with metastatic property (38, 40, 41). Immunohistochemical analysis showed that similar to CD133, CXCR4 was expressed by a small number of localized cells in either the primary or metastatic site (Figure 7B-D, right panels). Together, these results demonstrate that OSIC-like CD49<sup>f</sup>-CD133<sup>+</sup> cells consistently represent small proportion of transformed cells in both the primary and metastatic lesion, whereas CXCR4<sup>+</sup> cells could be a potential tumorigenic subpopulation together with CD49<sup>f</sup>-CD133<sup>+</sup> cells to initiate osteosarcoma growth and induce metastasis. Moreover, OSFC-like CD49<sup>f</sup><sup>+</sup> progeny, making up the tumor bulk whether formed in the primary or metastatic site, could be the progeny derived from the localized OSIC-like parental population.

## Discussion

The identity of the cells giving rise to OSFC has been elusive. By defining the lineage relationship between OSFC and their parental cells using an inverse lineage-tracking strategy, we determined that the CD49<sup>f</sup><sup>+</sup> progeny, which constitute the osteosarcoma mass, are derived from CD49<sup>f</sup>- or CD49<sup>f</sup>-CD133<sup>+</sup> cells (Figures 4A; 6B, right panel). CD49<sup>f</sup> (also called integrin  $\alpha 6$ ) has been used as a CD34-like protein to identify early MSC progenitors with increased clonogenicity in mice (31). Moreover, studies from Rich's group found high levels of CD49<sup>f</sup> expression in surgical biopsy specimens of glioblastoma multiforme (32). We found that although the expanded CD49<sup>f</sup><sup>+</sup> progeny shorten the time of tumor formation (Figures 1, 3), their tumorigenicity and drug resistance are reduced compared to the same features of their parental CD49<sup>f</sup>- cells (Figure 4C-G). Thus,

expansion of the CD49f<sup>+</sup> population is driven by the enhanced self-renewal of their parental CD49f<sup>-</sup> cells, while the limited tumorigenicity of these progeny may be related to expression of the CD49f marker. CD49f<sup>-</sup>CD133<sup>+</sup> cells appear important because they have a greater capacity than CD49f<sup>-</sup> cells to generate CD49f<sup>+</sup> OSFC (Figures 6B vs. 4A) and induce tumors *in vivo* more readily (75%) than the CD49f<sup>-</sup> (56%), CD133<sup>+</sup> (42%), CD49f<sup>+</sup> (44%), CD133<sup>-</sup> (33%), or CD49f<sup>-</sup>CD133<sup>-</sup> (0%) subpopulation (Figures 4C vs. 5F vs. 6B). Those CD49f<sup>-</sup>CD133<sup>+</sup> cells are consistently found as a small fraction within UT2 xenografts, primary tumors, or metastatic lesions and produce an abundance of more differentiated CD49f<sup>+</sup> progeny (Figures 5-7). Moreover, CD49f<sup>-</sup>CD133<sup>+</sup> cells co-exist with a small subpopulation expressing CXCR4, a marker possessing metastatic property of cancer stem cells (38-41), in both the primary and metastatic sites of osteosarcoma patient samples (Figure 7). Altogether, these findings suggest that CD49f<sup>-</sup>CD133<sup>+</sup> cells possess the OSIC-like properties of self-renewal and tumorigenicity, and are capable of initiating and sustaining osteosarcoma growth (Supplemental Figure 3).

The phenotype of cancer stem cells has become increasingly complex, despite the publication of well-designed and properly executed studies (10, 11, 19). The role of CD133 as a robust marker of cancer stem cells is a case in point (18, 19). In some studies, only CD133<sup>+</sup> cells are able to initiate tumors *in vivo*, whereas in others CD133<sup>-</sup> cells also possess tumor-initiating potential (11, 19, 22, 23). Our analysis demonstrates that whereas CD49f<sup>-</sup>CD133<sup>+</sup>, CD49f<sup>-</sup>, CD49f<sup>+</sup>, CD133<sup>+</sup>, and CD133<sup>-</sup> subpopulations can all form osteosarcoma *in vivo*, CD49f<sup>-</sup>CD133<sup>+</sup> cells possess the strongest tumorigenicity and have a substantial capacity to differentiate to CD49f<sup>+</sup> cells (Figures 4-6). Interestingly, while CD49f<sup>-</sup>CD133<sup>+</sup> cells retain the capacity for adipogenic differentiation, their strong tumorigenicity is associated with decreased osteogenic potential (Figure 6B-F). This inverse relationship is observed during the transition from the low tumorigenicity of U2OS cells to the high tumorigenicity of UT2 cells induced by serial transplantation (Figure 1), in which osteogenic but not adipogenic differentiation marker genes are decreased in UT2 or CD49f<sup>-</sup> cells (Figure 6D-F). Thus, our studies reveal that the gain of tumorigenicity correlates with a diminished osteogenic potential in OSIC-like CD49f<sup>-</sup>CD133<sup>+</sup> cells, while the decreased tumorigenicity of OSFC progeny is associated with more differentiated osteogenic features. What, then, is the mechanism of the inhibited osteogenic fate of CD49f<sup>-</sup>CD133<sup>+</sup> cells? One explanation is that CD133 expression promotes the attachment of stem cells to their niche (18, 19), where they could preserve self-renewal potential by sustaining an aberrant differentiation program that represses osteogenic potential, as we hypothesize (Supplemental Figure 3). This hypothesis, although attractive, will require rigorous testing with CD49f<sup>-</sup>CD133<sup>+</sup> versus CD49f<sup>-</sup>CD133<sup>-</sup> cells and more differentiated CD49f<sup>+</sup> progeny.

Currently, the mechanisms of CD49f<sup>-</sup>CD133<sup>+</sup> contributing to OSIC properties remain unknown. Several groundbreaking studies with mouse transgenic models have shown the importance of the Wnt, Hedgehog, Notch, BMI1, and TGF- $\beta$  pathways in activating and maintaining cancer stem cells (19, 37, 42, 43). We found that in UT2 cells generated by serial transplantation (Figure 1), the molecules crucial to mediating Wnt, Notch, and TGF- $\beta$  signaling pathways are up-regulated, compared to findings in MSC and/or U2OS cells (Figure 2B-H). This suggests that in UT2 cells, CD49f<sup>-</sup>CD133<sup>+</sup> subpopulation maintains

these pathways that are known to be associated with cancer stem cells. Hence, determining such molecular basis for self-renewal of the CD49<sup>f</sup>-CD133<sup>+</sup> cells will be an important step toward devising targeted therapeutics that inhibit the tumorigenicity of CD49<sup>f</sup>-CD133<sup>+</sup> cells.

Recent studies from Iwakuma's group show that by using human osteosarcoma KHOS/NP (transformed with Kirsten murine sarcoma virus) and MNNG/HOS (transformed with carcinogenic nitrosamine) cells, both CD117<sup>+</sup> and Stro-1<sup>+</sup> subpopulations exhibit OSIC properties, whereas Stro-1 likely contributes more than CD117 (4). We found that neither CD117<sup>+</sup> nor Stro-1<sup>+</sup> cells show appreciable tumorigenicity compared to CD133<sup>+</sup>, CD133<sup>-</sup>, CD49<sup>f</sup>-, CD49<sup>f</sup>+, or CD49<sup>f</sup>-CD133<sup>+</sup> cells (Figures 5E vs. 4C and 6B). Moreover, whereas unsorted KHOS/NP cells induced tumor formation (Supplemental Table 1), CD117<sup>+</sup>CD49<sup>+</sup> cells isolated from KHOS/NP cells still lack tumorigenicity (0/4 mice; 500 cells/mouse), in contrast to the CD117<sup>-</sup>CD49<sup>f</sup>- population, which induced tumor formation in 2/4 mice tested. We suggest that the discrepancies between Iwakuma's work and ours may result from the differences in cellular background. However, we do not rule out the contribution of the Stro-1<sup>+</sup> marker to the OSIC properties of CD49<sup>f</sup>-CD133<sup>+</sup> cells, as our initial studies indicate that the Stro-1<sup>+</sup> subpopulation isolated from UT2 cells does possess some tumorigenicity. Further dissection of the CD49<sup>f</sup>- subpopulation among primary osteosarcoma cells, using a serial single cell clonogenic assay and human-to-mouse xenotransplantation, should determine the still-missing counterpart(s) of the OSIC-like CD49<sup>f</sup>-CD133<sup>+</sup> cells, leading to identifying of OSIC. Such studies could, in turn, point the way to treatments that eradicate osteosarcoma by expunging its root cause.

Our studies show that the gain of strong tumorigenicity, seen with OSIC-like CD49<sup>f</sup>-CD133<sup>+</sup> cells, correlates with a diminished osteogenic fate. This new phenotype distinguishes CD49<sup>f</sup>-CD133<sup>+</sup> subpopulation from their progeny, CD49<sup>f</sup>+, which compose the bulk of the tumor mass and possess more differentiated osteogenic features with limited tumorigenicity. Moreover, CD49<sup>f</sup>+ progeny remain capable of inducing osteosarcoma *in vivo*. This finding provides new insight that may help to resolve the current controversy over whether some daughter cells or marker-negative counterpart subpopulations of cancer stem cells possess limited tumorigenicity *in vivo*. Hence, these studies advance our understanding of cancer stem cell properties and suggest new approaches to targeted therapy in patients with osteosarcoma.

## Materials and Methods

### Patient samples, cells, and cell culture

Three osteosarcoma patient samples taken before chemotherapy were provided by CHLA Pathology Tissue Bank in accord with the protocol approved by the CHLA Committee on Clinical Investigations. Human osteosarcoma cell lines U2OS, MG63, SAOS-2 were from ATCC (Manassas, VA, USA) and cultured as described (30). UT1, UT2, and UT3 cells were derived from the second, third, and fourth human-to-mouse xenotransplantations of parental U2OS xenograft tissues as described (30). Other primary cells and cell culture are detailed in Supplemental Information.

### **Flow cytometric analysis and cell sorting**

Flow cytometric analysis was performed as described (44), with details in Supplemental Information.

### **Xenotransplantation**

Cells ( $5 \times 10^2$  to  $1 \times 10^7$ ) were injected subcutaneously into the shoulder of 4-6 week old BALB/c nude mice or 7-week old NOD/SCID mice (Jackson Laboratory, Bar Harbor, Maine). All mouse work was performed according to guidelines under protocols approved by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee. More details of animal work are described in Supplemental Information.

### **Intrafemoral implantation**

Seven-week old BALB/c nude mice were anesthetized with a mixture of xylazine and ketamine. The knee of mice was bent and placed facing the experimenter. A 26-gauge needle was then used to perforate the femur head and, through the punctured hole once the needle was removed, a second needle coupled to a 1 ml syringe containing cells ( $1 \times 10^5$  in 20  $\mu$ l of PBS) was carefully introduced into the medullary cavity of the femur. Control groups were injected with PBS. The surgical procedure was completed with a stitch of the knee skin.

### **Drugs and drug resistance test**

Anti-osteosarcoma drugs (36), including cisplatin (Pharmachemie B.V., Haarlem, The Netherlands) and doxorubicin (Ben Venue Laboratories, Benford, OH), as well as other anti-cancer toxins, including taxol (Bristol-Myers Squibb, Princeton, NJ) and idarubicin (Hisun Pharmaceutical, Hangzhou, China), were diluted to final concentrations with corresponding culture medium. Drug resistance assay is detailed in Supplemental Information.

### **Tumor processing and histology**

Tumor processing, hematoxylin-eosin (HE) staining, and immunohistochemical assay were performed by CHLA Pathology Core, as described (30).

### **Sphere culture**

Cells ( $3 \times 10^5$  /ml) were plated into 6-well plate coated with 1.5% Noble Agar (Difco, Detroit, MI) as described (45), with details in Supplemental Information.

### **Microarray and Cluster analyses**

Microarray and cluster analyses were performed as described (30), with more details in Supplemental Information.

### ***In vivo* and *in vitro* tracking of genetic lineage**

Cells were injected subcutaneously into NOD/SCID mice or cultured in dishes. The xenograft tissues or cells were harvested for assessing changes in surface immunophenotypes with FACS using CD49f, CD133, CD117, or Stro-1 antibodies.

## Osteogenic and adipogenic differentiation

As described (46, 47), Alizarin Red S (ARS) and Oil Red O (ORO) (Sigma) were used for differentiation evaluation (see Supplemental Information).

## Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR, using QuantiTect™ SYBR Green PCR kit (Qiagen, Inc., Valencia, CA), is detailed in Supplemental Information.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

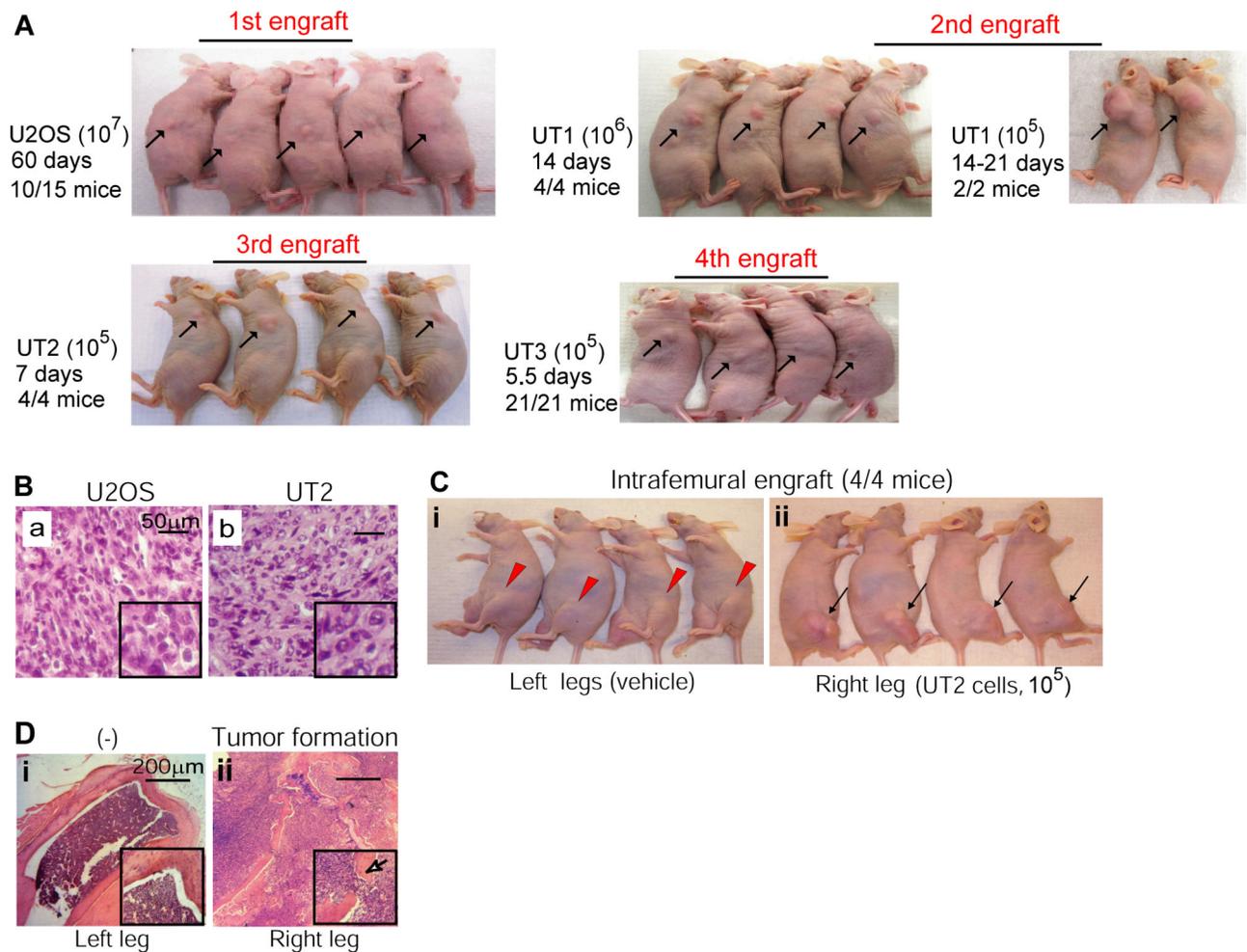
This work was supported by grants from the National Institutes of Health (R01 CA120512 and ARRA-R01CA120512 to Lingtao Wu).

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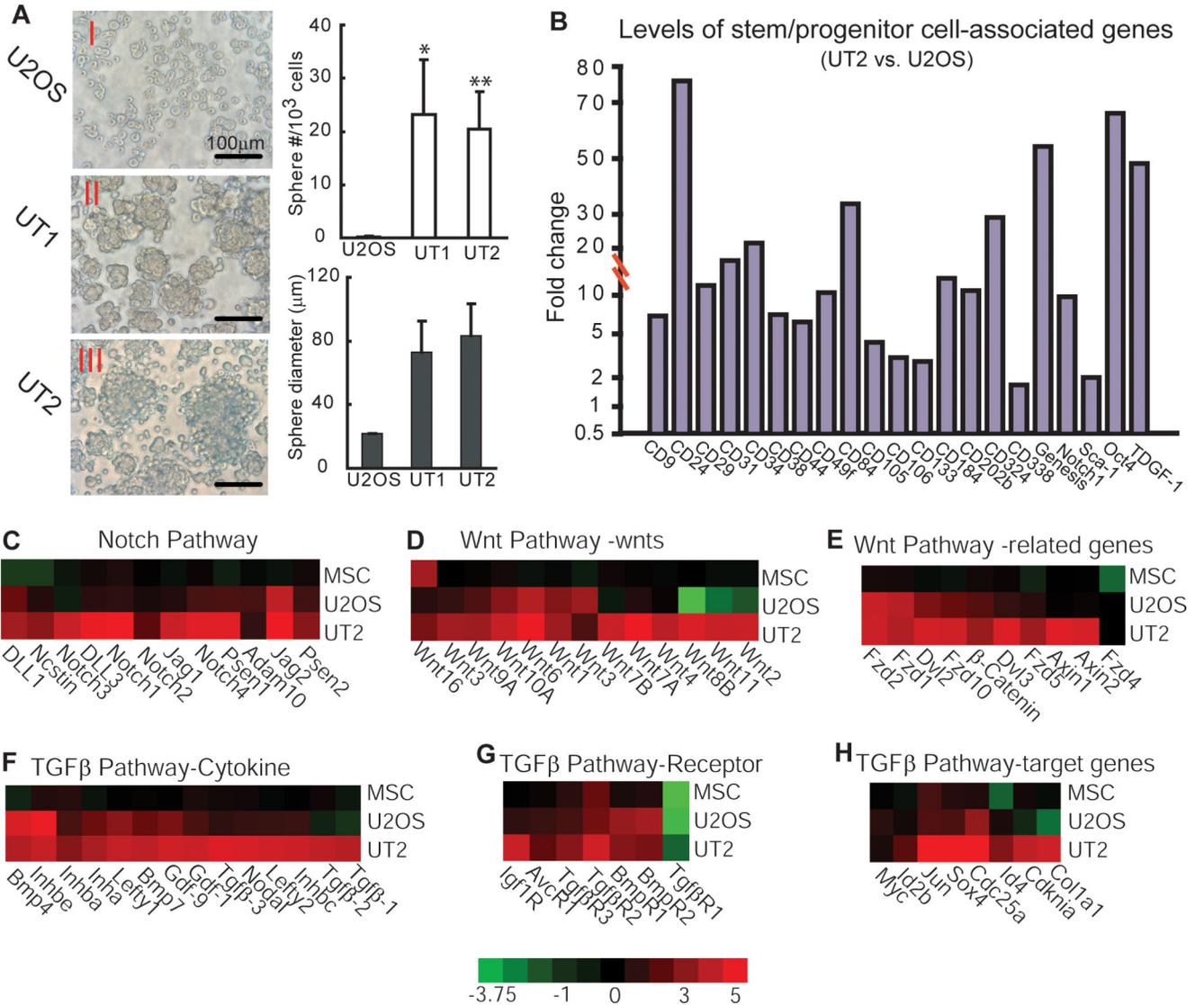
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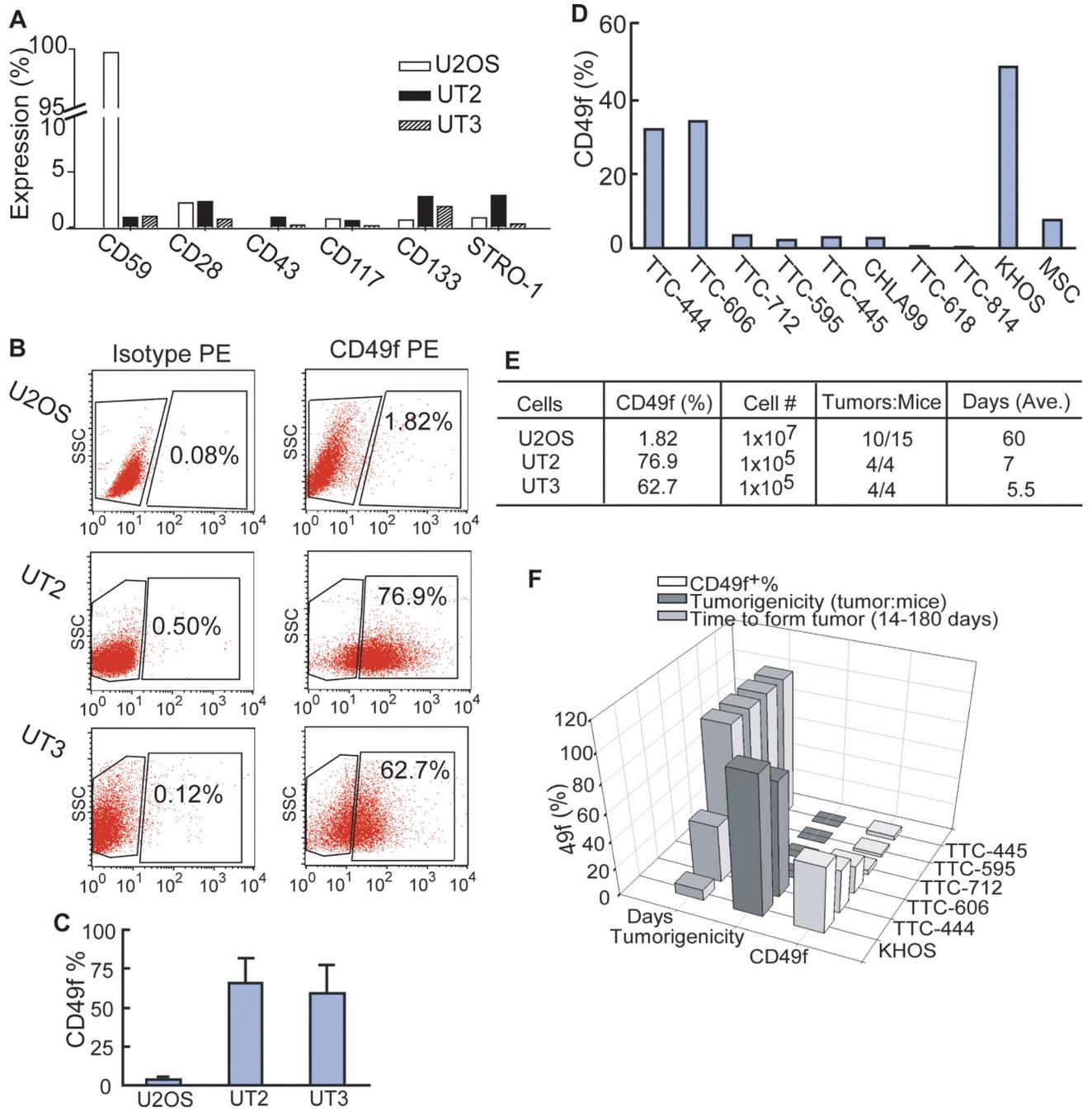
**Figure 1. Serial xenotransplantation enriches self-renewal and tumorigenicity of osteosarcoma cells**

(A) Self-renewal and tumorigenicity were assessed by the amount of cells injected and the time of tumor formation. Cells from the first U2OS xenograft were re-transplanted to yield the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generations of U2OS cells (designated as UT1, UT2, and UT3). (B) HE staining determined that UT2 cells retained histological features of U2OS cells, as reflected by cellular polymorphism, nuclear hyperchromasia, and mitotic activities, together with osteoid formation that recapitulated the hallmark histological phenotype of the parental osteosarcoma. (C) UT2 cells remained capable of tumorigenicity in the bone. Tumor formation occurred in the right legs of mice with injection of UT2 cells (section ii). (D) HE staining of osteosarcoma xenograft formed in the femurs of the right legs (panel C), focally breaching the cortex, and extending into soft tissue (arrow and inset).



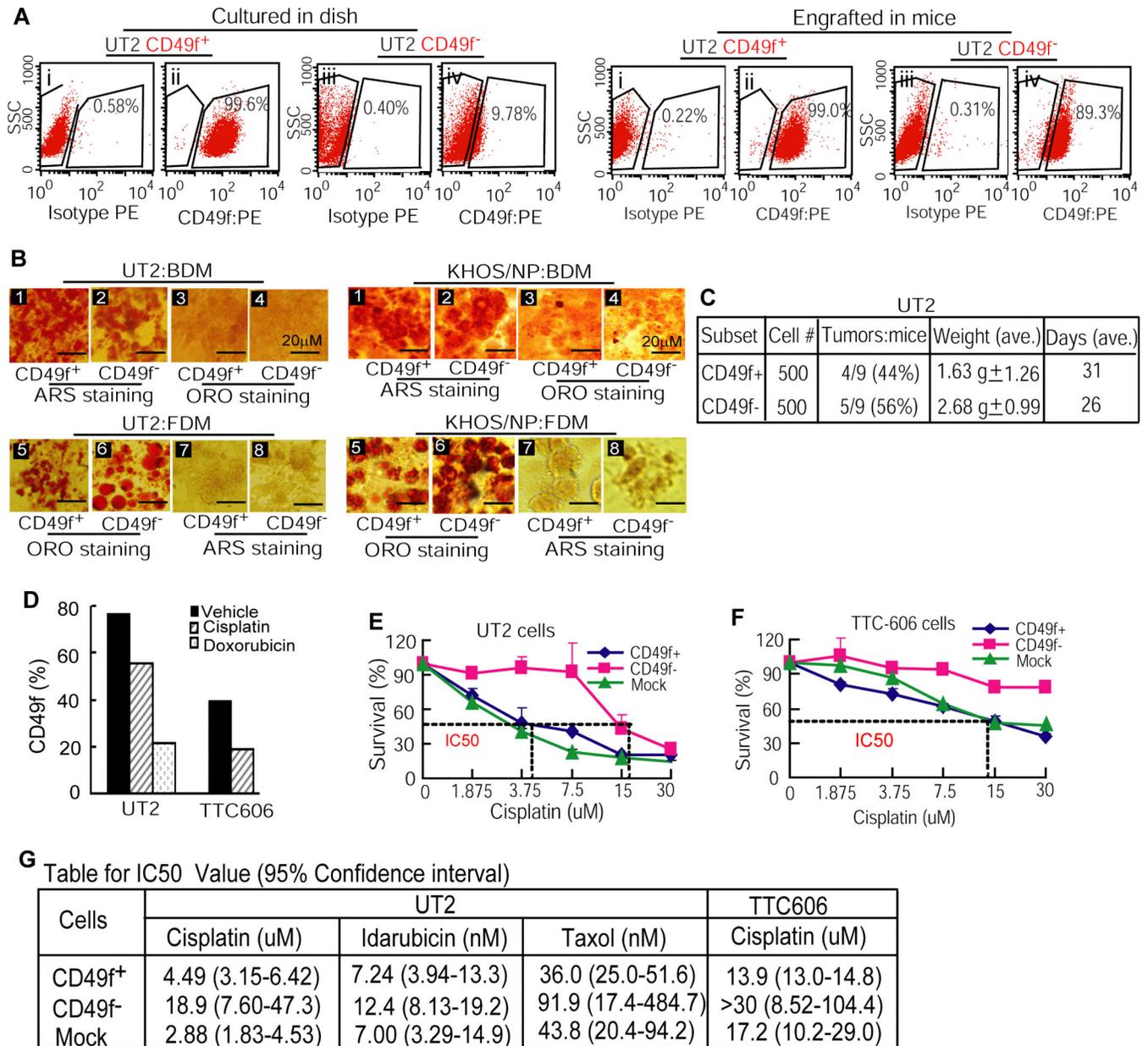
**Figure 2. Enriched OSIC activities correlate with accelerated sphere formation and expression of stem/progenitor cell-associated genes**

(A) Microscopy analysis of spheres cultivated in suspension for 12 days. Representative phase-contrast micrographs of spheres were shown with 400x magnification. UT1 or UT2 vs. U2OS: \*P<0.05; \*\*P<0.01. (B) Up-regulated expressions of stem cell/progenitor marker genes in UT2 cells compared to parental U2OS cells. (C-H) Serial transplantation up-regulated expressions of signaling molecules in the Notch, Wnt, and TGFβ pathways. The color bar represents the expression ratio on a log-2 scale (UT2 vs. U2OS or MSC).



**Figure 3. Enhanced OSIC self-renewal and tumorigenicity induce a progressive expansion of CD49f<sup>+</sup> cells forming the osteosarcoma mass**

(A-C) Expression levels of CD59, CD28, CD43, CD117, CD133 and STRO-1 (panel A) as well as CD49f (panels B, C) were determined by FACS in U2OS, UT2 and UT3 cells. (D) FACS analysis of CD49f expression in normal (MSC), KHOS/NP, and 8 different primary osteosarcoma cells. (E & F) Inverse relationship between CD49f levels vs. time for tumor formation in serially transplanted (panel E) and the first transplanted cells (panel F).



**Figure 4. CD49f<sup>-</sup> cells with greater tumorigenicity and drug resistance generate CD49f<sup>+</sup> subpopulation**

(A) CD49f<sup>-</sup> cells differentiated to CD49f<sup>+</sup> subpopulation in culture (9.78%; left panel) and in NOD/SCID mice (>89%, right panel). (B) Both CD49f<sup>-</sup> and CD49f<sup>+</sup> cells isolated from UT2 (left panel) or KHOS/NP cells (right panel) showed osteogenic and adipogenic potential, as judged by ARS and ORO staining. Cross staining was used as negative control (sections 1, 2 vs. 3, 4; sections 5, 6 vs. 7, 8). (C) CD49f<sup>-</sup> cells showed greater tumorigenicity than CD49f<sup>+</sup> cells. (D) The CD49f<sup>+</sup> subpopulation was decreased in UT2 and primary osteosarcoma TTC606 cells treated with cisplatin or doxorubicin. (E & F) CD49f<sup>-</sup> subpopulation isolated from UT2 (panel E) or TTC606 cells (panel F) resisted cisplatin treatment. Percentages of surviving cells were shown relative to the IC<sub>50</sub> value for

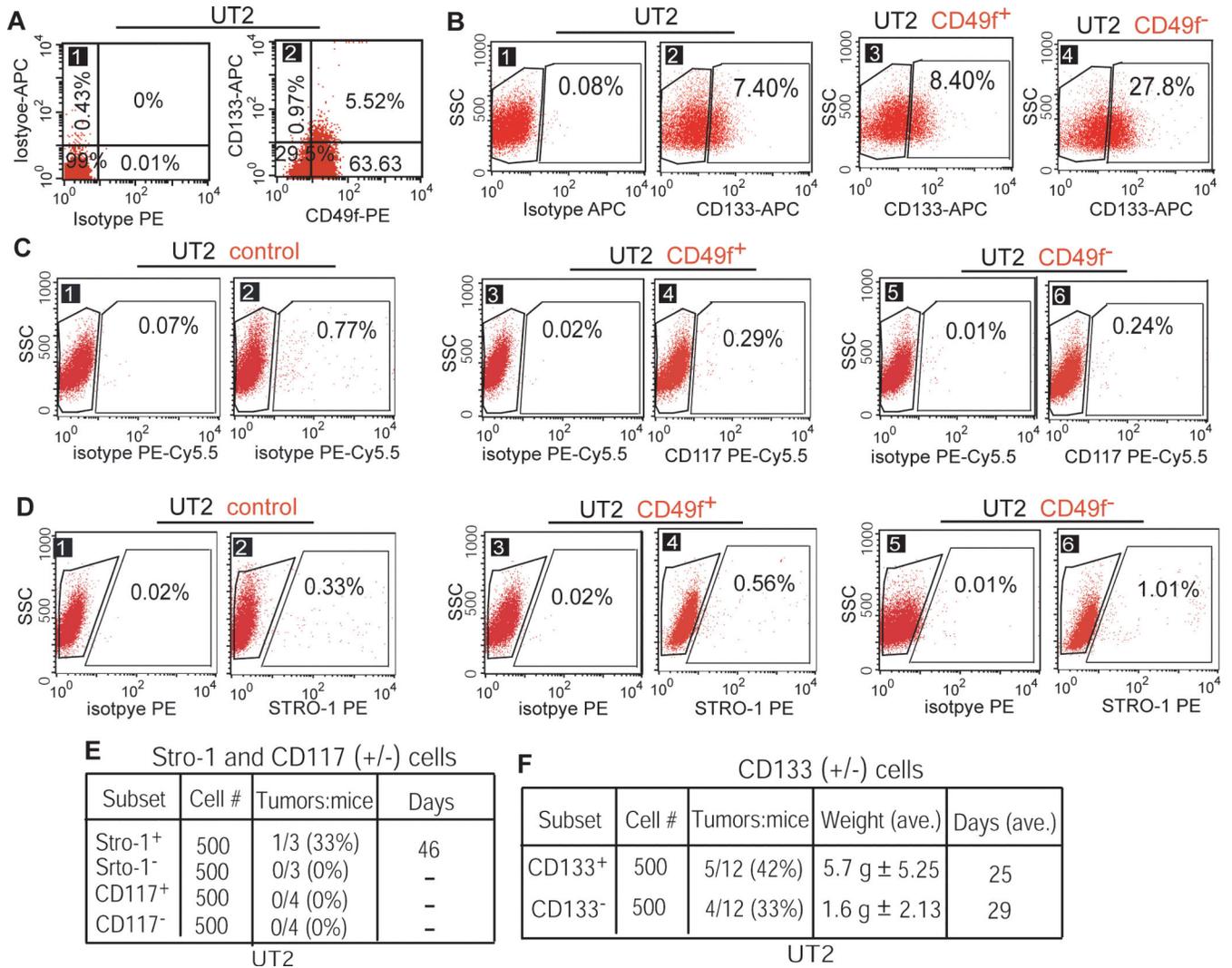
each of cell populations. (**G**)  $IC_{50}$  values for  $CD49f^+$  or  $CD49f^-$  cells treated with different drugs for 48 hr. Data are means with 95% confidence intervals.

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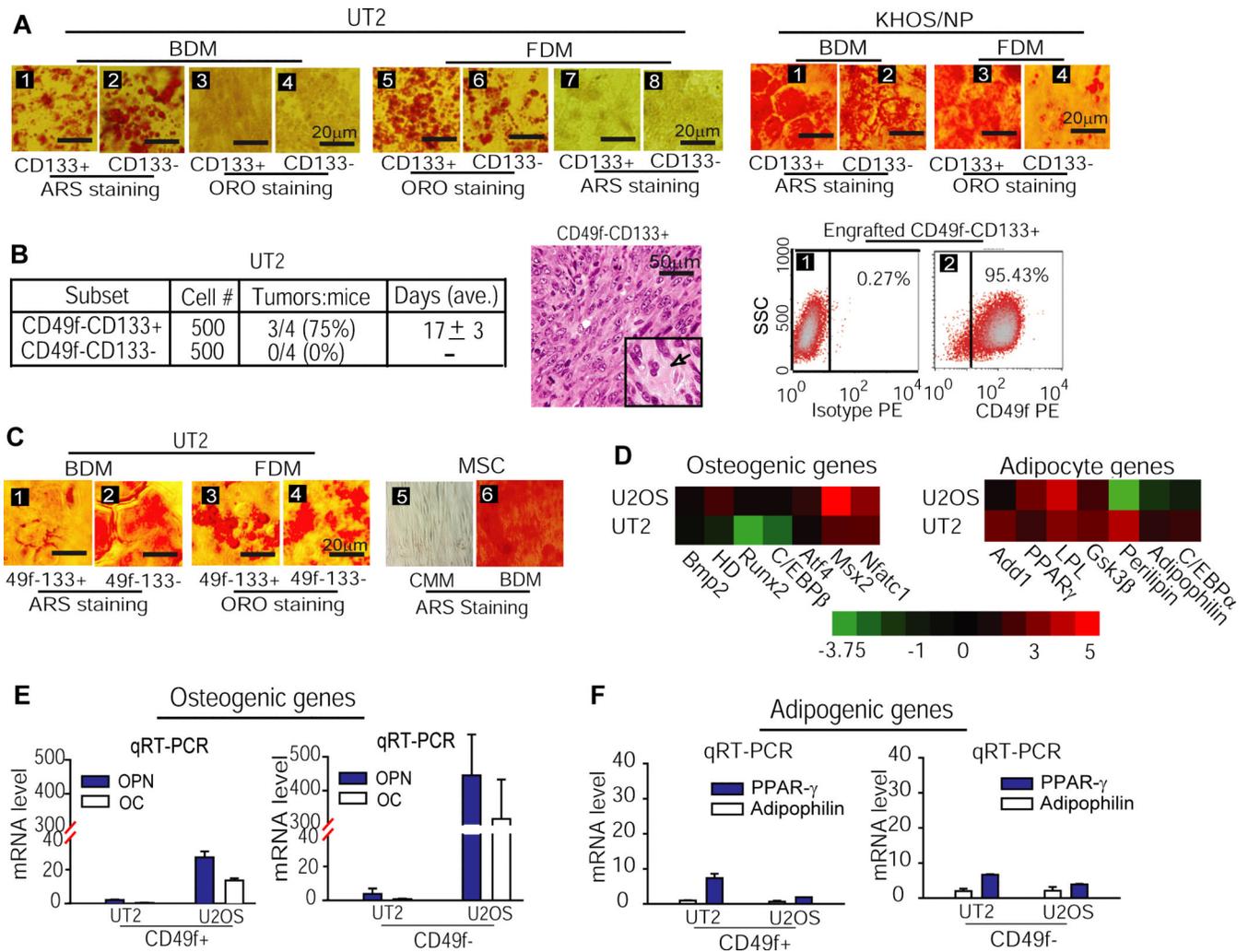
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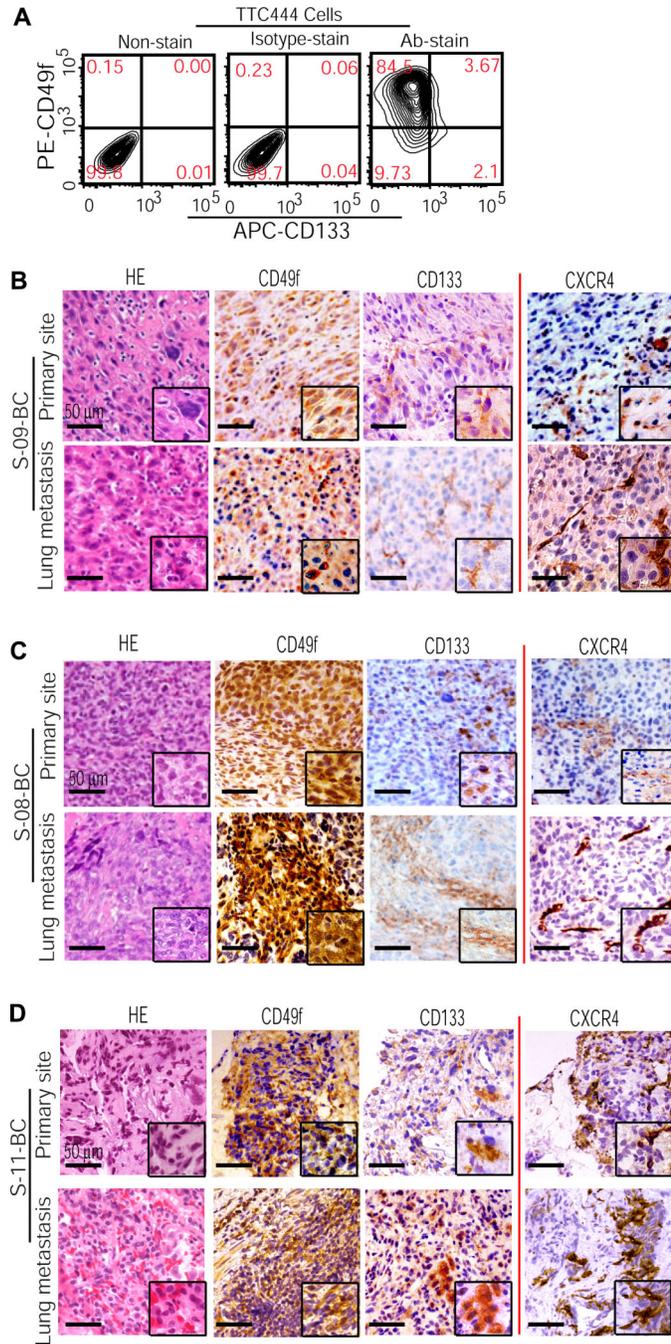
**Figure 5. CD49f<sup>-</sup> cells generate the CD133<sup>+</sup> subpopulation**

(A) FACS analyses of frequencies of the CD49f<sup>-</sup>CD133<sup>+</sup>, CD49f<sup>-</sup>CD133<sup>-</sup>, CD49f<sup>+</sup>CD133<sup>+</sup>, and CD49f<sup>+</sup>CD133<sup>-</sup> subpopulations in UT2 cells. (B) CD49f<sup>-</sup> cells differentiated to CD133<sup>+</sup> subpopulation (27.8%) after 14 days of culture from basal level of 7.4% in UT2 cells, compared to CD49f<sup>+</sup> cells with a slight increase (8.4%). (C & D) CD117<sup>+</sup> (panel C) and Stro-1<sup>+</sup> (panel D) subpopulations remained essentially unchanged after culture of CD49f<sup>+</sup> or CD49f<sup>-</sup> cells for 14 days. (E) CD117<sup>+</sup> cells failed to initiate tumor formation and Stro-1<sup>+</sup> cells showed low tumorigenicity. (F) CD133<sup>+</sup> cells showed higher tumorigenicity than CD133<sup>-</sup> cells.



**Figure 6. Strong tumorigenicity of CD49f<sup>-</sup>CD133<sup>+</sup> cells correlates with inhibited osteogenic capacity**

(A) Both CD133<sup>+</sup> and CD133<sup>-</sup> subpopulations of UT2 (left panel) or KHOS/PN cells (right panel) displayed osteogenic and adipogenic differentiation, as judged by ARS and ORO staining. (B) CD49f<sup>-</sup>CD133<sup>+</sup> cells but not CD49f<sup>-</sup>CD133<sup>-</sup> cells formed tumors in NOD/SCID mice (left panel); HE staining of CD49f<sup>-</sup>CD133<sup>+</sup> xenograft cells showed the histological features of U2OS cells with osteoid (extra-cellular matrix) formation, as reflected by spindle cells with a vesicular nucleus often containing prominent nucleoli, frequent mitotic activities, and focal areas of osteoid formation (middle panel); engrafted CD49f<sup>-</sup>CD133<sup>+</sup> cells differentiated to CD49f<sup>+</sup> cells in mice (right panel). (C) While both CD49f<sup>-</sup>CD133<sup>+</sup> and CD49f<sup>-</sup>CD133<sup>-</sup> cells showed a similar capacity for fat differentiation (sections 3 and 4), CD49f<sup>-</sup>CD133<sup>+</sup> cells showed a reduced potential for bone differentiation, compared to CD49f<sup>-</sup>CD133<sup>-</sup> cells and MSC (sections 1 vs. 2 and 6). (D) Microarray analysis revealed a decreased expression of osteogenic marker genes in UT2 cells. (E & F) qRT-PCR analysis of osteogenic (panel E) and adipogenic marker gene expressions (panel F) in CD49f<sup>-</sup> and CD49f<sup>+</sup> subpopulations.



**Figure 7. The OSIC-like CD49<sup>f</sup>-CD133<sup>+</sup> phenotype identifies a small fraction of transformed cells in patient samples of either primary or metastatic osteosarcoma**  
**(A)** FACS analysis of CD49f<sup>-</sup>CD133<sup>+</sup> versus CD49f<sup>+</sup> cells in TTC444 cells. Ab, antibody.  
**(B-D)** HE staining of the paired tissue samples from both primary and concordant metastatic pulmonary sites in 3 osteosarcoma patients. Parallel immunohistochemical assay detected the expression of CD49f, CD133, and CXCR4 in these samples.