Supporting Information

A) Supplemental Materials and Methods

Culture of human primary cells and KHOS/NP cells. Human osteosarcoma cells derived from patient samples were provided by CHLA Pathology Tissue Bank (TTC444, TTC606, TTC712, TTC595, TTC445, CHLA99, TTC618, and TTC814) and cultured as described (1). KHOS/NP cells transformed with Kirsten murine sarcoma virus (ATCC) were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% penicillin at 37°C with 5% CO_2. Normal human primary mesenchymal stem cells (MSC), purchased from the Tulane University Center for Gene Therapy (TUCGT) under a material transfer agreement, were cultured with MEMα medium with 16.5% FBS, following standard protocols provided by TUCGT as described (1).

Flow cytometric sorting of subpopulations. Flow cytometric analysis was performed by using fluorescence-activated cell sorting (FACS) with FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Different subpopulations of cells were isolated with FACSAria sorting based on the defined surface immunophenotypes. Cells were labeled with different anti-human antibodies, including PE-CD49f (Clone-GoH3, BD Biosciences PharMingen, San Diego, CA), FITC-CD49f (Clone-GoH3, BD Biosciences), CD117 (Clone YB5.B8, BD Biosciences), Stro-1 (Clone-STRO-1, RD systems, Minneapolis, MN), and CD133 (Clone-AC133, Miltenyi Biotech, Germany). Secondary antibodies were R-PE-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL), APC-conjugated goat anti-mouse IgG1 (Southern Biotech), PE/CY5.5-conjugated goat anti-mouse IgG (H+L) (Southern Biotech). Corresponding isotypes conjugated to irrelevant antibodies were used as

controls. For isolation of rare population, e.g., the $CD49f^{-}CD133^{+}$ subset, cells were first suspended in PBS with 0.5% FBS, labeled with anti-CD49f antibodies, and mixed with magnetic microbeads to separate $CD49f^{-}$ and $CD49f^{+}$ subsets first by use of magnet sorter (Miltenyi Biotech, Germany). The subsequent $CD49f^{-}CD133^{+}$ and $CD49f^{-}CD133^{-}$ cells were further sorted on FACSAria using corresponding antibodies. DAPI staining was used to remove dead cells during flow cytometry analysis. The cell purity was evaluated by flow cytometry and the viability was assessed by trypan blue staining. Cells with >80% viability were used for *in vivo* tumorigenic analysis.

Animal works: For injection of small amount of cells, 5 x 10^2 sorted cells were suspended in ECM gel (Sigma, St. Louis, MO) at 1:1 ratio (v/v) at 4°C. Mice were sacrificed at the indicated times. Mice without identified tumor were monitored and sacrificed at 3 months post-injection of cells from different cell lines or 6 months post-injection of primary cells.

Sphere culture. Cells were grown for 12 days in defined serum-free RPMI medium consisting of N2 supplement with 1% methylcellulose (20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 100 μg/ml transferring, 5 μg/ml insulin; Invitrogen, Carlsbad, CA), 10 ng/ml bFGF (Invitrogen), and 10 ng/ml EGF (Invitrogen). Both EGF and bFGF were replaced every other day. Sphere formation was evaluated using the inverted phase-contrast microscope, and single sphere with a diameter larger than 50 μm was counted.

Drug resistance assay. The XTT based proliferation kit II (Roche, Palo Alto, CA) was used in drug resistance assay. Cells were plated into 96-well microtiter plates in triplicate. After 48 hr of drug treatment, the samples were measured with a Multiskan Spectrum

Microplate Spectrophotometer (Thermo scientific, Hudson, NH) using a 450-nm absorbance wavelength and a 655-nm reference wavelength. The concentration of drug inhibition for 50% of cells (IC_{50}) was calculated with Dose-Effect Analysis software with Microcomputers.

Microarray and cluster analysis. MSC, U2OS, and UT2 cells were subjected to HGU133 plus-2 arrays (Affymetrix, Santa Clara, CA, USA). Expression levels of all genes were first normalized against the levels of β-actin expression within the each of the individual array chips. The β-actin-derived expression levels of all genes in control chip were then used as a standard of 1-fold against β-actin-derived expression levels of all genes in different testing chips, respectively. Genes showing ≥ 2-fold changes in expression level were considered to be significantly altered and were subjected to further analysis. The microarray data of selected probe sets were then subjected to cluster analysis using the Gene Cluster (UCB&LBNL, Michael Eisen's lab). The fold-change in signal intensity vs. the average signal intensity of control tissue was analyzed and plotted on a log-2 scale.

Differentiation assays. Different subpopulations of cells were plated into 96-well plates and cultured with the appropriate medium. After reaching 70-80% confluence, the cells were washed and cultured for 21 days with either bone differentiation medium [(BDM; corresponding culture medium supplemented with 10 nM dexamethasone (Sigma, # D2915), 20 mM β-glycerolphosphate (Sigma, # G9891), 50 μM L-Ascorbic acid 2-phosphate (Sigma, # A8960)], or fat differentiation medium [(FDM; corresponding culture medium supplemented with 0.5 μM dexamethasone, 0.5 μM Isobutylmethylxanthine (Sigma, # I5879), 50 μM Indomethacin (Sigma, # I7378)]. Cells were then fixed with 10% buffered formalin and stained with Alizarin Red S (ARS; Sigma) or Oil Red O (ORO; Sigma) as described (2, 3). For internal

controls, cells cultured in FDM were cross-stained with ARS, whereas cells cultured in BDM were cross-stained with ORO.

qRT-PCR. cDNAs were generated from DNase I–treated RNA (iScript; Bio-Rad, Hercules, CA), and qRT-PCR was performed with QuantiTectTM SYBR Green PCR kit. The housekeeping gene GAPDH was used as an internal control. All reactions were performed with an Eppendorf epGradient Mastercycler (Eppendorf, Hamburg, Germany). Two negative controls were carried in parallel through all steps of the experiments. Standard curves for cDNA were composed of three 10-fold dilutions of control cDNA. The primers and amplification conditions are summarized in Supplemental Table 4.

B) Supplemental tables/figures

Cell line	Tumorigenicity	Cell #	Tumors:mice	Days (ave.)
KHOS	High	$1x10^7$	5/5	14
U2OS	Low	$1x10^7$	10/15	60
MG63	No	$1x10^7$	0/3	-
SAOS-2	No	$1x10^7$	0/3	-

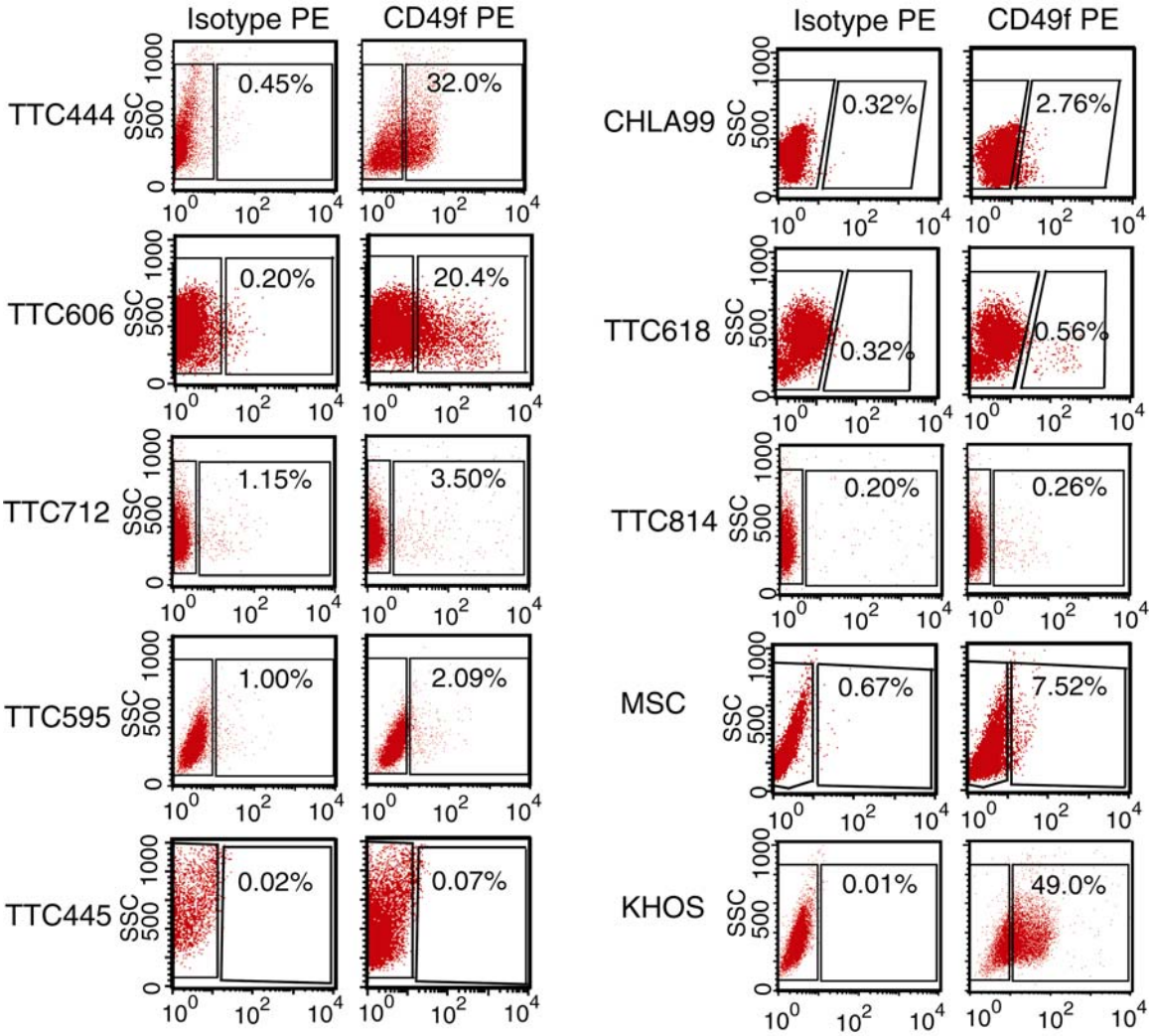
Supplemental Table 1. Tumorigenic capacity of osteosarcoma cells from different osteosarcoma cell lines.

Primary cells	Tumorigenicity	Cell #	Tumors:mice	Days (ave.)
TTC595	No	$1x10^7$	0/2	-
TTC712	No	$1x10^7$	0/2	-
TTC606	No	$1x10^7$	0/2	-
TTC444	Yes	$1x10^7$	5/6	75
TTC441	No	$1x10^7$	0/2	-
TTC445	No	$1x10^7$	0/6	-

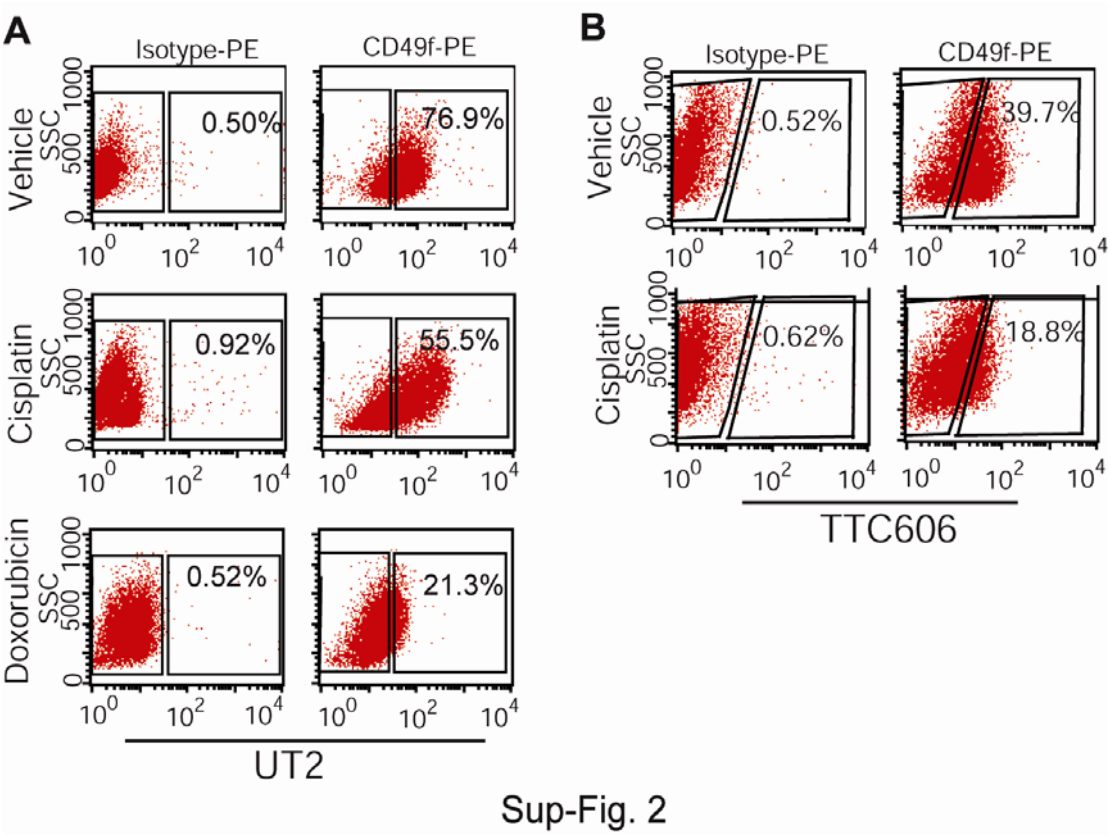
Supplemental Table 2. Tumorigenic capacity of different primary osteosarcoma cells.

Fold change (UT2 vs. U2OS)	Probe set #	Gene and gene family
>2	31492	Genes involved in the major signal transduction (not shown) and those pathways (genes > 10-1,000 folds; see below).
>10	12610	Vascular endothelial growth factor, transmembrane protein, solute carrier family, ring finger protein, interleukin, cyclin-dependent kinase, and alkaline phosphatase.
>100	1867	Integrin, fibroblast growth factor, mitogen-activated protein kinase, zinc finger protein, chromosome open reading frame, collagen, F-box protein, CD14, CD24, CD52, CD53, CD6, CD84, CD86, and CD93.
>1000	24	Zinc finger protein 598, G protein-coupled receptor kinase 5, T cell receptor beta chain, nuclear receptor subfamily 6, and dual specificity phosphatase 6.

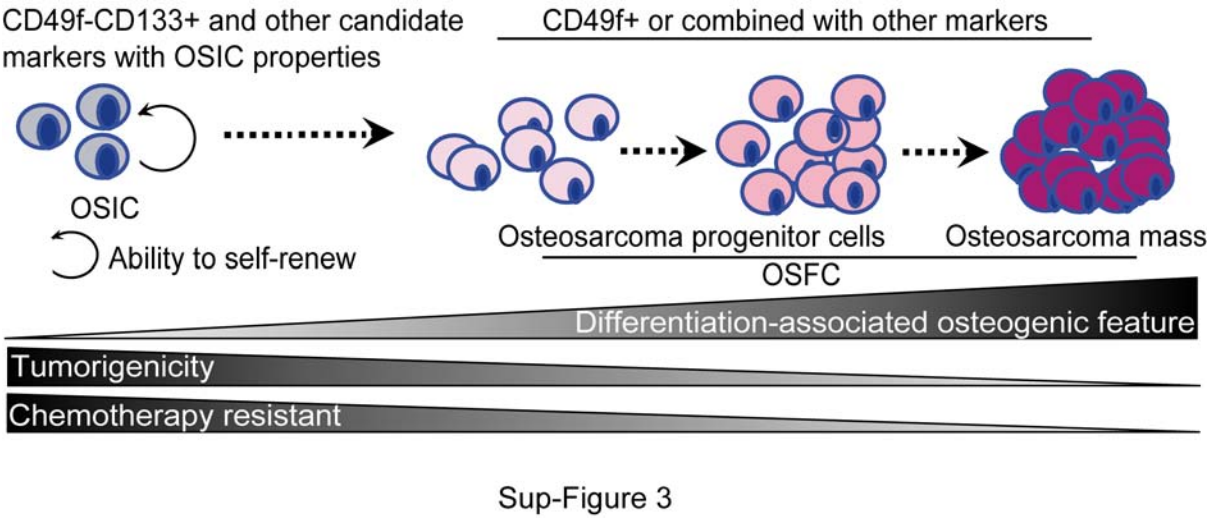
Supplemental Table 3. Up-regulated gene expression in UT2 cells. Profiling of global gene expression was performed by comparing the levels of genes in UT2 cells with the levels of parental U2OS cells. Genes whose expression levels differ at least ≥ two-fold than U2OS cells were shown.



Supplemental Figure 1. FACS analysis of CD49f levels in different primary osteosarcoma cells, normal human MSC, and KHOS/NP osteosarcoma cell line.



Supplemental Figure 2. FACS analysis of CD49f subpopulation in UT2 (A) or TTC606 (B) cells treated with cisplatin or doxorubicin.



Supplemental Figure 3. Model of osteosarcoma initiation and formation. OSIC-like $CD49f^{-}CD133^{+}$ cells exhibit the capacity to self-renew and to initiate tumor growth by producing an abundant of more differentiated OSFC progeny.

Name	GenBank no.	Forward	Reverse
osteopontin	X13694.1	tgaaacgagtcagctggatg	tgaaattcatggctgtggaa
osteocalcin	NM_199173.4	agcaaaggtgcagcctttgt	gcgcctgggtctcttcact
PPAR-γ	NM_005037.5	gcggagatctccagtgatatc	tcagcgactgggacttttct
adipophilin	NM_001122.2	ggcagagaacggtgtgaagac	tctggatgatgggcagagc
GAPDH	NM_002046.3	gtcatccatgacaactttgg	gagcttgacaaagtggtcgt

Supplemental Table 4. Primers used for qRT-PCR amplification. Initial denaturization (95°C for 15 min) followed by consecutive 40 thermal cycles (95°C, 30 s; 58°C, 30 s; and 72°C, 30 s).

References

1. Luo P, Yang X, Ying M, Chaudhry P, Wang A, Shimada H, et al. Retinoid-suppressed phosphorylation of RARalpha mediates the differentiation pathway of osteosarcoma cells. Oncogene. 2010 May 13;29(19):2772-83.
2. Zhang W, Deng ZL, Chen L, Zuo GW, Luo Q, Shi Q, et al. Retinoic acids potentiate BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. PLoS One. 2010;5(7):e11917.
3. Maxson S, Burg KJ. Conditioned media cause increases in select osteogenic and adipogenic differentiation markers in mesenchymal stem cell cultures. J Tissue Eng Regen Med. 2008 Mar-Apr;2(2-3):147-54.