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Journal of Cellular Biochemistry 116:1719–1729 (2015)

Journal of Cellular Biochemistry

CD133$^+$CD44$^+$ Cells Mediate in the Lung Metastasis of Osteosarcoma

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ABSTRACT

CD133 and CD44 are commonly used markers of cancer stem cells (CSCs), which are characterized by their ability for self-renewal and tumorigenicity. However, the clinical value and significance of CD133 and CD44 in lung metastasis of osteosarcoma (OS) remains controversial. The purpose of this study was to investigate whether CD133$^+$CD44$^+$ cells mediates the metastasis of OS. We identified the CD133$^+$CD44$^+$ cells in lung metastatic lesions and OS cell lines, and next demonstrated CD133$^+$CD44$^+$ cells were more aggressive in sphere formation, migration and invasiveness compared with CD133$^+$CD44$^-$, CD133$^-$CD44$^+$, or CD133$^-$CD44$^-$ cells. We finally sorted the CD133$^+$CD44$^+$ and CD133$^-$ CD44$^-$ cells from Saos-2 cell lines, after intratibial xenograft in nude mice, these cells developed primary tumors, and CD133$^+$CD44$^+$ cells are more potential to form lung metastatic tumors. Thus we concluded that CD133$^+$CD44$^+$ cells may mediate in the lung metastasis of OS. J. Cell. Biochem. 116: 1719–1729, 2015.

KEY WORDS: CD133; CD44; OSTEOSARCOMA; LUNG METASTASIS; CANCER STEM CELLS

Osteosarcoma (OS), as the most common, malignant primary bone tumor, typically occurs in children and adolescents [Arndt and Crist, 1999], with an incidence of approximately 3 per million per year in the general population, and 8–11 at 15–19 years of age [Ottaviani and Jaffe, 2009; Ritter and Bielack, 2010]. Despite the involvement of multidisciplinary treatment, including aggressive surgical resection and intensive multiagent chemotherapy [Damron and Pritchard, 1995], 80% of patients would develop recurrence or metastasis after surgical resection [Marina et al., 2004]. The 5-year survival rate of these patients remains less than 40% [Briccoli et al., 2010]. Metastatic lesions at presentation and histological response to preoperative chemotherapy are considered as reliable and reproducible prognostic indicators, as metastasis is the most powerful prognostic indicator [Gorlick and Khanna, 2010]. It is of substantial urgency to identify molecular markers related with the increased potential of metastasis.

Cluster of differentiation 133 (CD133) is recognized as a marker for cancer stem cells (CSCs) [Wu and Wu, 2009]. CD133, as a member of pentaspan transmembrane glycoproteins [Weigmann et al., 1997; Yin et al., 1997], recently has been supposed to be a negative prognosis indicator related with metastasis. Recently, Tan et al. [2014] found CD133 expression was associated with poor differentiation and lymph node metastasis in lung cancer, and Zhang et al. [2014] also reported that A549 CD133$^+$ cells exhibited characteristic of high liver metastatic potential in lung adenocarcinoma. It is also reported clear cell renal cell carcinoma patients with a higher CD133 mRNA in peripheral blood mononuclear cells had a significantly higher recurrence rate than those with a low CD133 mRNA [Feng et al., 2014]. Silinsky et al. [2013] also found CD133$^+$ and CD133$^+$ CXCR4$^+$ cancer cells were correlated with the presence of lymph node metastasis in colorectal cancer, which is one of the strongest negative prognostic factors for colorectal cancer patients. In our previous study, we also have found the expression of CD133 in OS tissues and it was correlated to lung metastasis and poor prognosis in OS patients [He et al., 2012].

Recently, Fujiwara et al. [2014] found that high expression of CD133 in OS was significantly correlated with poor prognosis, and silencing of miR-133a with locked nucleic acid reduced cell invasion, while administrated locked nucleic acid -133a along with chemotherapy in OS-bearing mice, lung metastasis was suppressed and the survival time was prolonged.

Aina He and Xiaojing Yang contributed equally to this work.
The authors declared that they have no conflicts of interest.

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Manuscript Received: 9 October 2014; Manuscript Accepted: 6 February 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 March 2015
DOI 10.1002/jcb.25131 • © 2015 Wiley Periodicals, Inc.

Another cell surface marker, CD44, is a plasma membrane glycoprotein that binds to the extracellular matrix component hyaluronan and has been shown to be involved in metastasis formation in a variety of tumors. CD44 exists on the surface of both hematopoietic cells and non-hematopoietic cells including subsets of leucocytes, erythrocytes, and epithelia [Aruffo et al., 1990; Gunthert et al., 1991; Gunthert, 1993]. CD44 is also present in mesenchymal cells such as fibroblasts, smooth muscle cells, and glial cells of the central nervous system [Kuryu et al., 1999]. As CD44 reacts with the extracellular matrix, several published reports have suggested that CD44 expression is related to metastatic potential, prognosis, and the biologic properties of human malignancies [Boldrini et al., 2010]. It has been identified that CD44 enhances tumor formation and lung metastasis in OS and is an additional predictor for poor patient outcome [Gvozdenovic et al., 2013].

However, detailed analysis by integration of both CD133 and CD44 as markers was rare. We hypothesize that CD133 in combination with CD44 could be more accurate in defining OS that were solely responsible for metastasis. In this study, we found CD133 and CD44 were more intensively expressed in lung metastatic leisure of OS and $CD133^{+}CD44^{+}$ OS cell lines had more efficient sphere formation ability. Our data showed that $CD133^{+}CD44^{+}$ OS cells had enhanced migratory and invasive ability by wound-healing assay and transwell invasion assays, and the consistent results were confirmed in the in vivo experiments. Therefore, we performed the association between CSCs marker CD133, CD44 and the lung metastasis of OS.

METHODS

LUNG METASTATIC LEISURE COLLECTION

OS patients with resectable lung metastatic leisure underwent thoracotomy for resection of suspected pulmonary metastasis, the tissues were collected. Fifty OS tissues were obtained from the department of pathology, the Sixth People's Hospital, Shanghai Jiao Tong University (Shanghai, China) from 2010 to 2011, after their written informed consent. None of the patients received any neoadjuvant therapy. Ethical approval for the study was provided by the local independent ethics committee and informed consent was obtained from each patient or patient's guardian.

HISTOLOGICAL EXAMINATION

Sections were fixed and embedded in paraffin, followed by hematoxylin and eosin (HE) staining and subsequent light microscopy. A pathologist blinded to the study evaluated the microscopy findings.

DOUBLE IMMUNOHISTOCHEMICAL LABELING

Sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide. Then, the sections were processed in 10 mmol/L citrate buffer (pH 6.0) and heated to 121°C in an autoclave for 20 min to retrieve the antigen. Ten percent of goat serum was applied for 1 h at room temperature to block any non-specific reactions. The sections were then incubated overnight at 4°C with anti-CD133 (1:100; Abcam, Hong Kong), anti-CD44 (1:100; Abcam). Sections were followed by a mixture of FITC- and TRITC-conjugated secondary antibodies for 2 h at 4°C. The stained sections were examined with a Leica fluorescence microscope (Olympus BX41, Japan).

WESTERN BLOTTING ANALYSIS

Protein (50 μg) prepared from OS patients' lung metastatic and non-metastatic leisures were loaded per lane and electrophoresed in SDS–PAGE, and then transferred onto polyvinylidene difluoride Immobilon-P membrane (Bio-Rad, CA) using a transblot apparatus (Bio-Rad). The membranes were blocked in 10 mmol/L Tri–HCl (pH 8.0), 150 mmol/L NaCl, and 0.05%Tween 20 (TBST) with 5% (w/v) non-fat milk at room temperature, followed by overnight incubation at 4°C with primary antibodies diluted in TBST (1:1,000 for CD133, Abcam; 1:1,000 for CD44, Abcam; 1:1,000 for β-actin, Cwbiotec, Beijing, China). After washing with TBST, the membranes were incubated for 1 h with an HRP-conjugated secondary antibody diluted 1:5,000 in TBST, and the labeled proteins were detected by using the enhanced chemiluminescence reagents and exposed to the film.

CELL CULTURE

Three OS cell lines MG-63, Saos-2, U2OS (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies), supplemented with 10% fetal bovine serum (FBS; GibCo BRL, Grand Island, NY), 2 mM L-glutamine, 100 units/ml penicillin-G, and 100 mg/ml streptomycin at 37°C and 5% CO_2.

SINGLE CELL SUSPENSIONS PREPARATION

We chose four OS patients with lung metastasis and the lung tissue of each patient were minced and incubated overnight in DMEM with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 μg/ml of both penicillin and streptomycin (Cellgro, Mediatech, Inc., Manassas, VA) in a Heracell CO_2 incubator at 37°C and 5% CO_2. Lung tissue cells were purified using 5 ml Ficoll-Paque PLUS solution (GE Healthcare, USA) to remove dead cells and then stained with antibodies against CD133 and CD44.

FLOW CYTOMETRY

To measure the proportions of $CD133^{+}CD44^{+}$ cells in single cell suspensions from lung metastatic leisure and human OS cell lines MG63, Saos-2, U2OS, cells were detached using 0.02% EDTA in phosphate-buffered saline (PBS), counted and washed in PBS. At least 105 cells were incubated with CD133/1 (AC133)-VioBright FITC (1:100, Miltenyi Biotec, CA) and CD44-PE (1:100, Miltenyi Biotec) in 4°C for 60 min in the dark. After washing steps, the labeled cells were analyzed by flow cytometry (Beckman Coulter, CA).

CELLS IMMUNOFLUORESCENCE STAINING

Cells cultured in 6-well plates were fixed in 4% paraformaldehyde for 30 min at 4°C, washed in PBS, treated with PBS supplemented with 1% BSA for 1 h at room temperature and then stained with CD133 antibody (1:100, Abcam) at 4°C overnight. Goat anti-Rabbit IgG-FITC (Cwbiotec) was used as a second antibody, followed with CD44 antibody (1:100, Miltenyi Biotec) at room temperature for 2 h.

The nuclei were stained with DAPI. Cells were then washed and observed under the fluorescence microscope (Olympus BX41).

MAGNETIC BEADS ISOLATION

Selection of CD133+ cells was performed using a CD133 MicroBead Kit (Miltenyi Biotec) according to the instructions. Next, $CD133^+CD44^+$ and $CD133^+CD44^-$ cells were isolated from the CD133+ cell population using CD44 MicroBeads (Miltenyi Biotec), $CD133^-CD44^+$ and $CD133^-CD44^-$ cells were isolated from the $CD133^-$ cell population using CD 44 MicroBeads.

SPHERE ASSAYS

$CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells were plated at a density of 60,000 cells/well in 6-well ultra low attachment plates (Corning, Inc., Corning, NY) in DMEM cell medium, supplemented with 1% methylcellulose, progesterone (10 nM), putrescine (50 mM), sodium selenite (15 nM), transferrin (13 mg/ml), insulin (10 mg/ml; Sigma) and human EGF (10 ng/ml) and human bFGF (10 ng/ml; Sigma). Fresh aliquots of EGF and bFGF were added every other day. After culture for 72 h, spheres were visible at inverted phase-contrast microscope (Nikon TS 100, Nikon) larger than 0.1 mm were counted. The experiments were repeated at least three times.

SCRATCH WOUND-HEALING ASSAY

Migration ability was determined using a scratch wound-healing assay. $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells were seeded and grown to confluence, and then scratches were made on the cell layer with a pipette tip running across the dishes. Plates were washed twice with fresh medium to remove non-adherent cells. Three to four different locations were visualized and photographed at 0 and 24 h later under a phase-contrast inverted microscope (Olympus BX41). The distance between the two edges of the scratch was measured [Li et al., 2011].

MATRIGEL INVASION ASSAY

Cell invasion was performed using 24-well transwells (8 mm pore size, Corning, Inc.) coated with matrigel (1 mg/ml, BD, NJ, USA) in triplicate. $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells (10^5 per well) were seeded in the upper chambers in culture media containing 0.2% FBS, and the lower chambers were filled with 500 μl 10% FBS medium to induce cell migration. Following incubation for 24 h, cells inside the chamber were wiped off with a cotton swab, invading cells were stained with Giemsa (Lexiang Biotec, Shanghai, China) and examined under microscopy (Olympus BX41). Cells in at least six random microscopic fields (200×) were counted to determine relative invasive potential.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA of $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells were extracted using TRIzol reagent (Ambion, Austin, TX) and treated with RNase-free DNase (DNase I, Ambion) to remove potential genomic DNA contaminants. One microgram total RNA was reverse-transcribed with RETROscript™ two-step RT-PCR system (Ambion). Reactions were performed according to the manufacturer's instructions by using SYBR green PCR supermix (Sangon Biotec, Shanghai, China) in a single-color RT-PCR detection system (Stratagene, Santa Clara, CA). Gene expression level (mRNA) of octamer-binding transcription factor 4 (Oct-4), NANOG, and metastasis-related receptor C-X-C chemokine receptor type 4 (CXCR4) were normalized to that of GAPDH and β-actin transcript. Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology Information, USA) were used to design primer pairs for RT-PCR reactions (Table I).

TABLE I. Please provide the caption for Table I

Gene (GenBank accession)	Sequence (5′–3′)	Tm (°C)	Location
Oct 4 (NM_002701)	CTTGAATCCCGAATGGAAAGGG	61	42–63
	GTGTATATCCCAGGGTGATCCTC		205–183
NANOG (NM_024865)	TTTGTGGGCCTGAAGAAAACT	61	83–103
	AGGGCTGTCCTGAATAAGCAG		198–178
CXCR4 (NM_003467)	TGACGGACAAGTACAGGCTG	61	215–234
	AGGGAAGCGTGATGACAAAGA		277–257
GAPDH (NM_002046)	AAGGTGAAGGTCGGAGTCAAC	61	7–27
	GGGGTCATTGATGGCAACAATA		108–87
β-actin (NM_001017992)	CTCGACACCAGGGCGTTATG	61	116–135
	CCACTCCATGCTCGATAGGAT		229–209

INTRATIBIAL HUMAN OS XENOGRAFT MODEL IN SCID MICE

Sixteen male nude (BALB/c nu/nu) mice, 4- to 6-week old, were purchased from the Chinese Academy of Sciences and housed under pathogen-free conditions in individual ventilated cages. Sterile food and water were provided ad libitum. All animal studies were approved by the local Committee for Animal Experiments. Prior to injection, 16 nude mice were randomized to two groups. A total of 5×10^5 $CD133^+$ $CD44^+$ or $CD133^-$ $CD44^-$ cells SaoS-2 cells in 100 μl PBS were injected intramedullary into the tibia of individual mice were orthotopically injected into the medullar cavity of the tibia of individual mice.

The health status of the mice was monitored three times a week and primary tumor growth examined by calculating the tumor volume from the measurements of the legs with a caliper. Tumor volume was calculated from caliper rule measurements of the width and the length of the tumor-bearing tibia using the following formulas: leg volume = length × $(\text{width})^2 \times 0.5$; tumor volume = leg Volume on day X − leg Volume on day 0; (X = 7,14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days after tumor cell injection). All mice were sacrificed on experimental day 84 and the organs were prepared as reported [Yang et al., 2003]. In vivo lung metastatic capability was evaluated in nude mice, and whole lungs were resected and photographed.

STATISTICS

Comparisons of the experimental groups were performed using the independent-samples *t*-test. Statistical analyses were conducted using SPSS 16.0. Data were expressed as the mean ± SEM. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

DETECTION OF $CD133^+$ AND $CD44^+$ CELLS IN LUNG METASTATIC LEISURE

To identify whether $CD133^+$ or $CD44^+$ cells were involved in the lung metastasis of OS, we collected lung metastatic leisures from OS patients who received lung surgery (Fig. 1A). We investigated the

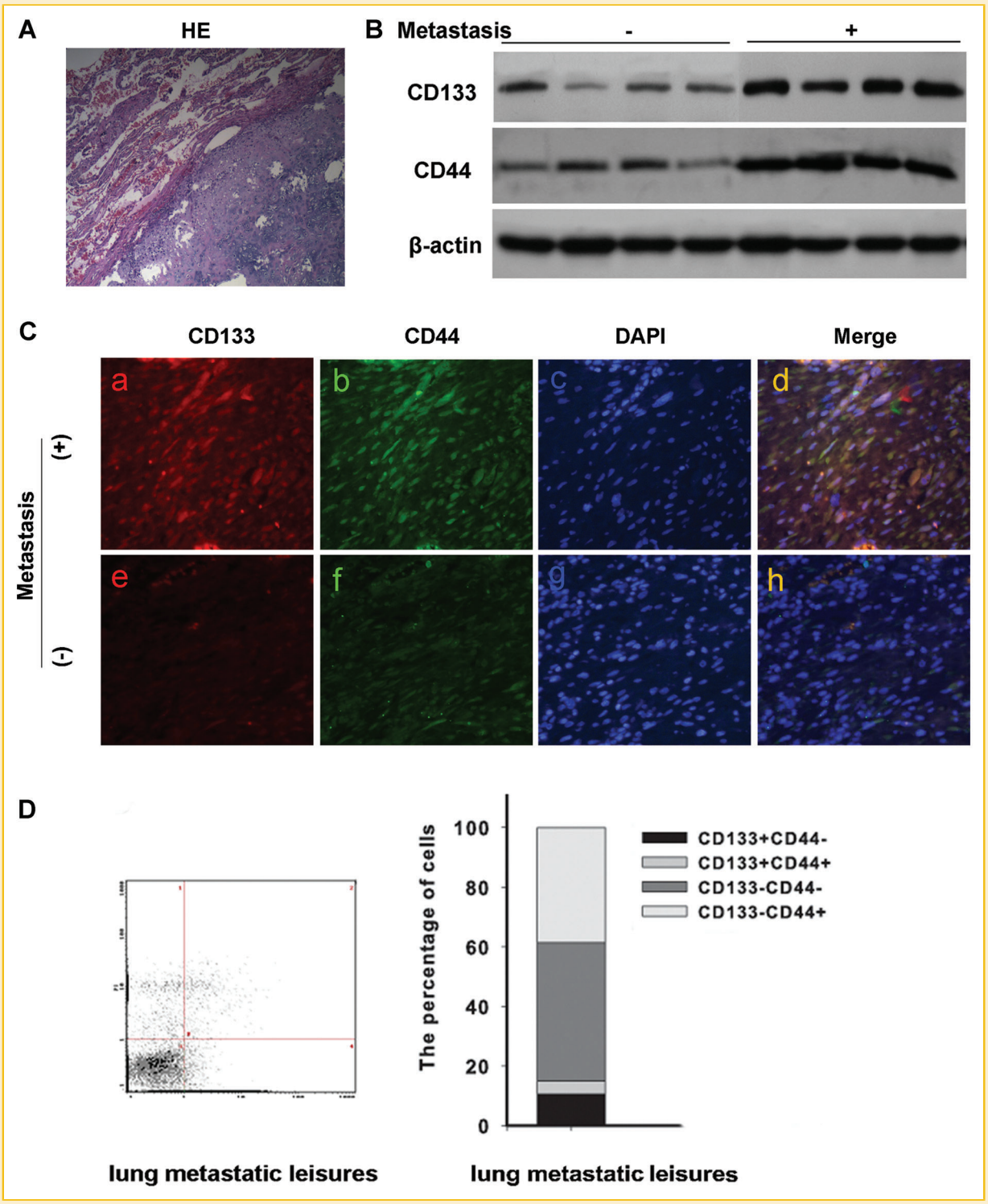

Fig. 1. Detection of $CD133^+$ and $CD44^+$ cells in lung metastatic OS leisure. A: HE staining confirming the lung metastatic OS leisure. B: The expression of CD133 and CD44 protein in four lung metastatic and four non-metastatic tissues, analyzed by Western blotting. β-actin was used as an internal control. C: A double immunohistochemical labeling of CD133 and CD44 in metastatic and non-metastatic leisures. D: Detection of $CD133^+$ and $CD44^+$ cells in single cell suspensions obtained from lung metastatic leisures by flow cytometry.

relative levels of CD133 and CD44 expression in metastatic and non-metastatic leisures, and found CD133 and CD44 were more intensively expressed in lung metastatic leisure (Fig. 1B). A double immunohistochemical labeling was performed on metastatic and non-metastatic leisures (Fig. 1C). The results showed that CD133 and CD44 were remarkably elevated on metastatic leisures, compared with the non-metastatic leisures (Fig. 1C). Moreover, CD133 and CD44 were co-expressed in the same tissue (Fig. 1C). Further, $CD133^+$ and $CD44^+$ cells were also identified among single cell suspensions obtained from lung metastatic leisures (Fig. 1D), suggesting that they may be important seeding cells of lung metastasis.

DETECTION OF $CD133^+CD44^+$ CELLS IN OS CELL LINES

To confirm the existence of $CD133^+CD44^+$ cells in OS, we examined the expression of CD133 and CD44 in OS cell lines by flow cytometry. As shown in Figure 2, the percentage of $CD133^+CD44^+$ subgroup cells in MG-63, Saos-2, U2OS were 4.40 ± 0.52, 3.76 ± 0.45, and 3.66 ± 0.78, respectively (Fig. 2A). Following isolation using magnetic beads, we sorted $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells from OS cell line Saos-2 by MACS, and further confirmed by immunofluorescence staining (Fig. 2B).

SPHERE FORMATION ABILITY OF $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$, AND $CD133^-CD44^-$ CELLS

Sphere formation assay was then performed to determine the self-renewing capability. We observed that $CD133^+CD44^+$ cells were more efficient in generating spheres in all of the three OS cell lines (Fig. 3).

MIGRATION ABILITY OF $CD133^+$ $CD44^+$, $CD133^+$ $CD44^-$, $CD133^-$ $CD44^+$, AND $CD133^-$ $CD44^-$ CELLS

Migration ability of the two cells was examined by a wound-healing assay, as shown in Figure 4. Following incubation of physically wounded cells for 24 h, $CD133^+CD44^+$ cells had traveled a significantly longer distance than $CD133^+CD44^-$, $CD133^-CD44^+$, $CD133^-CD44^-$ cells ($P < 0.05$), respectively in MG-63, Saos-2, U2OS (Fig. 4).

INVASIVENESS OF $CD133^+$ $CD44^+$, $CD133^+$ $CD44^-$, $CD133^-$ $CD44^+$, AND $CD133^-$ $CD44^-$ CELLS

To analyze invasiveness, we performed transwell invasion assays using cell culture inserts covered with extracellular matrix components. 9.00 ± 1.16 $CD133^+CD44^+$ cells while only 5.00 ± 0.06 $CD133^-CD44^-$ cells per field traveled through the membranes in MG-63, and 12.00 ± 1.53 versus 5.00 ± 0.58 in Saos-2, 11.67 ± 1.76 versus 6.00 ± 0.58 in U2OS, respectively ($P < 0.05$, Fig. 5).

mRNA EXPRESSION OF OCT-4, NANOG AND CXCR4 IN $CD133^+$ $CD44^+$ CELLS AND $CD133^-$ $CD44^-$ CELLS

As CD133 and CD44 have been considered as CSC markers in many kinds of tumors, we detected mRNA expression of the stem gene Oct-4 and NANOG, and also metastasis-associated receptor CXCR4 by using RT-PCR in $CD133^+CD44^+$ population and its counterpart

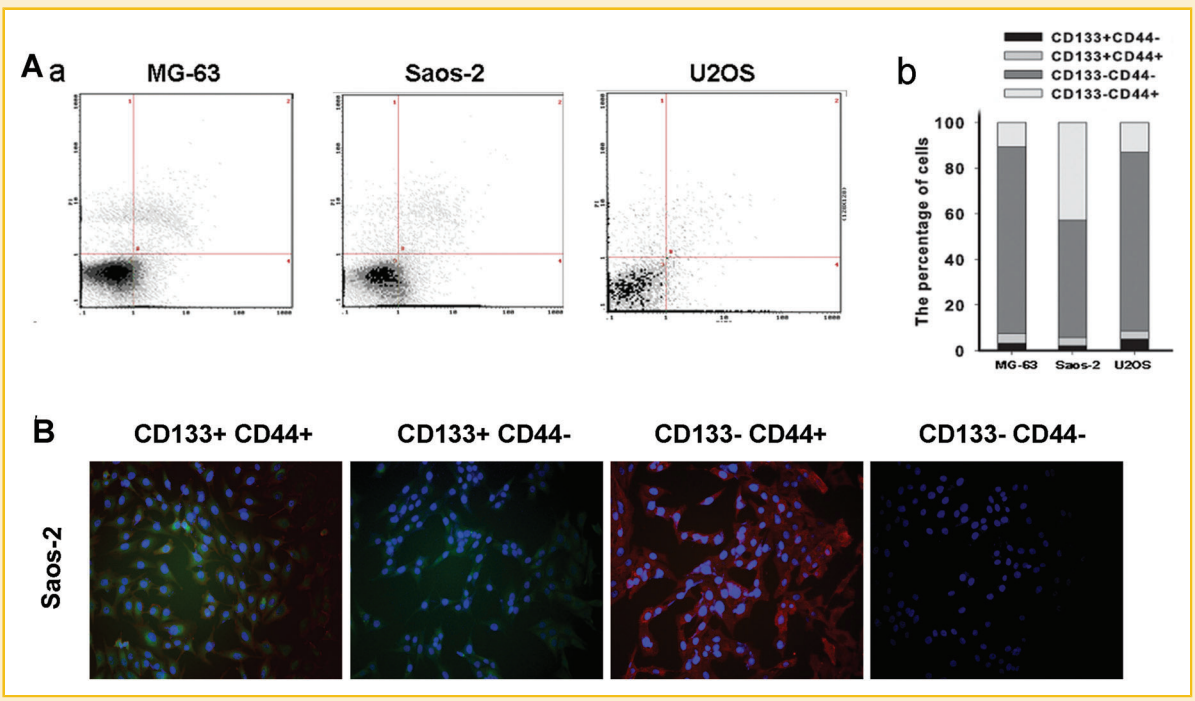

Fig. 2. Detection of $CD133^+CD44^+$ cells in OS cell lines. A (a): Flow cytometry analysis of $CD133^+CD44^+$ cells in MG-63, Saos-2, U2OS cell lines. (b): Percentages of $CD133^+CD44^+$ in the three cell lines. B: $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$ cells and $CD133^-CD44^-$ Saos-2 cells under fluorescence light (magnification, 200×). Data are shown as the mean ± SEM from three separate experiments. $^*P < 0.05$.

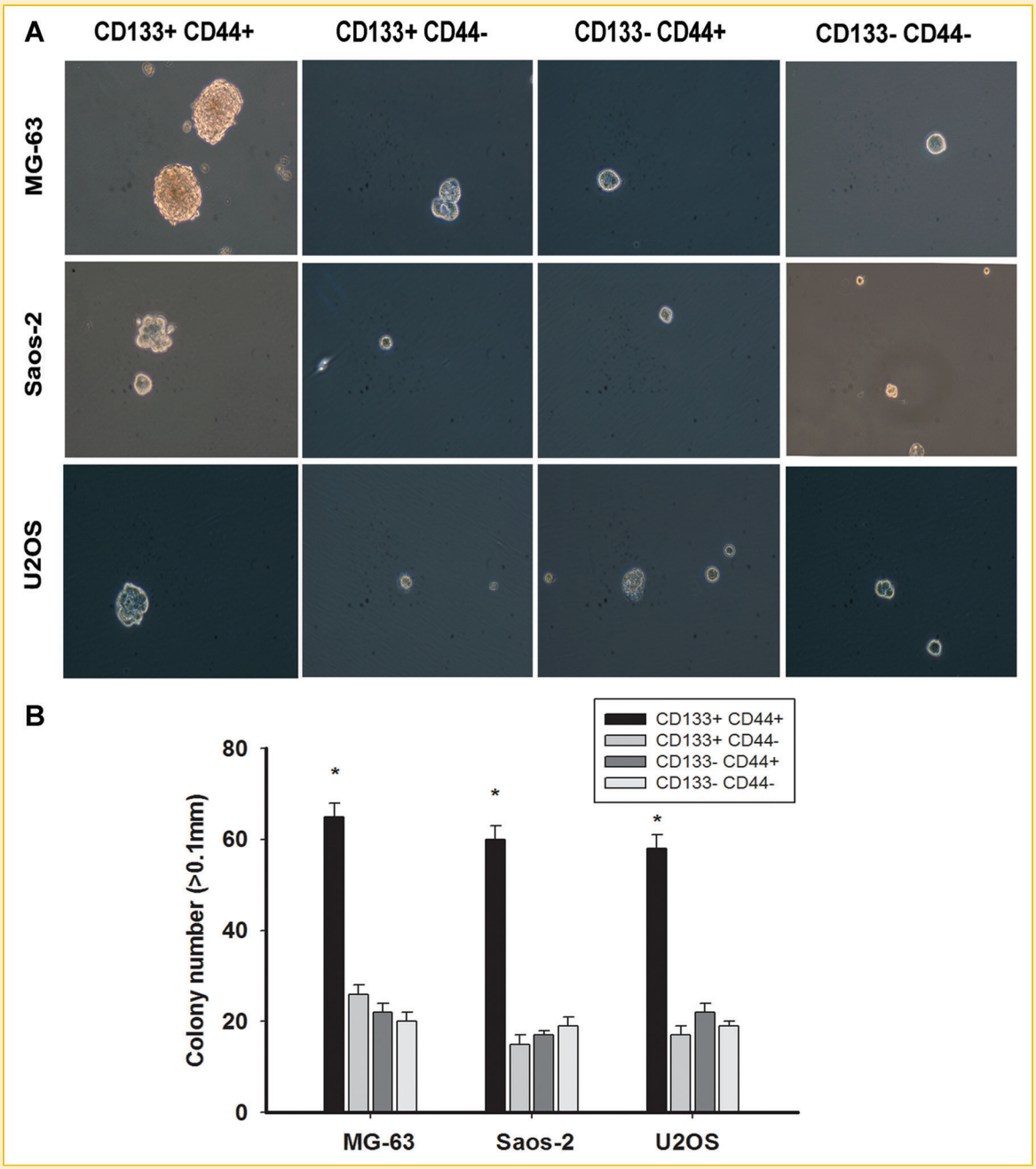

Fig. 3. Sphere clusters formed by $CD133^+CD44^+$ cells in OS cell lines. A: Sphere clusters formed by $CD133^+CD44^+$ cells after 7days in MG-63, Saos-2, U2OS, respectively (original magnification 200×); $CD133^+CD44^-$, $CD133^-CD44^+$, and $CD133^-CD44^-$ cells did not form spheres after 7 days in MG-63, Saos-2, U2OS. B: The number of colonies with a diameter larger than 0.1 mm was quantified after 3 days of culture. Data are shown as the mean ± SEM from three separate experiments. $^*P < 0.05$.

$CD133^-CD44^-$ cells. The results revealed the mRNA expressions of Oct-4, NANOG and CXCR4 were significantly higher in $CD133^+CD44^+$ population (Fig. 6).

$CD133^+$ $CD44^+$ CELLS EFFICIENTLY INITIATE TUMORS WITH A HIGH FREQUENCY OF METASTASIS

The in vitro experiments showed that $CD133^+CD44^+$ SaoS-2 cells had enhanced migratory and invasive ability. We therefore compared the growth and metastasis potential of $CD133^+$ $CD44^+$ and $CD133^-$ $CD44^-$ SaoS-2 cells in vivo. OS model was established by injecting $CD133^+$ $CD44^+$ or $CD133^-$ $CD44^-$ Saos-2 cells into the intratibia of the nude mice. $CD133^-$ $CD44^-$ SaOS-2 cell-derived tumors developed more slowly and the first osteoblastic lesions became visible 2 weeks after those in mice injected with $CD133^+$ $CD44^+$ SaoS-2 cells. $CD133^+$ $CD44^+$ SaOS-2 cells xenografts with a mean primary tumor volume of $310.6 \pm 35.0\ mm^3$ were significantly

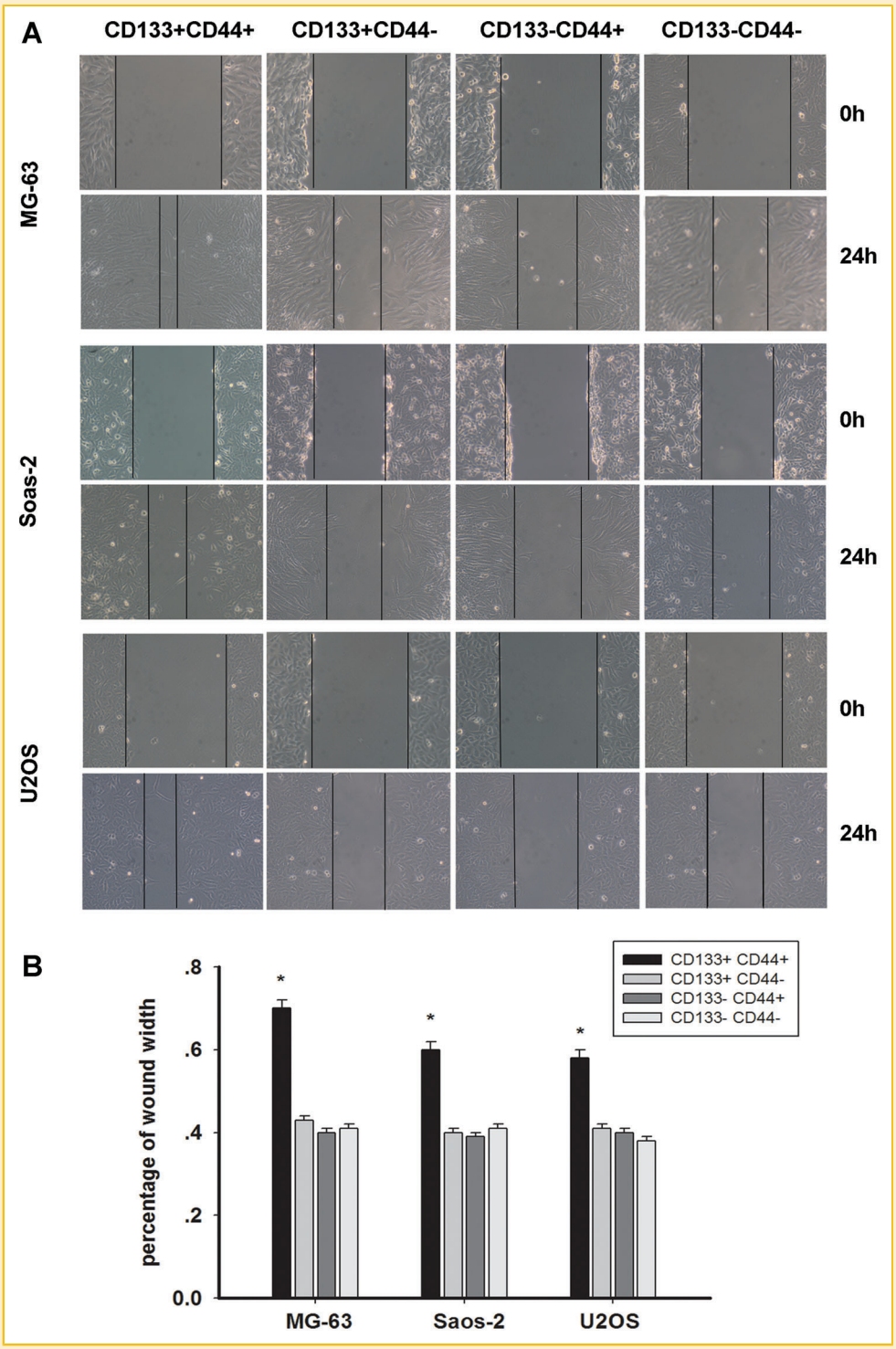

Fig. 4. Migration ability of $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$ cells and $CD133^-CD44^-$ cells. A: The distance between the edges of the scratch became increasingly narrow in the $CD133^+CD44^+$, $CD133^+CD44^-$, $CD133^-CD44^+$ cells and $CD133^-CD44^-$ cells. There were notable differences between $CD133^+CD44^+$ cells and $CD133^+CD44^-$, $CD133^-CD44^+$ cells or $CD133^-CD44^-$ cells at 24 h, respectively, in MG-63, Saos-2, U2OS cells. B: The percentages of wound width in MG-63, Saos-2, U2OS. Data are shown as the mean ± SEM from three separate experiments. $^*P < 0.05$.

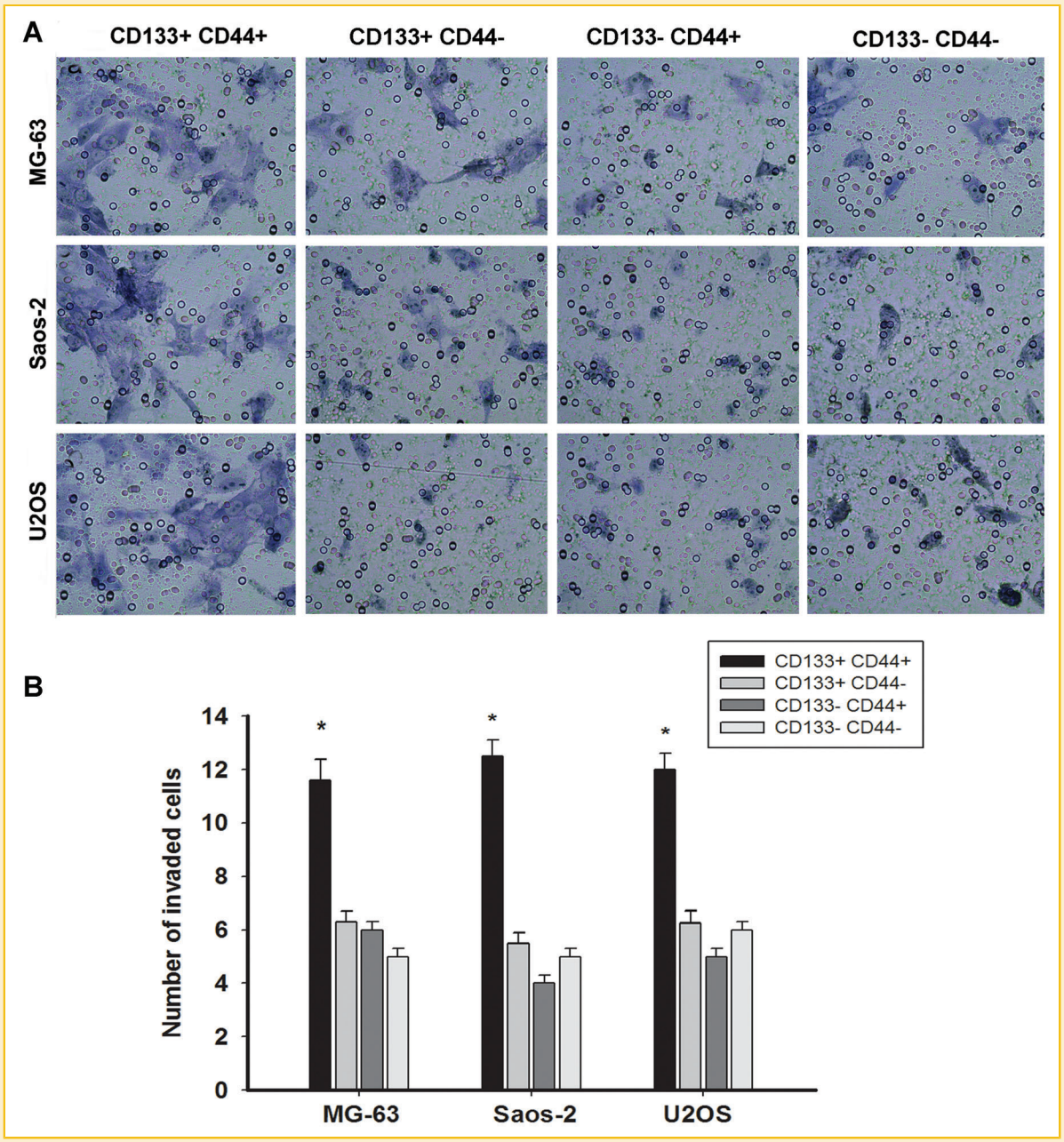

Fig. 5. Quantitative measurements of invaded cells. A: The membranes were stained with Giemsa after removal the cells inside the chambers. Magnification, 400×. B: Quantitative measurement of invaded cells. Data were showed as mean ± SEM from four separate experiments. $^{*}P < 0.05$.

($P < 0.05$) larger than $CD133^{-}$ $CD44^{-}$ ($159.2 \pm 27.3\ mm^3$) xenografts (Fig. 7A,C). Strikingly, when the animals were sacrificed after 12 weeks, six out of eight mice developed lung metastases in $CD133^{+}$ $CD44^{+}$ group, whereas only one of the lungs of mice treated with $CD133^{-}$ $CD44^{-}$cells Saos-2 cells metastases (Fig. 7B,D).

DISCUSSION

The purpose of this study was to investigate whether $CD133^{+}CD44^{+}$ mediates the metastasis of OS. We first identified that CD133 and CD44 co-expressed in lung metastatic lesions and OS cell lines. Next, $CD133^{+}CD44^{+}$ cells were demonstrated have more aggressive in sphere formation, migration and invasiveness than $CD133^{+}CD44^{-}$, $CD133^{-}CD44^{+}$, or $CD133^{-}CD44^{-}$ cells. We finally sorted the $CD133^{+}$ $CD44^{+}$ or $CD133^{-}$ $CD44^{-}$ cells from Saos-2 cell lines, after intratibial xenograft in nude mice. These cells developed primary tumors, and $CD133^{+}CD44^{+}$ cells are more potential to form lung metastatic tumors. Thus we concluded that $CD133^{+}CD44^{+}$ cells may mediate the lung metastasis of OS.

Recent findings suggest that tumors are comprised of heterogeneous cell populations, and only a small fraction of these are tumorigenic with the ability to self-renew and produce phenotypically

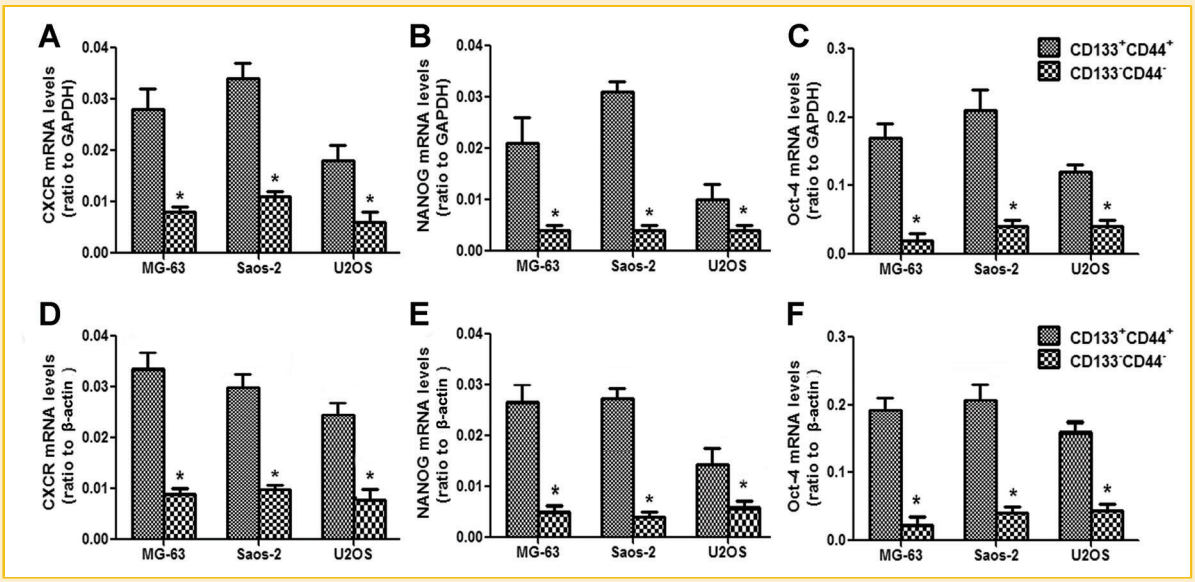

Fig. 6. RT-PCR analysis of CXCR4 (A), NANOG (B), and Oct-4 (C) to GAPDH and CXCR4 (D), NANOG (E), and Oct-4 (F) to β-actin in $CD133^{+}CD44^{+}$ and $CD133^{-}CD44^{-}$ cells. Data were showed as mean ± SEM from three independent experiments. $^{*}P < 0.05$.

diverse tumor cell populations. Cells in this fraction are called CSCs or tumor-initiating cells (TICs). CSCs have been identified from many types of cancer. They share several similarities with normal adult stem cells including sphere-forming ability, self-renewability, expression of stem cell surface markers and transcription factors. CSCs have also been proposed to be responsible for cancer metastasis, however, scarce evidence for their metastatic potential has been provided.

Early in 2008, Tsuchida et al. [2008] showed that a side population of cells could be isolated by exposure of the HOS OS cell line to the chemotherapy agent cisplatin. Although the cisplatin-treated side

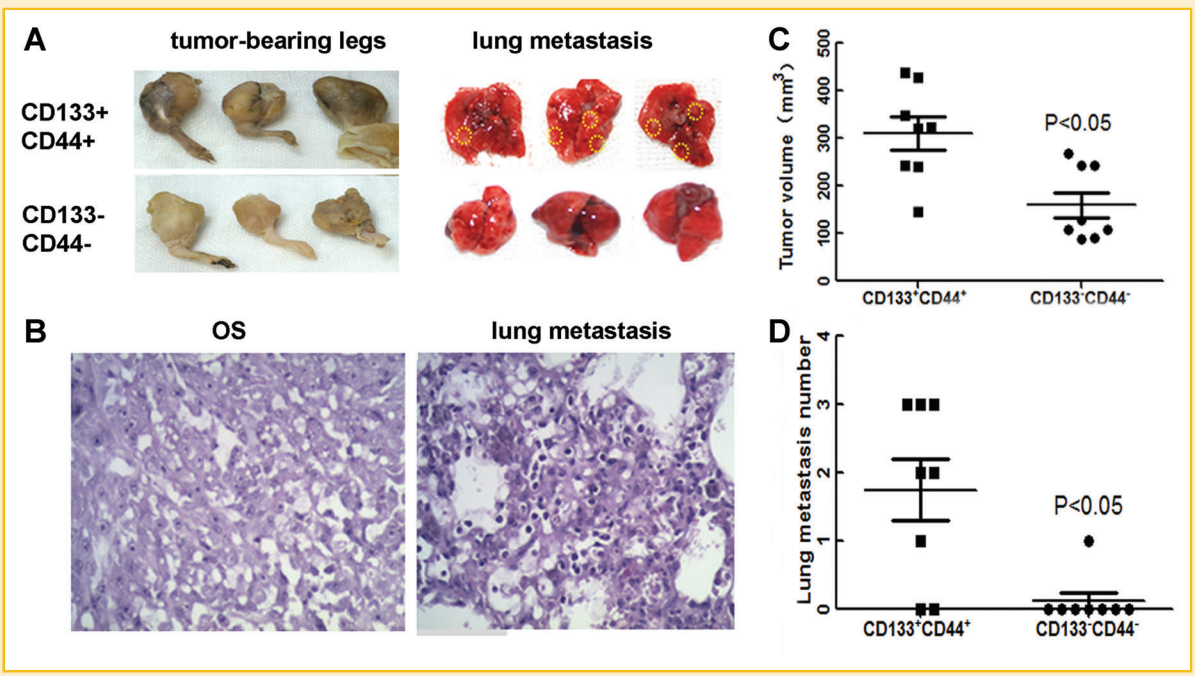

Fig. 7. $CD133^{+}CD44^{+}$ cells efficiently initiate tumors with a high frequency of metastasis. A, Left: Representative images of tumor-bearing legs after intratibial injection of $CD133^{+}CD44^{+}$ or $CD133^{-}CD44^{-}$ Saos-2 cells. Right: Representative images of the lungs of mice injected with $CD133^{+}CD44^{+}$ or $CD133^{-}CD44^{-}$ Saos-2 cells. B: HE staining confirming the OS model and lung metastasis. C: Primary tumor volume in respective mice 12 weeks after tumor cell injection. D: Quantification of pulmonary metastases prepared after sacrifice in week 12 after tumor cell injection. Data show means ± SD of triplicates from one experiment representative of three experiments performed.

population of cells demonstrated several CSCs features including expression of stem cell-associated genes, colony growth, and tumor formation, side population of cells from untreated cells failed to form tumors suggesting that side population not necessarily identification of a sarcoma CSC. In contrast, Yang et al. [2011] derived side population of cells from primary OS cell lines and found that side population, as well as non-side population cells, were capable of tumor formation thus questioning the validity of side population of cells as a specific CSCs marker. Fujii et al. [2009] found out sarcospheres from OS cell line MG63 showed the stem-like properties with the ability of self-renewal, and increased expression of the stem cell-related genes such as Nanog, OCT3/4 SOX2 and DNA repair enzyme genes, MLH1 and MSH2. However, in vivo experiment comparing the ability of tumor formation was not done in their research. Subsequently, Tirino et al. [2008] identified and isolated a small $CD133^+$ subpopulation in OS cell lines SAOS2, U2OS, and MG-63. Compared to $CD133^-$ cells, $CD133^+$ cells exhibited increased abilities in self-renew, proliferation, sphere formation, and higher expression of the stem cell-associated gene OCT3/4. They further evaluated 21 patient-derived sarcoma tissues and found variable expression of CD133 [Tirino et al., 2011]. However, the CD133 fraction from OS cell lines were incapable of tumor formation when injected into non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice which leads one to question the validity of CD133 expression in identifying a tumorigenic CSC population in OS. In our previous research [He et al., 2012], we also found the expression of CD133 in OS tissues, and furthermore, it was found to be correlated to lung metastasis and poor prognosis in OS patients.

More recently, Adhikari et al. [2010] examined the expression of two mesenchymal stem cell markers, CD117 and Stro-1, and discovered that mouse and human OS cell lines positive for both CD117 and Stro-1 readily formed spherical colonies, more readily formed tumors in a nude mouse model in comparison to cells negative for both CD117 and Stro-1, had higher metastatic potential in an orthotopic NOD/SCID mouse model. This study has been the first to specifically identify a subpopulation of OS cells with the hallmark features of a CSC: self-renewal capacity and the ability to reconstitute the original cellular make up of a tumor or cell population upon serial transplantation. Besides CD117 and Stro-1, other cell surface markers have been identified, in addition to expressing CD133, the human OS cell lines Saos-2, OSA-1, OSA-2, and OSA-3 also expressed nestin, a marker for neural stem cells and brain CSC, suggesting that nestin and CD133 might be used as co-markers for identifying OS CSCs [Veselska et al., 2008].

It is important to notice that in numerous types of human carcinomas CD44 is a common marker for CSCs [Zoller, 2011]. CD44 has been implicated in tumor metastasis [Gunthert et al., 1991]. The commonest type of CD44 is the "standard" or hematopoietic variant (CD44s or CD44H), which is expressed by some mesodermal cells and carcinoma cells. CD44 has many variant forms, which are generated by alternative splicing of at least 10 exons named v1–v10 [Arch et al., 1992]. The contribution of CD44 to OS progression and metastasis was conflicting. Boldrini et al. [2010] found the overall survival rates for CD44H-positive and negative OS patients were similar, while Gvozdenovic et al. [2013] also found that OS patients with high levels of CD44 expression tended to have shorter overall survival. Kim et al. [2002] also found the survival rate in the CD44v5-positive group was significantly lower than that in the CD44v5-negative group. Furthermore, CD44V6 expression was also found to be associated with metastasis and overall survival [Kuryu et al., 1999; Deng et al., 2013]. It remains to be further research about the mechanism of these results.

In this study, we verified the stem of $CD133^+CD44^+$ cells in OS and investigated its potential role in metastasis in vitro and in vivo, which would make the identification of such new therapeutic targets possible, with the final goal to develop treatment strategies that eradicate metastases, the major cause of death in OS. Validation of the stem cell-like properties of the $CD133^+CD44^+$ population of cells in a broader panel of primary, patient-derived OS cells would further strengthen the utility of CD133 and CD44 marker expression as specific markers for the identification, isolation, and therapeutic targeting of this unique population in OS.

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