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Identification of Stem Cell Related Gene Expression from the Osteosarcoma Cell Core Side

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Osteosarcoma is the most frequent primary malignant bone tumor with higher incidences in children and adolescents. Despite clinical evolutions, patients with osteosacoma have had a poor prognosis. There has been increasing evidence that cancer is a stem cell disease. This study sought to isolate and characterize cancer stem cells from human osteosarcoma with relevant literature reviews. Here we show that the emerging evidence suggests osteosarcoma should be regarded as a differentiation disease such as stem cell disease. Two human osteosarcoma cell lines were cultured in non-adherent culture conditions as sarcospheres. Sarcospheres were observed using histomorphology and alkaline phosphatase (ALP) staining. Expression of the embryonic stem cell marker was analyzed with use of reverse transcriptase-PCR. Sarcospheres could be reproduced consistently throughout multiple passages and produced adherent osteosarcoma cell cultures. Expression of stem cell-associated genes such as those encoding Nanog, octamer-binding transcription factor 3/4, sex determining region Y box 2 , c-Myc and ALP indicated pluripotent stem-like cells. These results support the extension of the cancer stem cell theory to include osteosarcoma. Understanding the cancer stem cell derived from human osteosarcoma could lead to the evolution of diagnosis and treatment for osteosarcoma patients.

Key Words Osteosarcoma, Neoplastic stem cells

INTRODUCTION

Osteosarcoma, a primary malignant neoplasm derived from bone mesenchymal tissue, is prevalent in childhood and adolescence and one of the most common primary bone malignancies [1]. It is clinically known to have high grade malignant potentials with a tendency of local recurrence as well as distant metastasis in an early stage. Osteosarcoma tends to metastasize to lung most frequently, and metastatic osteosarcoma has high mortality rates [2].

Despite the fact that latest evolution in surgical technique and chemotherapeutic agents have improved treatment success rate, the most recent 10-year long-term survival rate still remains a standstill at 65 percent [3]. Consequently, a novel therapeutic approach is in a desperate need to replace current remedies, which have already reached its limits.

A stem cell is defined by two distinctive features, which are self-replicating capacity and pluripotency [4]. A cancer stem cell, with self-renewal and pluripotent capacities, is present in a small number within a cancerous tumor at first and continues to produce atypical offspring cells which eventually occupy most of cancerous tumor [5]. Similar to stem cells, these cancer stem cells are responsible for oncogenesis, cancer growth, local recurrence, and distant metastasis [6]. Recent researches have reported that stem-like cancer cells are closely associated with pathogenesis of leukemia, brain cancer, and breast cancer [7-11]. Moreover, stem cell marker, such as octamer-binding transcription factor 3/4 (Oct3/4), Nanog, and sex determining region Y box 2 (SOX2) were expressed in various human cancer cells [12]. However, there has not been any previous evidence on pathogenesis and oncogenesis of osteosarcoma. Investigation on relationship between cancer stem cell and oncogenesis of osteosarcoma is believed to yield critical evidences in understanding pathogenesis and overcoming limitations of current remedies.

In the current study, cell lines derived from human osteo-

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sarcoma were used to identify cancer stem cells in osteosarcoma. Isolation of cancer stem cells from human osteosarcoma cell lines is anticipated to contribute to the understanding of cancer pathogenesis, growth, recurrence, metastasis, prognosis, and novel therapeutic modalities in osteosarcoma.

MATERIALS AND METHODS

Human osteosarcoma cell line culture

Human osteosarcoma cell lines, Cal-72 (Duetsche Sammlung von Zellkulturen und Mikroorganismen, Braunschweig, Germany) and SaOs-2 (American Type Culture Collection, Manassas, VA, USA) were purchased for cell preparation. After plating in cell density of $2 \times 10^5/cm^2$, all cell lines were expanded in mesenchymal stem cell growth media (Clonetics, Walkersville, USA), which is Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine 200 mM, penicillin 25 U/mL, and streptomycin 25 μg/mL under condition of 37°C and 5% CO_2. After the cell expansion, subcultivation in a split ratio of 1:5 was performed to yield enough cell concentrations. After dropping osteosarcoma cell lines yielded from the primary culture onto a non-adhesive 6 well culture plate containing 1% methylcellulose to separate the cell lines to cell concentrations of 10^5/mL in each well, the cell lines were cultured in a osteogenic differentiation media (ODM) (GibcoBRL®, Gaithersburg, MD, USA), which is DMEM supplemented with β-glycrophosphate 10 mM, dexamethasone 100 nM, ascorbic acid-2-phosphate 0.05 mM, without 10% FBS under condition of 37°C and 5% CO_2. by changing the media every 3 to 4 day. In addition, human bone marrow derived mesenchymal stem cell were simultaneously cultivated under the same conditions for the control group. This study was approved by the Institutional Review Board of Jeju National University Hospital (IRB No. 2007-1).

Sarcosphere culture

After harvesting sarcosphere, it was cultivated in ODM containing 10% FBS on adhesive cell culture plates. After the formation of osteosarcoma cell monolayers, it was again cultivated in ODM without 10% FBS on non-adhesive 6 well culture plates in order to verify formation of sarcosphere. For each sarcosphere, above-mentioned cultivation process was repeated for 7 to 10 generations.

Morphologic analysis

Sarcosphere formation was visualized under an inverted phase contrast microscope on day 3, 5, 7, an 14 after cultivation. Detected stained with alkaline phosphatase (ALP) and visualized under an inverted phase contrast microscope.

ALP staining

Solution in a mix with 1 mL of sodium nitrite solution and 1 mL of FBB-alkaline solution for two minutes was blended with 45 mL of deionized water. Then, the solution was slowly mixed with 1 mL of naphthol AS-BI alkaline solution, 25 mL of citrate solution, 65 mL of acetone, and 8 mL of 37% formaldehyde to prepare a fixation solution. After fixing the sample in the fixation solution for 30 seconds, it was rinsed with deionized water for 45 seconds; then, alkaline dye mixture was added for reaction in shaded environment for 15 minutes. After rinsing with deionized water, it was stained with neutral red solution and observed under phase contrast microscope.

Immunofluorescent staining

After the deparaffinization process, prepared slides were rehydrated and rinsed with distilled water solution. After processing under methyl alcohol and 0.3% H_2O_2 for eight minutes to block intrinsic peroxidase, it was rinsed three times in 0.1 M PBS for 3 minute each. It was processed with 0.2% hyaluronidase from bovine testes (H3757; Sigma Aldrich, St. Louis, MO, USA) under 37°C for 30 minutes to recover antigens; then, it was again rinsed three times in 0.1 M PBS for 3 minute each. After processing with blocking solution (LSAB kit K0681; DACO, Santa Clara, CA, USA) at room temperature for 30 minutes in order to block nonspecific antigen-antibody binding, blocking solution was removed using a filter paper. Monoclonal anti-human ALP (Sigma Aldrich) was processed for 24 hours at temperature of 4°C after in 1 M PBS in a ratio of 1:100 to 1:200. The first antibody was processed with fluorescence-labeled secondary antibody, and the expression was observed using Olympus IX inverted phase contrast microscope (Olympus, Tokyo, Japan) and pictured with Olympus DP71 camera (Olympus).

Reverse transcriptase (RT)-PCR

After extraction of sacospheres on day 14 and 21 of cultivation, RNA was separated using RNAase-free DNase-processed RNeasy mini kit (Qiagen Sciences, Valencia, CA, USA). RNA was semi-quantified at 260 OD using spectrophotometry. First-strand cDNA was formulated using Oligo $(dT)_{12-18}$ primer (Invitrogen Life Technologies, Carlsbad, CA, USA), 1.5 μg total RNA, and SuperScript II RNase H Reverse Transcriptase (Invitrogen Life Technologies), and the target cDNA was amplified using Platinum Taq DNA Polymerase (Invitrogen Life Technologies). PCR was performed using AccuPower® PCR PreMix (Bionner, Daejeon, South Korea) through GeneAmp PCR system (Perkin-Elmer 9600; Norwalk, CT, USA). The primer was formulated using Nanog, SOX2, ALP, c-Myc, a gene expressed in malignant caner with low differentiation and embryonic stem cell, osteocalcin, a gene associated with osteogenic differentiation, and Oct3/4, a gene representative of pluripotency and self-renewal ability of stem cells; in addition, β-actin cDNA was simultaneously amplified for comparison (Table 1).

Preliminary work was done to ensure and optimize annealing temperature at each step of PCR and PCR cycles and to establish linear ranges, which are described in Table 1. Then, PCR products were electrophoresed on ethidium-bromide

Table 1. Primer sequences used in RT-PCR

Target transcript	Primer sequence	Annealing temperature (°C)	PCR cycles	Predicted size (bp)
c-Myc	F : 5'-CAG AGG AGG AAC GAG CTG AAG CGC-3' R : 5'-TTA TGC ACC AGA GTT TCG AAG CTG TTC G-3'	60	35	228
SOX2	F : 5'-GGT TAC CTC TTC CTC CCA CTC CAG-3' R : 5'-TCA CAT GTGC GAC AGG GGC AG-3'	60	35	193
Oct3/4	F : 5'-TGG AGA AGG AGA AGC TGG AGC AAA-3' R : 5'-GGC AGA GGT CGT TTG GCT GAA TAG-3'	60	35	485
Nanog	F : 5'-TCC TCC TCT TCC TCT ATA CTA AC-3' R : 5'-CCC ACA ATC ACA GGC ATAG-3'	60	35	228
Osteocalcin	F : 5'-GGC AGC GAG GTA TGA AGA GAC-3' R : 5'-CTG GAG AGG AGC AGA ACT GG-3'	60	30	284
ALP	F : 5'-TGG AGC TTC AGA AGC TCA ACA CCA-3' R : 5'-ATC TCG TTG TCT GAG TAC TCG TCC-3'	65	35	454
β-actin	F : 5'-GTG GGG CGC CCC AGG CAC CAG GGC-3' R : 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'	60	25	540

F, forward, sense primer; R, reverse, antisense primer; SOX2, sex determining region Y box 2; RT-PCR, Reverse transcription-PCR; Oct3/4, octamer-binding transcription factor 3/4; ALP, alkaline phosphatase; bp, basis point.

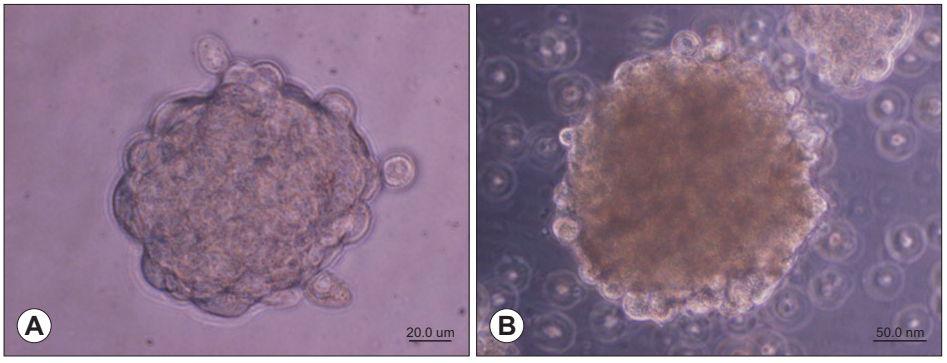

Figure 1. Phase contrast images of sarcospheres from human osteosarcoma cell lines (ALP stainng). (A) Representative sarcosphere from the Cal-72 human osteosarcoma cell line. Bar represents 20 μm. (B) Sarcosphere from the SaOs-2 human osteosarcoma cell line. Bar represents 50 μm.

containing 1.5% agarose gel at 100 V, and the results were pictured with a digital camera (DP 71; Olympus, Tokyo, Japan).

RESULTS

CAL-72 and SaOs-2 human osteosarcoma cell lines produced sarcospheres mostly within 7 days of cultivation (Fig. 1). One point five percent of cells in the CAL-72 cell line and the 1.0% of cells in SaOs-2 cell lines formulated sarcospheres. However, human bone marrow-derived mesenchymal stem cells did not survive longer than a week under non-adhesive culture conditions without FBS. Sarcospheres formulated from human osteosarcoma cell lines such as CAL-72 and SaOs-2 were able to develop into osteosarcoma cancer cells under a normal adhesive culture condition but remained as sarcospheres under a non-adhesive culture conditions (Fig. 2). Despite multiple trials through several generations, sarcospheres and monolayer cancer cells failed to grow out of their original states under non-adhesive culture conditions. Under normal monolayer culture conditions, sarcospheres developed at the same speed to reach enough cell concentration even in the 7th generation as seen in 1st generation, and such cultivation time was similar in each generation.

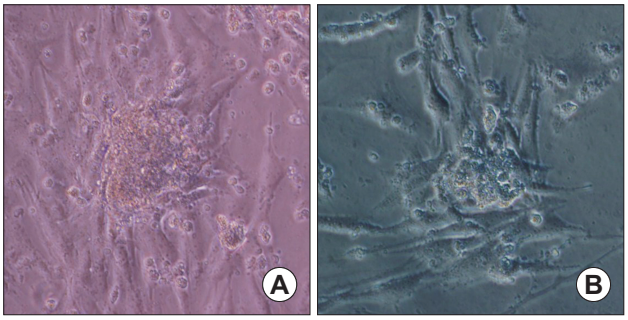

Figure 2. Adherent cancer cells expanded from the spheres when sarcosphere reattached to adherent plate after removal from the non-adherent culture (ALP stainng). (A) Adherent cells expanding from Cal-72 sarcosphere (original magnification ×10). (B) Adherent cells extending from SaOs-2 sarcosphere (original magnification ×10).

The ALP staining results revealed that more than 90% of the cells within sarcospheres originated from CAL-72 and SaOs-2 cell lines showed high degree of expression against

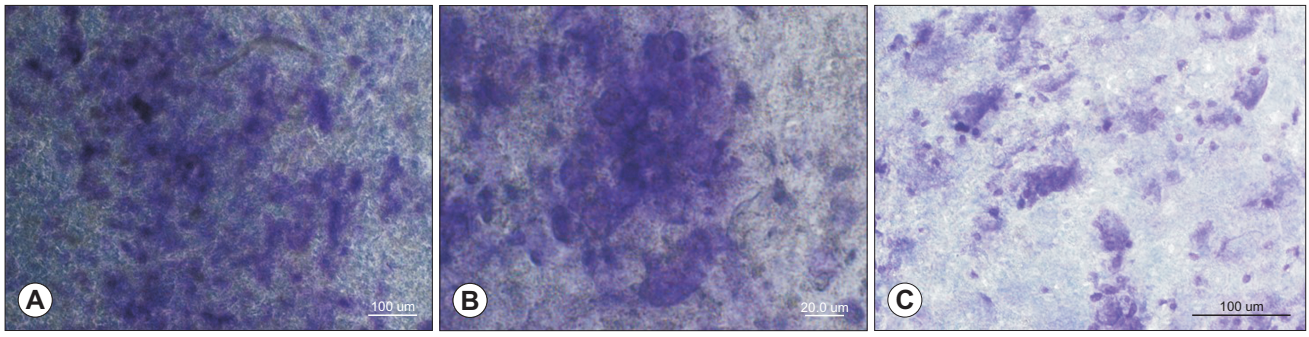

Figure 3. Alkaline phosphatase staining revealed intense staining in > 90% of cells within sarcospheres. In the adherent monolayer culture, 30% of the cells stained positively. (A) Sarcosphere from the Cal-72 cell line. Bar represents 100 μm. (B) Sarcosphere from the SaOs-2 cell line. Bar represents 20 μm. (C) Cal-72 adherent cells. Bar represents 100 μm.

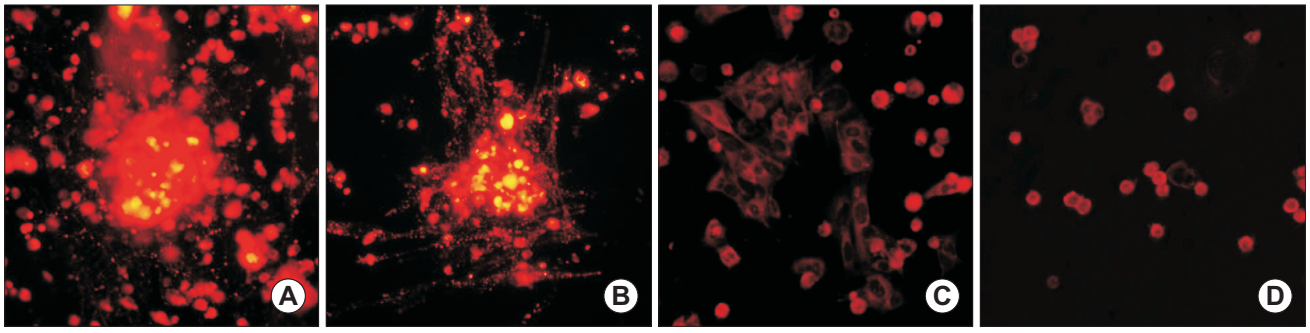

Figure 4. Immunofluorescent staining for alkaline phosphatase showed intensive expression in sarcospheres. Adherent osteosarcoma cell culture showed weak positive staining. A sparse staining was observed in human bone marrow-derived mesenchymal stem cells. (A) Sarcosphere from the Cal-72 cell line (original magnification ×10). (B) Sarcosphere from the SaOs-2 cell line (original magnification ×10). (C) Cal-72 adherent cells (original magnification ×15). (D) Human bone marrow-derived mesenchymal stem cells (original magnification ×10).

ALP (Fig. 3A and 3B). On the other hand, only 30% of the cells from adhesive cell lines showed positive expression, and such degree of expression was similar to the control human bone marrow-derived stem cells with positive expression in only 35% of the cells (Fig. 3C).

Immunofluorescence staining was carried out using anti-ALP antibodies. The cells within sarcospheres originated from CAL-72 and SaOs-2 cell lines indicated high degree of ALP expression in both nucleus and cytoplasm (Fig. 4A and 4B). Such high expression against ALP was seen consistently and repeatedly throughout multiple passages. Expression against ALP in both nucleus and cytoplasm was also observed in monolayer cultivation of sarcosphere (Fig. 4C); however, fewer cells showed relatively lesser degree of expression compared to the sarcospheres. Bone marrow derived mesenchymal stem cells representing, the control group, indicated even weaker ALP expression than in monolayer cancer cells (Fig. 4D).

RT-PCR results revealed that Oct3/4, Nanog, SOX2, c-Myc, and ALP were all strongly overexpressed in sarcospheres originated from CAL-72 and SaOs-2 cell lines (Fig. 5). Even though Oct3/4, Nanog, SOX2, c-Myc, and ALP were all expressed in monolayer cancer cells, their degree of

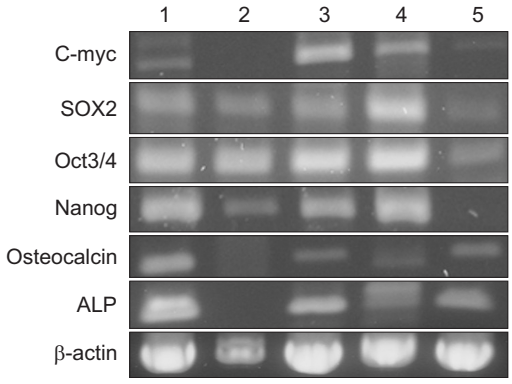

Figure 5. RT-PCR for the stem cell related genes in osteosarcoma sphere cultures and monolayer cultures compared as bone marrow-derived mesenchymal stem cell cultures. 1: Cal-72 sarcospheres, 2: Cal-72 monolayer adherent cells, 3: SaOs-2 sarcospheres, 4: Saos-2 adherent cells, 5: Human bone marrow-derived mesenchymal stem cells (control). Oct3/4, octamer-binding transcription factor 3/4; SOX2, sex determining region Y box 2; ALP, alkaline phosphatase; RT-PCR, reverse transcriptase-PCR.

expression of relatively weaker than sarcospheres. In bone marrow-derived mesenchymal stem cells, Oct3/4, SOX, ALP were weakly expressed, but c-Myc and Nanog did not show any expression.

DISCUSSION

The purpose of the current study was to establish a platform to identify the nature of cancer stem cells which are known to be responsible for growth, recurrence, and distant metastasis of osteosarcoma by inducing cancer stem cells from human osteosarcoma cell lines.

It has been already proven that only small fractions of cells vigorously proliferate in tumors and cultured cancer cells [13]. From such findings, the concept that cancer is composed of both cancer stem cells with strong proliferative power and their progeny cells with limited proliferative power was on the rise. Consequently, stem-like cells needed to demonstrate their proliferative power, long-term self-renewability, and productive capacity of progeny cells with capability to differentiate into primary cells [4,14].

There have been numerous attempts to extract cancer stem cells out of solid tumor, and among those attempts, a number of experiments utilizing sarcospheres are well described [8,15-18]. Most cells growing in non-adhesive culture media deprived of serum tend to perish or age out due to its harsh culture environment [19-21]. The fact that sarcospheres repeatedly succeed to be cultured through serial passages when migrated from non-adhesive culture media to adhesive culture media reflects its one to one relationship between sarcospheres and stem cells [14].

The sphere culture technique was used to formulate sarcospheres from human osteosarcoma cells lines in this study. The sarcospheres repeatedly formed their identical sarcospheres when cultured through serial passages more than seven generations. When monolayer cultivation of sarcospheres is performed on adhesive culture media, normal osteosarcoma monolayer cells were successfully cultured, and their cultivation time to reach enough cell concentrations was similar in each passage. When a monolayer of cultured cancer cells was transferred to non-adhesive culture media, formation of the sarcospheres were reproduced through serial passages. These results confirm self-renewal and self-regeneration of sarcospheres originated from human osteosarcoma cells to prove that the sarcospheres contain similar phenotypes as their preceding stem cells.

ALP is normally present as various isoenzymes in various parts of body, such as hepatic tissues and bone tissues, and abundant in osteoblast; furthermore, pluripotent germ cells possess especially high activity of ALPs [22]. Consequently, activity of ALP, which represents cellular undifferentiated state, is widely used as a biomarker for embryonic stem cells and embryonic germ cells [23,24].

Immunofluorescence staining, using anti-ALP in the current research, revealed that human osteosarcoma sarcospheres show extremely strong expression of ALP relative to cancer cell monolayer and bone marrow-derived mesenchymal stem cells. Such high activities of ALP show that cells within sarcospheres maintain a similar undifferentiated state as seen in embryonic stem cells.

Oct3/4, a homeoprotein transcription factor of POU family, is expressed within the inner cell mass in the early stage and essential for maintaining pluripotency; however, its expression decreases as differentiations progresses [17,25,26]. After maturation, Oct3/5 is not observed in somatic cells except for type A spermatogonium, testicular seminoma, teratoma and primitive progenitor cells [27]. Nanog is a newly discovered homeoprotein transcription factor, which is generally expressed in embryonic stem cells [28]. Expression of Nanog maintains the stem cell phenotype without leukemia suppressors and enables self-renewal and propagation of cells [29,30]. Even though Nanog is expressed in not only embryonic stem cells but also teratoma and germ cell tumors, its expression in somatic cells has never been reported [17,25,29]. Transcriptions factors, such Nanog and Oct3/4, are essential for cell differentiation, and interactions between Nanog and Oct3/4 is critical to maintain pluripotency of stem cells [31]. SOX2, a SOX family transcription factor, is crucial in maintaining self-renewal of undifferentiated embryonic stem cells, and regulates development of embryos, and ultimately determines cell identity [32]. Furthermore, when SOX2 is co-expressed with Oct3/4 it is a central transcription factor associated with immortality in induced pluripotent stem cells (iPS) [33]. SOX2 is mostly expressed in embryonic stem cells as well as colon [34]. c-Myc is a proto-oncogene, associated with self-replication process and also used as a biomarker for embryonic stem cells [35].

In the current study, expression of Oct3/4, Nanog, c-Myc, and ALP was analyzed using RT-PCR. These four markers were strongly expressed in human osteosarcoma sarscospheres, compared to the control groups, which are cancer cell monolayer and bone marrow-derived mesenchymal stem cells. In sarcospheres, expression of Oct3/4, Nanog, and SOX2 is indicative of pluripotency of stem cells, self-renewal and self-reproduction of embryonic stem cells, and self-regeneration of undifferentiated cells. In addition, expression of c-Myc indicated undifferentiated state of the cells, and totipotency is confirmed by strong expression of ALP in ALP staining and immunofluorescence staining.

Osteosarcoma is a malignant cancer cell with bone-marrow mesenchymal origin, characterized by clinical, histologic, and molecular diversity and formation of abnormal bone matrix [1]. Bone marrow-derived mesenchymal stem cells can differentiate into all three germ layers and form bone matrix when induced for bone differentiation [36]. These similarities between osteosarcoma and bone marrow-derived mesenchymal stem cells infer to that development of osteosarcoma cell may be in response to abnormal differentiation of mesen-

chymal stem cells [37].

In the current study, comparing osteosarcoma sarcospheres to human bone marrow-derived mesenchymal stem cells as a control group, the result indicated that mesenchymal stem cells did not survive in non-adhesive culture conditions and showed weaker activity in ALP staining and immunofluorescence staining than osteosarcoma sarcospheres; in addition, RT-PCR results demonstrated mild expression of Oct3/4 and ALP but a lack of Nanog expression in human bone marrow-derived mesenchymal stem cells. Therefore, such results indicated that bone marrow-derived mesenchymal stem cells are more committed adult stem cell relative to cancer stem cells. In other words, cancer stem cells are more primitive undifferentiated stem cells with capacity of self-replication, totipotency and pluripotency as seen in embryonic stem cells. Furthermore, de-differentiation process of bone marrow-derived mesenchymal stem cells is thought to contribute to development of osteosarcoma cancer stem cells, evidence of which is shown by a higher expression of SOX2, a pluripotency-specific transcription factor, in sarcospheres than the control group as seen in our study.

Sarcospheres derived from human osteosarcoma cell lines in serum deprived non-adhesive culture conditions confirmed that they were, in fact, cancer stem cells, and from our research results, application of cancer stem cell theory is plausible in understanding of human osteosarcoma. Because stem cells show less multidrug resistance and higher resistance to chemotherapy compared to adult cells, novel therapeutic modalities are in emergent need for treatment of osteosarcoma, which now can be considered a stem cell disease, unlike current chemotherapies only targeting gross size reduction. Therefore, identification of cancer stem cells within osteosarcoma using Oct3/4, Nanog, and ALP markers and antibodies may open up areas for future researches in selective treatment methods at a molecular level.

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CONFLICTS OF INTERESTS

No potential conflicts of interest were disclosed.

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