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

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Research Article

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Abstract

Background: Osteosarcoma is the most frequent primary malignant bone tumor with higher incidences in children and adolescents. Despite clinical evolutions, patients with osteosarcoma 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.

Methods: 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-Polymerase Chain Reaction (RT-PCR).

Results: Sarcospheres could be reproduced consistently throughout multiple passages and produced adherent osteosarcoma cell cultures. Expression of stem cell-associated genes such as Nanog, octamer-binding transcription factor 3/4 (OCT 3/4), sex determining region Y box 2 (SOX2), C-myc and ALP indicated pluripotent stem-like cells.

Conclusion: These results support the extension of cancer stem cell theory to include osteosarcoma. Understanding the cancer stem cell of human osteosarcoma could lead to the evolution of diagnosis and treatment for osteosarcoma patients.

Background

Osteosarcoma, a primary malignant neoplasm derived from bone mesenchymal tissue, is prevalent in childhood and adolescence and one of the most common primary bone cancer [1]. It is clinically known to have high grade malignant potentials with a tendency of local recurrence as well as distant metastasis in 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, a cancer 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]. 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 osteosarcoma 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.

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, Vanassas, MA, USA) were purchased for cell preparation. After plating in cell density of $2\times10^5/cm^2$, all cell lines were expanded in mesenchymal stem cell growth media (referred to as MSCGM; Clonetics, Walkersville, USA), which is Dulbecco's modified eagle's medium (referred to as DMEM) supplemented with 10% fetal bovine serum, L-glutamine 200 mM, penicillin 25 U/mL, and streptomycin 25 μg/mL under condition of 37℃ 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 (referred to as 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% fetal bovine serum under condition of 37℃ 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.

Sarcosphere culture

After harvesting sarcosphere, it was cultivated in ODM containing 10% fetal bovine serum on adhesive cell culture plates. After the formation of osteosarcoma cell monolayers, it was again cultivated in ODM without 10% fetal bovine serum 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

After formation of sarcosphere, sarcosphere was observed under an inverted phase contrast microscope at cultivation 3rd, 5th, 7th, and 14th day. At cultivation 7th and 14th day, sarcosphere was stained with

ALP and detected under an inverted phase contrast microscope.

ALP staining (kit No. 86-C Sigma, St. Louis, MO, USA)

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 0.1M phosphate buffered solution (PBS) for 3 minute each. It was processed with 0.2% Hyaluronidase (from Bovine tests, Type VIII, Sigma, H3757) under 37°C for 30 minutes to recover antigens; then, it was again rinsed three times in 0.1M PBS for 3 minute each. After processing with blocking solution ((LSAB kit, DACO, K0681, USA) under 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) was processed for 24 hours under temperature of 4°C after diluting in 1M PBS in a ratio of 1:100 to 1:200. The first antibody was processed with fluorescence-labeled secondary antibody (Alexa 488 nm-550 nm) under manual instruction, and the expression was observed using Olympus IX inverted phase contrast microscope and pictured with Olympus DP71 camera.

RT-PCR

After extraction of sarcosphere at cultivation 14th and 21st day, total ribonucleic acid (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 complementary deoxyribonucleic acid (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, Carlsbad, CA, USA), 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).

Table 1
Primer sequences used in RT-PCR

Target transcript	Primer sequence	Annealing temperature	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°C	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°C	35	193
OCT3/4	F : 5'-CTG AGG GCC AGG CAG GAG CAC GAG-3' R : 5'-CTG TAG GGA GGG CTT CGG GCA CTT-3'	60°C	35	485
Nanog	F : 5'-AGG GTC TGC TAC TGA GAT GCT CTG-3' R : 5'-CAA CCA CTG GTT TTT CTG CCA CCG-3'	60°C	35	228
Osteocalcin	F : 5'-GGC AGC AGG TAG TGA AGA GAC-3' R : 5'-GGC AAG GGG AAG AGG AAA GAA G-3'	60°C	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°C	35	454
ß-actin	F : 5'-GTG GGG CGC CCC AGG CAC CAG GGC-3' R : 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'	60°C	25	540
(F: forward, sense primer, R: reverse, antisense primer)				

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 containing 1.5% agarose gel at 100V, and the results were pictured with a digital camera.

Results

CAL-72 and SaOs-2 human osteosarcoma cell lines produced sarcospheres mostly within 7 days of cultivation (Fig. 1). 1.5% of cells in CAL-72 cell line and 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 fetal bovine serum. 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 condition (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, sarcosphere 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.

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 ALP (Fig. 3A, 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 group, which was human bone marrow-derived stem cells and showed 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 expression in both nucleus and cytoplasm against ALP (Fig. 4A, 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 cell, the control group, indicated even weaker 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 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 some fractions of cells vigorously proliferate in in-vitro and in-vivo cultures of cancer cells [12]. 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 differentiability into primary cells [4, 13].

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, 14–17]. Most cells growing in non-adhesive culture media deprived of serum tend to perish or age out due to its harsh culture environment [18–20]. 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 [13].

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. Above-mentioned results of the study 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 [21]. Consequently, activity of ALP, which represents cellular undifferentiated state, is widely used as a biomarker for embryonic stem cells and embryonic germ cells [22, 23].

Immunofluorescence staining, using ALP staining and anti-ALP antibodies 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 similar undifferentiated state as seen in embryonic stem cells.

Oct3/4, homeoprotein transcription factor in POU family, is expressed within the inner cell mass in the early stage and essential in maintaining pluripotency; however, its expression decreases as differentiations progresses [16, 24, 25]. After maturation, Oct3/5 is not observed in somatic cells except for type A spermatogonium, testicular seminoma, teratoma and primitive progenitor cells [26]. Nanog is a newly discovered homeoprotein transcription factor, which is generally expressed in embryonic stem cells [27]. Expression of Nanog maintains its stem cell phenotype without leukemia suppressors and enables self-renewal and propagation of cell lines [[28, 29]]. 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 [16, 24, 28]. Transcriptions factors, such Nanog and Oct3/4, are essential in cell differentiation, and interactions between Nanog and Oct3/4 is critical to maintain pluripotency of stem

[30]. SOX2, a SOX family transcription factor, is crucial in maintaining self-renewal of undifferentiated embryonic stem cells, regulates develops of embryos, and ultimately determines cell identity [31]. Furthermore, when SOX2 is co-expressed with OCT4 in suppression of Oct3/4, it is a central transcription factor associated with immortality in induced pluripotent stem cell (iPS) [32]. SOX2 is mostly expressed in embryonic stem cells as well as colon [33]. C-myc is a proto-oncogene, associated with self-replication process and also used as a biomarker for embryonic stem cells [34].

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 study with sarcospheres, expression of Oct3/4, Nanog, and SOX2 confirmed pluripotency of stem cells, self-renewal and self-reproduction of embryonic stem cells, and self-regeneration of undifferentiated cells, respectively. 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 [35]. 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 mesenchymal stem cell [36].

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 condition 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 in our study.

Conclusion

Sarcospheres derived from human osteosarcoma cell lines in serum deprived non-adhesive culture condition 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

Abbreviations

ALP: alkaline phosphatase; RT-PCR: reverse transcriptase polymerase chain reaction; OCT 3/4: octamer-binding transcription factor 3/4; SOX 2: sex determining region Y box 2; DMEM: Dulbecco's modified eagle's medium; ODM: osteogenic differentiation media; PBS: phosphate buffered solution; RNA: ribonucleic acid; cDNA: complementary deoxyribonucleic acid

Declarations

Ethical approval and consent to participate

This study was approved by the Institutional Review Board (ethics committee) of Jeju National University Hospital (IRB No. 2007-1).

Consent for publication

Not applicable.

Availability of data and materials

All data included in this manuscript will be available upon corresponding author approval.

Competing interests

The authors declare no financial conflict of interest with regard to the content of this report.

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Authors Contribution

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Conducting Experiments: Kwang Woo Nam, DONG KEE JEONG

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Data Interpretation: Kwang Woo Nam, DONG KEE JEONG, Chaemoon Lim, Young Ho Roh

Manuscript Preparation: Chaemoon Lim, Young Ho Roh, Seung Jin Yoo

The author(s) read and approved the final manuscript.

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Figures

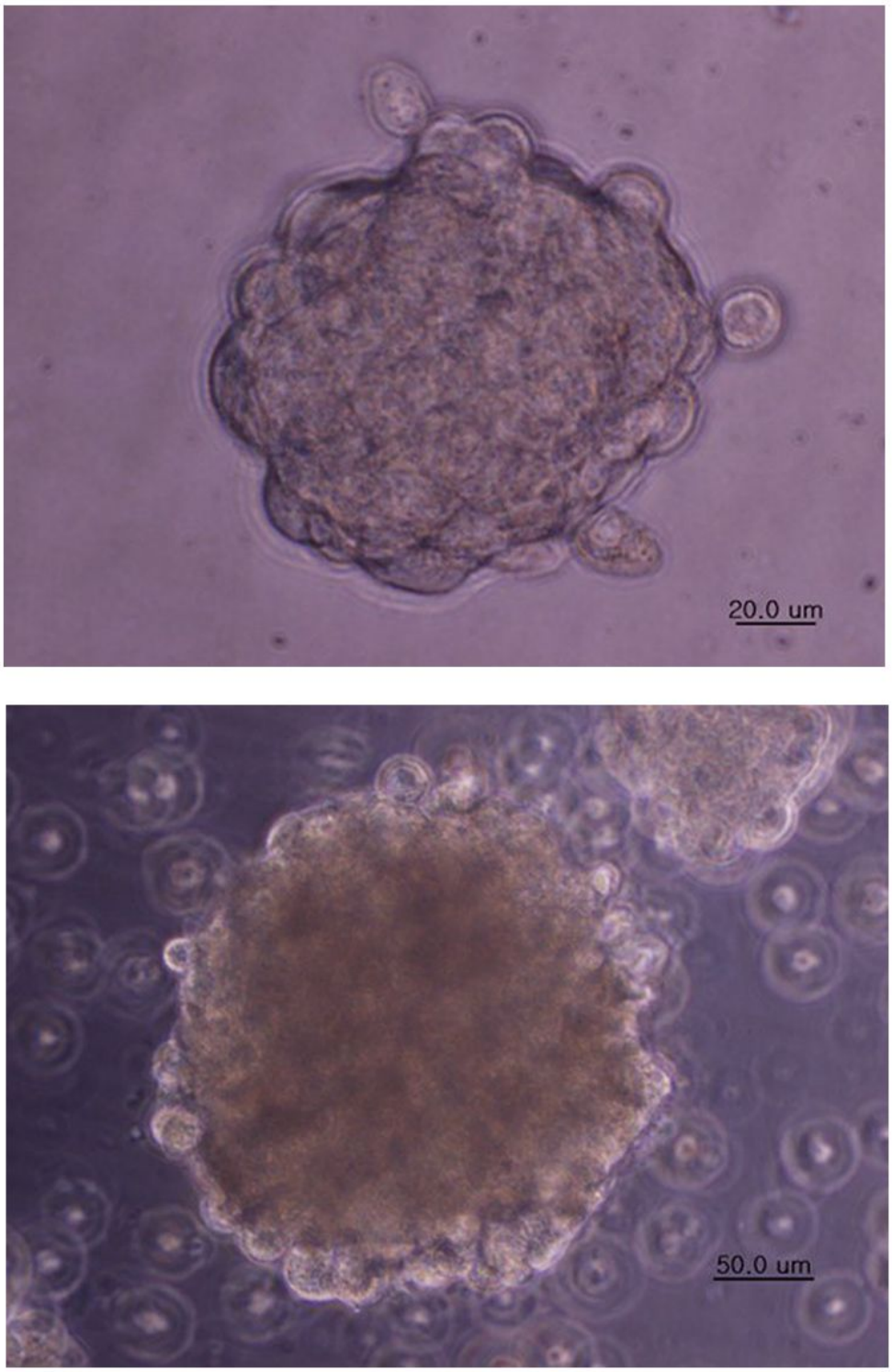

Figure 1

Phase contrast images of sarcospheres from human osteosarcoma cell lines. A. Representative sarcosphere from Cal-72 human osteosarcoma cell line. Bar represents 20 μm. B. Sarcosphere from SaOs-2 human osteosarcoma cell line. Bar represents 50 μm.

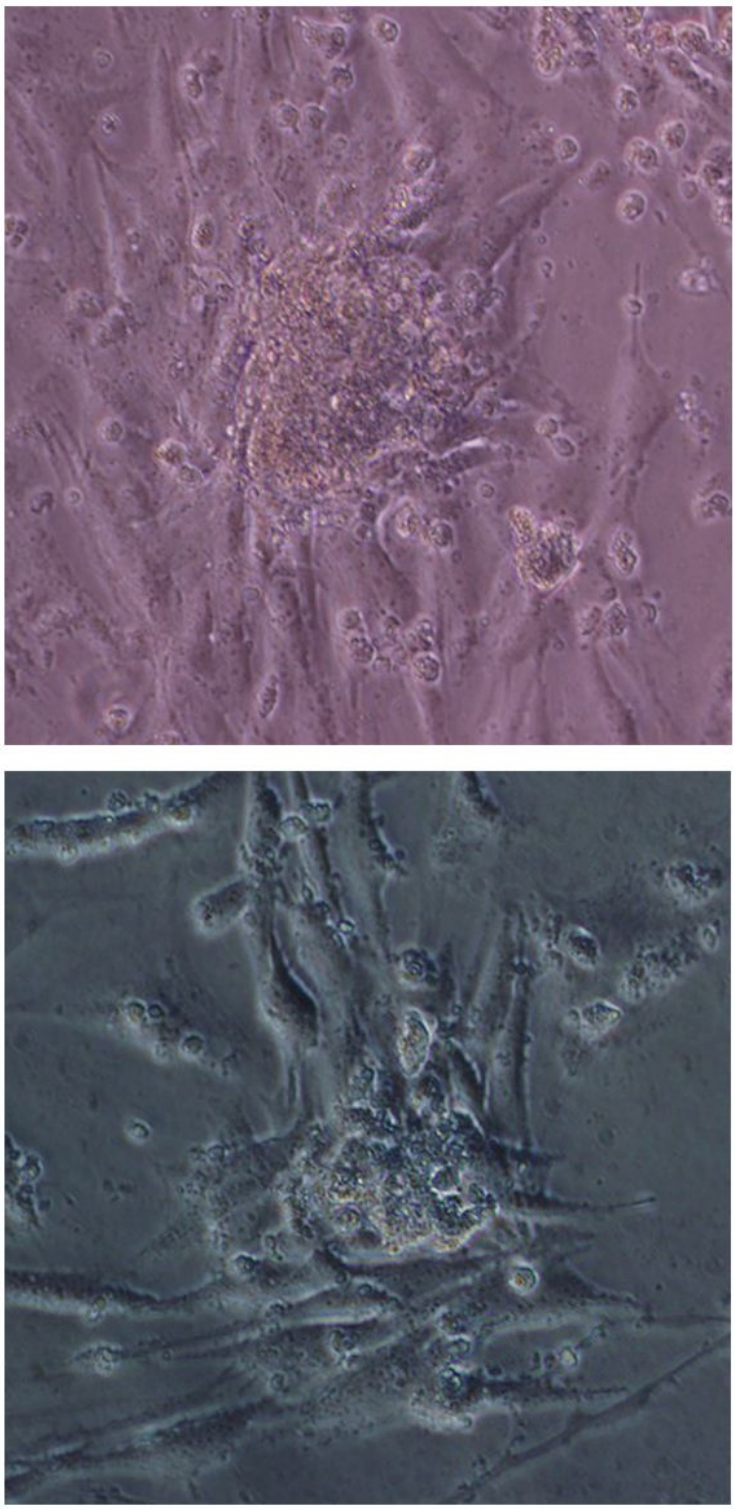

Figure 2

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

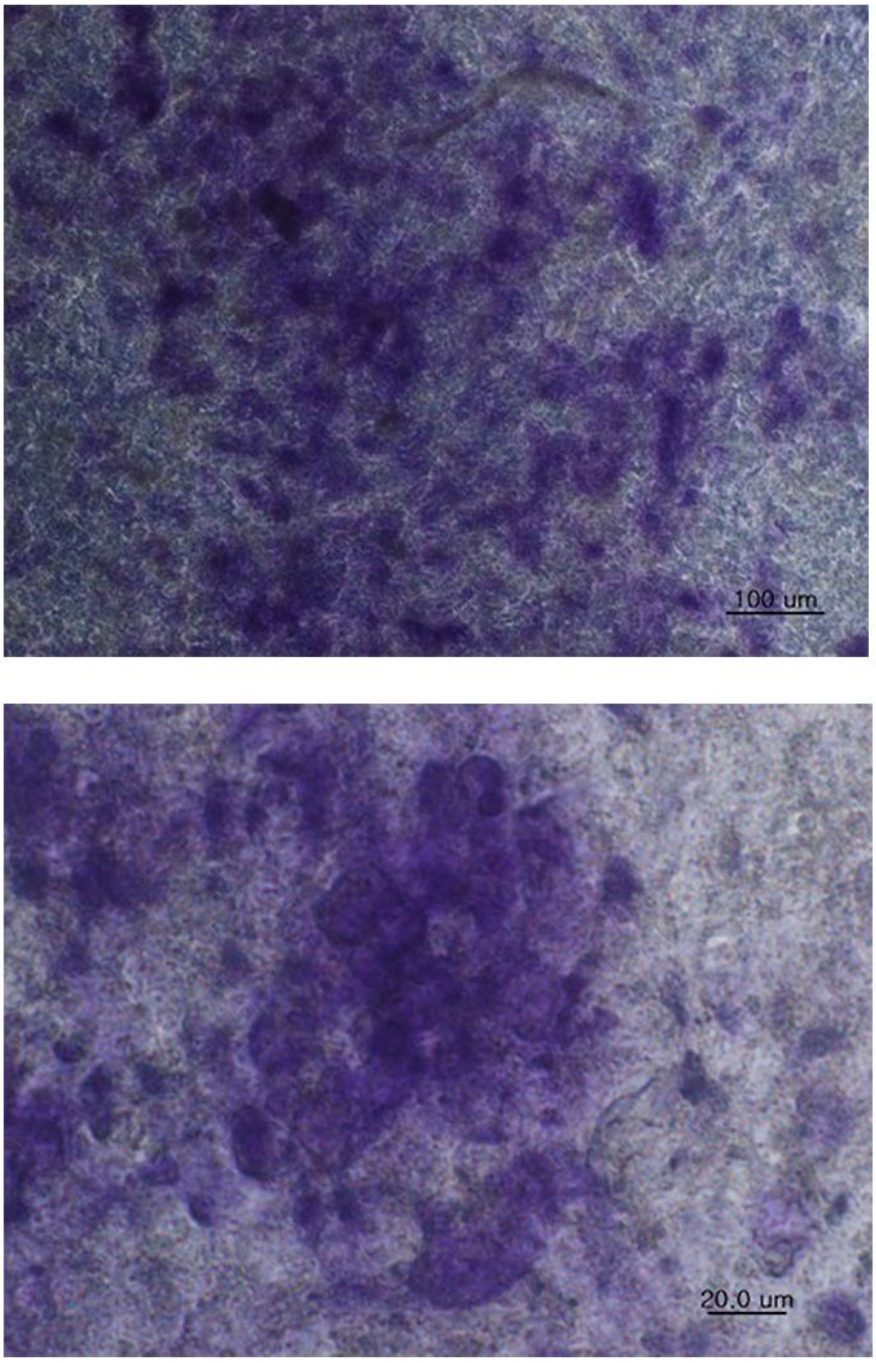

Figure 3

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 Cal-72 cell line. Bar represents 100 μm. B. Sarcosphere from SaOs-2 cell line. Bar represents 20 μm. C. Cal-72 adherent cells. Bar represents 100 μm.

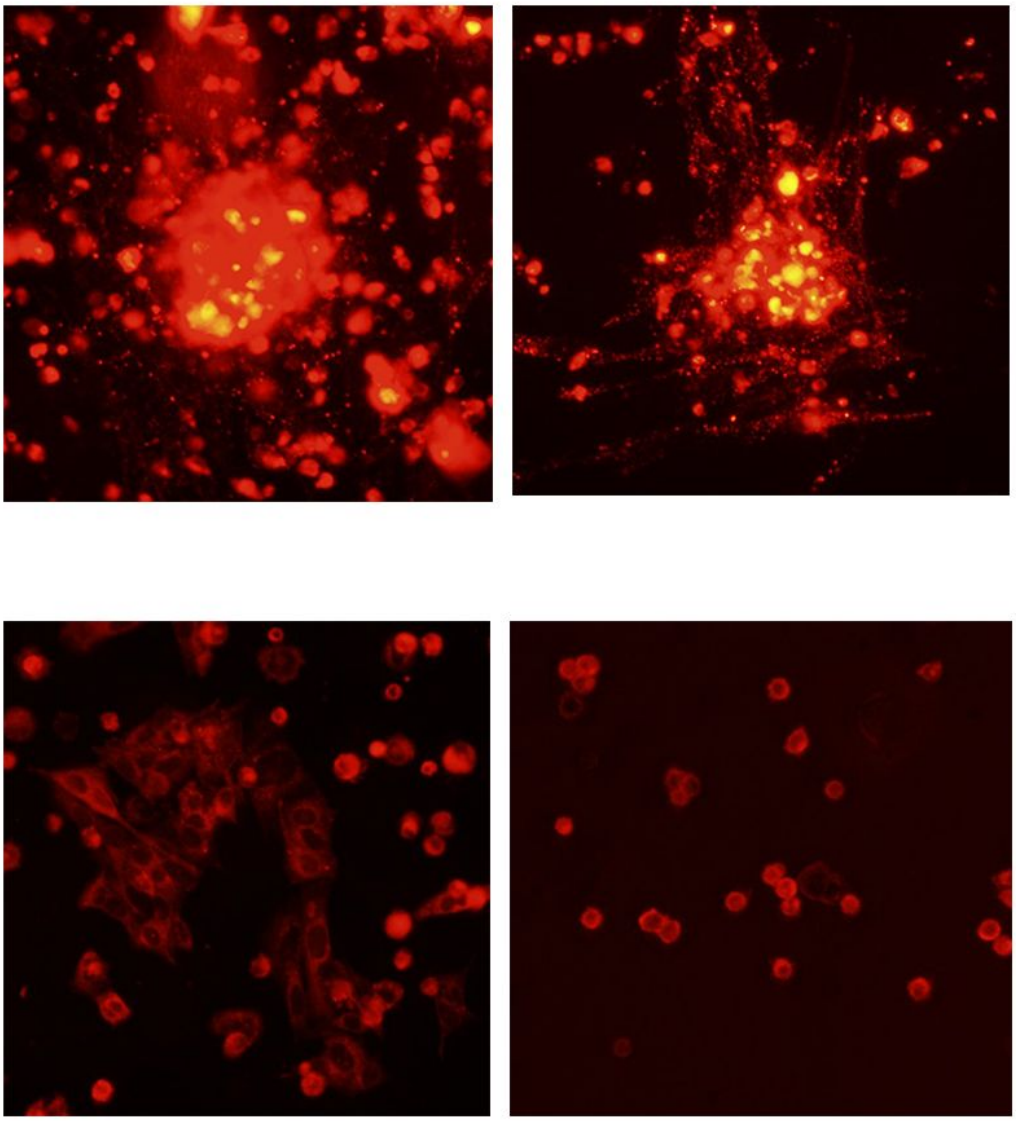

Figure 4

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 Cal-72 cell line. Original magnification 10×. B. Sarcosphere from 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×.

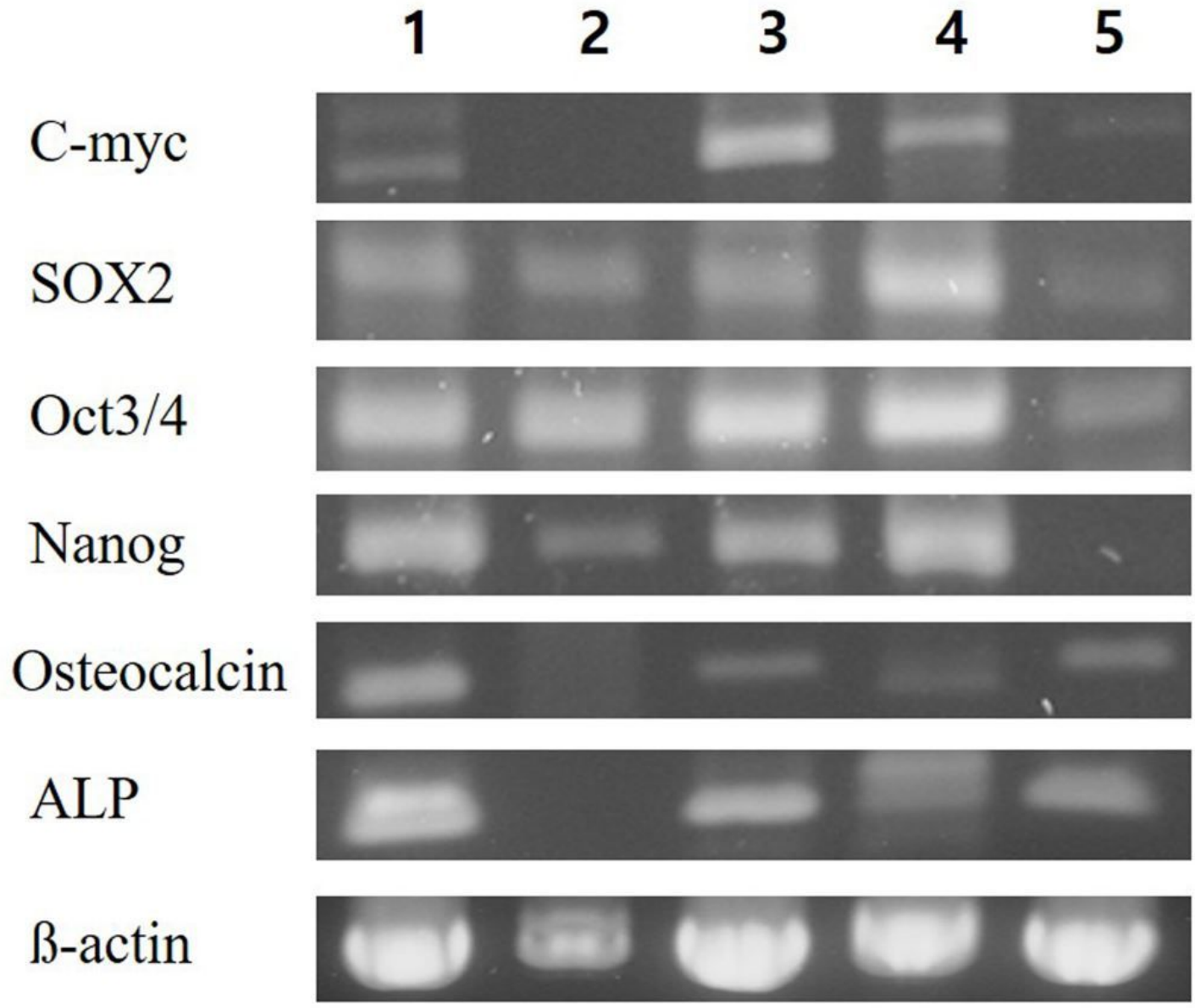

Figure 5

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 (as control)