Isolation of stem-like cells from human MG-63 osteosarcoma cells using limiting dilution in combination with vincristine selection

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To isolate stem-like cells from the human MG-63 osteosarcoma cell line, different subpopulations of MG-63 cells were cloned by limiting dilution and passaged to obtain different sublines. The subline with highest clonogenicity was identified using a proliferation assay, cell-cycle analysis, and soft-agar colony-forming assay. The sublines were further selected in serum-free medium containing 20 ng/ml vincristine to identify cells that could form suspended sarcospheres. Identified cells were then characterized based on morphology, cell surface markers, adipogenic and osteogenic differentiation, and tumorigenicity in nude mice. A total of 19 holoclones that could be stably passaged were obtained. Sublines A1, A3, and D1 were markedly different from other sublines and the parental cell line. Subline D1 not only had a higher colony-forming efficiency and formed larger colonies, but also possessed a shorter latency of tumorigenesis in vivo. After subline D1 was cultured in suspension in medium containing vincristine, a highly enriched subpopulation of cells that could form sarcospheres and be stably passaged were obtained. These cells, designated as MG-63-M expressed multiple markers of multipotent or embryonic stem cells and possessed the capacity for self-renewal, multilineage differentiation, and significant multi-drug resistance. Thus, our results suggest that a subpopulation of stem-like cells can be isolated from human MG-63 osteosarcoma cell line.

Keywords: MG-63 cells; Holoclone; Tumor stem-like cells, Vincristine

At present, the biological mechanism behind osteosarcoma formation is unclear. However, many researchers accept the ‘cancer stem cell’ hypothesis that there is a small number of self-renewing cancer cells with properties reminiscent of normal stem cells within a tumor. Cancer stem cells divide asymmetrically. The vast majority of cells derived from cancer stem cells are ordinary cells. A few, however, possess drug resistance and high self-renewal and proliferative capacity, which may explain the poor prognosis, recurrence, and metastasis of most tumors even after chemotherapy. Gibbs et al.1 successfully isolated a small subpopulation of stem-like cells from a bone sarcoma using a neurosphere culture system. These stem-like cells are capable of forming suspended spheres, express key markers of embryonic stem cells, and possess the capacity of self-renewal and multilineage differentiation.

In the present study, we have isolated tumor stem-like cells from the human MG-63 osteosarcoma cell line. Limiting dilution of MG-63 cells has been first performed to isolate holoclones with high clonogenicity. Several osteosarcoma cell sublines with high tumorigenicity have been identified. The sublines are then selected in serum-free medium containing a low concentration of vincristine to identify cells that are capable of forming suspended sarcospheres. A highly enriched sub-population, designated as MG-63-M, with the capacity of self-renewal, multi-lineage differentiation, high tumorigenicity and significant multi-drug resistance has been characterized.

Materials and Methods

Cell culture and clone derivation

The MG-63 cell line was purchased from the American Type Culture Collection (ATCC, Beijing, China). The scheme developed for clone isolation is depicted in Fig. 2. Cultures were incubated in a humidified tissue culture incubator in a 37.5% CO2.
atmosphere and were fed with fresh H-DMEM supplemented with 15% fetal bovine serum (Hyclone, USA) and 1% non-essential amino acids every 4 days. Nineteen individual sublines were isolated from attached spheroids by picking cells that developed with cloning cylinders.

**Cell proliferation**

Eight sublines were prepared as single cell suspensions and seeded in 96-well plates at 1000 cells/well in 0.1 ml H-DMEM containing 10% FBS. On days 1 through 7, culture medium was removed and 10 µl cells counting kit-8 (cck-8) was added. The cells were incubated for 2 h at 37°C. The plates were agitated for 15 min and the optical density of the solution in the wells was measured at 450 nm (Bio-TEK, USA).

**Determination of capacity for anchorage-independent growth in vitro**

Subconfluent cultures were harvested, suspended in 0.3% agarose at a concentration of 5×10^3 cells per ml, and seeded in six-well ultralow attachment plates (Corning Inc., USA) which contained a basal layer of 0.5% agarose. Colonies were counted after 2 weeks under a microscope at 10× magnification.

**Sphere formation assay**

MG-63 and MG-63-M cells were plated at a density of 6 × 10^4 cells/well in 6-well ultra low attachment plates (Corning, Inc.,) in N_2 medium with 1% methylcellulose. N_2 medium consisted of high-glucose Dulbecco’s Modified Eagle Medium (H-DMEM) with transferrin (25 mg/ml), insulin (20 mg/ml), vincristine (20 ng/ml), human recombinant epidermal growth factor (EGF; 20 ng/ml), and basic fibroblast growth factor (bFGF; 20 ng/ml) mixed with an equal volume of 2% methylcellulose. Fresh aliquots of EGF and bFGF were added every other day. After 6 and 12 days of culturing, colonies were counted by inverted phase contrast microscopy (Leica DC 300F). T sphere assay was performed three times. All reagents were purchased from Sigma.

**RNA isolation and reverse transcription-PCR**

Total RNA was extracted from cells using TRIzol reagent. RNA was reverse transcribed using the cDNA Synthesis Kit (Fermenters, France). cDNA was amplified using 1 µl of the reverse transcriptase reaction products in 20 µl with 10 pmol of the primers for 35 cycles. Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing and 60 s of extension at 72°C. The PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The primer sequences used for cDNA amplification will be provided on request. β-Actin was used as the internal control in all reactions.

**Flow cytometry analysis**

Cells were washed in 0.1% bovine serum albumin in phosphate buffered saline (PBS) at 48°C and incubated with 10 ml indicated antibody. Cells were then washed and assayed with a FACS Vantage (Becton Dickinson, USA) or Epics XL (Beckman-Coulter). Cells were incubated with antibodies against CD133, CD34, CD117, CD24, CD90, CD44, CD29, CD105, or CD271 (Santa Cruz, USA) on ice for 30 min, followed by incubation with a compatible fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 30-45 min.

**Immunofluorescence staining**

Cells were grown on glass coverslips for 48 h, fixed in ice-cold methanol or 4% paraformaldehyde at 4°C for 10 min, washed with PBS and blocked at room temperature for 30 min with 1% normal goat serum. Primary antibody incubation followed at room temperature for 30 min. After washing thoroughly with PBS, cells were further probed with FITC-tagged secondary antibody. Coverslips were mounted in anti-fade solution and viewed by confocal microscopy. The list of primary antibodies used and their specific dilutions will be provided upon request.

**Differentiation staining**

Cells derived from bone sarcoma spheres were grown on an adhesive substrate in adipogenic and osteogenic media for 14 days. Lipid-induced cells were fixed with 10% formalin, washed, and stained with oil red “O” for 30 min. Cells in osteogenic medium were fixed in ethanol, followed by von Kossa stain and exposed to sunlight for 30 min. Then cells were washed three times in distilled water and counterstained with sodium thiosulfate for 2 min.

**Results**

**Holoclone isolation**

A total of 60 holoclones were isolated in 96-well plates. Only 19 continued to grow in culture flasks and the others underwent spontaneous apoptosis or could not be passaged. The holoclones obtained were passaged eight to twelve times. Three sublines then
underwent spontaneous death and could not be further passaged. Cells of the remaining sublines were significantly different in gross morphology from the parent cells.

**Cell proliferation**

Eight sublines showing representative morphological characteristics were selected and growth curves were generated. Cells passaged four to sixteen times were used. In each experiment, cells of the same generation were used. The doubling time of subline A3 was significantly shorter than that of other sublines. As the passage number increased, the proliferation rate of subline D1 increased gradually (Fig. 1).

**Cell morphology**

Most sublines consisted of cells that had no significant changes in morphology when compared with the parental cells. The majority of cells had a short spindle or polygon shape, showed no contact inhibition, and were arranged densely and piled up on each other with a large cell body and slender cytoplasmic processes. Cells of sublines A3 and D1 had smaller cell bodies (Fig. 2). Cells of sublines A1 and F1 had an elongated spindle shape, showed a moderate degree of contact inhibition, and tended to form a single layer, with a small cell body, abundant cytoplasm, and short and thick cytoplasmic processes.

**Colony-forming efficiency**

Subline D1 formed the highest number of colonies. Most notably, the number of large colonies formed by subline D1 was significantly higher those of other sublines. The colony-forming ability of subline A3 was significantly higher than that of the parental cell line. Fourteen days after inoculation, the colony-forming efficiencies of sublines A3 and D1 were 19.5% and 24.2% (Fig. 3), respectively. The large-colony-forming efficiencies of subline A3 and the parental cell line were 3% and 14.3%, respectively.

**Cell cycle analysis**

Subline A3 contained more cells in the DNA synthesis phase (S) than did the parental cell line (31.6% vs. 7.23%). In contrast, the majority of cells of subline D1 (85.5%) were arrested in the G0/G1 phase. Moreover, subline D1 contained more cells in the S-phase than the parental cell line (13.5% vs. 7.23%).

![Fig. 1](image1.png)

**Fig. 1**—Growth curves of different sublines of MG-63 cells [Cell proliferation assays were carried out over a period of 7 days. The experiment was conducted in triplicate and average number of cells was plotted against time (days)]

![Fig. 2](image2.png)

**Fig. 2**—Colony morphologies in MG-63 cell line [(A and B): Two examples of colonies produced by the MG-63 line 7 days after plating indicate that their early colony morphologies are holoclones; and (C through F): Morphologies of holoclone sublines]

![Fig. 3](image3.png)

**Fig. 3**—Colony-forming efficiency of MG-63, D1 subline, and MG-63-M cells were 14.3%, 24.2%, and 75.6%, respectively.
Sarcosphere formation

MG-63 cells were centrifuged, harvested, and inoculated in serum-free medium containing vincristine. After 48 to 72 h of culture, only a few sarcospheres of unequal size formed and grew in suspension. The majority of adherent cells that could not form sarcospheres died due to drug intolerance. In MG-63 cells cultured in serum-free medium without vincristine, sarcosphere formation was delayed by approximately 12 to 24 h and the number of adherent cells was significantly higher than those in cells cultured in medium containing vincristine. Two days after passage, the formation of secondary spheres could be seen. These newly formed sarcospheres gradually grew in size and had a regular morphology. The subline that could be serially passaged as sarcospheres was designated as "MG-63-M" (Fig. 4). When first-passage MG-63-M cells were used to conduct soft agar colony formation assay, the colony-forming efficiency was as high as 75.6% (Fig. 3).

RT-PCR

RT-PCR analysis showed that markers specific for pluripotent stem cells (CD133) or embryonic stem cells (OCT3/4, nestin, and Nanog) were highly expressed in MG-63-M cells. The mRNAs for multi-drug resistance protein 1 (MDR1) and ATP-binding cassette superfamily G member 2 ABCG2 (ABCG2) were also highly expressed in MG-63-M cells (Fig. 5).

Flow cytometry detection of markers of different types of stem cells

The expression of surface markers of mesenchymal stem cells (CD105, CD90, CD44, and CD 29), hematopoietic stem cells (CD34 and CD133), and epithelial stem cells (CD24) were analyzed by flow cytometry. The results showed that CD34, CD31, and CD105 were not detectable on either MG-63 and MG-63-M cells, whereas CD90 and CD44 were strongly expressed on both types of cells at similar levels. The expression level of CD29 was slightly higher on MG-63-M cells than on MG-63 cells, whereas the expression level of CD24 was slightly lower on MG-63-M cells than on MG-63 cells. The expression level of pluripotent stem cell marker CD133 was much higher on MG-63-M cells than on MG-63 cells. RT-PCR analysis also confirmed these results (Fig. 6).

Adipogenic and osteogenic induction

MG-63-M and MG-63 cells were cultured in adipogenic and osteogenic media, respectively. Fourteen days after adipogenic induction, oil red "O" staining of adipogenic cultures was performed. Many lipid droplets were observed in the cytoplasm of induced MG-63-M cells. Twenty-four days after osteogenic induction, von Kossa staining of osteogenic cultures was performed. The formation of bone nodules was observed in MG-63-M cells. In contrast, although positive staining was observed in MG-63 cells, no significant bone nodule formation was noted (Fig. 7).

Proliferation of MG-63-M cells

The doubling time of MG-63-M cells was approximately 22.8 h. Newly formed stem-like cells

Fig. 4—Phase contrast images of monoclonal sarcospheres formed from self-renewing cells from bone sarcomas [Monolayer cultures of cells isolated from bone sarcomas were seeded at clonogenic density into N2 medium with 20 ng/ml vincristine and cultured for 2 weeks. Under these conditions, spheres formed at a frequency of 1/1000 cells. (A): N2 medium with 20 ng/ml vincristine after 7 days; (B): N2 medium with 20 ng/ml vincristine after 2 weeks; (C): Representative image of a sarcosphere in the suspension culture. Cells within the sphere show a compact undifferentiated morphology; and (D): Sarcosphere removed from the suspension culture and allowed to attach to a substratum. Adherent cell scan be seen expanding from the sphere]
were observed 24 to 48 h after passage and gradually increased in number. The exponential growth of MG-63-M cells occurred during the period between three and five days after passage.

**Immunofluorescence staining**

Immunofluorescence staining of suspended sarcospheres grown in serum-free medium was performed. The results showed that sarcospheres highly expressed pluripotent stem cell marker CD133, embryonic stem cell markers OCT3/4, nestin, and Nanog, multidrug resistance gene products MDR1 and ABCG2, and proliferation marker Ki-67 (Fig. 8).

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**Fig. 6**—Percent immunofluorescence staining of MG-63 cells (left) and MG-63-M cells (right) [(A through C): Analysis of mesenchymal markers of stemness CD90, CD44, and CD105; (D and E): Analysis of CD24, an epithelial marker, and CD133, a marker for pluripotent stem cells. Each panel is a single representative of three independent experiments]

**Fig. 7**—(A): MG-63-M cells were incubated in osteogenic media and analyzed for mineralization by von Kossa staining; and (B): MG-63-M cells were incubated in adipogenic media and analyzed for lipid vacuoles by oil red “O” stain [Both cultures showed focal staining for osteogenic and adipogenic differentiation]

**Fig. 8**—(A and B): Phase contrast images of typical spheroids generated in culture from MG-63-M cells; (C): Immunofluorescent staining for ABCG2 expression indicating multidrug resistance of MG-63-M cells; and (D through I) Immunofluorescence staining for Ki-67, CD133, OCT4, vimentin, nestin, and Nanog expression indicating self-renewing M spheroids]
Cells were implanted into the subcutaneous region of the inner thigh of nude mice. Under the operating microscope, it was observed that the tumors were gray in color, had obscure boundaries, and were surrounded by pseudocapsules. The cut face of the tumors had a scaly appearance. H & E staining of tumor sections showed that the tumors exhibited pathological features of malignant osteosarcomas.

**Discussion**

Previous studies have shown that MG-63 cells are not monoclonal but are heterogeneous in origin. Many researchers believe that purification of monoclonal cell strains from cell lines is essential for the study of cells. To obtain monoclonal stem-like cell strains from MG-63 cells, we first identified several subpopulations of holoclones. Each holoclone originated from a round- or oval-shaped dense colony of cells. Holoclone cells from breast cancers, gliomas, and other solid tumors tend to form small colonies and have high clonogenicity and strong adhesion abilities. Our findings suggested that the osteosarcoma cells that formed holoclones were tumorigenic. We also found that cultured monoclonal cells did not always retain their unique shape and characteristics, but underwent re-differentiation or automatic subcloning after passage over several generations. For this reason, cells of the same generation were used in each experiment in the present study (all within 20 passages or less).

Soft-agar colony-forming ability, which reflects the loss of anchorage dependence, is an important parameter for evaluation of the tumorigenic potential of malignant tumor cells. A cell migration, invasion, adhesion, and outgrowth assay is the most commonly used and most reliable method for determination of cell motility, as this reflects the ability of tumor cells to detach from the extracellular matrix in the primary tumor, disseminate, and colonize distant organs in vivo. In this study, we found that the proliferative rate of subline D1 was relatively slow during early passages, but increased with generation number. Its invasion and migration ability was not significantly different from that of the parental cell line. Cell cycle analysis revealed that the percentage of cells in G0/G1 phase was significantly higher in cells of subline D1 than in cells of other sublines. Moreover, subline D1 possessed significantly higher colony-forming capacity than other sublines and the parental cell line. Compared to other sublines, the colonies formed by subline D1 were not only greater in number, but also larger in size. Therefore, we speculate that cells of subline D1 may be the true tumorigenic cells in the MG63 line.

A growing body of research suggests that solid tumors consist of stem cells, fast proliferative progenitor cells or precursor cells, and maturely differentiated cells. In this study, we used an improved method to isolate tumor stem-like cells that possessed high tumorigenicity and drug resistance from monoclonal subline D1 derived from MG-63 cells. The cell cycle-specific drug vincristine was used in this study to isolate tumor stem-like cells that we designated MG-63-M cells. Earlier, Yu et al. successfully isolated a highly enriched subpopulation of tumor stem cells from the human glioma cell line U87 using a similar method.

The gross morphology of the tumor stem-like cells we isolated was basically the same as that of tumor stem cells previously isolated from brain tumors, breast tumors, and osteosarcomas. The tumor stem-like cells obtained from suspended spheroids of MG-63 cells have many attributes in common with both normal stem cells and glioblastoma stem cells. Similar to those reported previously, the tumor stem-like cells we characterized expressed many mesenchymal stem cell markers (CD29, CD44, CD45, and CD105). Moreover, these cells also expressed many genes associated with the mesoderm, ectoderm, and endoderm germ layers. Sarcospheres highly expressed the key markers of embryonic stem cells: Oct-3/4, nestin, and Nanog. In contrast, these genes were expressed at extremely low levels in MG-63 cells. Pluripotent stem cell marker CD133 was highly expressed in MG-63-M cells (>90%), but barely detected in MG-63 cells. Further experiments showed that although these stem cell markers were highly expressed in small sarcospheres formed at early stage, their expression levels decreased gradually with the increase in sphere size and cell number, indicating that tumor cells gradually became more heterogeneous. These results suggest that original stem cell-like cells undergo a transition from asymmetric to symmetric division and consequently generate many heterogeneous progeny cells.

Oct-3/4 is a member of the POU family of homeobox transcription factors. In the early embryo, it is expressed in the inner cell mass and is critical for maintaining the pluripotency of embryonic stem cells. During subsequent embryonic growth and...
development, its expression is gradually downregulated\textsuperscript{12}. In adults, it is expressed only in type A spermatogonia, testicular germ cell tumors, teratocarcinomas, and some progenitor cells\textsuperscript{16}. Similarly, the expression of the Nanog gene is barely detectable in somatic cells\textsuperscript{16,17}. Nanog gene activation is essential for control of self-renewal and maintenance of pluripotency in embryonic stem cells\textsuperscript{18}. In the present study, we found that the Nanog gene was highly expressed in MG-63-M cells, suggesting that MG-63-M cells, similar to embryonic stem cells also possessed pluripotency. Oct 3/4, Nanog, and nestin are required to maintain differentiation potential and self-renewal of murine embryonic stem cells\textsuperscript{19}. If these proteins play similar roles in tumor stem-like cells, they will be ideal molecular targets for directed therapy of osteosarcomas.

Overexpression of P-glycoprotein (Pgp) has been detected in many malignant tumors. Generally, MDR1/Pgp expression is predictive of poor prognosis as it is associated with the malignant transformation of many tumors\textsuperscript{20}. A variety of stem cells express ABCG2, which may protect these cells from exogenous and endogenous toxin-induced damage\textsuperscript{21}. Moreover, ABCG2 can transport some differentiation-promoting molecules out of cells to maintain progenitor cells in an undifferentiated state\textsuperscript{22}. ABCG2 is expressed in leukemia, germ cell tumor, breast cancer, prostate cancer, and lung cancer cells and is thought to be a conservative marker of tumor stem cells\textsuperscript{23,24}.

Many researchers speculate that ABCG2 expression in cancer stem cells or side population cells may be partly responsible for poor outcome of patients with malignant tumors. In the present study, we found that tumor stem-like cells isolated using a low concentration of vincristine exhibited significant drug resistance. RT-PCR indicated that mRNAs for the multidrug resistance gene Pgp/MDR1/ABCB1 and multidrug resistance-associated protein genes MRP1/ABCC1 and MXR/BCRP/ABCG2 were highly expressed in MG-63-M cells. This result provided support for the cancer stem cell hypothesis and might explain why a considerable number of osteosarcoma patients show remarkable resistance to chemotherapy. A recent study showed that the survival rate of patients treated only with chemotherapy was only 20\%\textsuperscript{25}.

The cancer stem cell hypothesis provides an explanation for the etiology of many heterogeneous and highly malignant tumors. In this study, we successfully isolated a subpopulation of tumor stem-like cells from a human osteosarcoma cell line by harvesting spheroids formed in serum-free culture medium containing a low concentration of vincristine. Based on our results, we believe that initial identification of holoclones with high tumorigenicity followed by further enrichment is an effective approach for isolation of tumor stem cells. The tumor stem-like cells obtained in this study not only possessed the capacity of self-renewal and multilineage differentiation, but also had higher tumorigenicity and more significant drug resistance than those obtained using the conventional method. Thus, it is plausible to speculate that this subpopulation of cells plays a crucial role in the recurrence and chemotherapy resistance in osteosarcomas and, therefore, represents an important target for treatment of osteosarcomas.

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