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Effects of Microgravity on Cancer Stem Cells Cultured in a NASA Developed Bioreactor: Chemotherapeutic Implications.

Sarah Elizabeth Daron Kelly

Effects of Microgravity on Cancer Stem Cells Cultured in a NASA Developed Bioreactor: Chemotherapeutic Implications.

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by

Sarah Elizabeth Daron Kelly

Thesis Committee

Jagan V. Valluri, Ph.D., Committee Chairperson
Pier Paolo Claudio, M.D., Ph.D., Committee Member
Vincent E. Sollars, Ph.D. Committee Member
Marshall University, Huntington, WV

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Abstract

The purpose of this study was to investigate the effects of simulated microgravity on cancer cell growth. Simulated microgravity is a condition in which cells are in constant free-fall and in which they are able to grow suspended without the shear and stress. Microgravity allows for important insights into the cellular mechanism and may be key in understanding why some cells thrive in this condition and others go into apoptosis. Cancer stem cells (CSCs) are considered a subset of the bulk tumor responsible for initiating and maintaining cancer. SAOS-2 is a human bone cancer cell line which consists of a heterogeneous population [10% of CD133(+) cells and 90% CD133(-)]. CD133 is a transmembrane pentameric glycoprotein and a surface marker for progenitor cells including neural and embryonic stem cells, and it is expressed in cancers, including some leukemias and brain tumors. We isolated CD133(+) cells from the SAOS-2 cell line by using a MACSorting system which magnetically sorts CD133(+) cells with the aid of an antibody against CD133 conjugated to a magnetic bead. SAOS-2 positivity to CD133 was assessed by flow cytometry. One million SAOS-2 CD133(+) MACSorted cells were cultured, in a Hydrodynamic Focusing Bioreactor (HFB) which was developed by NASA at the Johnson Space Center for modeling microgravity conditions. This experiment showed an increase of cell growth compared to the normal gravity control. We observed a 15-fold proliferation of the CD133(+) cellular fraction with cells that were cultured for 7-days at optimized conditions. Additionally, 100% of the harvested cells were found to be CD133(+), indicating that the HFB had selected for the SAOS-2 undifferentiated cellular fraction. CSCs are thought to be responsible for the formation and growth of neoplastic tissue and are naturally resistant to chemotherapy also in part due to their quiescent nature. We also tested the hypothesis that the microgravity environment could sensitize the CD133(+) resistant osteosarcoma cells to various chemotherapy agents at clinically relevant doses. SAOS-2 cells were cultured in three chemotherapy treatments: cisplatin, doxorubicin, and methotrexate. Toxicity and viability assays were performed on CD133(+) cells, CD133(-) cells, total SAOS-2 population, and on cells that were cultured in the HFB for 5-days with these different chemotherapies. Interestingly, we found, by FACS analysis and MTT assay, that the microgravity environment greatly sensitized the CSCs, which are normally resistant to chemotherapy treatments, to become susceptible to various chemotherapeutic agents. This experiment paves the way to less toxic and more effective chemotherapeutic treatments in patients.

Dedication

I would like to dedicate this to my Aunt Madeleine and Uncle Okie. I know that you do not always understand my dreams and goals but you have always been supportive and have encouraged me to follow my dreams. Knowing that you are proud of me has given me immeasurable strength to follow my heart, and for that I could never repay you both.

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Abbreviations

1G	1 (Normal) Gravity
AML	Acute myeloid leukemia
ANOVA	Analysis of Variance
BMSSC	Bone Marrow Stromal Stem Cells
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CDDP	Cisplatin
CSCs	Cancer Stem Cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOX	Doxorubicin
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HARV	High Aspect Ratio Vessel
HFB/HDFB	Hydrodynamic Focusing Bioreactor

IACUC	Institutional Animal Care and Use Committee
LD_{50}	Lethal Dose, drug concentration resulting in 50% inhibition of viability
MACSorted	Magnetic-Activated Cell Sorting
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NASA	National Aeronautics and Space Administration
PBS	Phosphate buffered saline
PE	Phycoerythrin
PS	Phospholipid Phosphatidylserine
RCCS	Rotary Cell Culture System
RCM	Rotary Culture Max
RNA	Ribonucleic Acid
RPMI 1650	Roswell Park Memorial Institute medium
RWPV	Rotating Wall-Perfused Vessel
RWV	Rotating Wall Vessel
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SPARC	Secreted Protein Acidic and Rich in Cysteine
T-IC	Tumor Initiating Cell

Chapter I: General Introduction

1.1 Cancer Stem Cells

Cancer stem cells (CSCs) are considered a subset of the bulk tumor responsible for initiating and maintaining cancer. Neoplasm's or tumors may be viewed as tissue consisting of heterogeneous population of cells that differ in biological characteristics and potential for self-renewal [1]. Normal stem cells are characterized by three properties: 1) capability of self-renewal; 2) strict control on stem cell numbers; 3) ability to divide and differentiate to generate all functional elements of that particular tissue [2]. Compared to normal stem cells, cancer stem cells are believed to have no control on cell number. The cancer stem cell hypothesis asserts that solid tumors are maintained exclusively by a rare fraction of cancer cells with stem cell properties [3]. Thus, these cells should represent the primary therapeutic target in order to achieve complete eradication of the tumor. However, according to the model of clonal evolution of tumor cells, cancer is formed through the accumulation of genetic mutations in cells and gradual selection of clones [4, 5]. Therefore, the tumor is regarded as abnormal tissue that descended from a single cell through continuous accumulation of genetic errors and various epigenetic changes. However, several experiments carried out during the last decades have shown that not every tumor cell is a tumor initiating cell (T-IC) and that as many as 10 million tumor cells are required to transplant a new tumor from an existing one [5-8]. This evidence suggested the possibility that tumor cells may exist in a hierarchical state in which only a small number of those cells possess tumor initiating

potential.

The existence of cancer stem cells was first proven in the context of acute myeloid leukemia [9]. More recently, this principle has also been extended to other tumors, such as breast and brain cancer [10, 11]. Additionally, cancer stem cells have been reported to be the only tumorogenic population in many forms of solid tumors; being only a minor fraction among the bulk of more differentiated cells in the tumor [3, 12-14]. These cells appear to be capable of asymmetric division and self-renewal like that of normal stem cells (Fig. 1), and are unlimited in there proliferative potential which is required for tumor development and maintenance [5, 7, 15-19]. This small population of cancer stem cells (CSC) has been identified in adult and pediatric tumors [6, 8, 10, 11, 20-22].

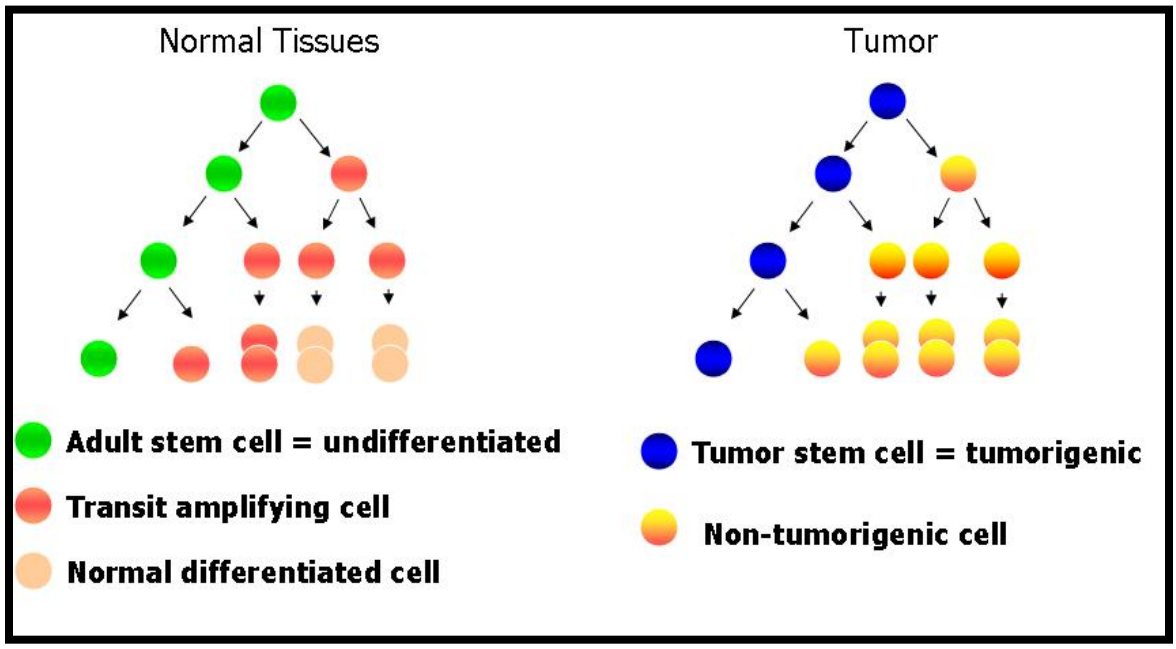


Figure 1. A representation of asymmetrical cell division in normal tissue along side of tumorigenesis.

The recent discovery of CSCs has played a pivotal role in changing our view of carcinogenesis and chemotherapy [23-25]. At present, reduction in size of a tumor is considered as a response to the treatment. However, tumors often initially respond to treatment only to recur again (Fig. 2). Certain tumors are highly resistant to chemotherapy and other forms of treatment and although aggressive treatments destroy the majority of the cancerous cells, a small fraction of the cells survive and often regenerate into even larger masses of tumor cells [15, 26, 27]. This may explain why traditional chemotherapies can initially reduce tumor but fails to eradicate it, allowing eventual recurrence [1, 3, 14, 26, 28]. This may be an explanation for the recurrence if the treatment is only targeting cancer cells and not able to target the cancer stem cells.

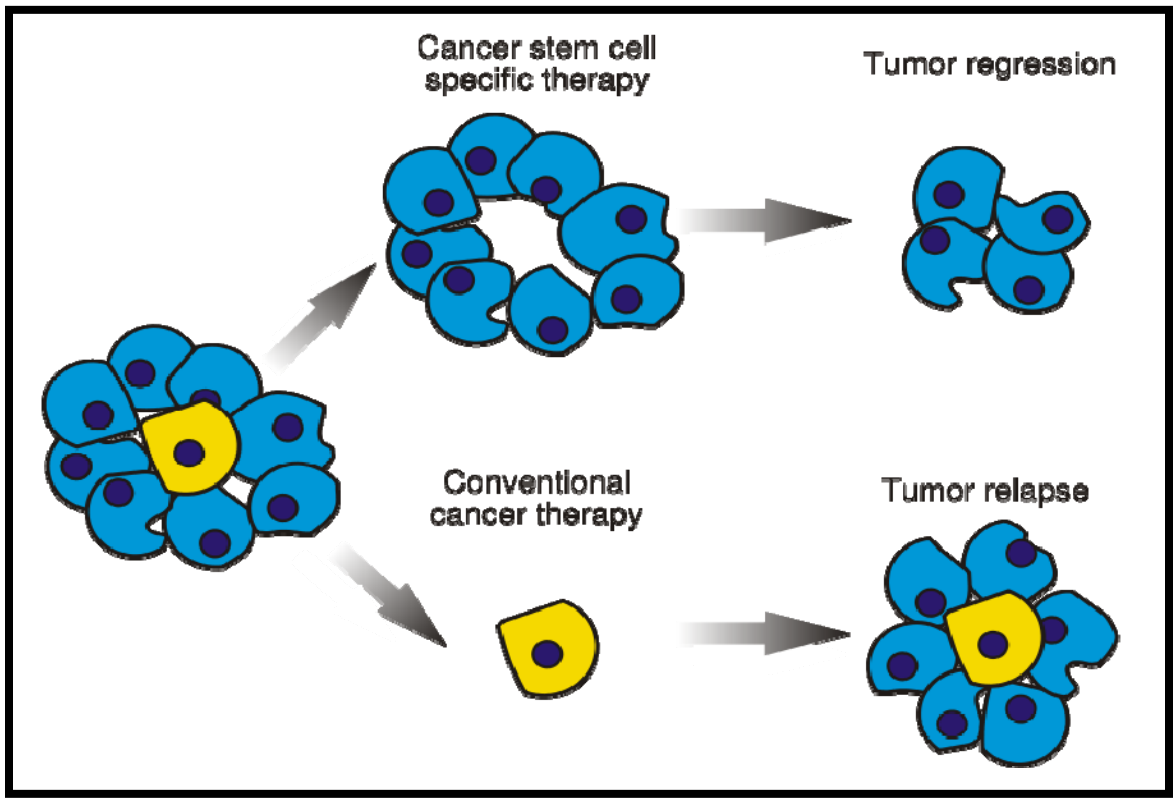


Figure 2. A diagram illustrating the distinction between cancer stem cell targeted (above) and conventional (below) cancer therapies (Adapted from Peter Znamenskiy http://en.wikipedia.org/wiki/File:Cancer_stem_cells_text_resized.svg)

CSCs are more resistant to therapy for two reasons. One is their quiescence nature, but they also have increased expression of anti-apoptotic proteins and drug efflux transporters [1, 29-33]. Cancer treatments available today mostly exploit the proliferative and metastatic potentials of the cancer cells; therefore, the majority of treatments are targeted at rapidly dividing cells and at molecular targets that represent the bulk of the tumor. Additionally, if a drug affects the growth of only a minor population within the tumor, there will be only a minimal decrease in the growth of the tumor in the short term and an increased risk for adaptations and resistances to occur in the long term. Theoretically, identification and characterization of the cancer stem cells may allow the development of treatment modalities that will target the cancer stem cells rather than the rapidly dividing cells in the cancer and eradication of the tumor altogether.

1.2 Cell Markers

1.2.1 Stem Cell Markers

Several surface cellular markers have been recently used to identify CSCs. One such marker is CD133 which is a transmembrane pentameric glycoprotein (Fig. 3), also known as AC133 or Prominin 1. The function of CD133 is unknown, it is expressed by hematopoietic progenitor cells as well as neural and embryonic stem cells, and it is expressed in cancers, including some leukemias and brain tumors [34, 35]. These CD molecules are a cluster of differentiation molecules present on the surface of various cells within the human body. The use of this marker for cancer research is becoming increasingly more popular.

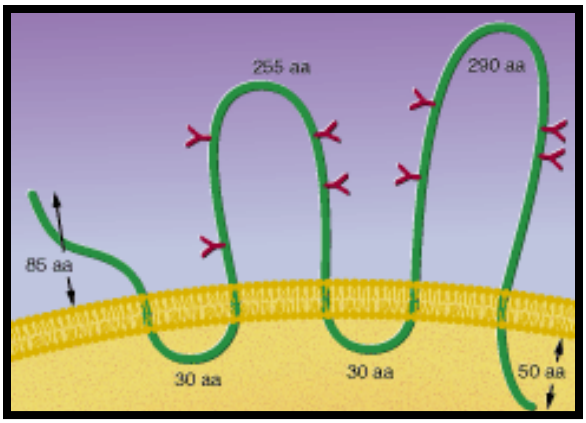


Figure 3. A structure model of CD133 proposed by Miraglia S. *et al.* (1997). This protein has an extracellular N-terminus, 5 hydrophobic transmembrane domains, 2 small cytoplasmic loops, 2 large extracellular loops and a cytoplasmic C-*terminus*.

Also several other markers have been known for there importance. CD34 is a cell surface glycoprotein and functions as a cell-adhesion factor. In this role, CD34 mediates the attachment of stem cells to their niche, extra-cellular matrix or directly to stromal cells. CD34 expression is found in various malignant tumors [36, 37]. CD38 is another cluster of differentiation molecule and it is found on the surface of many white blood cells in the immune system [38]. The CD38 protein has been found in leukemia, myelomas, solid tumors, as well as bone metabolism [38]. CD38 also functions in cell adhesion, signal transduction and calcium signaling [38]. Acute myeloid leukemia (AML) cells observed as CD34(+) and CD38(-) are in an undifferentiated and primitive form and have been shown to engraft in immune-compromised mice [39].

CD117, also called KIT or C-kit receptor, is a cytokine receptor, tyrosine kinase type III, expressed on the surface of hematopoietic stem cells as well as other cell types.

This receptor binds to the cytokine stem cell factor (SCF) that promotes cell growth in certain cell types, and mutations of this receptor have been associated to some types of cancer [40]. CD117 is an important cell surface marker used to identify certain types of hematopoietic progenitors in the bone marrow [40]. In addition, the gene encoding CD117 is frequently over expressed and amplified in germ cell tumors, leukemia and gastrointestinal stromal tumors [40, 41].When this receptor binds to SCF, it forms a dimer, which activates cell signaling by second messengers. Signaling through CD117 has been shown to play a role in cell survival, proliferation, and differentiation [42].

Stro-1 is frequently used to identify bone marrow stromal cell precursors and bone marrow stem cells [43, 44]. The Stro-1 antibody has been recently used as a single reagent to isolate human bone marrow stromal stem cells (BMSSC). This is due to Stro-1 being restricted specificity to surface molecule expressed by clonogenic BMSSC, with little or no reactivity to hematopoietic stem/progenitor populations or mature stromal elements [45]. It is considered also an early marker of embryonic stem cells. The role of Oct-4 as one of the essential pluripotent genes for embryonic stem cells is well documented [46], several studies have suggested roles of this gene in sustaining self-renewal capacity of adult somatic stem cells. Oct-4, one of the earliest transcription factors of the embryo, is critically involved in the self-renewal of undifferentiated embryonic stem cells and has been recently shown involved in bladder tumor progression [47].

Osteocalcin is a non-collagenous protein found in bone and dentin. It is secreted by osteoblasts, and it is thought to play a role in mineralization and calcium ion homeostasis [48]. It is often used as a biochemical marker for the bone formation

processes and for mesenchymal stem cells [49, 50]. RunX-2 has been shown to be essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. RunX-2 is a transcription factor associated with osteoblasts differentiation that can bind DNA. Recently it has been shown that Sox-9 is dominant over RunX-2 function in mesenchymal precursors that are destined for a chondrogenic lineage during endochondral ossification [51].

1.2.2 Cellular Activity Markers

Other molecules involved in many different aspects of cellular activity were also markers of interest. Endoglin is a molecule that has been postulated to be involved in the cytoskeletal organization affecting cell morphology and migration [52]. Integrins are instead cell surface receptors that interact with the extracellular matrix (ECM) and act as cell signaling molecules. They define cellular shape, mobility, and also help regulate the cell cycle. These proteins play a role in the attachment of cells to other cells, and also aide in the attachment of a cell to the ECM. The signals integrins convert travel both ways in the cells. Signals travel outside-in: transducing information from the ECM to the cell, and inside-out: "exposing" the status of the cell to the extracellular world. Cell interaction and attachment to the ECM is a basic requirement to build a multicellular organism. One of the most important functions of surface integrins is their role in cell migration and homing [53, 54]. Another protein that also mediates cell-matrix interactions is Secreted Protein Acidic and Rich in Cysteine (SPARC). SPARC is a protein that, mediating cell-matrix interactions, plays a role in modulation of cell

adhesion, differentiation, and angiogenesis [55].

1.3 Microgravity

Conventional methods for cell culturing are based on methods that allow cells to attach to the bottom of a cell culture dish and as cells settle due to gravity they begin to grow in a monolayer fashion but do not fulfill all the original functions of the tissue from which they are originating. In contrast, the cellular constructs within the mammalian body are three-dimensional.[56]. These cell in microgravity are not the same as a low attachment dish because they attach to each other to form a three-dimensional. Microgravity (μG) is a sustained free falling condition, in which these cells never reach the bottom and the cells in time begin to adapt to this new environment giving them the possibility to grow in all three dimensions [56]. On Earth, microgravity conditions can be produced by dropping from a tower or by parabolic flights in an airplane. However, the duration of microgravity obtained by these methods is too short for cells to exhibit obvious changes in growth and development. Simulated microgravity bioreactors provide this environment on Earth without time restriction.

Prolonged exposure of humans and experimental animals to the altered gravitational conditions of space flight has adverse effects on different cellular systems [57]. The effects of microgravity environment on tumor growth and carcinogenesis are unknown and this research field still lacks systematic investigation on microgravity induced gene expression, which is the key information needed to ultimately unfold the mechanism behind microgravity-induced diseases. There are several types of ground-based reactors that simulate microgravity effects. Various designs of rotating-wall vessels

have been used to create a three-dimensional culture environment with variable shear stress and microgravity simulating that in space [56]. The hydrodynamic focusing bioreactor (HFB) or sometimes labels as (HDFB) by (Fig 4), has many advantage to the rotating wall vessel RWV, such as the lack of formation of air bubbles, and it offers possibilities for controlling the location of the cell formations.

The effects of simulated microgravity on gene expression of human osteosarcoma cells cultured in a NASA-developed hydrofocusing bioreactor (HFB) were studied. The HFB (Celdyne, Houston, TX) developed by NASA at the Johnson Space Center, is a fluid filled dome, which rotates at a specified speed to provide a unique hydrofocusing capability that will allow for an extremely low-shear culture environment (Fig. 5). The NASA - designed bioreactors have been successfully used on Earth and in Space to enable investigators to use simulated or actual microgravity, respectively, to study the role of gravity on the formation of three-dimensional mammalian cell tissue models and production of bio-products. This reduction in structural energy used by the cell can be transferred to the production of secondary activities such as increased growth rates. In this study it was discovered that CSCs are stimulated to proliferate when they are placed in microgravity conditions and it has been tested that the absence of gravity will sensitize the CSCs or tumor stem cells to chemotherapeutic agents.

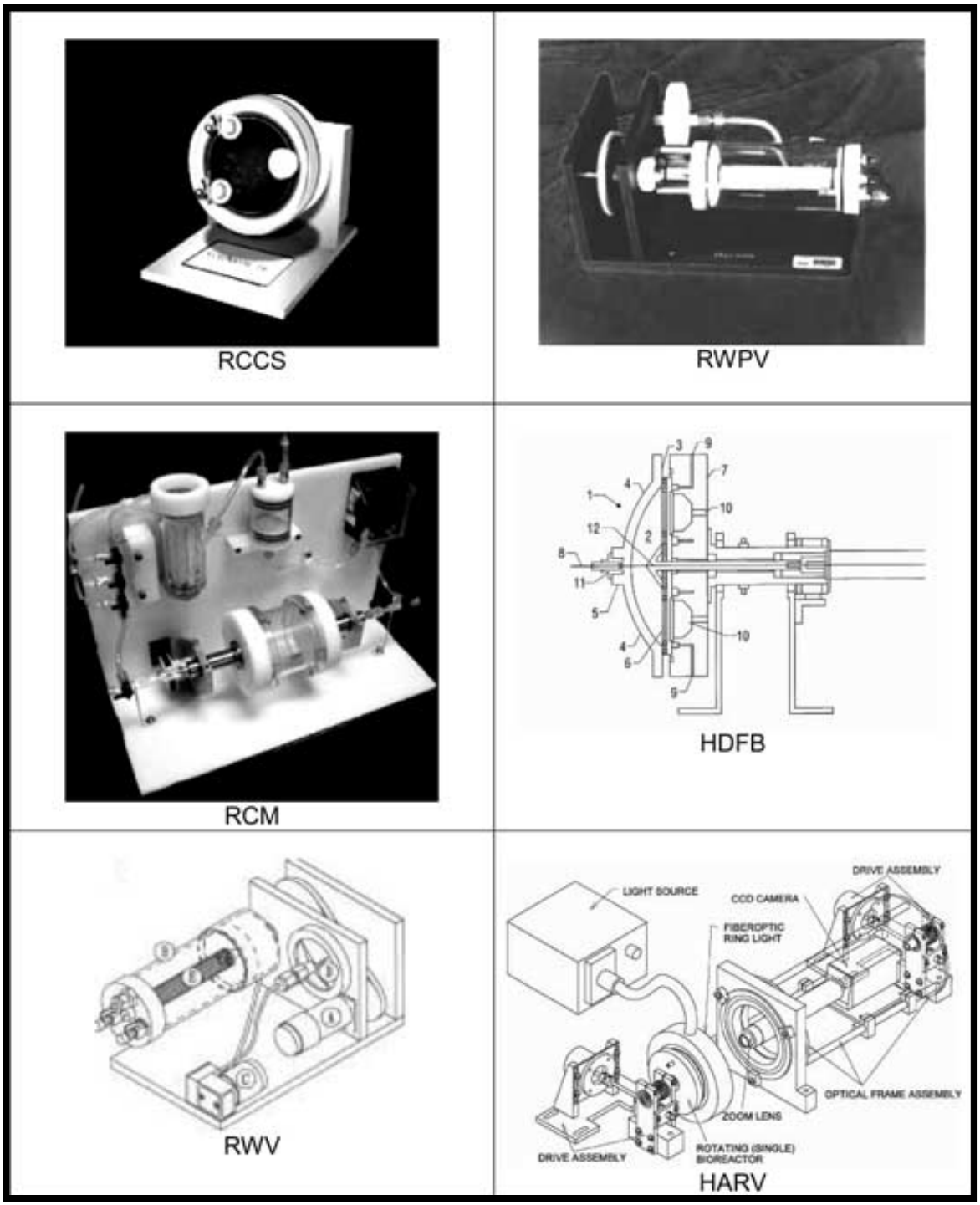


Figure 4. Different types of rotating wall vessel bioreactor systems. RCCS = rotary cell culture system; RWPV = rotating wall-perfused vessel; RCM = rotary culture max; HDFB = hydrodynamic focusing bioreactor; RWV = rotating wall vessel; and HARV = high aspect ratio vessel. By Mihailova et al. (2006)

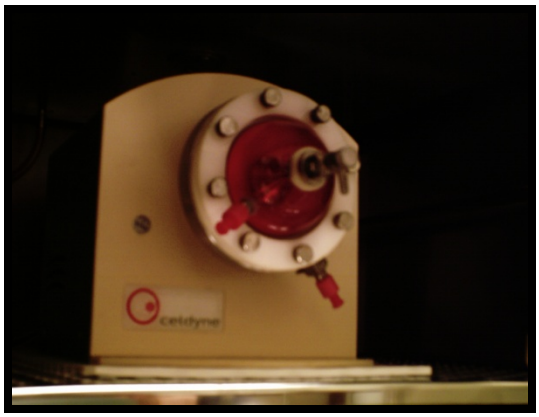


Figure 5. Photograph of the HFB in use.

1.4 Chemotherapy

Effective therapies for cancer patients require a thorough understanding of mechanisms leading to tumor development and drug resistance. Cancer treatments available today are mostly targeted at its proliferation potential and its ability to metastasize, and hence the majority of treatments are targeted at rapidly dividing cells and at molecular targets that represent the bulk of the tumor. This may explain the failure of treatments to eradicate the disease or the recurrence of cancer. Although current treatments can reduce the size of the tumor, these effects are transient and usually do not improve patient's survival outcomes [58]. There are three aspects to consider in tumors where CSCs play a role. First, the mutation of normal stem cells or progenitor cells into cancer stem cells can lead to the development of the primary tumor. Second, during chemotherapy, most of the primary tumor cells may be removed, but if cancer stem cells are not eradicated, they become refractory cancer stem cells and may lead to recurrence of the tumor. Third, the cancer stem cells may migrate to distal sites from the primary tumor and cause metastasis [59]. Theoretically, identification and characterization of the

cancer stem cells may allow the development of treatment modalities that target the cancer stem cells rather than rapidly dividing cells in the cancer.

One of the least studied tumors, but the one to which children and young women pay the most expensive toll is osteosarcoma. Patients with osteosarcoma generally receive a combination of chemotherapy followed by surgery on the tumor to improve their chances of survival. Chemotherapy is usually given to control the spread of the disease and to reduce the tumor making surgery more manageable. Unfortunately, these treatments often have serious side effects, especially in children, because they can harm a broad range of normal cells. Recent findings suggest that osteosarcomas are derived from cancer stem cells that function as the "root" of the tumor.

In this study, the effects of cisplatin, methotrexate and doxorubicin on osteosarcoma cells were tested, because those are the commonly used drugs to combat this disease [60]. These drugs have different mechanisms of action. Cisplatin is an inorganic compound that is believed to kill cancer cells by binding to DNA, causing double-strand DNA breaks and interfering with its repair mechanism, eventually leading to cell death. Cisplatin is a cell cycle phase specific drug (S-Phase) [61].

Methotrexate and its active metabolites compete for the folate-binding site of the enzyme dihydrofolate reductase. Folic acid must be reduced to tetrahydrofolic acid by this enzyme for DNA synthesis and cellular replication to occur. Methotrexate functions by competitive inhibition of the enzyme, leading to blockage of tetrahydrofolate synthesis, depletion of nucleotide precursors, and inhibition of DNA, RNA and protein synthesis. Therefore, also methotrexate is a cell-cycle specific drug that acts during DNA synthesis [62].

Doxorubicin is an anthracycline antibiotic produced by the fungus *Streptomyces peucetius*. Doxorubicin's mechanism of action is DNA damaging. It damages DNA by intercalation of the anthracycline portion, metal ion chelation, or by generation of free radicals. Doxorubicin has also been shown to inhibit DNA topoisomerase II, which is critical to DNA function. However, it has been shown that its cytotoxic activity is cell cycle phase-nonspecific [63].

Current therapy for osteosarcoma includes neoadjuvant chemotherapy, surgery, and postoperative (adjuvant) chemotherapy [64, 65]. Doxorubicin has been proven effective in adjuvant chemotherapy regimens after surgery in preventing and/or reducing recurrence and metastasis rates in osteosarcoma patients that are operable [65, 66].

1.5 Project objectives

The goal of this study is to characterize the growth of CSCs in simulated microgravity as a model for cancer treatment development. This model will be used to select the best experimental condition required to culture CSCs in a microgravity-based bioreactor. The bioreactor will be used to simulate cancer stem cells growth in absence of microgravity, test for specific cell markers, and sensitivity of the CSCs to various chemotherapeutic agents.

The Specific Aims of this study are therefore:

1. To culture CSCs under microgravity conditions and characterize their proliferation and differentiation.
2. Examine the expression of specific cellular markers.
3. To enhance cancer stem cells chemo sensitivity to various chemotherapy agents using microgravity (μG) conditions and to characterize the biological mechanisms that drive cancer stem cells resistance to apoptosis induced by chemotherapy.

Chapter II: Materials and Methods

2.1 General Culture Methods

2.1.1 Cell Culture

SAOS-2, HOS, and U2OS (human osteosarcoma), T98G and U87MG (human glioblastoma), Du145 and LNCap (human prostate adenocarcinoma), WI38 and H23 (human lung fibroblast and lung adenocarcinoma, respectively), Hep3b (human hepatocarcinoma) and Hela (human cervical cancer) cells were obtained by the American Type Culture Collection (ATCC, Manassas, VA). Mewo and HO-1 cells (human melanoma) were kindly provided by Dr. Paul B. Fisher (Virginia Commonwealth University, VA) and HN12 and HN30 cells (human head & neck squamous carcinoma) were kindly provided by Dr. George Yoo (Wayne State University, MI). Cells were grown in the ATCC-recommended media (either RPMI 1640 or D-MEM) at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. All media was supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Hyclone, Logan, UT), 100-μg/mL penicillin, 100-μg /mL streptomycin (Invitrogen, Carlsbad, CA).

2.1.2 Hydrodynamic focusing bioreactor (HFB) cell culture

A hydrodynamic focusing bioreactor (HFB) cell culture system (Celdyne, Houston TX) is on loan from NASA and was used to culture cells. Cells were counted and a range of $1X\ 10^5 – 1.2\ X\ 10^7$ cells were placed in the 40 mL rotating chamber for 3-

days to 7-days set at 25 rpm with airflow set at 20%. Cells were then removed and counted again using trypan blue exclusion to determine cellular viability. The cells were either pelleted for future research or labeled with florescent markers for characterization.

2.2 General Antibody Labeling

2.2.1 Antibody labeling for Cell Sorting

Up to 2 $X10^7$ cells were sorted either by a magnetic-activated cell sorting (MACSorting) system, which consists of magnetic beads conjugated to an antibody against CD133 (Miltenyi, Auburn, CA), or by flow cytometry (FACS Aria, BD Bioscience, San Jose, CA). In brief, cells were harvested using 0.25% trypsin, pelleted and labeled with CD133/1 biotin and CD133/2-PE. Cells were washed and labeled with antibiotin magnetic beads, and then passed through a magnetic column where the CD133(+) cells were retained, while unlabelled cells pass through the column. The CD133(+) retained cells were eluted from the columns after removal from the magnet. Positive and negative cells were then analyzed by FACS for purity.

2.2.2 Flow Cytometry Studies

Cells were analyzed by the antigenic criteria using anti-CD133 (prominin1) (Milteny Biotech, Auburn, CA), -CD34 (Milteny Biotech, Auburn, CA); -SPARC, -Sox-9, -RunX-2, -Osteocalcin, -Integrin-ß1, -Endoglin, -Stro-1 (all from SantaCruz, Santacruz, CA), -CD117/c-Kit (BD Bioscience, San Jose, CA), and –Oct-4 (Cell Signaling, Danvers, MA) and with the secondary antibodies goat antimouse (FITC) and

mouse anti goat (PE conjugated) (Santa Cruz, CA),. Cells were detached using 0.02% EDTA in PBS and pelleted (10 min at 300 xg), washed in 0.1% BSA in PBS at 4°C and incubated in a solution of 1μg antibody + 9 μL 0.1% BSA in 1X PBS. Cells were washed in the same solution once and were processed for sorting (FACS Aria, BD Bioscience, San Jose, CA). Propidium iodide stained cells were also analyzed with a fluorescence-activated cell sorter (FACS Aria, BD Bioscience, San Jose, CA), and the data were analyzed using the Flow-Jo program (BD Biosciences, San Jose, CA). Cell cycle was analyzed by flow cytometry as follows: following staining with a primary antibody against CD133 (Milteny Biotec, Auburn, CA), cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed in PBS, then left for 1-hour in PBS/milk 6%. Then cells were stained with a DNA staining solution (0.1 % Triton X-100, 0.1 % sodium citrate, 1 mg/mL RNAse A and 50 mg/mL propidium iodide) for 2-hours at room temperature in the dark. Isotypes and non-probed cells were used as controls. Multiple cell cycle analysis studies were performed using FACS Aria to obtain a reproducible model experiments on growing cells.

2.2.3 Immunofluorescence

$2X10^4$ cells were seeded on two-well micro-chamber slides (Nunc, Naperville, IL). On the day cells were stained, they were washed with PBS and blocked in a mixture of 0.5% BSA, 2 mM EDTA, and FcR blocking reagent (Miltenyi, Auburn, CA) for 20 minutes. The primary antibodies were incubated for 20 minutes on ice, washed with PBS twice, and a secondary anti-mouse or anti-rabbit conjugated with FITC or PE (e-Bioscience, San Diego, CA) antibodies were used at a dilution of 1:200 and were

incubated on ice for 15 minutes. Negative controls were performed with secondary antibodies only. The slides were examined under an inverted Olympus IX70 microscope (Olympus America, Inc. Melville, NY). Fluorescence images were captured with Sensicam QE camera (Cooke Co., Auburn Hills, MI) and operated with SlideBook 3.0 software (Intelligent Imaging Innovations Inc., Denver, CO).

2.3 General Assays

2.3.1 Soft agar assay and sphere assay

Cells were counted and 5x 10^3 SAOS-2 and CD133(+) and CD133(-) SAOS-2 cells were plated in 6-well culture dishes in 3 mL of soft agar growth medium; 500 μL of medium per well were added every 2-days. The spheres formed were counted under the microscope. Cells were also plated in ultra-low attachment dishes (Corning, Lowell, MA catalogue# 3262), and the number of spheres for each well was evaluated after 7-days and 14-days of culture. The ultra-low attachment surface has a neutral hydrophilic hydrogel coating, which greatly reduces binding of attachment proteins. This minimizes cells attachment and spreading.

2.3.2 *In vitro* growth characteristics and chemo sensitivity

To determine the *in vitro* growth rate of each cell line, 30,000 cells/cm^2 were seeded in the appropriate tissue culture media. Cells were then harvested and counted by trypan blue dye exclusion every 24-hours, and doubling time was calculated during the exponential phase of growth (from 48-hours to 96-hours after seeding). For saturation

density, from day 4 onwards, the medium was changed daily, and cells were counted every 2-days until they stopped growing. The degree of cisplatin, doxorubicin, and methotrexate resistance was expressed by lethal dose of the drug concentration resulting in 50% inhibition of viability (LD_{50}) of the various cell lines. LD_{50} was calculated by assessing cell viability using trypan blue exclusion staining and confirmed by MTT assay. Cisplatin (Alexis Biochemicals, San Diego, CA), doxorubicin (Sigma Aldrich, St. Louis, MO) and methotrexate (MP Biomedicals, Solon, OH) were dissolved to a stock concentration of 100 μg/mL, 50 μg/mL, and 20 μg/mL, respectively and diluted in normal saline immediately before the experiments. Cells were treated with the chemotherapeutic agent for 1-hour, and then were washed and fresh culture medium was added. Cell viability was assessed 24-hours after treatment. Or the cells were treated continuously for 24-hours with the chemotherapeutic agent, and their viability was assessed after treatment. To determine the LD_{50} values 30,000 cells/cm^2 were seeded in their growth medium containing 10% FBS, and after 24-hours the medium was replaced with medium containing 10% FBS and no drug (control) or with different concentrations of the chemotherapy drugs. After 48-hours, cells were harvested and counted by trypan blue dye exclusion to estimate the percentages of cell death compared with the appropriate control, which were then used to calculate the LD_{50} values.

2.3.3 MTT Assay

$1X10^4$ exponentially growing cells were seeded in 100 μL of medium in 96-microwell, flat-bottomed plates. For each of the variants tested, 7 replicas were performed. Following 24-hours in culture (to allow the cells to attach and resume

growth), 20 μL of different concentrations of the drug were added to each well containing untreated cells. Normal saline (PBS) was added to the controls. Cells were exposed to the drugs either for 24-hours continuously or for one hour time intervals, as indicated. At the end of drug exposure, the CDDP, MTX, or DOX treated cells as well as parallel control cells were incubated for 3 hours with 50 μL of MTT solution (5 mg/mL). After incubation, culture medium in each well was discarded and replaced with 50 μL of DMSO. After 10 minutes shaking, the absorbance of each well was determined by a spectrophotometer at 510 nm. The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Chemotherapeutic drugs LD_{50} was determined as a chemotherapeutic drugs concentration showing 50% of cell survival as compared with the control cells. The experiments were replicated 7 times.

2.3.4 In vivo tumor formation assay

Animal care and humane use and treatment of mice were in strict compliance with: (*a*) institutional guidelines; (*b*) the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996); and (*c*) the Association for Assessment and Accreditation of Laboratory Animal Care International. An animal protocol was approved from the institutional IACUC committee for this study. Tumors were generated by the subcutaneous injection of HOS, SAOS-2, and H23 cells into nude mice (male and female NU/NU-*nu*BR outbred, isolator-maintained mice, 4–5 weeks of age from Charles Rivers, Wilmington, MA).

The nude mice were injected along the right dorsal flanks (one flank/mouse) with $2X10^6$ of CD133(+), CD133(-), and CD133(+) HFB cultured HOS or SAOS-2, or $2X10^6$ of CD133(-), total population and CD133(+) HFB cultured H23 cells/flank. The CD133(+) and CD133(-) cells were selected using a CD133 antibody coupled to magnetic-activated cell sorting (MACSorted) system. The CD133(+) HOS, SAOS-2, or H23 cultured HFB cells were selected by culturing the HOS, SAOS-2, or H23 cells for 5-days in the HFB. The expression of CD133 on the cells was confirmed by flow cytometry before injecting the cells into nude mice. Animal weight was monitored weekly. Tumor growth was followed by measuring the longest axis of the tumor and the axis perpendicular to this with a caliper. Tumor volume was calculated with the formula tumor volume = $(\text{length})*(\text{width})^2/2$.

2.5 Statistical analysis

The results for each variant in the different experimental designs represent an average of 4 to 5 different experiments. The data of 7 or more measurements were averaged; the coefficient of variation among these values never exceeded 10%. Mean values and standard errors were calculated for each point from the pooled normalized to control data. Statistical analysis of the significance of the results was performed with a 1-way ANOVA.

Chapter III: Results

3.1 Osteosarcoma stem-like cells proliferate in the Hydro-Focusing Bioreactor (HFB)

Simulated microgravity is a condition in which cells are in constant free fall and in which they are able to grow in an anchorage independent manner. Since the effects of microgravity on tumors are unknown, this study sought to investigate the effects of simulated microgravity, using the HFB. This allowed for absence of shearing stress on the growth capacity of tumor cells of different embryonic origin.

It was found that when a known number of SAOS-2 cells were seeded in the HFB, only a small fraction of these cells survived the treatment, independently from the cellular density seeded in the bioreactor. The cell count of viable SAOS-2 cells recovered after 5-days of culture in the HFB in three separate experiments was dramatically reduced (Fig. 6).

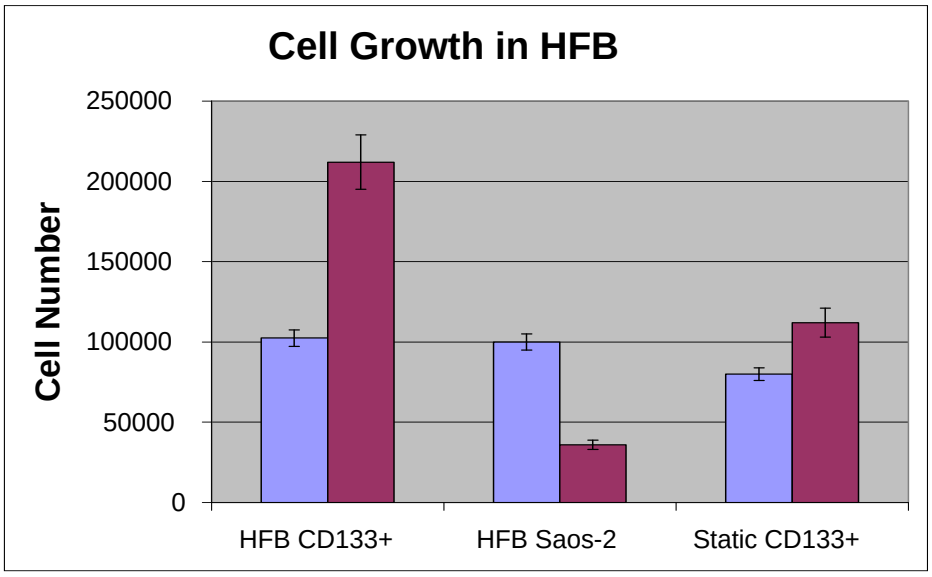


Figure 6. Trypan blue exclusion cell count of SAOS-2 cells (osteosarcoma cells) cultured in the HFB. Blue bars indicate the number of cells placed in the bioreactor on day 1. Purple bars indicate the number of viable cells recovered after 5-days of culture in the bioreactor.

Interestingly, the SAOS-2 cells that were recovered from the bioreactor formed spheres and appeared to be significantly smaller than a sample from a plate of adherent normal gravity (1G) cultured SAOS-2 cells harvested by trypsin treatment. Cells grown in the HFB for 5-days show spheres derived from SAOS-2 cells (Fig. 7).

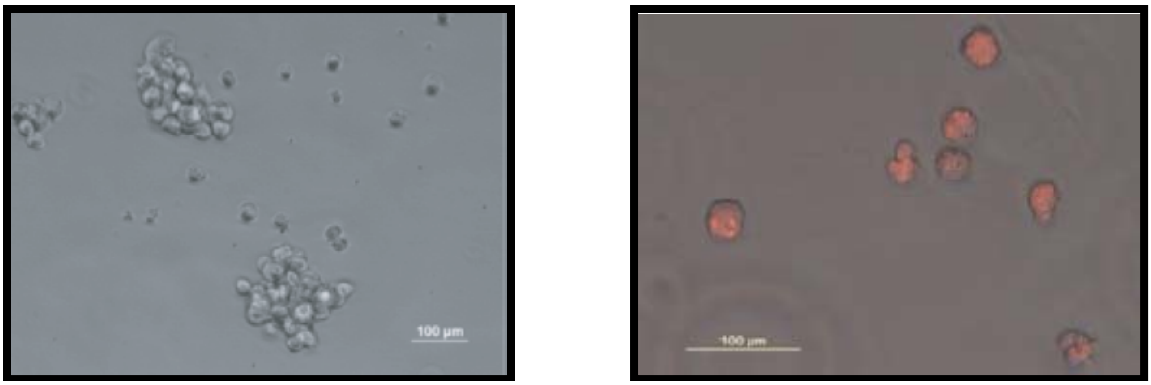


Figure 7. Left Bright field contrast phase image (20X) of 3-D culture (spheres) formed from SAOS-2 cells that were grown in the HFB for 5-days. Right shows an image of CD133 immunofluorescence staining (40X) of SAOS-2 cells grown in the bioreactor for 5-days.

The cells apparent change in morphology and ability to form spheres in the HFB environment indicates that the HFB selected cells could be cancer stem-like cells. This is supported by others that have demonstrated the presence of cancer stem-like cells within primary bone sarcomas, as well as the osteosarcoma cell lines MG-63, OS-521, 0S-01-187, OS-99-01, and the chondrosarcoma cell line CS-828 [67]. To test this hypothesis immunophenotyping was performed on these cells, and found that the SAOS-2 HFB selected cells were 100% positive to CD133, a typical stem cell marker of mesenchymal origin (Fig. 7).

Since the SAOS-2 cells that were recovered from the bioreactor were all CD133(+), the next step was to determine whether the CD133(+) cells not only survive microgravity but may possibly be stimulated to proliferate in the microgravity environment. To this end, CD133(+) cells were selected from the SAOS-2 cells using a MACSorting system and placed CD133(+) cells into the HFB for 5-days. Interestingly, SAOS-2 cells MACSorted with an antibody against CD133 and cultured in the bioreactor for 5-days increased in number by two-fold (Fig. 6).

After a few trials of cell culture optimization, we were able to achieve a 15-fold increase in proliferation of CD133(+) cancer stem-like cells from the parental SAOS-2 cells over a 7-day period. This was achieved by stopping the reactor every 24-hours and gently mixing the culture 10 times in an orthogonal manner over a period of one minute, which allowed for redistribution of the media in the dome and mixing of the nutrient with the proliferating cells (Fig. 8). This shows that it is possible to select and proliferate a specific population contained in the SAOS-2 parental cell line when following an optimized protocol of cell culture in the HFB. Similar results were achieved using various other cancer cell lines HOS, U2OS, and H23 cells.

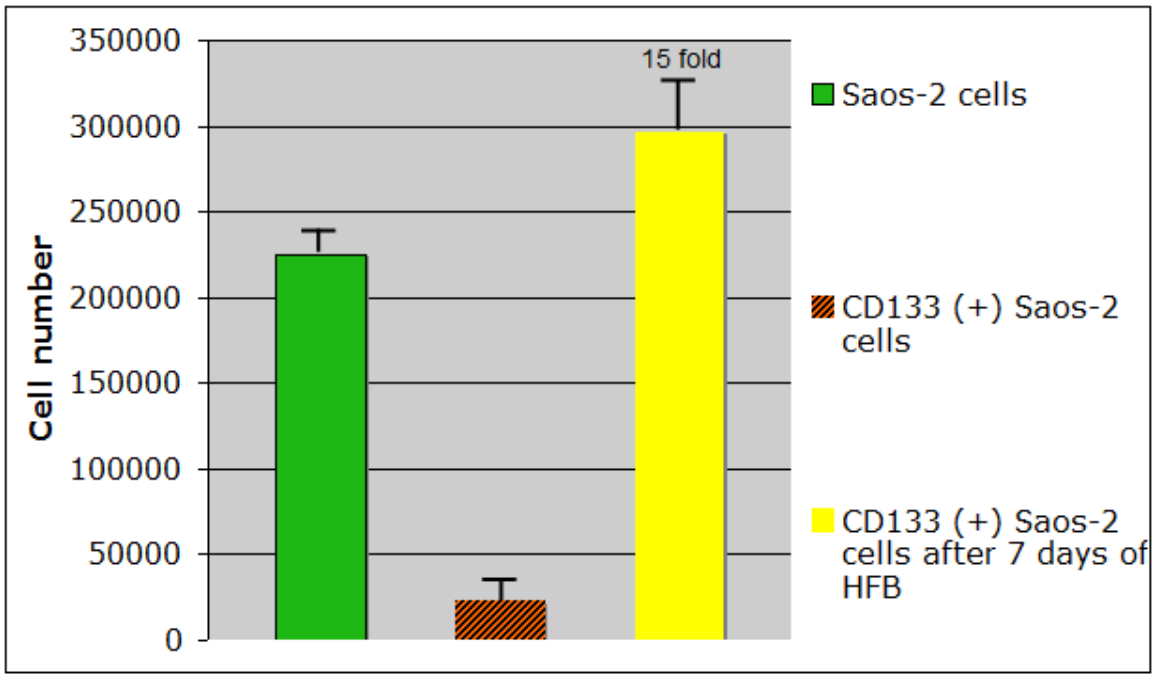


Figure 8. Trypan blue exclusion cell counts of an optimized experiment of HFB culture of SAOS-2 cells in the HFB after 7-days. CD133(+) SAOS-2 cells cultured in the bioreactor were selected and proliferated 15-fold after 7-days. Green bar indicates the number of SAOS-2 cells seeded in the HFB. Yellow bar indicates the number of viable CD133(+) SAOS-2 cells recovered after 7-days of optimized culture in the bioreactor. Orange bar indicate the number of CD133(+) cells present in the parental SAOS-2

population. The data is representative of three separate experiments yielding comparable results.

3.2 CD133(-) osteosarcoma cells die by apoptosis in the Hydro-Focusing Bioreactor (HFB)

Since only a small fraction of the osteosarcoma SAOS-2 as well as HOS cells placed in the HFB, were recovered after 3-days or 5-days of culture, this study tested whether these cells were being negatively selected by the HFB pushing them to undergo apoptosis. The SAOS-2 cells cultured in the HFB for 3-days and 5-days versus the CD133(+) MACSorted cells cultured in the same conditions were analyzed by Annexin-V.

Apoptosis is an active organized cell death process characterized by morphological and biochemical features occurring at different stages. Once triggered, apoptosis proceeds with different kinetics that are cell type specific, this culminates with cell disruption and formation of apoptotic bodies. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin-V, a protein with a high affinity for PS. The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. As such, Annexin-V can be used for the easy, flow cytometric identification and sorting of cells in the early stages of apoptosis.

This study analyzed SAOS-2 cells cultured for 3-days or 5-days in the bioreactor and found that SAOS-2 cells cultured in the HFB for 3-days or 5-days die by apoptosis as shown by a flow cytometric assay of Annexin-V and propidium iodide staining of the

samples (Fig. 9 B and D). The cells that were cultured for 3-days in the HFB were found to have 24% of the cells in apoptosis. Interestingly, the fraction of cells dying by apoptosis increased to 62% after 5-days of culture in the HFB, confirming the initial observation (Figs. 6-9). Most importantly, samples of CD133(+) MACSorted cells grown in the HFB for 5-days didn't show such an increase in apoptosis by Annexin-V staining when compared to the control sample (Figs. 10E and F). In the same culture conditions in the HFB, the CD133(+) cells proliferate while the CD133(-) cells instead die by apoptosis.

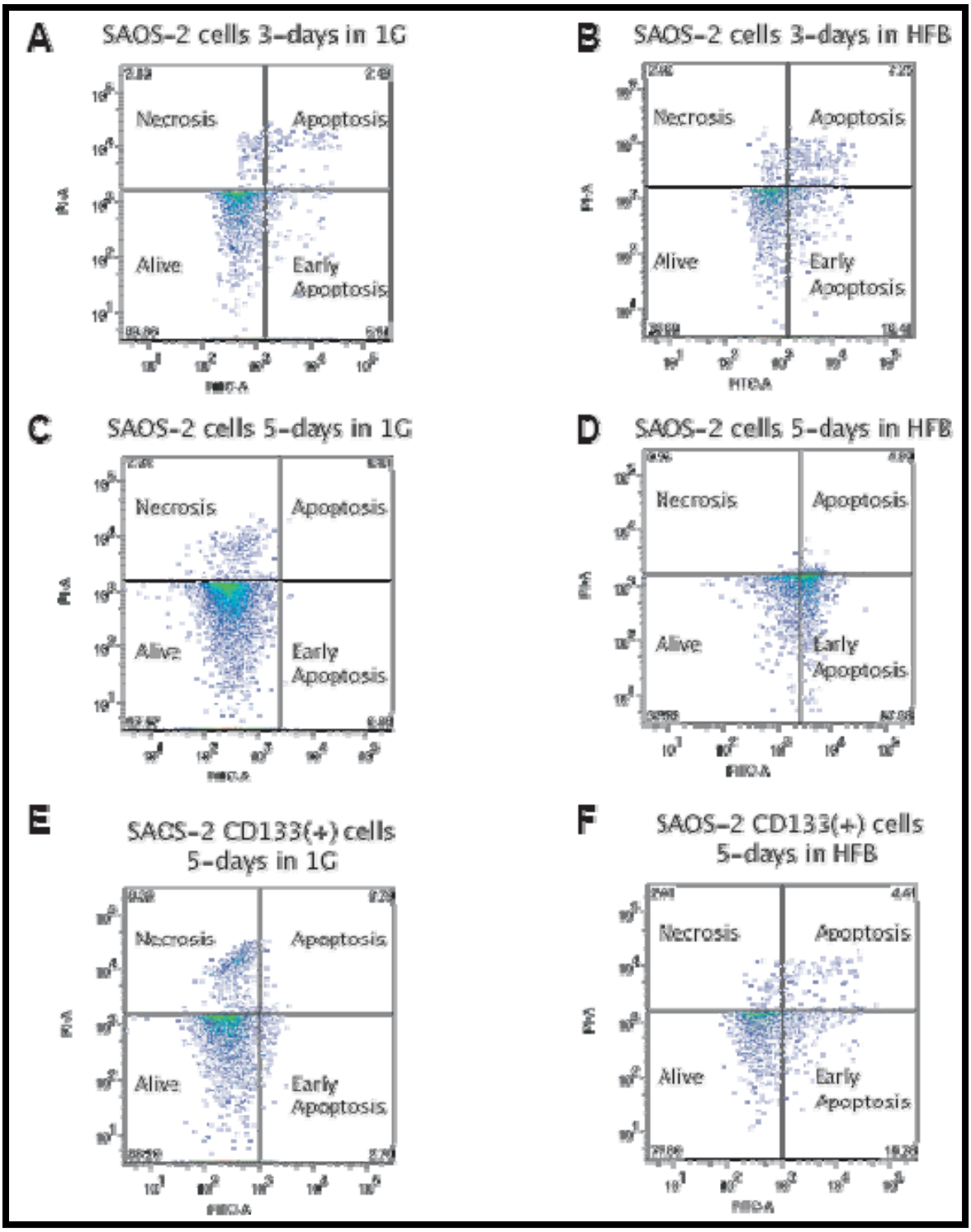


Figure 9. SAOS-2 cells die by apoptosis after 5-days of culture in the hydrofocusing bioreactor as evidenced by Annexin-V staining/Facs analysis. A) Annexin-V staining of SAOS-2 cells cultured in 1G for 3-days. The analysis allows to distinguish in the diagram between living cells (lower left quadrant), early apoptotic cells (lower right quadrant), apoptotic cells (upper right quadrant), and necrotic cells (upper left quadrant). B)

Annexin-V staining of SAOS-2 cells cultured in the HFB for 3-days. C) Annexin-V staining of SAOS-2 cells cultured in 1G for 5-days. D) Annexin-V staining of SAOS-2 cells cultured in the HFB for 5-days. E) Annexin-V staining of CD133(+) MACSorted SAOS-2 cells which were cultured in 1G for 5-days. F) Annexin-V staining of CD133(+) MACSorted SAOS-2 cells which were cultured in the HFB for 5-days.

	Alive	Early Apoptosis	Apoptosis	Necrosis
A	89.86	5.51	2.49	2.13
B	73.68	16.41	7.25	2.66
C	97.57	0.06	0.01	2.34
D	37.38	57.38	0.36	4.89
E	88.2	2.7	0.79	8.32
F	77.8	15.78	4.41	2.01

This study also investigated this phenomenon by measuring the activity of caspase by using a colorimetric caspase kit that measures the activity of caspase-3 in the samples. Caspases are essential in cells for apoptosis and failure of apoptosis is one of the main contributions to tumor development. Caspase-3 is an effector-caspase that cleaves other protein substrates within the cell, to trigger the apoptotic process.

This study found that SAOS-2 cells cultured in the HFB for 5-days showed an increase of 1.6-fold in the caspase-3 activity detected (Fig. 10), which is an indicator of the apoptotic index in these cells.

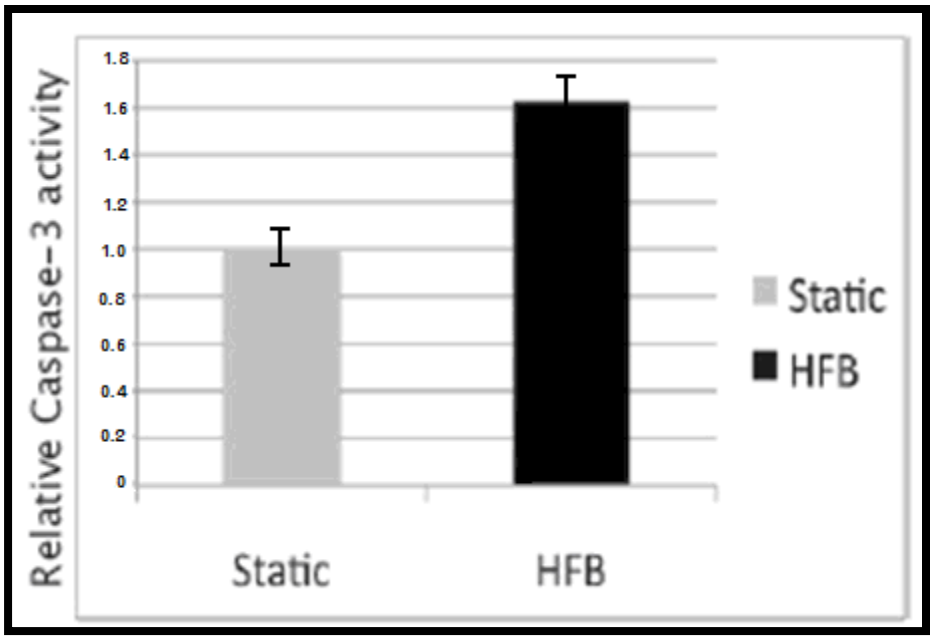


Figure 10. Relative caspase-3 activity of SAOS-2 cells cultured for 5-days in the HFB compared to SAOS-2 cells grown in static 1G condition.

3.3 Stem cell markers expression increase following culture in the Hydro-Focusing Bioreactor

Perhaps the most intriguing capability conferred by the rotating, low-shear bioreactor system is the opportunity to study, under controlled conditions, the interaction of cells of a given type or the interaction of one cell type with another when cells in suspension are free to form their own associations. This study wanted to analyze the expression levels of various markers related to the development of stem cells of mesenchymal origin. The expression of the analyzed genes affected by three-dimensional growth overall amplified as illustrated by the increased production of the cell adhesion molecules by cells in spheroids.

The expression of CD133, CD34, CD38, Osteocalcin, SPRAC, Sox-9, RunX-2, Stro-1, CD117/c-Kit, Oct-4, Endoglin, and Integrin-ß1 was examined by flow cytometry.

The percent of fluorescence (the index of the expression level of the examined marker) of the various markers were examined in cells cultured in static condition or cells cultured in the HFB for 5-days (Fig. 11). The expression levels of the examined markers increased after 5-days of culture in the HFB compared to the expression levels of the same marker on cells cultured in 1G static condition (attached to the culture dish and under normal gravity conditions). Interestingly, the SAOS-2 cells grown in the HFB showed the highest relative increase in the expression levels of Sox-9, CD133, Osteocalcin, Integrin-ß1, Sparc, RunX-2, and Endoglin (94.7%, 89.3%, 82%, 81.8%, 81.3%, 79%, and 76%, respectively), when compared to SAOS-2 cells cultured under normal gravity conditions. Oct-4, CD117, Stro-1, and CD34 also showed an increase in their expression (75.9%, 67.5%, 47.6%, 22.36%, respectively) compared to the 1G growth versus SAOS-2 cells grown in simulated microgravity for 5-days. This study found no change in the expression levels of CD38 in the same conditions. The experiment has been repeated 5 times with comparable results and standard deviations were calculated and are reported on the diagram as error bars. Following the growth in the HFB for 5-days SAOS-2 cells were stained for CD133, Sox-9, SPARC, and CD117 to show the positivity to these stem cell markers by immunofluorescence (Fig 12).

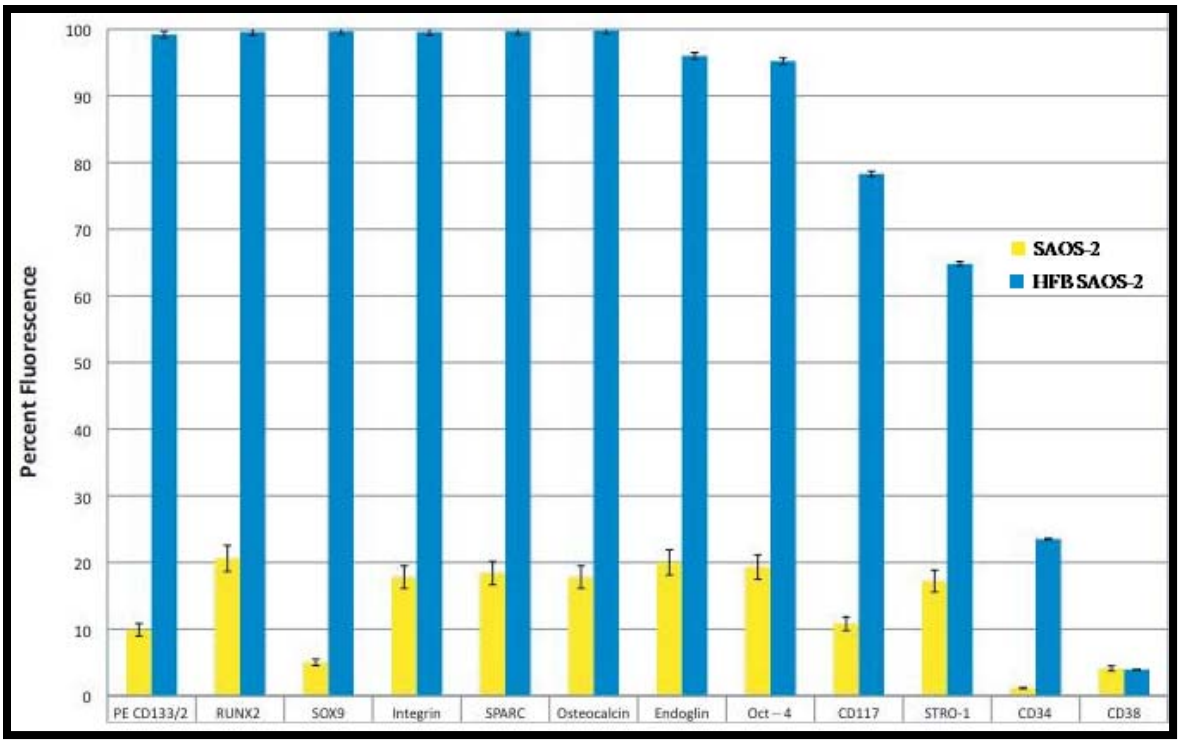


Figure 11. Fluorescence index of various stem cell and differentiation markers examined in cells cultured in 1G static condition, and after culture in the bioreactor for 5-days. Yellow bars indicate the basal levels of expression of the various markers in parental SAOS-2 cells cultured in normal 1G gravity condition (control). Blue bars indicate the expression levels of the various markers in parental SAOS-2 cells that were cultured in microgravity condition for 5-days, resulting in a population of selected and proliferated CD133(+) SAOS-2 cells.

	CD133/2	RUNX2	SOX9	Integrin	SPARC	Osteocalcin
Saos-2	9.9	20.6	5	17.8	18.4	17.8
HFB Saos-2	99.2	99.6	99.7	99.6	99.7	99.8
	Endoglin	Oct3/4	CD117	STRO1	CD34	CD38
Saos-2	20	19.3	10.8	17.2	1.14	4.08
HFB Saos-2	96	95.2	78.3	64.8	23.5	3.9

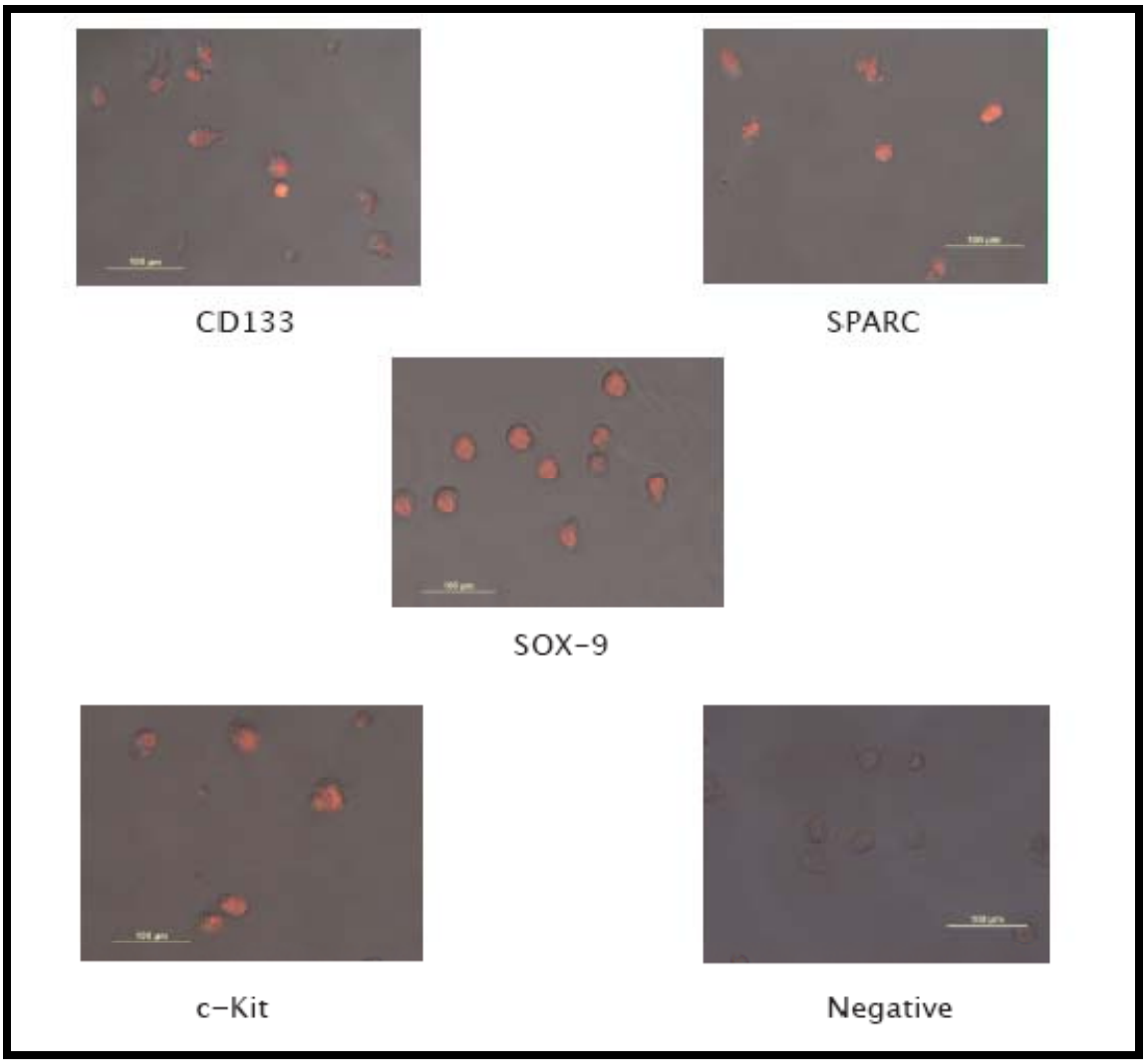


Figure 12. Immunofluorescence staining (40x) and positivity of SAOS-2 cells to CD133, Sox-9, SPARC, and CD117/c-Kit following growth in the bioreactor for 5-days.

3.5 CD133(+) cells grow three dimensionally in the Hydro-Focusing Bioreactor and form spheres.

The SAOS-2 CD133(+) enriched osteosarcoma cells proliferate and assemble three-dimensionally as sarcospheres after 3-days culture in the bioreactor, which is yet another characteristic of stem cell growth. This study shows at 10X and 40X magnification power,

respectively, the sarcospheres grown in the bioreactor after 3-days of culture in simulated microgravity (Figs. 13A and 13B). Interestingly, the CD133(+) enriched SAOS-2 cells that were grown in simulated microgravity were able to re-establish the parental cell line, which is constituted by about 10%±6.8 CD133(+) cells and 90%±6.8 CD133(-) cells, after a period of one week then seeded into irradiated culture dishes, which allow for adherent cells to attach to the plastic environment (Figs. 13C and 13D). In the reconstituted cell culture some not-adhering cells were noticeable, which we speculated were the stem-like cells or CSCs.

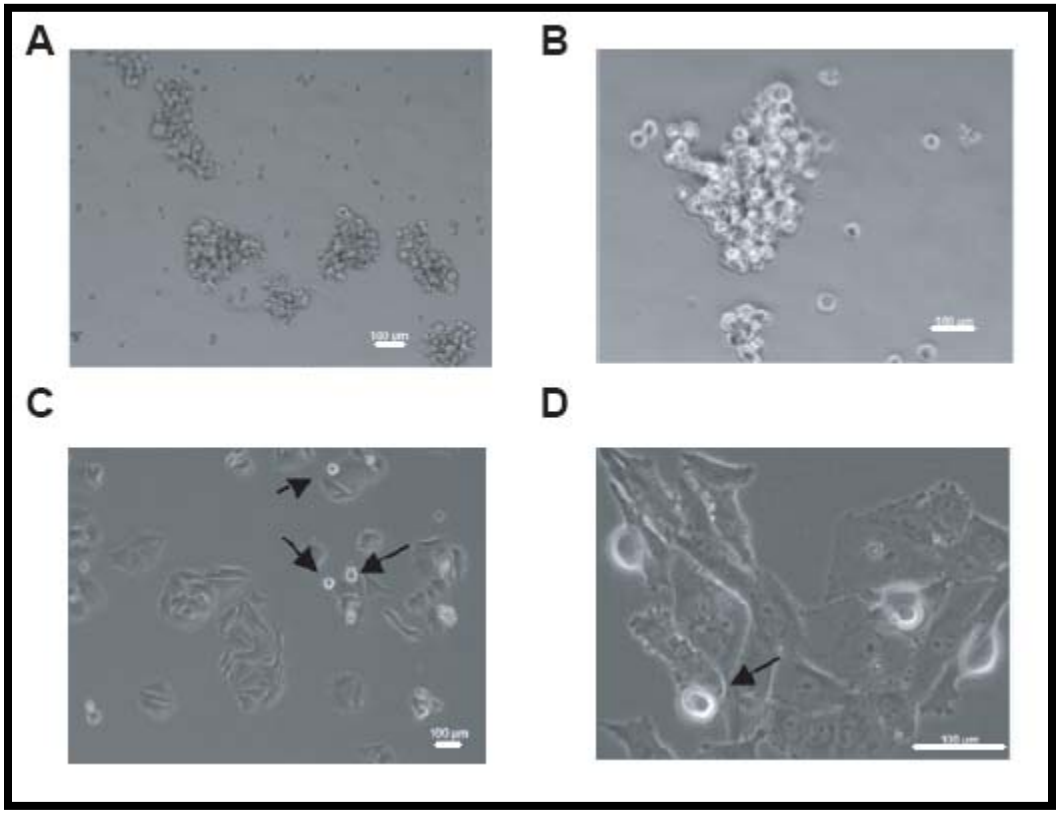


Figure 13. Phase contrast images of spheres formed by HFB SAOS-2 CD133(+) enriched osteosarcoma cells. All scale bars are 100 µm. A) HFB grown SAOS-2 osteosarcoma cells CD133(+) are able to proliferate and assemble three-dimensionally as sarcospheres after 3-days of culture in the bioreactor (10X magnification). B) HFB grown SAOS-2 osteosarcoma cells CD133(+) are able to proliferate and assemble three-dimensionally as sarcospheres after 3-days of culture in the bioreactor (40X magnification). C) HFB grown SAOS-2 osteosarcoma cells CD133(+) seeded into an irradiated plastic culture dish, reconstitute the normal

attached phenotype of the parental SAOS-2 cells after one week of culture (10X magnification). Arrows point at CD133(+) SAOS-2 cells showing a rounded and weakly attaching phenotype. D) HFB grown SAOS-2 osteosarcoma cells CD133(+) seeded into an irradiated plastic culture dish, reconstitute the normal attached phenotype of the parental SAOS-2 cells after one week of culture (40X magnification). Arrow points at CD133(+) SAOS-2 cells showing a rounded and weakly attaching phenotype.

This study also tested the ability of CD133(+) SAOS-2 MACSorted cells to form sarcospheres if placed in ultra low-attaching dishes. Their ability to form spheres was less efficient when compared to those cells that have been cultured in the HFB, but they were still able to form spheres (Figs 14A and 14B). The SAOS-2 CD133(+) MACSorted enriched cells were also tested for the ability to attach to tissue culture dishes and to reconstitute the parental SAOS-2 cell line. As expected, the MACSorted CD133(+) enriched cells placed in attaching tissue culture dishes after being cultured in ultra low-attaching dishes for two weeks, reconstituted the parental SAOS-2 cell line after one week of culture by attaching to the dish, manifesting a flattened and differentiated phenotype over time (Figs 14C and 14D). The SAOS-2 cell line was reconstituted after 10-days of inoculation of CD133(+) enriched cells in adhering tissue culture dishes (Fig. 14E).

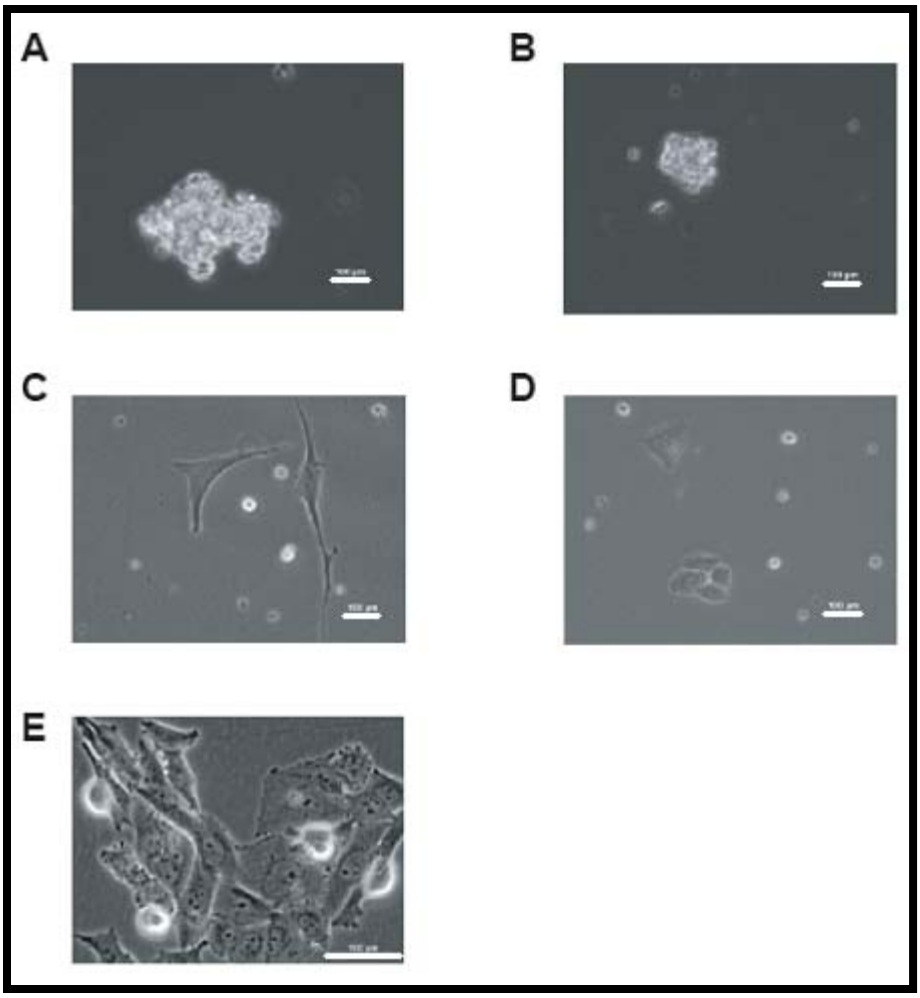


Figure 14. Phase contrast images of spheres formed by MACSorted CD133(+) SAOS-2 osteosarcoma cells in ultra low-attaching dishes. All scale bars are 100 μm. A) MACSorted CD133(+) SAOS-2 cells assemble three-dimensionally as sarcospheres after 2 weeks of culture in ultra low-attaching dishes (20X magnification). B) Another example of MACSorted CD133(+) SAOS-2 cells forming sarcospheres after 2 weeks of culture in ultra low-attaching dishes (10X magnification). C) SAOS-2 osteosarcoma cells deriving from three-dimensional cultures grown in ultra low-attaching dishes seeded into attaching dishes shows adherent and differentiated phenotype after 3-days. D) SAOS-2 osteosarcoma cells deriving from three-dimensional cultures grown in ultra low-attaching dishes seeded into attaching dishes show an adherent and differentiated phenotype after 5-days. E) SAOS-2 osteosarcoma cells deriving from three-dimensional cultures grown in ultra low-attaching dishes seeded into attaching dishes show an adherent and differentiated phenotype after 10-days.

An additional indication that two distinct populations are identifiable in the SAOS-

2 cell line is the evidence that CD133(+) MACSorted SAOS-2 cells have enhanced ability to grow in soft agar when compared to the parental cells. The cells were plated in 6-well plates performing 6 replicas of each experimental point. Equal number of SAOS-2 cells and of CD133(+) SAOS-2 cells (5x 10^3 cells/well) were seeded in 6 replicas into a six-well dish. The efficiency of CD133(+) SAOS-2 cells to grow in soft agar and to assemble in three-dimensional structures is much higher than that of the parental SAOS-2 cells. Six hundred and forty ± 240 colonies were counted from the soft-agar 6-well dishes seeded with the CD133(+) SAOS-2 enriched cells compared to only 20±12 colonies counted from the soft-agar dishes seeded with the CD133(-) SAOS-2 cells. Interestingly, 120±80 colonies were counted from the soft-agar dishes seeded with the parental SAOS-2 cells, suggesting that the ability of forming colonies in soft-agar could be due to the presence of a fraction (consistently, about 10%±6.8) of stem-like cells within the SAOS-2 cell line (Table 1). The soft-agar assay performed on the parental SAOS-2 cells formed spheres, but with very low efficiency when compared to the enriched CD133(+) cells (Figs 15A and 15B).

Table 1. Average number of colonies counted in 6-well dishes in a triplicate experiment.

	Parental SAOS-2	CD133(+)	CD133(-)
Total number of colonies	120±80	640±240	20±12

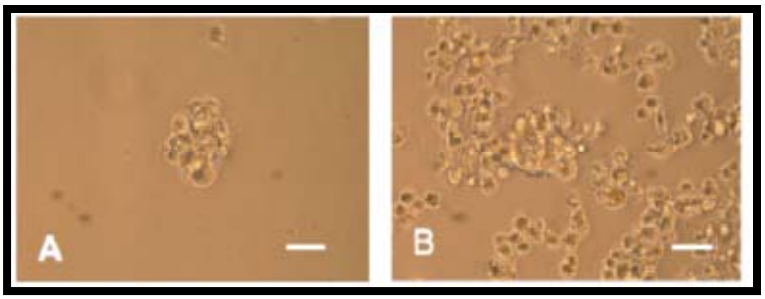
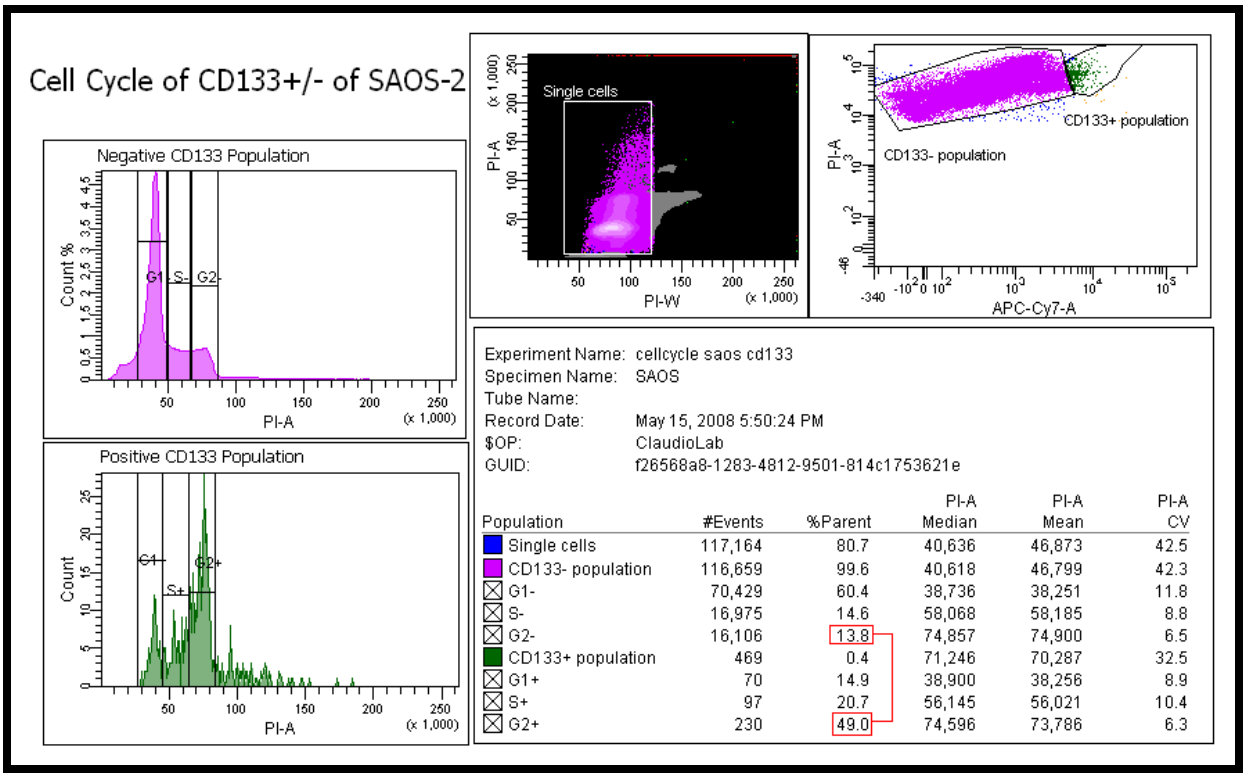


Figure 15. A) Contrast phase microscopy image of a soft agar assay of parental SAOS-2 cells. B) Contrast phase microscopy image of a soft agar assay of HFB CD133(+) enriched SAOS-2 cells.

Further evidence that there are two distinct populations in the SAOS-2 cell line, which appears to be composed of CD133(+) and CD133(-) cells, is that these two different populations have a distinct cell cycle profile. A flow cytometry analysis of SAOS-2 cells grown in 1G culture cells was performed. Cells were sorted by their CD133 status and stained with propidium iodide. CD133(-) cells exhibited a different cell cycle profile with respect to the CD133(+) population. The flow cytometric assay of SAOS-2 cells demonstrated that the CD133(-) SAOS-2 population had 13.8% of cells in the G2/M phase of the cell cycle, while the CD133(+) cells showed an increased fraction (49%) of cells in the G2/M phase of the cell cycle (Fig. 16). Comparable results were obtained in parallel and repeated experiments.



Population	#Events	%Parent	PI-A Median	PI-A Mean	PI-A CV
Single cells	117,164	80.7	40,636	46,873	42.5
CD133- population	116,659	99.6	40,618	46,799	42.3
G1-	70,429	60.4	38,736	38,251	11.8
S-	16,975	14.6	58,068	58,185	8.8
G2-	16,106	13.8	74,857	74,900	6.5
CD133+ population	469	0.4	71,246	70,287	32.5
G1+	70	14.9	38,900	38,256	8.9
S+	97	20.7	56,145	56,021	10.4
G2+	230	49.0	74,596	73,786	6.3

Figure 16. Flow cytometry analysis of SAOS-2 cells grown in 1G static culture sorted by their CD133 status and stained with propidium iodide showing two distinct populations, the CD133(+) and CD133(-) SAOS-2 cells with a different cell cycle profile.

To determine if the CD133(+) cells were increasing their proliferation due to the effect of microgravity an antibody for Ki-67 was used and examined through flow cytometeric assay. The antibody Ki-67 reacts with a human nuclear antigen that is present in proliferating cells, but absent in quiescent cells [68]. SAOS-2 cells grown in the bioreactor for 5-days were stained with anti-CD133 and the proliferation marker anti-Ki-67 demonstrated increased proliferation in the HFB compared to the SAOS-2 cells grown in 1G culture (Fig. 17). The fraction of SAOS-2 CD133(+) cells exhibited increased expression of Ki-67, 14.27% to 53.98%, when cultured in 1G versus CD133(+) cells cultured for 5-days in the HFB. Comparable results were obtained in three parallel experiments.

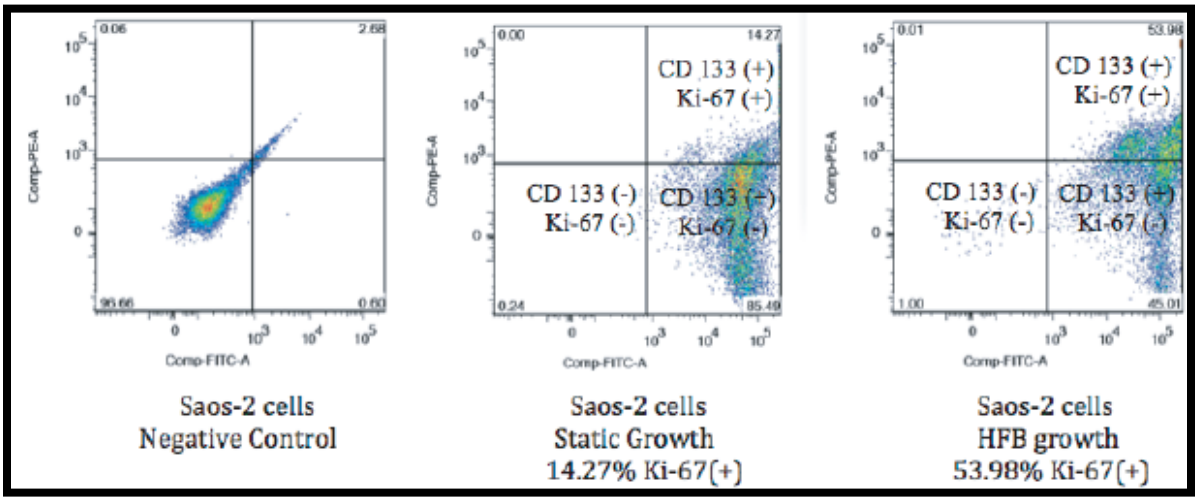


Figure 17. Immunophenotype of SAOS-2 cells grown in 1G versus HFB culture showing that the SAOS-2 cells grown for 5-days in simulated microgravity contain the majority of the cells stained for Ki-67.

3.6 CD133(+) cells derived from the H23 cell line selected in the HFB grow in nude mice.

It has been recently shown that CD133 cellular positivity is necessary for the engraftment of certain tumor stem-like cells in nude mice [69]. Hammati et al. demonstrated that neurospheres derived from pediatric tumors could engraft, migrate, proliferate, and differentiate in neonatal rat brains. These results were broadened when *in vivo* serially transplantable mouse brain tumors were generated from an intracranial injection of human glioblastoma multiforme–derived neurosphere cultures [70]. Interestingly, in the Galli et al. report although 100% of the primary intracranial injections generated tumors, only 50% of subcutaneous injections developed into a cancer, which highlights the importance of microenvironment for brain tumor development [70]. Various tumor cell lines were characterized and screened for CD133

expression and found that the various cell lines tested contained a diverse percentage of CD133(+) cells (Table 2).

Even more recently it has been shown that AML cells that exhibit CD34+/CD38- cancer-stem-like cells are able to migrate [39] and grow *in vivo* generating metastasis and show decreased sensitivity to chemotherapy [71]. In particular, Lapidot et al. fractionated AML cells on the basis of cell-surface-marker expression and found that the leukemia-initiating cells that could engraft SCID mice to produce large numbers of colony-forming progenitors were CD34+/CD38-; however, the CD34+/CD38+ and CD34- fractions contained no cells with these properties [39, 72, and 73]. The various tumor cell lines were also screened for the expression of CD34, and CD38 (Table 2).

Table 2. Percentage of positivity to CD133, CD34 and CD38 of the various cell lines tested.

Cell Line	**Tumor type**	**% CD133 (+)**	**%CD34(+)**	**%CD38(+)**
HOS	Human osteosarcoma	37.7±7.8	5.58±2.2	0.52±0.07
SAOS-2	Human osteosarcoma	10.86±6.8	1.14±0.13	4.08±0.08
U2OS	Human osteosarcoma	1.06±0.05	70.5±0.6	0.51±0.06
T98G	Human glioblastoma	0.3±0.1	0.4±0.2	0.2±0.01
U87MG	Human glioblastoma	0.2±0.1	0.8±0.3	0.4±0.2
Du145	Human prostate adenocarcinoma	0.6±0.3	0.3±0.05	1.2±0.03
LNCap	Human prostate adenocarcinoma	1±0.6	1.9±0.3	0.4±0.2
WI38	Human lung fibroblast	2.4±1	1.6±0.5	0.5±0.2
H23	Human lung adenocarcinoma	5.36±0.75	5.18±1.1	0.4±0.13
Hep3b	Human hepatocarcinoma	96.8±0.6	0.6±0.03	0.1±0.01
Hela	Human cervical carcinoma	0.8±0.7	2.5±1	0
Mewo	Human melanoma	0.5±0.1	0	0
HO-1	Human melanoma	1.4±0.6	0.8±0.2	0.3±0.1
HN-12	Human H&N squamo cellular carcinoma	0.4±0.3	0.2±0.1	0.3±0.1
HN-30	Human H&N squamo cellular carcinoma	0.1±0.1	0.2±0.1	0.2±0.1

Because it has been recently shown that CD133 cellular positivity is necessary for the engraftment of certain tumor stem-like cells in nude mice [69], this study tested CD133(+) osteosarcoma cells to evaluate if they would grow *in vivo* by engrafting nude mice on their flank. A series of six equally distributed female and male *nude* mice per group (CD133-; CD133+, and CD133+/HFB cells) were injected with $2X10^6$ HOS cells in the presence of matrigel on their flank. The cells were either MACSorted for CD133(+) and CD133(-) from a static culture of HOS cells, or were enriched and proliferated in the HFB for 5-days. Before injecting the cells were immunophenotyped by flow cytometry for the expression of CD133, CD34, and CD38. The CD133(+) and CD133(-) were checked for purity along with cells enriched by the HFB and found to be 99.7% pure on average.

Interestingly, HOS cells did show the highest percentage of CD133 positive cells (37.7%±7.8). Additionally, HOS cells were shown to contain a high percentage of CD38(-) within the CD34(+) cells (97.62%±1.2) (Table 3). It was also found 30.19%±0.5 CD38(-) /CD34(+) HOS cells were within the CD133(+) population. Comparable results were obtained in parallel and repeated experiments.

Table 3. Percentage of positivity to CD133, CD34, and CD38 of the various cell lines injected in mice.

Cell Line	CD133(+)	CD34(+)	CD38(+)	CD38(+) within CD34(+)	CD34(+) within CD38(+)	CD38(-) within CD34(+)	CD34(-) within CD38(+)
HOS	37.7	5.58	0.52	2.38	38.46	97.62	61.54
SAOS-2	10.86	1.14	4.08	61.75	70.26	48.25	29.74
U2OS	1.06	70.5	0.51	0.79	80.85	99.21	19.15
H23	5.36	5.18	0.4	48.2	46.56	51.81	53.44

Cell Line	CD34(+) within CD133(+)	CD38(+) within CD133(+)	CD38(-) within CD133(+)	CD34(-) within CD133(+)	CD34(+)/ CD38(-) within CD133(+)	CD34(+)/ CD38(+) within CD133(+)
HOS	1.99	13.03	86.97	98.01	30.19	6.49
SAOS-2	10.42	23.26	76.74	89.58	11.66	25.27
U2OS	87.07	52.03	47.97	12.93	73.93	14.46
H23	22.24	42.37	57.63	77.76	21.36	89.58

Unfortunately, against expectations, none of the mice injected with the HOS cells grew tumors after 6 weeks of observation in two separate sets of experiments. Therefore, the experiment was repeated using the SAOS-2 cell line in which we found 10.86% CD133(+) cells. A series of six equally distributed female and male *nude* mice per group (CD133-; CD133+, and CD133+/HFB cells) were injected with $2X10^6$ SAOS-2 cells in the presence of matrigel on their flank. The cells were either MACSorted for CD133(+) and CD133(-) from a static culture of SAOS-2 cells, or were enriched and proliferated in the HFB for 5-days. Before injecting the cells, the expression of CD133, CD34, and CD38 were checked by flow cytometry. Again, CD133(+), CD133(-) and HFB cultured cells were 99.7% pure on average across the groups as asserted by flow cytometry. SAOS-2 cells did contain an average of 1.14%±0.13 CD34(+) cells and 4.08±0.08 CD38(+) cells. SAOS-2 cells were also shown to contain a high percentage of CD38(-) within the CD34(+) cells (48.25%±0.5) (Table 3) and 11.66%±0.5 of CD38(-)/CD34(+)

cells within the CD133(+) SAOS-2 population. However, those mice did not grow tumors after 6 weeks of observation in two separate sets of experiments.

It was decided to repeat the test using the H23 human lung adenocarcinoma cells, a cell line that had been previously used in the lab to generate tumor xenografts on the flank of *nude* mice [74]. A series of six equally distributed female and male *nude* mice per group were injected with $2X10^6$ H23 cells (CD133-; CD133+/HFB cells; and total H23 population) in the presence of matrigel on their flank. Due to the small population of CD133(+) cells (5.36%) MACSorted CD133(+) population was not taken. The cells were either MACSorted for CD133 negativity from a static culture of H23 cells, or were enriched and proliferated in the HFB for 5-days. The expression of CD133, CD34, and CD38 was checked by flow cytometry before injecting the cells into the mice Purity of CD133(-), and HFB cultured were 99.7% pure on average and total H23 was found to have 5.36% CD133(+) population on average.

H23 cells contained 5.36% of CD133(+) cells, 5.18% CD34(+), and 0.4± CD38(+) cells. H23 cells were shown to contain a high percentage of CD38(-) within the CD34(+) cells (51.81%) (Table 3) and 21.36% CD38(-)/CD34(+) of H23 cells within the CD133(+) population. Mice injected with the HFB cultured H23 cells grew tumors after 6 weeks from the injection while the mice injected with the CD133(-) H23 cells didn't show any sign of palpable or measurable tumor formation (Figs. 18 and 19). The nude mice injected with the CD133(+) derived from a culture of H23 cells in the HFB for 5-days grew in *nude* mice more rapidly establishing bigger tumors than the H23 cells from a static culture, leading to the idea that the HFB had selected and proliferated tumor stem cells with the ability to engraft *in vivo*.

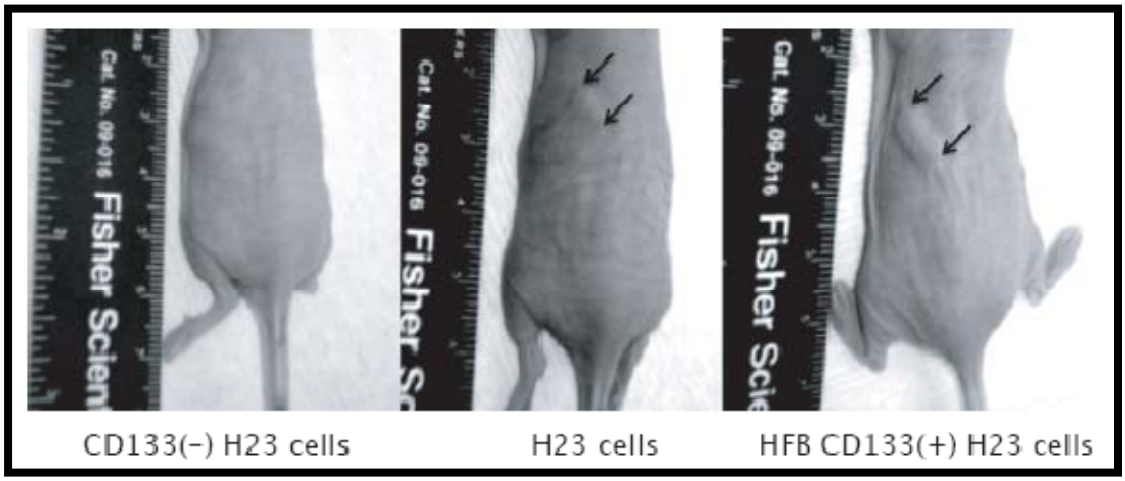


Figure 18. Pictures taken of *nude* mice. CD133(-) H23 cells were sorted using an antibody against CD133 coupled to magnetic beads. CD133(+) cells were obtained by 5-days of culture in the HFB. Mice show tumor formation capacity of $2X10^6$ cells injected. Left panel, shows lack of tumor formation in a mouse injected with CD133(-) cells. Central panel shows tumor formation in a mouse injected with H23 cells harvested from a static 1G culture. Left panel shows a considerable bigger tumor formation in a mouse injected with CD133(+) cells selected from a 5-days culture of the H23 cells in the HFB.

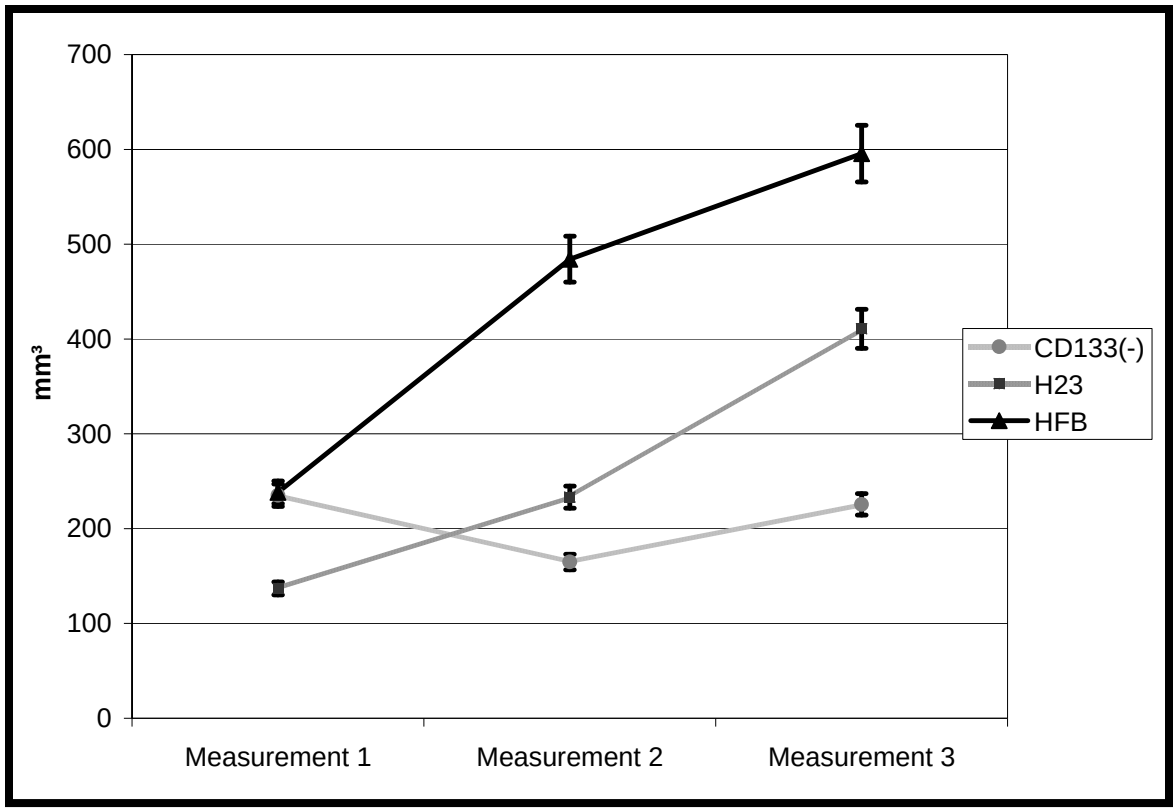


Figure 19. *Nude* mice tumor growth measurement. CD133(-) H23 cells showed no growth, the HFB cultured were CD133(+) cells showed the greatest growth. $2X10^6$ cells were injected.

3.7 Simulated microgravity enhances apoptosis and sensitizes cancer stem cells to chemotherapy.

This study tested the ability of microgravity to sensitize the osteosarcoma cells to various chemotherapy agents used in cancer therapy. It was discovered that the microgravity environment sensitized the CD133(+) resistant osteosarcoma cells to various chemotherapy agents at clinically relevant doses. The sensitivity of osteosarcoma cells to cisplatin, doxorubicin, and methotrexate, which are the drugs of choice for this tumor type in the clinical settings, was examined. SAOS-2 cells were grown for 5-days in the HFB and then $1x10^4$ cells were plated in 96-well dishes and were subjected, along

with a comparable number of cells of the other samples, to 5, 10, and 15 μg/mL of cisplatin for 24 hours and then subjected to MTT assay (Fig. 20). Equal amounts of cells were used for the doxorubicin and methotrexate treatments. For methotrexate treatments, the cells were subjected to 4, 11, 22, and 45 μg/mL of methotrexate for 24 hours and then subjected to MTT assay. For doxorubicin treatments the samples were treated with 0.5, 1.1, and 2.2 μg/mL of doxorubicin for 24 hours and then subjected to MTT assay. The MTT assay is a convenient colorimetric assay that assesses the number of viable cells versus the number of dead cell in a given sample. The CD133(+) cells grown in the HFB increased their sensitivity to the cisplatin, doxorubicin, and methotrexate treatments (Figs. 20, 21, and 22). These phenomena may be due to the fact that the cancer stem cells were stimulated to proliferate by culture in the HFB environment. The CD133(+) cells that were not subjected to HFB conditions appear to be resistant to the same treatment regiment. Similar results were achieved by treating the cells with the chemotherapeutic agents for 1-hour and subjecting the samples to MTT assay 24-hours later. This data has been confirmed with other techniques such as measurement of cell viability by trypan blue exclusion cell-count before and after chemotherapy treatment as well as with flow-cytometry analysis of Annexin-V.

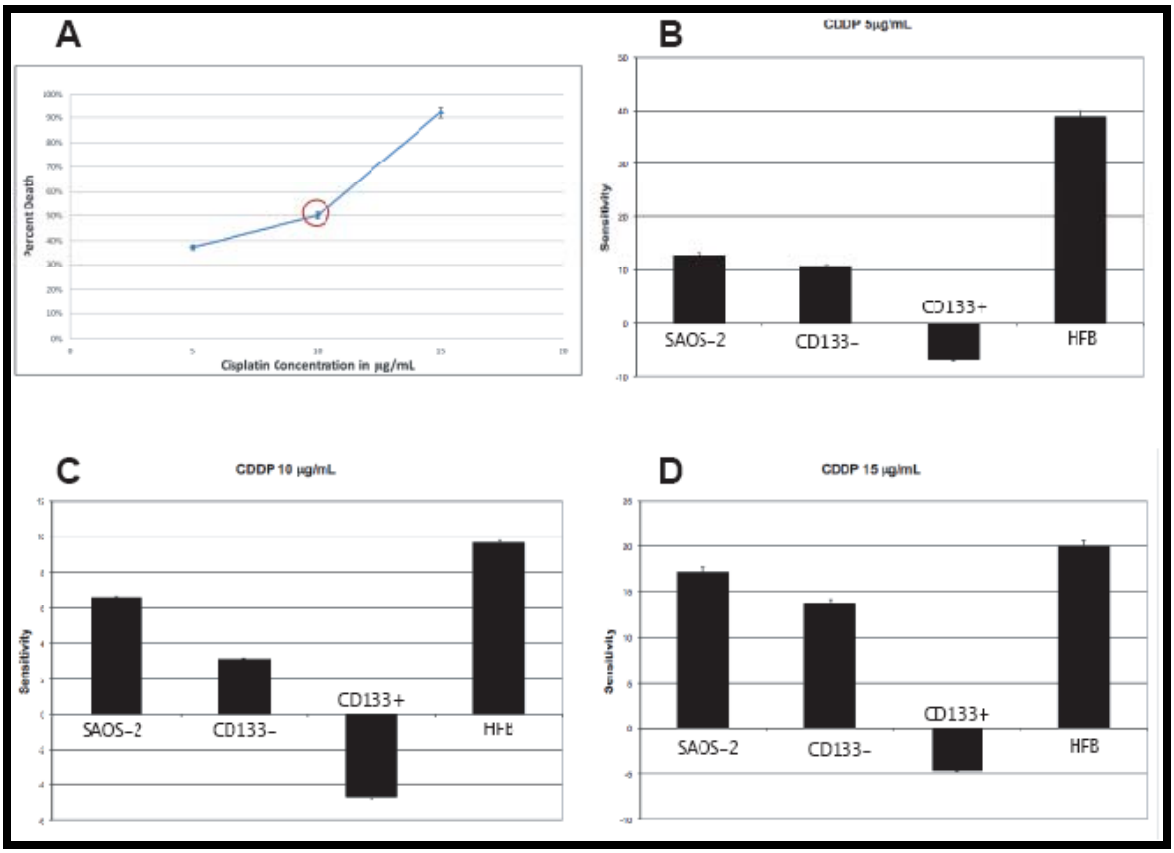


Figure 19. Sensitivity of SAOS-2 cells to cisplatin following growth in simulated microgravity. A) LD_{50} for cisplatin determined for the SAOS-2 cells. An LD_{50} of 10µg/mL for cisplatin was determined exposing the SAOS-2 cells to a 24-hours treatment using the MTT assay. B) Histogram showing the sensitivity of SAOS-2 cells to 5µg/mL of cisplatin following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead. C) Histogram showing the sensitivity of SAOS-2 cells to a clinically relevant dose of 10µg/mL of cisplatin following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead. D) Histogram showing the sensitivity of SAOS-2 cells of 15µg/mL of cisplatin following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead.

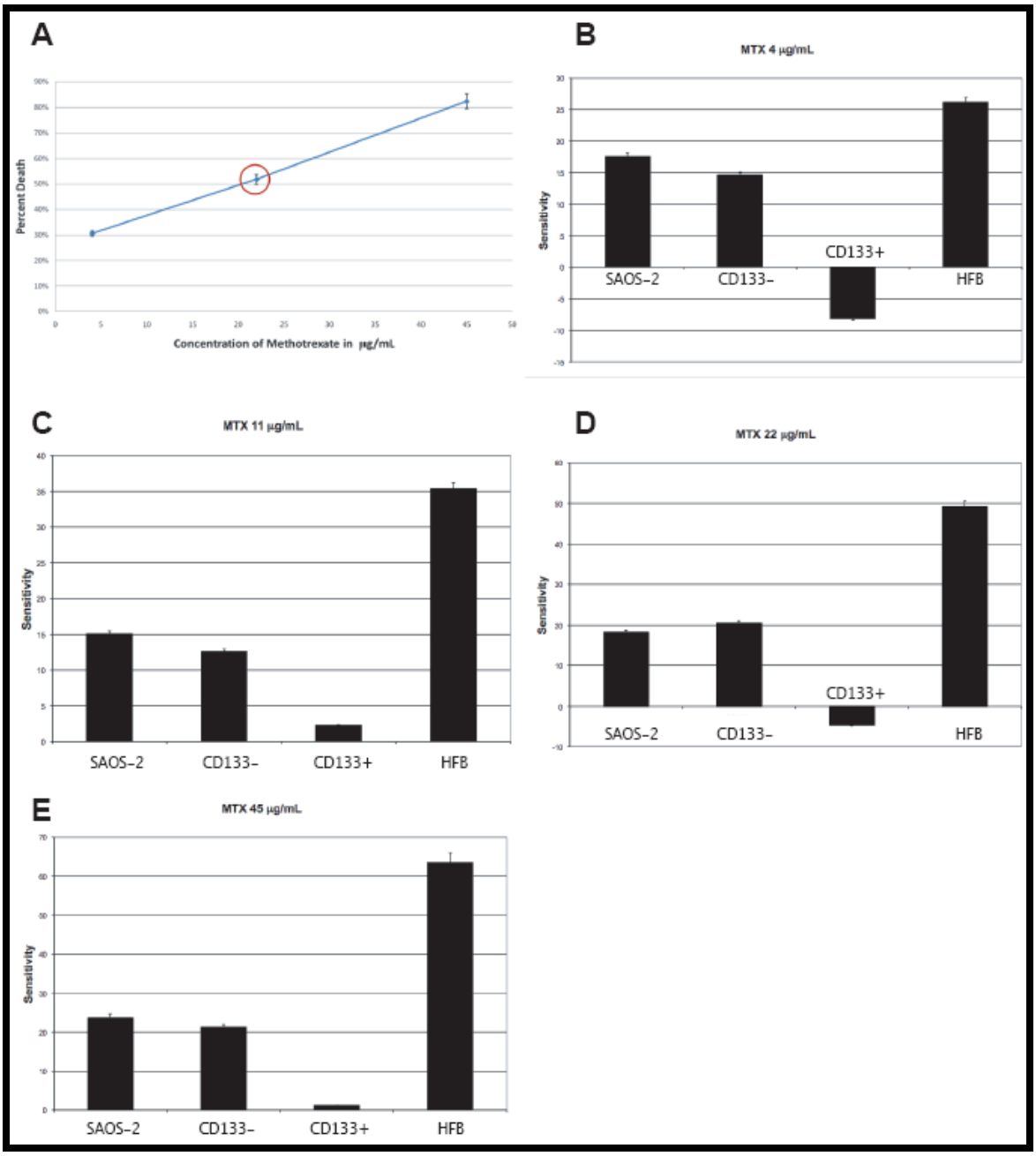


Figure 20. Sensitivity of SAOS-2 cells to methotrexate following growth in simulated microgravity. A) LD_{50} for methotrexate determined for the SAOS-2 cells. An LD_{50} of 22µg/mL for methotrexate was determined exposing the SAOS-2 cells to a 24-hours treatment to the drug, using the MTT assay. B) Histogram showing the sensitivity of SAOS-2 cells to 4µg/mL of methotrexate following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead. C) Histogram showing the sensitivity of SAOS-2 cells to 11µg/mL of methotrexate following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and

selected with the HFB culture system are greatly sensitive, instead. D) Histogram showing the sensitivity of SAOS-2 cells to a clinically relevant dose of 22μg/mL of methotrexate following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead. E) Histogram showing the sensitivity of SAOS-2 cells to 45μg/mL of methotrexate following a 24-hours treatment, using an MTT assay. CD133(+) cells are resistant to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead.

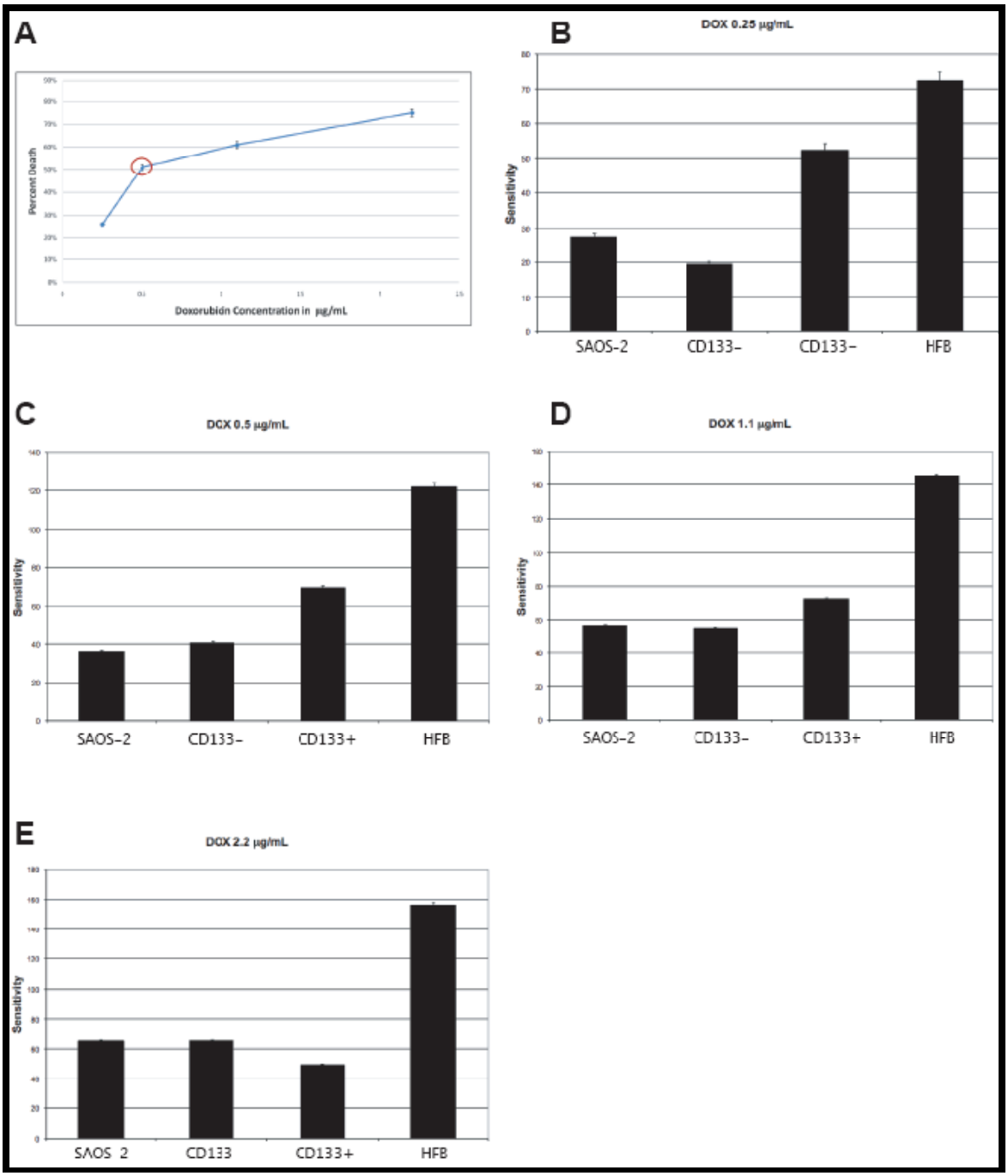


Figure 21. Sensitivity of SAOS-2 cells to doxorubicin following growth in simulated microgravity. A) LD_{50} for doxorubicin determined for the SAOS-2 cells. An LD_{50} of 0.5µg/mL for doxorubicin was determined exposing the SAOS-2 cells to a 24-hours treatment to the drug, using an MTT assay. B) Histogram showing the sensitivity of SAOS-2 cells to 0.25µg/mL of doxorubicin following a 24-hours treatment, and using an MTT assay. CD133(+) cells are sensitive to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are even more sensitive to the treatment, instead. C) Histogram showing the sensitivity of SAOS-2

cells to 0.5μg/mL of doxorubicin following a 24-hours treatment, using an MTT assay. CD133(+) cells show sensitivity to the clinically relevant dose of 0.5 μg/mL of doxorubicin, however the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are greatly sensitized to the chemotherapy treatment. D) Histogram showing the sensitivity of SAOS-2 cells to a dose of 1.1μg/mL of doxorubicin following a 24-hours treatment, and using an MTT assay. The CD133(+) cells show sensitivity to a dose of 1.1μg/mL of doxorubicin, however the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitized to the chemotherapy treatment. E) Histogram showing the sensitivity of SAOS-2 cells to 2.2μg/mL of doxorubicin following a 24-hours treatment, and using an MTT assay. CD133(+) cells are sensitive to the chemotherapy treatment, but the CD133(+) SAOS-2 cells proliferated and selected with the HFB culture system are sensitive, instead.

Interestingly, the CD133(+) osteosarcoma cells had an overall resistance to any dose of the cisplatin and methotrexate treatments tested, whether above or below the assessed LD_{50}. However, the cancer cells stimulated to grow in the HFB, which are CD133(+), showed enhanced cell death even at doses well-below the LD_{50}, suggesting that environments in which lack of gravity could be simulated may be used to lower the necessary dose of chemotherapy treatments for patients.

Surprisingly, the CD133(+) osteosarcoma cells are sensitive to treatments with doxorubicin even at doses lower than the LD_{50}. However, the CD133(+) cancer cells stimulated to grow in the HFB, showed enhanced cell death also at doses well-below the clinically relevant doses that correspond to the LD_{50} for this drug, again suggesting that the microgravity environment could be used to enhance treatment outcomes at lower doses and may reduce and/or eliminate the notorious highly toxic effects of doxorubicin.

The apoptotic effect of the HFB on the SAOS-2 cells were investigated by measuring the activity of caspase-3 by using the colorimetric caspase kit to measures the activity of caspases-3 in the samples grown in the HFB for 5-days and treated with 10 μg/mL of CDDP for 24-hours. Caspase-3 activity was measured 24-hours after the

chemotherapy treatment and according to the data previously presented. The SAOS-2 cells cultured in the HFB for 5-days showed an 3.5-fold increase of relative caspases-3 activity detected following CDDP treatment vs. the 2-fold of increased caspases-3 activity detected in SAOS-2 cells treated with the same amount of CDDP (10 μg/mL), compared to static growth condition, indicating that the HFB stimulated the CDDP-induced apoptotic index of the SAOS-2 cells (Fig. 23).

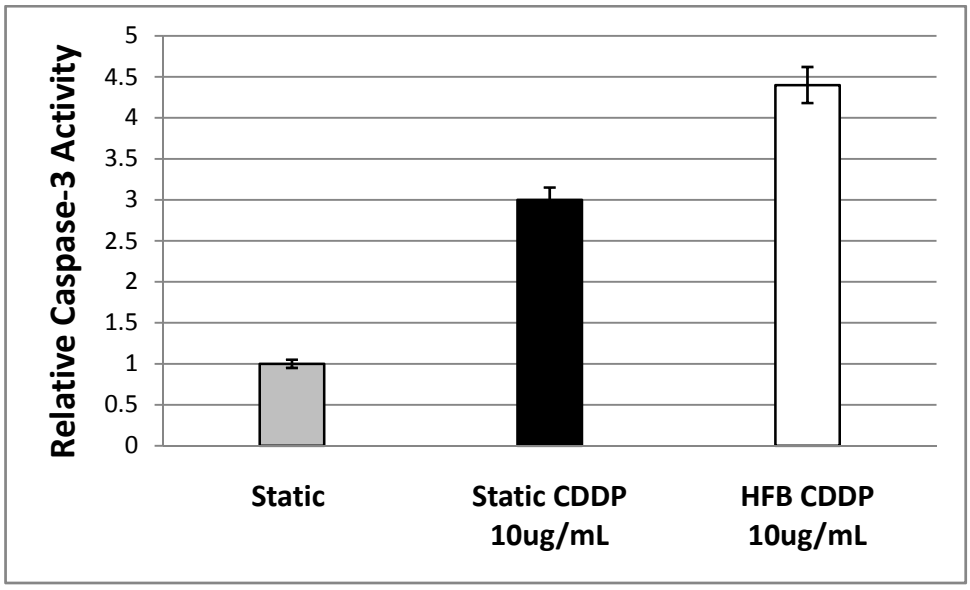


Figure 22. Graphic representation of the relative caspase-3 activity of SAOS-2 cells exposed to CDDP after growth stimulation in simulated microgravity. SAOS-2 cells grown for 5 days in the hydrofocusing bioreactor and treated with 10 μg/mL of CDDP die by apoptosis as evidenced by a caspases-3 colorimetric assay. The white bar illustrates the caspase-3 activity recovered from SAOS-2 cells grown in the HFB and then treated with CDDP for 24 hours. The black bar illustrates the caspase-3 activity recovered from SAOS-2 cells grown in static normal (1G) condition and then treated with CDDP for 24 hours, and the grey bar indicates the basal caspases-3 activity of SAOS-2 cells grown in static 1G condition.

The data presented here clearly show that the HFB conditions greatly sensitize the cancer stem cells to various chemotherapeutic agents. Importantly, it shows that the cancer stem cells, which are normally resistant to chemotherapy treatment, become sensitive even at low doses of the noxious drugs.

Chapter IV: Discussion

One of the biggest obstacles of stem cell research results from the small number of stem cells isolated from tissues. This limits research conducted on normal adult stem cells as well as on cancer stem cells from liquid or solid tumors. Of course, this issue may be overcome by *in vitro* propagation of stem cells (cancerous or not) to obtain a sufficient number of cells to develop novel therapies. The purpose of this study was to select the best experimental condition required to stimulate cancer stem cells proliferation, using the HFB, and then test for specific cell markers and sensitivity of CSCs to various chemotherapy agents.

The HFB showed that it was capable in 5-days and 7-days to increased cell growth compared to the 1G control. A 15-fold proliferation of the CD133(+) cellular fraction was observed with cells that were cultured for 7-days at optimized conditions in the HFB. Additionally, 100% of the harvested cells were found to be CD133(+), indicating that the HFB had selected for the SAOS-2 undifferentiated cellular fraction. In order to characterize and investigate the cells grown in the HFB, the expression levels of various adhesion molecules and stem cell markers before and after culturing the cells in simulated microgravity were assessed. Interestingly, the expression levels of several stem cell markers other than CD133 were also increased after a 5-day culture period in the HFB. The markers CD133, CD34, CD38, CD117/c-Kit, Sparc, Sox-9, RunX-2, Stro-1, Osteocalcin, Endoglin, Integrin-ß1, and OCT-4, all showed increased of expression after a 5-day run when compared to static growth control conditions. The increased expression of the tested markers following exposure of the cells to the simulated microgravity

environment, of the HFB, points toward various scenarios. One of the likely scenarios is that simulated microgravity could epigenetically regulate the expression of genes involved in cell-cell adhesion and migration, which are expressed during embryogenesis.

It was important to determine what was causing this selection of CSCs in the HFB. Apoptosis was the first mechanism looked at and was determined to be an important factor, causing the death of the CD133(-) population. This was confirmed by Annexin-V assay on unsorted SAOS-2 in cultured in the HFB for 3-days and 5-days compared to CD133(+) cells cultured in the HFB for 5-days (Figs 10A-10F).

It was also important to evaluate the proliferation of the CD133(+) population cultured in the HFB. Cell cycle analysis showed that CD133(+) cells are in a G2/M arrest (Fig. 17). This shows that the CD133(+) cultured in 1G conditions do not proliferate but remain in a quiescent state. Cell counts show an increase in growth, however, it is important to confirm that the unsorted cells in the HFB, the enriched population of CD133(+), were truly the cells responsible for proliferation. This was accomplished by staining cells for CD133(+) and Ki-67. The results showed that the cells grown in the HFB expressed more of the proliferation marker Ki-67 then that of MACSorted CD133(+) 1G control (Fig. 18).

Since CSCs are thought to be responsible for initiating the tumor. It has been shown that CD133(+)/CD34(+) cells have a higher engraftment capacity than their CD133(-)/CD34(+) counterpart [75, 76]. Expanding evidence highlighted the role of CD133 as a marker of CSCs in various human tumors [34] in conjunction with other stem cell markers. CSCs are significantly enriched in CD133(+) subpopulations derived from human colon cancer and hepatocellular carcinomas, as shown by their potential to both

self-renew and differentiate, to form colonies, and proliferate *in vitro*, and by their ability to reform the original tumor phenotype when transplanted either subcutaneously or into the renal capsule of immunodeficient mice [77-79]. This study found CD133 highly expressed (85%±0.6 positivity) in a cell line of hepatocarcinoma (Hep-3b). Additionally, CD133(+) CSCs have been recently shown to play a critical role in the initiation process of bone metastasis. In fact, multivariate analysis showed that CD133 expression is an independent predictor of bone metastasis [80], supporting the importance of CD133 as a central defining factor in the CSC phenotype. This lead to injections of *nude* mice with the CD133(+) derived from a culture of H23 cells in the HFB for 5-days, CD133(-), and total H23 population. This study found that HFB cultured cells grew in *nude* mice more rapidly establishing bigger tumors than the H23 cells from a static culture, showing that the HFB had selected and proliferated tumor stem cells with the ability to engraft *in vivo*.

As stated previously, some types of tumors are highly resistant to chemotherapy and other forms of treatment. Although aggressive treatments can destroy the majority of the cancerous cells, a small fraction of them remain and often later regenerate into even larger masses of tumor cells that are even more resistant treatment [3, 14, 24]. It is important to have a thorough understanding of mechanisms leading to tumor development and drug resistance to arrive at effective therapies for cancer patients.

It is widely accepted that cancer stem cells are responsible for tumor recurrence after chemotherapy or irradiation therapy. Although it is still not clear whether the cancer stem cells are derived from original tissue-derived stem cells, bone marrow stem cells or mature cells that have undergone a dedifferentiation process, it has been suggested that novel strategies for successful cancer therapy should focus on the elimination of cancer

stem cells [29].

Osteosarcoma is a highly resistant tumor to conventional therapies, it was for this reason that this study investigated whether osteosarcoma cell lines contain a subpopulation of putative stem cells that could be targeted for anti-cancer therapy. The importance of this work is summarized by the unique ability of the HFB to proliferate stem cells and to sensitize them to low doses of chemotherapy. The CD133(+) osteosarcoma cells, which are chemotherapy resistant to the various drugs, showed an increase sensitivity to the same drugs at much lower doses than the LD_{50} and this sensitization switch was operated by the microgravity environment.

This study used the HFB to stimulate cancer stem cells proliferation, and then tested for specific cell markers and sensitivity of the CSCs to various chemotherapy agents. Using the HFB a novel technique has now been formulated to select and propagate cancer stem cells. This will likely prove to be an invaluable tool in furthering stem cell research, embryonic and adult, as well as offering a rapid screening method to test chemotherapeutic agents that are more affective against cancer stem cells. Additionally, the data presented in this study clearly showed that CSCs, which are normally resistant to chemotherapy treatment, become sensitive also at low doses of chemo treatment. This could be an important advancement in the therapeutic options of oncologic patients, allowing for lower doses of chemo agents administered as well as for higher response rates.

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Curriculum Vitae

Name: Sarah Elizabeth Daron Kelly
Birth Place: Cincinnati, OH
Current Residence: Huntington, WV

Present Positions
Graduate student and researcher, Biomedical Sciences, School of Medicine, Marshall University, Huntington, WV

Education
MS Biological Sciences, Marshall University, Huntington, WV
BS Biological Sciences, Marshall University, Huntington, WV

Teaching Experience
Lab Instructor, Principles of Biology, Clearwater Christian College, 2003
Substitute Lecturer, Human Biology BSC 105, Marshall University, 2008
Lab Instructor, Cell Biology BSC 322, Marshall University, 2008
Lab Instructor, Cell Biology BSC 322, Marshall University, 2009

Research Laboratory Experience
Research Assistant, Biology Department, Marshall University (2006-2009)

Research Grants
Graduate Research Fellowship Program - NASA West Virginia Space Grant Consortium (June 2008)
Charles C. Gould Scholarship- Marshall University (May 2008)
Travel Grant - American Society for Gravitational and Space Biology (October 2007)
Travel Grant - NASA West Virginia Space Grant Consortium (September 2007)
Graduate Research Fellowship Program - NASA West Virginia Space Grant Consortium (May 2007)
NASA Space Grant Scholarship – NASA (November 2006)
College of Science Tuition Waiver Scholarship Award - Marshall University (November 2006)

Posters
Undergraduate Research Day at the Capitol February 8th 2007
Presentation was given at West Virginia's state capital to the states delegates about the current research project on "Bioreactor Production of Plant Pharmaceuticals."

Sigma Xi April 28th 2007
Presentation was given at Marshall University to fellow students and faculty about the current research project on "Medicinal Plant Cell Cultures in Microgravity-based Bioreactor."

STaR Symposium September 17th 2007

Presentation was given at Waterfront Place Hotel in Morgantown, WV to fellow students and faculty in the state of West Virginia about the current research project on "Production of Bioproducts in a Microgravity-based Bioreactor."

American Society for Gravitational Space Biology 23rd Annual Meeting
October 25-28th 2007

Presentation was given at AMES Research Center to fellow students and faculty from around the world about the current research project on "Selection of Tumor Stem Cells from Tumor Cell lines."

Life in Space for Life on Earth June 22-27th 2008

This presentation was given at a conference in Angers, France. This conference was hosted by European Space Agency (ESA) along with International Society for Gravitational Physiology (ISGP), American Society for Gravitational Space Biology (ASGSB), and European Low Gravity Research Association (ELGRA). Presentation was given about "Rapid Selection and Proliferation of Cancer Stem Cells in a NASA Developed Microgravity Bioreactor."

Advances in Cell Differentiation and Development Symposium November 14th, 2008

Cell Differentiation and Development Center (*CDDC*) First Annual Symposium was held at Memorial Student Center, Marshall University where a Presentation was given to fellow students and faculty from the region on the "Selection of Tumor Stem Cells from Tumor Cell lines." This was a competitive poster competition where I received first place.

STaR Symposium April 13-14th, 2009

Presentation was given at Charleston Marriott Town Center in Charleston, WV to fellow students and faculty in the state of West Virginia about the current research project on "Rapid Selection and Proliferation of Cancer Stem Cells in a NASA Developed Microgravity Bioreactor: Chemotherapeutic Implications."

Publications

Rapid Selection and Proliferation of Cancer Stem Cells in a NASA Developed Microgravity Bioreactor. S. E. Kelly, Di Benedetto A., Valluri, J.V., Claudio, P.P. reviewing process June 2008 Gravitational and Space Biology

Protein Expression in Glycine max Cells under Microgravity Conditions. Jagan Valluri, W. Kelly, and S. Kelly October 2008 Gravitational and Space Biology

Associations

American Society for Gravitational Space Biology (ASGSB) 2007-2008, Student Member