Rapid Selection and Proliferation of CD133(+) Cells from Cancer Cell Lines: Chemotherapeutic Implications

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Abstract
Cancer stem cells (CSCs) are considered a subset of the bulk tumor responsible for initiating and maintaining the disease. Several surface cellular markers have been recently used to identify CSCs. Among those is CD133, which is expressed by hematopoietic progenitor cells as well as embryonic stem cells and various cancers. We have recently isolated and cultured CD133 positive [CD133(+)] cells from various cancer cell lines using a NASA developed Hydrodynamic Focusing Bioreactor (HFB) (Celdyne, Houston, TX). For comparison, another bioreactor, the rotary cell culture system (RCCS) manufactured by Synthecon (Houston, TX) was used. Both the HFB and the RCCS bioreactors simulate aspects of hypogravity. In our study, the HFB increased CD133(+) cell growth from various cell lines compared to the RCCS vessel and to normal gravity control. We observed a (+)15-fold proliferation of the CD133(+) cellular fraction with cancer cells that were cultured for 7-days at optimized conditions. The RCCS vessel instead yielded a (−)4.8-fold decrease in the CD133(+)cellular fraction respect to the HFB after 7-days of culture. Interestingly, we also found that the hypogravity environment of the HFB greatly sensitized the CD133(+) cancer cells, which are normally resistant to chemo treatment, to become susceptible to various chemotherapeutic agents, paving the way to less toxic and more effective chemotherapeutic treatment in patients. To be able to test the efficacy of cytotoxic agents in vitro prior to their use in clinical setting on cancer cells as well as on cancer stem cells may pave the way to more effective chemotherapeutic strategies in patients. This could be an important advancement in the therapeutic options of oncologic patients, allowing for more targeted and personalized chemotherapy regimens as well as for higher response rates.

Introduction
Neoplasms may be viewed as tissue consisting of a heterogeneous population of cells that differ in biological characteristics and potential for self-renewal [1]. The clonal nature of certain malignant tumors is well established [2]. According to the model of clonal evolution of tumor cells, cancer is formed through the accumulation of genetic changes in cells and gradual selection of clones [3,4]. Therefore, the tumor is regarded as abnormal tissue. The CSCs are naturally resistant to most current chemotherapeutic agents, paving the way to less toxic and more effective chemotherapeutic treatment in patients. To be able to test the efficacy of cytotoxic agents in vitro prior to their use in clinical setting on cancer cells as well as on cancer stem cells may pave the way to more effective chemotherapeutic strategies in patients. This could be an important advancement in the therapeutic options of oncologic patients, allowing for more targeted and personalized chemotherapy regimens as well as for higher response rates.

Citation: Kelly SE, Di Benedetto A, Greco A, Howard CM, Sollars VE, et al. (2010) Rapid Selection and Proliferation of CD133(+) Cells from Cancer Cell Lines: Chemotherapeutic Implications. PLoS ONE 5(4): e10035. doi:10.1371/journal.pone.0010035

Editor: Annarosa Leri, Harvard Medical School, United States of America

Received February 10, 2010; Accepted March 16, 2010; Published April 8, 2010

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Funding: This research was performed with funds received in part by the Cell Differentiation and Development Center (CDDC) at Marshall University, NIH-CA138510, NIH-CA140024, NIH-COBRE 5P20RR020180 (to P.P.C.); West Virginia NASA Space Grant Consortium (to S.K. and J.V.V.); WV-INBRE 5P20RR016477 (to D.A.P.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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chemotherapy due to their quiescent nature. This may explain why traditional chemotherapies can initially reduce the majority of the tumor bulk but fail to eradicate it in full, allowing eventual recurrence [1,11,17,18,22]. CSCs are more resistant to therapy not only secondary to quiescence, but also due to increased expression of anti-apoptotic proteins and drug efflux transporters. 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. This may explain the failure of treatments to eradicate the disease or to prevent recurrence of cancer. Additionally, if a drug affects the growth of only a minor population of cells, there will be only a minimal decrease in the growth of the tumor in the short term.

Theoretically, identification and characterization of the CSCs may allow the development of treatment modalities that target the cancer stem cells rather than the rapidly dividing cells in the cancer.

Prolonged exposure of humans and experimental animals to the altered gravitational conditions of space flight has adverse effects on different cellular systems. The effects of hypogravity environment on tumor growth and carcinogenesis are yet unknown and this research field still lacks systematic investigation on hypogravity induced gene expression, which is the key information needed to ultimately unfold the mechanism behind hypogravity-induced diseases. To this end, we have studied the effects of simulated hypogravity on human osteosarcoma cells cultured in the NASA-developed hydrofocusing bioreactor (HFB) and in the rotary cell culture system (RCCS). Various designs of rotating-wall vessels have been used to create a three-dimensional culture environment with variable shear stress and hypogravity simulating that in space [25]. The HFB (Celldyne, Houston, TX) developed by NASA at the Johnson Space Center, is a fluid filled dome, which rotates at a specified speed and has a conical spinner to provide a unique hydrofocusing capability that will allow for an extremely low-shear culture environment and a nylon gas transfer membrane [25]. The RCCS consists of a horizontally rotated culture vessel oxygenated by a flat silicone rubber gas transfer membrane [26]. The NASA-designed HFB bioreactor and the RCCS have been successfully used on Earth and in Space to enable investigators to use modeled or actual hypogravity, respectively, to study the role of gravity on the formation of three-dimensional mammalian cell tissue models and production of bio-products [25,27,28,29,30,31].

In this study we show that cancer stem cells are stimulated to proliferate when they are cultured in the hypogravity conditions produced by the HFB and that the reduction of gravity simulated in the HFB sensitizes CSCs to chemotherapeutic agents.

Results

Osteosarcoma stem-like cells proliferate in the Hydro-Focusing Bioreactor (HFB), but not in rotary cell culture system (RCCS)

Modeled hypogravity 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 hypogravity on tumors are unknown, we thought to investigate the effects of modeled hypogravity in the absence of shearing stress on the growth capacity of tumor cells of different embryonic origin. We have used an HFB developed by NASA at the Johnson Space Center, which is composed of a 50 mL fluid filled dome that rotates at a specified speed and that has an internal conical spinner that hydro-focuses the cell culture allowing for a low-shear environment.

To our surprise, we 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. Figure 1A shows that the number of viable SAOS-2 cells cultured for 5-days in the HFB was dramatically reduced.

Interestingly, SAOS-2 cells cultured in the bioreactor formed cell spheres and appeared to be significantly smaller than SAOS-2 cells cultured in dishers and harvested by trypsin treatment (data not shown). Figure 1B shows a 20 x bright field picture of spheres formed from SAOS-2 cells that were grown in the HFB for 5 days. Cells were removed from the HFB reactor and pictures were taken immediately using an inverted microscope using contrast phase.

Because of their apparent change in morphology and ability to form spheres in the HFB environment and because others have demonstrated the presence of CD133(+) cells within primary bone sarcomas, as well as the osteosarcoma cell lines MG-63, OS-521, OS-01-137, OS-99-01, and SAOS-2 and the chondrosarcoma cell line CS-828 [32,33], we hypothesized that the HFB selected cells were CD133(+). To test this hypothesis we immunophenotyped these cells with an antibody directed against CD133 (Miltenyi, Germany), and found that HFB selected cells were 100% positive to CD133, a typical stem cell marker of mesenchymal origin (Figure 1B, inset panel and Figure 1C). Since the SAOS-2 cells recovered from the bioreactor were all CD133(+) (98.8%) (Figure 1C), we wanted to determine whether the CD133(+) cells not only survived but also proliferated in the hypogravity environment. To this end, we selected CD133(+) cells from the SAOS-2 or HOS cell lines using a MACSorting system and challenged these CD133(+) cells to a 5-day run in the HFB. Interestingly, SAOS-2 or HOS cells MACSorted with an antibody against CD133 and cultured in the bioreactor for 5 days increased in number by two-fold (Figure 1A). Figure 1A shows the trypan blue exclusion cell counts of CD133(+) SAOS-2 viable cells recovered after 5 days of culture in the bioreactor.

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 seven-day period 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 (Figure 1D). Figure 1D shows that following an optimized protocol of cell culture in the HFB, it is possible to select and to proliferate a specific population contained in the SAOS-2 parental cell line. Similar results were achieved using various other cancer cell lines of different tissue origin and which express a variable amount of the CD133 marker (HOS, U2OS, T98G, U87MG, Du145, LNCap, WI38, H23, Hep3b, Hela, Mervo, HO-1 cells, HN12 and HN30), see Table 1, and data not shown.

Another bioreactor that simulates hypogravity, the 50 mL rotary cell culture system (RCCS) [26] manufactured by Synthecon (Houston, TX), was used to compare results generated using the HFB. In a parallel experiment in which we seeded an equal number of SAOS-2 cells in the same tissue culture incubator with identical conditions of rotor speed, CO2, temperature, and vessel volume (50 mL), the HFB increased CD133(+) cell growth from SAOS-2 cells compared to the RCCS vessel and to earth gravity control. 5 x 10^6 SAOS-2 cells were seeded in the HFB or RCCS bioreactors of which 350,000 (7%) were CD133(+) and 4,630,000 CD133(−) (Table 2). Culture media, oxygenation, speed, temperature and CO2 were kept consistently constant for the two culture systems and in the same incubator for a 7-days run. After a 7-day run, the vessels were harvested and cells were reacted
with an antibody fluoresceinated against CD133 (Miltenyi, Germany) and counted using a BD Facs Aria flow cytometer. An isotype antibody was used as control. From the comparison of the two bioreactor cultures, we showed that a \( (+15) \) fold increase in CD133(+) cells number (5,370,960 CD133(+) cells) was achieved using the HFB on a 7-day run. No proliferation of CD133(+) cells was observed in the culture derived from the RCCS culture vessel. The RCCS culture vessel showed instead a drastic reduction in the CD133(+) cell population \( (-4.8 \times 10^3) \) fold decrease] from the number of CD133(+) cells seeded in the bioreactors on day-0 (Table 2). In fact, the number of SAOS-2 CD133(+) cells decreased from 350,000 to 72,800 in a 7-day run in the RCCS, while the HFB grown SAOS-2 CD133(+) cells increased from 350,000 to 5,370,960 during an equivalent period of time.

Because of this dramatic difference in the growth of SAOS-2 CD133(+) cells in the two bioreactors generating hypogravity (HFB and RCCS), we wanted to verify if pH could be a variable causing the effects on cell growth observed. Therefore, we measured the pH of the media of the HFB and RCCS bioreactors kept in the same incubator with a CO2 level set at 5% and a constant temperature of 37°C before and after a run of 7-days. We compared the pH of the media from the HFB and RCCS vessels with the conditioned media of control cells grown in a Petri dish in normal gravity condition versus unspent D-MEM medium. No statistically significant difference was found among the different cell culture conditions by repeating the experiments three times. In fact, unspent medium showed a pH of 7.82±0.04. Medium from control SAOS-2 cells grown in a Petri dish in normal gravity conditions showed a pH of 7.96±0.23; medium from SAOS-2 cells grown in the HFB had a pH of 7.71±0.23; and medium from SAOS-2 cells grown in the RCCS bioreactor showed a pH of 7.9±0.11, demonstrating that the different growth of CD133(+)
HFB, the CD133+ measures the activity of caspases-3 in the samples. According to activity of caspases by using a colorimetric caspases kit that instead die by apoptosis.

Focusing Bioreactor (HFB)

Osteosarcoma cells die by apoptosis in the Hydro-Focusing Bioreactor vessel. Since only a small fraction of the osteosarcoma SAOS-2 as well as HOS cells placed in the HFB-culture system were recovered after 3 or 5 days of culture, we tested whether these cells were being eliminated from the initial population by causing them to undergo apoptosis. Therefore, we compared the rates of apoptosis of SAOS-2 cells cultured in the HFB for 3 and 5 days to MACSorted CD133+ cells cultured in the HFB for 3 and 5 days.

To this end we have 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 or 5-days die by apoptosis as shown by a flow cytometric assay of Annexin-V and propidium iodide staining of the samples. Most importantly, samples of CD133+ cells proliferate while the CD133- cells cultured in the HFB for 5-days didn’t show such an increase in apoptosis by Annexin V staining when compared to the control sample. In the same culture conditions in the HFB, the CD133+ cells proliferate while the CD133- cells instead die by apoptosis.

We have also investigated this phenomenon by measuring the activity of caspases by using a colorimetric caspases kit that measures the activity of caspases-3 in the samples. According to the data presented we found that SAOS-2 cells (total population) cultured in the HFB for 5 days showed an increase of 1.6-fold in the caspases-3 activity detected (Figure 2E and F), which is a measure of the apoptotic index in these cells.

Stem cell marker expression increases 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. We wanted to analyze the expression levels of various markers related to the development of stem cells of mesenchymal origin. The expression of CD133, CD34, CD38, Osteocalcin, Sparc, Sox-9, RunX-2, Stro-1, CD117/c-Kit, Oct3/4, Endoglin, and Integrin-ß1 was examined by flow cytometry. Figure 3A shows the percent of fluorescent cells of the various markers examined in SAOS-2 cells cultured in static condition and after culture in the HFB for 5-days. The expression levels and the number of cells expressing the examined markers increased after 5 days of culture in the HFB vessel compared to the cells cultured in normal gravity conditions. Interestingly, the SAOS-2 cells grown in the HFB showed the highest relative increase in the number of cells positive to 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. Oct3/4, CD117, Stro-1, and CD34 also showed an increase in the number of cell number positivity.

### Table 1. Percentage of positivity ± standard deviation to CD133 of the various cell lines tested.

| Cell Line      | Tumor type                      | % CD133 (+) | %CD34(+) | %CD38(+) |
|----------------|---------------------------------|-------------|----------|----------|
| HOS            | Human osteosarcoma              | 37.7±7.8    | 5.8±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  |

### Table 2. Comparison of CD133(+) and (−) cell counts ± standard deviation of SAOS-2 cells grown in the HFB or RCCS vessels.

| Device | Total cell Input | CD133(+) cell Input | Total cell Output | CD133(+) cell Output | Fold Change |
|--------|------------------|---------------------|-------------------|----------------------|-------------|
| RCCS   | 5,000,000        | 350,000             | 5,200,000         | 72,800               | (−) 4.8     |
| HFB    | 5,000,000        | 350,000             | 5,370,960         | 5,370,960            | (+) 15.3    |

doi:10.1371/journal.pone.0010035.t001

doi:10.1371/journal.pone.0010035.t002

SAOS-2 cells observed between the RCCS and HFB vessels was not due to changes in pH in the culturing media.
CD133(+) cells grow three-dimensionally in the Hydrofocusing Bioreactor and form spheres

The SAOS-2 CD133(+) enriched osteosarcoma cells proliferate and assemble three-dimensionally as spheroids after 3-days culture in the bioreactor, which is yet another characteristic of stem cell growth. Figures 4A and B show at 10 and 40× magnification power, respectively, the spheroids grown in the bioreactor after 3 days of culture in simulated hypogravity. Interestingly, the CD133(+) SAOS-2 cells that were grown in simulated hypogravity were able to re-establish the parental cell line (constituted by about 10%±6.8 CD133(+) cells and 90%±6.8 CD133(−) cells) after a period of one week when seeded into treated culture dishes, which allow for adherent cells to attach to the plastic environment. Figure 4C and D (10× and 40×, respectively) show the SAOS-2 CD133(+) cells proliferated and selected with the HFB that were subsequently grown in attaching tissue culture dishes. In the reconstituted cell culture some not-adhering cells were noticeable; these could be stem-like cells also known as cancer stem cells.

It is known that stem-like cells will form cell spheres when cultured in ultra low-attaching dishes. We therefore tested the ability of CD133(+) SAOS-2 MAC-sorted cells to form cell clusters if placed in ultra low-attaching dishes. Their ability to form clusters was less efficient when compared to those cells that have been cultured in the hydrofocusing bioreactor, but they were still able to form spheres. Figure 5A and B show spheres formed by SAOS-2 CD133(+) cells in ultra low-attaching dishes. We also tested the ability of the SAOS-2 CD133(+) MACSorted and enriched cells 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. Figure 5C and D shows adherent SAOS-2 cells derived from CD133(+) MACSorted cells grown in adherent tissue culture dishes. Figure 5E shows the SAOS-2 cell line reconstituted after 10-days of inoculation of CD133(+) enriched cells in adhering tissue culture dishes.

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 replicates of each experimental point. (75.9%, 67.5%, 47.6%, 22.36%, respectively) comparing 1G-growth versus SAOS-2 cells grown in simulated hypogravity for 5-days. We found no change in the number of CD38 positive cells in the same conditions. The experiment was repeated 5 times with comparable results and standard deviations were calculated and are reported on the diagram as error bars. Figure 3B shows an example of immunofluorescence staining and positivity of SAOS-2 cells to CD133, Sox-9, Sparc, and CD117/c-Kit following growth in the HFB vessel for 5 days.

Equal number of SAOS-2 cells and of CD133(+) SAOS-2 cells (5×10⁵ 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±00 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 we have in our laboratory (Table 3). Figure 6 shows an example of a soft-agar assay performed with the parental tumor cell line (Figure 6A) or with the CD133 (+) enriched fraction of SAOS-2 cells (Figure 6B). The parental SAOS-2 cells formed spheres, but with very low efficiency when compared to the enriched CD133 (+) cells.

Further evidence that there are two distinct populations in the SAOS-2 cell line, which appears to be composed of CD133(+) and (−) cells, is that these two different populations have a distinct cell cycle profile. We performed a flow cytometry analysis of SAOS-2 cells grown in 1G culture and found that SAOS-2 CD133(+) cells, which were sorted by their CD133 status and stained with propidium iodide, had a different cell cycle profile with respect to the CD133(+) population. Figure 6C shows a representative flow cytometric assay of SAOS-2 cells demonstrating 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. Comparable results were obtained in parallel and repeated experiments.

Interestingly, SAOS-2 cells grown in the bioreactor for 5 days and stained with an anti-CD133 (Miltenyi, Germany) and the proliferation marker anti-Ki-67 demonstrated increased proliferation of the cells compared to the SAOS-2 cells grown in 1G cultures. Figure 6D shows a representative flow cytometric assay of SAOS-2 cells demonstrating that the cells cultured in the HFB proliferate at a different rate than the total SAOS-2 cell population. In fact, the fraction of SAOS-2 CD133(+) cells, which are also positive to Ki-67, increased from 14.27% to 53.98% comparing cells cultured in 1G-growth versus those cells cultured for 5 days in HFB simulated hypogravity condition, respectively. Comparable results were obtained in parallel and repeated experiments.

Simulated hypogravity enhances apoptosis and sensitizes cancer stem cells to chemotherapy

GSCs are thought to be responsible for initiating and maintaining the disease. 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 treatment resistant [17,18,20]. Our recent discovery that cancer stem cells are stimulated to proliferate when they are placed in hypogravity conditions encourages the idea to test the ability of simulated hypogravity to sensitize tumor stem cells to chemotherapeutic agents.

We have therefore tested the ability of hypogravity to sensitize the osteosarcoma cells to various chemotherapy agents used in cancer therapy. We have discovered that the HFB-hypogravity environment sensitized the CD133(+) resistant osteosarcoma cells to various chemotherapy agents at clinically relevant doses. We have tested the sensitivity of osteosarcoma cells to cisplatin, doxorubicin and methotrexate, which are the drugs of choice for this tumor type in the clinical settings. SAOS-2 cells were grown for 5-days in the HFB and then harvested. 1×10^4 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 (Figure 7). The same numbers 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 of mitochondrial viability that assesses the number of viable cells versus the number of dead cell in a given sample. Figures 7, 8, and 9 show that the CD133(+) cells grown in the HFB increased...
their sensitivity to the cisplatin, doxorubicin, and methotrexate treatments, respectively. 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 the hypogravity environment appear to be resistant to the same treatment regimen. Similar results were achieved by treating the cells with the chemotherapeutic agents for 1 hour and subjecting the samples to an MTT assay 24 hours later (data not shown). These data have been confirmed with other techniques, such as measurement of cell viability by trypan blue exclusion cell-count before and after chemo treatment, as well as by flow-cytometry analysis (data not shown).

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 LD50. However, the cancer cells stimulated to grow in the bioreactor, which are CD133(+), showed enhanced cell death even at doses well-below the LD50, suggesting that environments in which lack of gravity

Table 3. Average number of colonies counted ± standard deviation in 6-well dishes in a triplicate experiment.

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

doi:10.1371/journal.pone.0010035.t003
Figure 6. The SAOS-2 cell line is composed of two distinct populations. A) Contrast phase microscopy of a soft agar assay of parental SAOS-2 cells. B) Contrast phase microscopy of a soft agar assay of HFB CD133(+) proliferated SAOS-2 cells. C) Propidium iodide flow cytometry analysis of SAOS-2 cells grown in 1-G culture sorted by their CD133 status showing two distinct populations: the CD133(+) and CD133(−) cells with a different
activity detected following CDDP treatment vs. the 3-fold of increased caspases-3 activity detected in SAOS-2 cells treated with the same amount of CDDP (10 μg/mL) in static growth condition. This indicates that the HFB stimulated a CDDP-induced apoptosis of the SAOS-2 cells (Figure 10).

The data presented here clearly show that the hypogravity environment greatly sensitized the CD133(+) cells to various chemotherapeutic agents. Importantly, we showed that the CD133(+) cells, which are normally resistant to chemo treatment, become sensitive even at low doses of the noxious drugs.

Discussion

Modeled hypogravity is a condition in which cells are able to grow in an anchorage independent manner. The hydrofocusing
Figure 8. Sensitivity of SAOS-2 cells to methotrexate following growth in simulated hypogravity. A) LD₅₀ for methotrexate determined for the SAOS-2 cells. An LD₅₀ of 22μg/mL for methotrexate was determined exposing the SAOS-2 cells to a 24-hours treatment to the drug, using the MTT assay. Comparable results were obtained with cell count by trypan blue exclusion. 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. doi:10.1371/journal.pone.0010035.g008
Figure 9. Sensitivity of SAOS-2 cells to doxorubicin following growth in simulated hypogravity. A) LD50 for doxorubicin determined for the SAOS-2 cells. An LD50 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. Comparable results were obtained with cell count by trypan blue exclusion. 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.
bioreactor HFB from Celdyne, Houston TX was developed by NASA at the Johnson Space Center. The HFB is composed of a 50 mL fluid filled dome, which rotates at a specified speed to provide a unique hydrofocusing capability, that in absence of gas bubbles allows for a low-shear culture environment in which the cells can grow in modeled hypogravity [25]. Another bioreactor that allows cells to grow in a three-dimensional manner is the rotatory cell culture system (RCCS) manufactured by Synthecon (Houston, TX). The RCCS is a 50 mL horizontally rotating culture vessel, which reduces the shear and turbulence generated by conventional stirred bioreactors minimizing mechanical cell damage and simulating aspects of hypogravity [25,26].

Prolonged exposure of humans and experimental animals to the altered gravitational conditions of space flight has diverse effects on various cellular systems [34,35,36,37,38,39]. However, the effects of hypogravity on tumor growth and carcinogenesis are yet unknown. The purpose of this study was to investigate the effects of modeled hypogravity on cancer cell growth.

The existence of cancer stem cells in several solid and hematopoietic tumors has recently been proven [40,41,42,43,44,45,46,47]. Moreover, it was demonstrated that cancer stem cells are also present in established cancer cell lines [32,49,49]. Importantly, it is widely accepted that cancer stem cells are responsible for tumor recurrence after chemo- 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 [50].

Because osteosarcoma is a highly resistant tumor to conventional therapies, we investigated whether osteosarcoma cell lines contain a subpopulation of putative stem cells that could be targeted for anti-cancer therapy. 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.

Stem cells have the potential to self-renew and generate a developmental hierarchy of differentiating progeny. The original culture methodology employed by Reynolds et al. [51] and Reynolds and Weiss [52] to show that the adult mammalian brain contains cells that give rise to neurosphere clones has been used to isolate and characterize cells suspected of possessing attributes of stem and progenitor cells. The Reynolds’ group showed that the stressful growth conditions of their system due to serum starvation allows dedifferentiation of certain cells, or selects for the most sensitive of SAOS-2 cells to 2.2 μg/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.

doi:10.1371/journal.pone.0010035.g009
primitive cells by eliminating the differentiated cells that are unable to survive.

In our study, the HFB on day 5 and day 7 increased CD133(+) SAOS-2 cell growth compared to the RCCS vessel and to the earth gravity control. We observed a (+)15-fold proliferation of the SAOS-2 CD133(+) cellular fraction with cells that were cultured for 7-days at optimized conditions in the HFB. The RCCS vessel instead showed a (−)-8-fold decrease in the CD133(+) cellular fraction respect to the HFB after 7-days of culture. Additionally, 100% of the cells harvested from the HFB were found to be CD133(+), indicating that the HFB had selected for the SAOS-2 undifferentiated cellular fraction. We speculate that the selective growth of CD133(+) cells observed using the HFB vessel could be due to its specific design. The presence of the conic spinner on the axis of rotation of the HFB vessel that hydrofocuses the cell culture allowing for a low-shear environment could be responsible of the selective growth in the HFB environment.

In order to characterize and investigate the cells grown in the HFB, we assessed the expression levels of various adhesion molecules and stem cell markers before and after culturing the cells in simulated hypogravity. The CD133(+) cells appeared healthy and interestingly, both the expression levels and the number of cells expressing several of the stem cell markers other than CD133 were increased after a seven-day run in the HFB. We found that after a seven-day run compared to static growth control conditions there were an increased number of SAOS-2 cells expressing CD133, CD34, CD38, CD117/Kit, Sparc, Sosx-9, RunX-2, Stro-1, Osteocalcin, Endoglin, Integrin-81, and OCT3/4. The fact that we found increased number of cells expressing the tested markers and increased overall expression levels of the same after culture of the cells in the hypogravity environment points toward various scenarios. One scenario is that hypogravity could epigenetically regulate the expression of genes involved in cell-cell adhesion and migration, which are expressed during embryogenesis. Other possible scenarios are that hypogravity could affect transcription or post-translational events such as proteasome activity. We are currently analyzing a data set from a gene expression microarray chip in which we studied osteosarcoma cells challenged to grow in HFB versus cells grown in static 1-G in order to better characterize the influence of hypogravity on cellular homeostasis.

The importance of our work is summarized by the unique ability of the HFB to proliferate cancer stem cells and to sensitize them to low doses of chemotherapy. The selection of CD133(+) SAOS-2 sub-population due to cell death of the CD133(−) population is demonstrated by the activation of Caspase-3 activity shown in figure 2 following culture of the SAOS-2 cells (total population) in the HFB for 5-days.

We have also shown that the CD133(+) osteosarcoma cells, which are chemo-resistant to the various drugs, became sensitive to these same drugs at much lower doses than the LD₅₀. In this work we choose to test the effects of cisplatin, methotrexate and doxorubicin on osteosarcoma cells, because those are the commonly used drugs to combat this disease [53]. These drugs have different mechanisms of action that can be recapitulated in direct or indirect DNA damage. [34,35,36].

Current therapy for osteosarcoma includes neoadjuvant chemotherapy, surgery, and postoperative (adjuvant) chemotherapy [57,58]. Doxorubicin has been proven effective as adjuvant chemotherapy regimens after surgery in preventing and/or reducing recurrence and metastasis rates in osteosarcoma patients that are operable [58,59]. Our findings that SAOS-2 cells in general, and CD133(+) SAOS-2 cells in particular, are more sensitive to doxorubicin than to cisplatin or methotrexate helps elucidate some the clinical practice setting. However, more importantly, we have demonstrated the existence of a chemo sensitization switch operated by the hypogravity environment and generated by the unique capabilities of the HFB vessel.

Using the HFB we have formulated a novel technique to select and propagate CD133(+) cells. This will likely prove to be an invaluable tool in furthering stem cell research, embryonic and adult, as well as developing a rapid screening method to test chemotherapeutic agents that are more affective against cancer stem cells. Importantly, we showed that the HFB bioreactor efficiently proliferated CD133(+) cells, while the RCCS rotating vessel did not. Additionally, the data presented in this study clearly show that the hypogravity environment, by stimulating CD133(+) to proliferate, sensitized the CD133(+) to the various chemotherapeutic agents tested.

Presently used chemotherapy drugs have a high rate of failure. Cell culture chemotherapy testing is being used to identify which drugs are more likely to be effective against a particular tumor type in patients. Cell culture drug testing is of value in any situation in which there is a choice between two or more treatments. This includes virtually all situations in cancer chemotherapy, whether the goal is cure or palliation. Often, results are obtained before the patient begins treatment. This kind of testing can assist in individualizing cancer therapy by providing information about the likely response of an individual patient’s tumor to proposed therapy. Presently, chemotherapy testing is performed on cancer cells from patients without prior separation and proliferation of the cancer stem cells from the bulk of tumor cells. Knowing which chemotherapy agents the patient’s cancer cells or cancer stem cells are resistant to is very important. Then, these options can be eliminated, thereby avoiding the toxicity of ineffective agents. Choosing the most effective agent can help patients to avoid the physical, emotional, and financial costs of failed therapy and experience an increased quality of life.

Importantly, we showed that the HFB bioreactor proliferated CD133(+) cells, which are the minority of the bulk of the tumor cells. It is known that aggressive treatments destroy the majority of the cancerous cells, however a small fraction of the cells, the cancer stem cells, survive and often regenerate tumor cells that are now chemo-resistant [23,24,60]. Therefore, to be able to test the efficacy of cytotoxic agents in vitro prior to their use in clinical setting on cancer cells as well as on cancer stem cells may pave the way to more effective chemotherapeutic strategies in patients.

Materials and Methods

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 hepatocellular carcinoma) 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 medium (either RPMI 1640 or D-MEM) at 37°C in a water-saturated atmosphere of 95% air and 5% CO2. All media were 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).
Three-dimensional bioreactor cell culture

A hydrodynamic focusing bioreactor (HFB) (Celdyne, Houston TX) and a rotatory cell culture system (RCCS) (Synthecon, Houston, TX) were used. Both vessels have a volume of 50 mL and a membrane that allows for gas exchange. Culture media, oxygenation, speed, temperature and CO2 were kept consistently constant for the two culture systems, which were placed in the same incubator. The vessels can rotate at adjustable speed on a fixed axis. Cells were counted and a range of $1 \times 10^5$–$1.2 \times 10^7$ cells were placed in the 40 mL rotating chamber of the HFB for 3- to 7-days set at 25 rpm. Cells were counted and the same number of cells was placed in the 50 mL rotating chamber for 3- to 7-days set at 25 rpm. Cells were then removed and counted again using trypan blue exclusion to determine cellular viability and cell number. The cells were either pelleted for future research or labeled with fluorescent markers for characterization.

Cell Sorting

Up to $2 \times 10^7$ cells were sorted either by a magnetic-activated cell sorting (MACS) 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 anti-biotin magnetic beads, and then passed through a magnetic column where CD133(+) cells were retained, while unlabeled cells passed 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.

Flow Cytometry studies

Cells were analyzed by the antigenic criteria using anti-CD133 (prominin1) (Miltenyi Biotec, Auburn, CA); -CD34 (Miltenyi Biotec, 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 –Oct3/4 (Cell Signaling, Danvers, MA). Briefly, cells were detached using 0.02% EDTA in PBS and pelleted (10 min at 1,000 rpm), washed in 0.1% BSA in PBS at 4°C and incubated in a solution of 1μg antibody +9 μL 0.1% BSA in 1× PBS. Cells were washed in the same solution once and were processed for sorting (FACS Aria, BD Biosciences, San Jose, CA). Propidium iodide stained cells were also analyzed with a fluorescence-activated cell sorter (FACS Aria, BD Bioscience, San Jose, CA). Cell cycle was analyzed by flow cytometry as follows: following staining with a primary antibody against CD133 (Miltenyi Biotec, Auburn, CA), and with the secondary antibodies goat anti-mouse (FITC) and mouse anti-goat (PE conjugated) (Santa Cruz, CA), cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed in PBS, then left for 60 min 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 h at room temperature in the dark. Isotypes and non-probed cells were used as controls. Multiple cell cycle analysis studies were performed to obtain a reproducible model experiments on growing cells.

Annexin-V assay

Annexin-V was analyzed with the Annexin-V/FTTC Kit (Bender MedSystems, Burlingame, CA) following manufacturer’s instructions. Cytometry was operated with a FACS Aria (BD Bioscience, San Jose, CA).

Soft agar assay and sphere assay

Cells were counted and $5 \times 10^2$ SAOS-2 and CD133 (+) and (−) SAOS-2 cells were plated in 6-well culture dishes in 3 mL of soft agar growth medium; 500 μL of medium per well was added every 2 days.

Formed spheres were counted under the microscope. Cells were also plated in ultra-low attachment dishes (Corning, Lowell, MA catalogue # 3926), and the number of spheres for each well was evaluated after 7 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.

Immunofluorescence

Two $10^5$ 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 incubated on ice for 15 minutes. Negative controls were performed with secondary antibodies only. The slides were viewed under an inverted Olympus IX70 microscope (Olympus America, Inc., Melville, NY). Fluorescence images were captured with a computer QE camera (Cooke Co., Auburn Hills, MI) and operated with SlideBook 3.0 software (Intelligent Imaging Innovations Inc., Denver, CO).

In vitro growth characteristics and chemosensitivity

To determine the in vitro growth rate of each cell line, 30,000 cells/cm² were seeded in the appropriate tissue culture media. Cells were then harvested and counted by Trypan blue dye exclusion every 24 h, and doubling time was calculated during the exponential phase of growth (from 48 to 96 h 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. LD50 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 mg/mL, 50 mg/mL, and 20 mg/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. Cells were also treated continuously for 24 hours with the chemotherapeutic agent, and their viability was assessed after treatment. The degree of cisplatin, doxorubicin, and methotrexate resistance was expressed by the drug concentration resulting in 50% inhibition of viability (LD50) of the various cell lines. To determine the LD50 values 30,000 cells/cm² were seeded in their growth medium containing 10% FBS, and after 24 h the medium was replaced with medium containing 10% FBS and no drug (control) or with different concentrations of the chemotherapy drugs. After 96 h, 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 LD50 values.
MTT Assay

$1 \times 10^4$ exponentially growing cells were seeded in 100 μL of medium in 96-well flat-bottomed plates. For each of the variants tested, 7 repeats were used. Following 24 hr 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 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 untreated cells were exposed to the drugs either for 24-hours continuously or for an hour time intervals, as indicated. 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 wavelength. The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Chemotherapeutic drugs' LD50 was determined as a chemotherapeutic drugs' concentration showing 50% of cell survival as compared with the control cells. The experiments were repeated in seven replicates.

Caspase-3 colorimetric assay

Caspase-3 activity was measured with a Caspases-3 colorimetric assay kit (Chemicon, (now Millipore, Billerica, MA) following manufacturer's instructions.

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Statistics

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.

Acknowledgments

The authors would like to thank NASA-JSC, Celdyne, and Wyle Labs for use of the HFB systems, particularly Dr. Steve Gonda, Chief Scientist for Biotechnology at NASA-JSC. We gratefully acknowledge the Marshall University Biochemistry and Microbiology Department and the Biology Department.

Author Contributions

Conceived and designed the experiments: PPC. Performed the experiments: SK ADB AG CMH. Analyzed the data: SK ADB CMH JV PPC. Contributed reagents/materials/analysis tools: VS PPC. Wrote the paper: CMH VS PD JV PPC.

Proliferation of CD133 Cells

MTT Assay

$1 \times 10^4$ exponentially growing cells were seeded in 100 μL of medium in 96-microwell, flat-bottomed plates. For each of the variants tested, 7 repeats were used. Following 24 hr 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 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 untreated cells were exposed to the drugs either for 24-hours continuously or for an hour time intervals, as indicated. 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 wavelength. The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Chemotherapeutic drugs' LD50 was determined as a chemotherapeutic drugs' concentration showing 50% of cell survival as compared with the control cells. The experiments were repeated in seven replicates.

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