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# *In vitro* Tumorigenicity and Stemness Characterization of the U87MG Glioblastoma Cell Line based on the CD133 Cancer Stem Cell Marker

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## Abstract

The cancer stem cells (CSC) hypothesis is currently the most widely accepted theory regarding tumor formation and self-renewal ability. The need to find pharmacological compounds or biological agents capable of eliminating CSC makes the search for methods of recognition and isolation of these cells a matter of great urgency.

The aim of the study was to separate CD133+ and CD133- cell subpopulations from the U87MG glioblastoma cell line by magnetic activated cell sorting (MACS), and to analyze the tumorigenic and stem cell differences among four sets of cells: the cell line grown in serum (monolayer group), the neurospheres of the same cell line, and the CD133+ and CD133- sorted cell fractions from the neurospheres.

Our results showed that the neurospheres and the CD133+ cells overexpressed stem cell marker genes such as SOX2, Nestin, Musashi1 and CD133, and formed more colonies in soft agar than the cell groups with less CD133 expression (monolayer and CD133- group). On the other hand the CD133- and the monolayer groups had similar expression levels of CD133, while the CD133- cells expressed higher levels of Nestin, SOX2 and Musashi1 than the monolayer cells did. In addition, cell migration assessed by the scratching test showed that the CD133- and the monolayer cells migrated more than the neurospheres and the CD133+ cells did. Moreover, the CD133- cells had a higher proliferation rate than the CD133+ cells and the neurospheres, with all of these three groups being cultured in the medium for neurospheres.

In conclusion, we might point out the presence of CSC in the CD133- group, as suggested by others. Therefore, CD133+ cells are not necessarily equivalent to CSC. Perhaps CD133+ cells present a higher probability of including CSC than CD133- do. Further functional and genetic analyses must be performed in order to reach optimal isolation of CSC.

**Keywords:** Glioblastoma; Cancer stem cell; CD133: Tumorigenesis

## Introduction

Glioblastoma Multiforme (GBM) is the most common tumor that emerges from the central nervous system. It has an incidence of 2–3 cases per 100,000 people in Europe and North America. GBM defies modern treatments such as surgery, radiotherapy [1] and chemotherapy [2] and consequently, median survival ranges from just 9 to 12 months [3]; and 5-year mortality rates are as high as 95%. GBM primary tumors can develop after a short clinical history and without any evidence of a precursor lesion [4]. In contrast, secondary tumors develop from the progression of low-grade astrocytomas.

The cancer stem cells (CSC) hypothesis [5] is, currently, the most widely accepted theory regarding tumor formation and self-renewal ability. It states that there are different tumorigenic phenotypes inside a tumor mass. One of these cell phenotypes is capable of generating new tumors if transplanted to a host and it is able to self-generate and regenerate the rest of the tumor cells [6,7]. This cell type is called CSC in accordance with the similarities found with stem cells. The fact that CSC are able to withstand a large number of drugs and treatments, and have the ability to regenerate the tumor mass after treatment, makes them a highly attractive target for new therapies and anti-tumorigenic drugs [8]. The need to find pharmacological compounds capable of eliminating CSC makes the search for methods of recognition and isolation of these cells for experimentation a matter of great urgency.

CSC show different growth patterns when compared to non-CSC. They are not only drug-resistant cells, but are also highly invasive, proliferative cells capable of neurosphere and colony formation [9-12]. These properties which make them aggressive in our nervous system, allow us to discriminate them from the rest of the cells within a tumor or a cancerous cell line grown in culture, and to experiment with them *in vitro*. CSC not only show a particular *in vivo* and *in vitro* behavior but are also genetically different from the rest of the tumor. CSC have protein and RNA patterns of expression similar to those of the normal stem cells. More precisely, CSC are capable of expressing genes and proteins of pluripotency such as SOX2, Nestin, Musashi, OCT4 and others [2,13,14].

Scientists have not been able to specifically determine the genetic or expression profiles of CSC. Some researchers have found that GBM cells that express Prominin-1 (CD133) on their membrane show CSC-like properties [8,10]. However, other results are not quite as conclusive [15]. The search for the definitive marker of a CSC is still

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ongoing, and it is more likely that we will have to use a series of markers within a cell, instead of just one, in order to label a cell as a CSC. Until this set of markers or a definitive marker is found, we can still isolate subpopulations from one or several markers and thus, obtain a CSC-enriched culture. The search for elucidating the presence of CSC in a tumor and isolating them from the rest of the cancerous cells has led to the discovery of several protein markers expressed by these cells on their membranes. Stem cell markers such as CD133 in GBM [11,13], and other proteins in different tumors, are used for positive selection [9] of these cells by Magnetic Activated Cell Sorting (MACS) or Fluorescence-Activated Cell Sorting (FACS). Isolation helps *in vitro* experimentation and culture enrichment of CSC [16,17].

CSC are able to grow *in vitro* under special circumstances under which other cancer cells tend to die or are unable to spread. When cancer cells grow in serum-free medium (SFM), with specific growth factors such as B27, Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF), they are able to expand unanchored to the plastic surface. It has been determined that under these circumstances CSC expansion and growth is improved, and CSC are able to proliferate in cell suspension cellular aggregates known as neurospheres [16-18].

Neurosphere culture cells show more CSC characteristics *in vitro* and *in vivo*; and have a higher expression of stem cell genes, proteins and markers than monolayer cell cultures [2]. Moreover, authors have shown that cancer cells with a CSC phenotype selected by the expression of CSC markers are able to form neurospheres easier than cancer cells that do not express stem cell markers [11].

Cells undergo a series of genetic changes until they transform into cancer cells, more than just generating stem cell characteristics. Tumors need an intraspecific organization to evade innate defenses. Signaling pathways such as Hedgehog, Notch, Wnt and others, have roles in development and progression of tumors and are critical for the generation, differentiation and drug-resistance of CSC [19]. The Sonic Hedgehog pathway (SHP) is one of the altered pathways in cancer progression [20]. SHP overexpression increases the growth of cancer cells. It has been observed that the inhibition of SHP in GBM CD133+ cells enhances their sensitivity to temozolomide, a chemotherapeutic agent [21]. Therefore, finding out which of the SHP regulators have their expression levels altered in CSC might contribute to therapy development.

Our working hypothesis is that the selection of cancer cells with the CD133 marker and their subsequent isolation and characterization will allow us to assess the ability of the different CD133 subpopulations as CSC. The aim of this study is to separate CD133+ and CD133- cell subpopulations from the U87MG glioblastoma cell line by magnetic activated cell sorting (MACS), and to analyze the tumorigenic and stem cell differences among four sets of cells: the cell line grown in serum (monolayer group), the neurospheres of the same cell line, and the CD133+ and CD133- sorted cell fractions from the neurospheres.

## Material and Methods

### Cell culture

U87MG is a human glioblastoma epithelial-like cell line, purchased from the European Collection of Cell Culture, ECACC (Salisbury, Wiltshire, United Kingdom). It was maintained in DMEM+L-Glutamax medium (Invitrogen, Barcelona, Spain). The medium was supplemented with heat-inactivated 10% fetal bovine serum (Sigma Aldrich Corporation, St Louis, MO, USA), 1% penicillin/streptomycin (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and 0.1% amphotericin B (Invitrogen, Barcelona, Spain). The cell line was grown at 37°C in a humidified atmosphere of 5% $CO_2$/95% air.

In order to form secondary neurospheres, U87MG cells were seeded in DMEM+F12 medium (Invitrogen, Barcelona, Spain), supplemented with 20 µg/ml of B27 supplement (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), 0.2 µg/ml of Epidermal Growth Factor, EGF (Sigma Aldrich Corporation, St Louis, MO, USA) and 0.2 µg/ml of basic Fibroblast Growth Factor, bFGF (Sigma Aldrich Corporation, St Louis, MO, USA). Sphere flasks were placed at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. The morphology of living cells was studied with an Olympus IX71 inverted microscope (Melville, NY).

### Flow cytometry

CD133 expression was assessed by flow cytometry. Neurospheres were disaggregated by trypsinization with mechanical help. 105 U87MG neurosphere forming cells were treated with an anti-CD133 antibody conjugated with fluorescein isothiocyanate, FITC (Miltenyi Biotech, Bergisch-Gladbach, Germany). 5x104 non treated U87MG cells were used as negative control for the experiment.

### Sorting of CD133 subpopulations by MACS (Magnetic-Activated Cell Sorting)

Magnetic cell sorting was used to separate the CD133+ cells from the U87MG neurosphere culture. Cells were pipetted up and down with a 20-200 µl pipette and 0.1% trypsin to disaggregate them. The supernatant was filtered through a 40 µm cell strainer to remove still-aggregated cells. The supernatant was then centrifuged and resuspended in Fcr blocking buffer (Miltenyi Biotech, Bergisch-Gladbach, Germany), MACS buffer (PBS, 2mM EDTA, 0.5% BSA) and anti-CD133 antibodies conjugated with magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) at 4ºC. After 30 min, cells were centrifuged and resuspended in MACS buffer. Finally, cells were sorted by a magnetic column following the manufacturer's instructions (Miltenyi Biotech, Bergisch-Gladbach, Germany). Following the MACS procedure, CD133+ and CD133- sorted fractions were cultured under sphere-forming cell conditions, for number and marker enrichment.

### Tumorigenicity assays

We carried out experiments that compared *in vitro* tumorigenicity of the U87MG monolayer cells, the U87MG neurosphere cells, and the CD133+ and CD133- sorted neurosphere cells. Each of the four populations was seeded in a 6-well plate and experiments were repeated 3 times on different days for statistical purposes.

**Colony formation assay under monolayer conditions:** A colony formation assay was performed for evaluating the clonogenicity of the cell population groups. After trypsinization and counting, U87MG cells were diluted and 200 of them were used to seed each well of a 6-well plate. The wells were filled with 5 ml of DMEM+L-Glutamax medium supplemented with 10% fetal bovine, 1% penicillin/streptomycin and 0.1% amphotericin B. Plates were grown at 37°C in a humidified atmosphere of 5% $CO_2$/95% air for 14-16 days. When colony number differences were evident, cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

**Soft agar colony formation assay:** In order to evaluate the ability of individual cell lines to grow in an anchorage-independent manner, cells were plated in soft agar. The bottom of each well contained 2 ml of 0.5% agarose and DMEM 2X (Sigma Aldrich Corporation, St Louis, MO, USA). This compact agar was covered with 2 ml of 0.2% agar and DMEM 2X with $5x10^3$ U87MG cells. The medium was changed every 3 days. After 21 days, the wells were stained with 0.003% crystal violet, and five areas were randomly selected from each well in order to count the approximate number of colonies.

**Scratching assay *in vitro* migration test:** Motility of the U87MG

cell populations was assessed by the scratching assay. Each group of U87MG cells was seeded in 27-well plates. When 70% confluence was achieved, a 100-1000 µl pipette tip was used to "scratch" the bottom of the wells. Photographs of the "wound healing" were taken at 0, 4, 8, 18, 26 and 48 h.

## CSC marker expression analysis

Gene expression of GLI1, Nestin, Musashi1, SOX2, CD133, and GAPDH was analyzed by quantitative RT-PCR. RNA from the U87MG monolayer, neurosphere, CD133- and CD133+ cell populations was isolated using the guanidine isothiocyanate reagent from the QuickPrep Total RNA Extraction Kit (Amersham Biosciences Corporation, Piscataway, NJ, USA), following manufacturer's instructions. 1 µg RNA was used for cDNA synthesis facilitated by SuperScript™ II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

Nestin, Musashi1 and CD133 stem cell markers [12], GLI1 [22], and GAPDH expression were assessed by RT-qPCR in the U87MG cell groups. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control of expression. Primer sequences were designed using Primer3 software (Table 1). All RT-qPCR reactions were carried out in an IQ5 multicolor RT-

| Gene | Sense primer 5'-3' | Antisense primer 5'-3' | Tm (ºC) |
|---|---|---|---|
| **Nestin** | GTGGCTCCAAGACTTCC | GCACAGGTGTCTCAAGG | 57,9 |
| **GLI1** | TTCCTACCAGAGTCCCAAGT | CCCTATGTGAAGCCCTATTT | 56,0 |
| **CD133** | GCTGATGTTGAAACTGCTTGAG | TGGTGCCGTTGCCTTGG | 66,0 |
| **Musashi1** | CCCTGGCTACACCTACC | AGGCAGTGAGAGGAATGG | 58,5 |
| **SOX2** | GGCAGCTACAGCATGATGCAGGAC | CTGGTCATGGAGTTGTACTGCAGG | 70,0 |
| **GAPDH** | AACGTGTCAGTGGTGGACCTG | AGTGGGTGTCGCTGTTGAAGT | 54,5 |

**Table 1:** SYBR green qPCR primers.

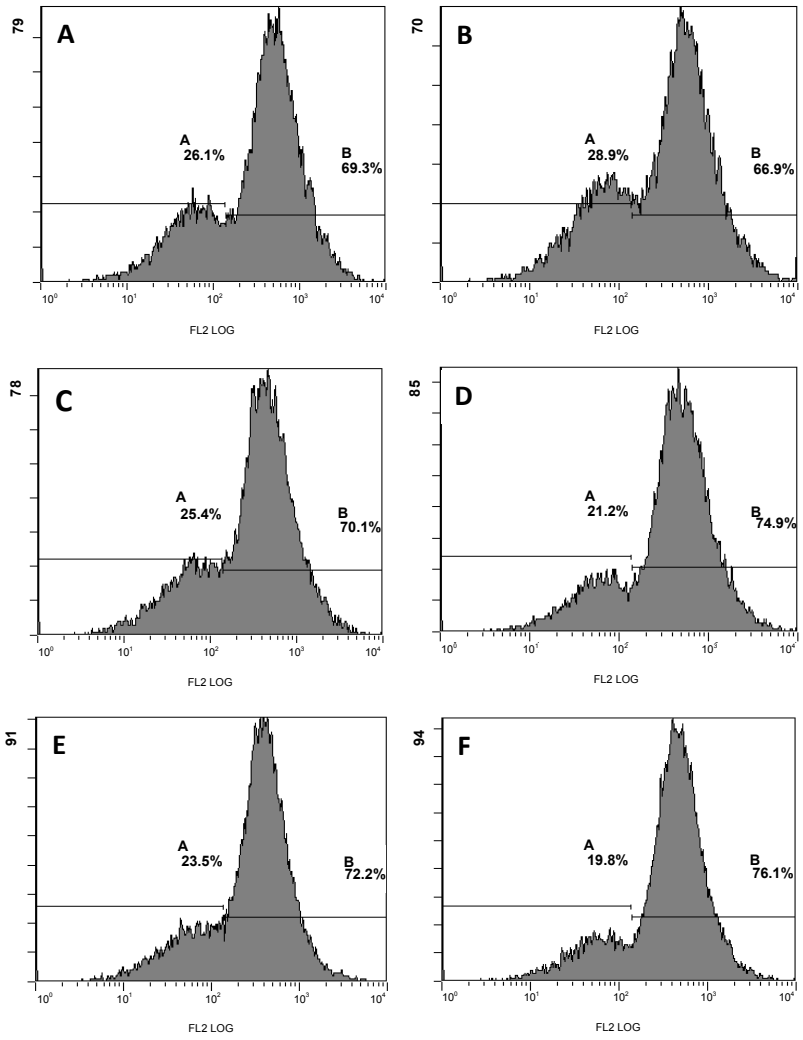


**Figure 1:** Cytometry results: A and B, neurospheres; C and D, CD133+ cells; E and F, CD133- cells. The A, C and E graphics correspond to the controls. B shows a 2.8% increase with respect to A (from 26.1% to 28.9%) due to CD133 expression. D and F show a decrease in the level of fluorescence which indicates null expression of CD133 cell marker.

qPCR detection system (Bio-Rad), with 2.5 µl of starting cDNA. For quantification, an efficiency corrected quantification model was applied. The derivative ratio values describe the relative expression change of the target gene relative to the GAPDH reference gene expression:

$$ratio = \frac{(E_{t\arg et})^{\Delta CT_{t\arg et}(control-sample)}}{(E_{ref})^{\Delta CT_{ref}(control-sample)}}$$

## Results

### U87MG cytometry

The U87MG cell line (monolayer cell group) grown in serum containing medium (SCM) was used to assess CD133 cell marker expression. Unfortunately, CD133 expression in this type of cell culture is too low for the cytometer to determine the exact level of expression (data not shown). To overcome the low CD133 expression, we decided to work with U87MG cells grown in serum free medium (SFM; neurosphere cell group). Flow cytometry detected that U87MG SFM cells had up to 2.8% of CD133+ cells (Figure 1). We proceeded to sort these cells by the MACS technique.

### U87MG magnetic cell sorting

The magnetic sorting of the U87MG SCM cells was a difficult task. The first attempts to sort cells by their expression of the CD133 cell surface marker produced very poor results. Cell sorting times were very long (6 h), with frequent blocking of the column that was used for the sorting, due to the amount of cells to be sorted. In the end, only a few CD133+ cells were obtained with each attempt.

In order to improve the number of CD133+ cells, the initial number of cells to be sorted was changed to less than 107, thereby avoiding the column blocking problem; however, the results did not improve. We decided to culture these few cells in order to amplify their number and with this, the percentage of CD133 expression. So, both CD133+ cells and a fraction of the CD133- cells obtained by MACS were cultured under SFM conditions. When the cultures were established, the cells were used for experimentation and for assessing CD133 cell marker expression by cytometry. Cytometry results showed that the CD133- culture had no CD133 expression, and that the CD133+ culture was also negative (Figure 1).

### Soft agar assay

The neurosphere group and the CD133+ were the groups that formed the most colonies. ANOVA statistical test showed that there were highly significant differences between groups; in order to determine which group presented differences the Tamhane's T2 post hoc test was performed. The test determined that CD133- and the monolayer group presented no significant differences in this assay. The same result was obtained for the CD133+ and the neurosphere group. However, highly significant differences were determined between both, the CD133+ and the CD133- group ($p$<0.001), and between the monolayer and the neurosphere group ($p$<0.001) (Figure 2).

### Colony formation assay

The CD133+ group formed the fewest number of colonies; the neurosphere cell group was the next. The CD133- and monolayer group had the highest number of colonies and these were compact, dense and presented the best appearance. Colony number data was analyzed by Kruskal Wallis pairwise comparisons. The test determined statistical differences between the CD133+ and CD133- groups ($p$=0.002) and between the neurospheres and monolayer groups ($p$=0.004). Highly significant differences were determined when comparing the CD133+ and the monolayer group ($p$<0.001) (Figure 3). The $p$ value obtained when comparing the CD133- and the neurosphere group was borderline to significant ($p$=0.054). The Bonferroni correction was not applied [23]; values represent median ± IC95.

### Scratching assay

The monolayer and the CD133- groups took the least time in filling the scratch, but after 26 h, the CD133- cells showed more scratch gaps in the middle area of the wells, where cells had less confluence. The monolayer group showed a more cohesive motility pattern, with the cells stretching over the whole perimeter of the scratch. The CD133+

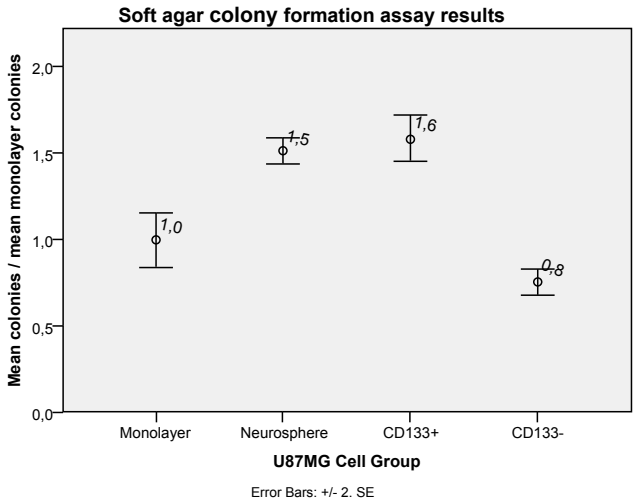


**Figure 2:** The mean value obtained from the wells of the agar assay shows statistical differences between the monolayer and CD133- groups, and the neurosphere and CD133+ groups. Data were analyzed using an analysis of variance (ANOVA) followed by Tamhane's T2 post hoc test.

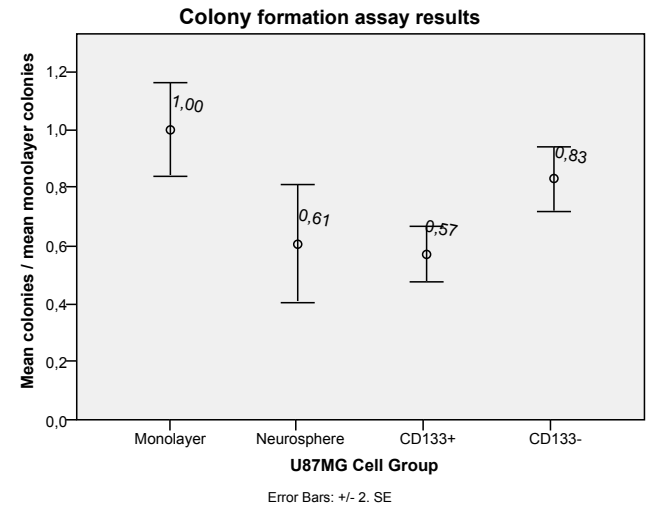


**Figure 3:** Colony formation assay in monolayer culture. The median value analyzed presents significant differences between the monolayer and both the neurosphere and CD133+ groups. There is also a significant difference between the CD133+ and CD133- groups. The CD133- and the neurosphere groups have a borderline significant $p$ value of 0.054. Data were analyzed using Kruskal Wallis test followed by Kruskal Wallis pairwise comparisons. The Bonferroni correction was not applied [23].

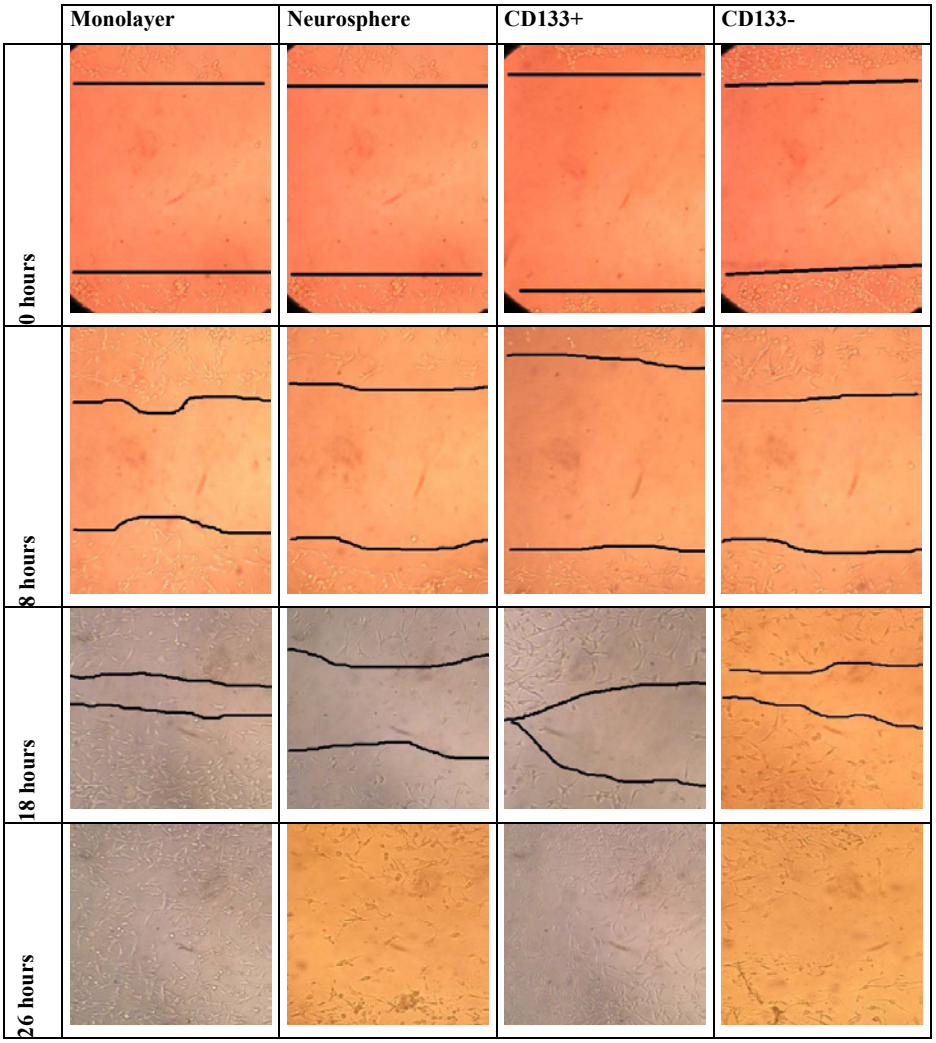


**Figure 4:** *In vitro* invasiveness test (scratching assay). During the first 18 h the monolayer and CD133- cell groups showed a faster motility. The CD133+ and the neurosphere groups advanced slowly in the margins of the scratch, but their margins were more diffuse with cells that were outside the perimeter and into the scratch. The monolayer group showed very clear and defined margins of motility, and their cells formed a much more compact web. After 26 h the monolayer, neurosphere and CD133+ groups had already covered the scratch. The CD133- group still had open areas in the middle of the wells. After 48 h, all the scratches were covered with cells and were impossible to recognize. All the photos were taken with the same augmentation.

cells and the neurosphere cells had slower migrating movement, but it was common to see cells that escaped the borders of the scratch into the wound. After 8 h, the border of both groups became much more diffuse than that of the monolayer or CD133- cells (Figure 4).

## RT-qPCR

CD133 RNA expression was assessed by RT-qPCR. All the cell groups showed expression of the cell marker CD133. The CD133+ and the neurosphere groups showed the highest expression of the Prominin-1 marker (Figure 5A). The stem cell marker genes: Musashi1 (Figure 5C), Nestin (Figure 5D) and SOX2 (Figure 5E), had no clear pattern of expression. Nestin had an expression pattern similar to that of CD133 RNA, being as much as two fold higher in the CD133+ and the neurosphere groups, with respect to the expression in the CD133- group. SOX2 presented very high levels of expression in the cells grown in serum-free medium, while Musashi doubled its expression in serum-free cells when compared to the monolayer group. GLI1 expression was higher in the neurosphere and CD133+ groups, although differences in GLI1 expression (Figure 5B) were not as marked as in other genes.

## Discussion

The results obtained in this work were dissimilar. While some of them supported previously expected data, others were unpredicted. The clonogenicity and motility assays (Figures 2-4) were diverse. The clonogenicity in soft agar assay (Figure 2) adjusted to expected results [24,25]. The cell groups with higher CD133 expression levels (neurosphere and CD133+ groups) were the ones that formed more colonies, while the cell groups with lower CD133 expression (monolayer and CD133- groups) were the ones in which lesser colonies were formed. However, colony formation under monolayer conditions (Figure 3) and the scratching assay (Figure 4) did not reflect that which other researchers had previously described [26-29]. The CD133+ and the neurosphere groups were less clonogenic and invasive than the CD133- and monolayer groups.

With regard to the scratching assay, we might be able to explain the

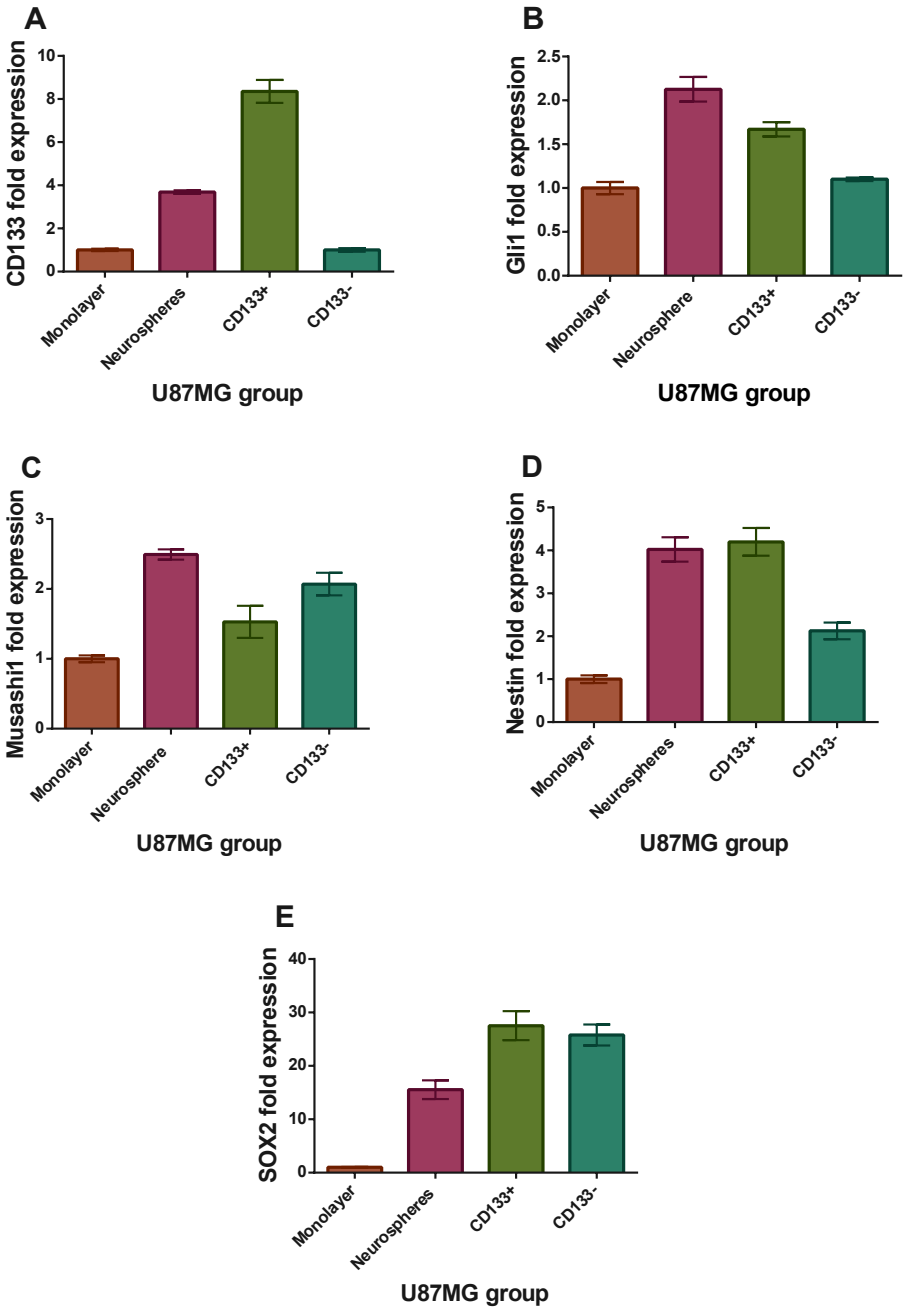


**Figure 5:** qPCR results show 8 fold expression in the CD133+ group (A) when compared with the CD133- and monolayer groups. Neurosphere cells present a high level of CD133 RNA expression. The nestin RNA expression (D) follows the Prominin-1 pattern, while Musashi (C) and GLI1 (B) have more expression in the neurosphere group. SOX2 expression (E) is higher in both of the sorted groups (CD133- and CD133+). Bars represent mean ± SEM.

unexpected results by focusing on the characteristics of the experiment, as it is not a quantitative one, and therefore, the results greatly depend on the researcher's ability to distinguish or interpret the cellular migration differences between groups.

The colony formation assay under monolayer conditions, as well as the scratching assay, showed that the groups which should have formed more colonies (CD133+ and neurosphere groups) [30] were the ones in which fewer colonies were formed. This could be because the ability of the cell lines to form colonies in the SFM groups (CD133+, CD133- and neurosphere) was abolished when using SCM and a monolayer culture was established for the assay. We definitely needed cells growing attached for obtaining colony formation and for the scratching assays. Therefore, serum in the cell media was necessary at this stage of the experiment. This might have created a type of environment that made it impossible for the CD133+ and neurosphere groups to display all their possibilities in terms of colony formation and cell migration.

RT-qPCR gene expression results were closer to the expected ones. When compared to the monolayer group, the CD133+ and the

neurosphere groups had higher levels of expression of Nestin, Musashi1, CD133 and SOX2 genes [13,14]. On the other hand, the CD133- and the monolayer groups presented almost the same expression levels of Nestin and CD133, as expected [13,14], but higher levels of the stem gene markers SOX2 and Musashi1. These results may be explained by three facts: first, CD133- cells may have changed to CD133+ during the passages of the culture after sorting; second, the existence of partially differentiated CSC in the CD133- group, which do not express CD133 but remain pluripotent and express stem gene markers [31]; and finally, it is also plausible that the sorting of CD133+ cells was not specific enough, leaving some of them in the negative fraction. The RT-qPCR results of the expression of GLI1 showed ambiguous differences among the groups. The neurosphere and the CD133+ groups had the higher levels of expression, 2 fold and 1.6 fold, when compared to the monolayer group. But the differences are not as evident as in the other RT-qPCR experiments.

With regard to the flow cytometry experiment, the soft agar colony formation and especially the RT-qPCR assays make us doubt the validity of the protocol set for the FACS technique. One question that might be taken into account is whether it is possible that a CD133- cell fraction can grow in SFM when previous studies have shown that CD133- cells have reduced ability to form neurospheres [2,25,30]. The sorted CD133- cells used in this analysis could grow in SFM, which lead us to the conclusion that, maybe, a CD133- cell does not necessarily have to be excluded from being a CSC. In the same way, a CD133+ group of cells does not necessarily mean a group of CSCs. Perhaps a higher probability of finding one CSC in a CD133+ is more convincing than just calling any CD133+ cell a CSC.

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