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Identification of Melanoma Stem Cells in Long-term Cultures and of SOX6 as a Specific Biomarker for these Stem Cells

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Abstract

Background: Melanoma is the most aggressive skin cancer and its resistance to existing therapies in advanced stages is already known. This resistance can be directly related to the presence of cancer stem cells. We developed an experimental model that involved study of isolates of melanoma lineages (TM1 and TM5), cells that had some "stem" characteristics, in order to better characterize them.

Materials and Methods: We used a specific culture medium containing: RPMI, basic fibroblast growth factor, epidermal growth factor, leukemia inhibitory factor and retinoic acid. Three lineages were used: melan-a, TM1 and TM5. Both melanoma lineages remained viable for 8 consecutive months in culture, melan-a lineage did not survive the changes within the microenvironment and selection with greater adaptive capacity was achieved. These cells presented some characteristics of stem cells and relationship with malignancy.

Results: These cells presented certain characteristics, including the formation of adherent or non-adherent spheres, quiescence, survival, expression of stem cells molecules and transdifferentiation in other cellular types. We called these TM1*dif* and TM5*dif* cells "fibroblasto-like, melanocyte-like, and neural-like." We observed that SOX6 is a specific biomarker for these cells as its expression was totally lost during the growth of long-term cultures. *In vivo*, tumors formed by TM5*dif* had higher malignancy with greater growth of the lesion and ulceration than control tumors (TM5ctr).

Conclusion: Our findings show that melanoma lineages, grown with specific media, in long-term culture, present a good protocol for the isolation of a subpopulation with some characteristics and behavior of stem cells. This can contribute to a better understanding of the role these cells play in melanomagenesis.

Keywords: Melanoma, stem cell, SOX6, biomarkers, long-term, culture, malignancy, cancer stem cell, CD133, nestin, c-Kit

Introduction

In the last few years, the role and the relevance of cancer stem cells in the understanding of cancer have been the object of intense investigations. Recent studies have reported the presence of cancer stem cells in several types of cancer, for example, leukemia [1-3], brain [4-6], melanoma [7-16], thyroid cancer [17], and breast cancer [18,19].

Experimental models to characterize cancer stem cells in cancer lineages and fresh tumors using short-term cultures have already been proposed [5,7,20]. However, cancer cells are remarkably unstable and it is difficult to observe relevant changes related to morphology, proliferation and protein expression in short-term cultures. Our work demonstrated that these instabilities decrease and are more readily observed and studied with the use of long-term cultures. The decrease in the instabilities of these cells likely occurs due to the selective mechanisms that favor a subpopulation of cells with greater capacity for survival and adaptation, as suggested in the theory proposed by Vineis and Berwick, Sztiller-Sikorska et al., and Stelling et al. [21-23].

Different studies have identified cancer stem cell subpopulations using different methods and markers. Melanomas have been identified by CD271, a marker for neural crest stem cells, by the chemoresistance mediator ABCB5$^+$ [24] or by Hoechst 33342 efflux and especially by CD44$^+$, CD133$^+$ and CD24$^+$ [25]. These cells comprise about 5-10% of the tumor population, and yet, execute important functions in maintaining tumor mass. These cells seem to have a greater migratory and invasive capacity than the rest of the population and are candidates to be metastatic tumor cells [26].

To isolate multipotent adult stem cells from the dermis of mammalian skin, it is necessary to use neural factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF) and retinoic acid in long-term cultures (over 14 months) to obtain cells with stem cell characteristics [27,28]. To isolate cancer stem cells of different lineages or of fresh tumors, use of the same specific growth factors mentioned above is still required [5,7].

The potential for dedifferentiation and transdifferentiation of stem cells, and most likely of cancer stem cells, can be explained by the kinetic model proposed by Lemoni et al. [22], where progenitor cells are reprogrammed by environmental stimuli. In fact, the ability to reprogram progenitor cells or already differentiated cells is very relevant to a better understanding of tumor growth and development. Takahashi and Yamanaka developed induced pluripotent stem cells (iPS cells) by transfections of factors such Oct-3/4, SOX2, KLF4, and c-Myc in fibroblasts [29], and showed that iPS cells can be generated from adult cells.

We suggest that cancer cells adopt their reprogramming capabilities over time as proteins that were silenced in the embryonic phase are expressed again; consequently, cancer cells adopt some characteristics of undifferentiated cells. We developed this work based on these theories.

The SOX family is comprised of approximately 20 transcriptional factors. These proteins regulate transcription in numerous ways by interacting directly or through coactivators to mediate gene activation and repression. It is known that the SOX family of transcriptional factors is involved in the development and normal physiology of numerous tissues. The SOX protein appears to coordinate maintenance of stem cell properties and the cell lineage's restriction and terminal differentiation. SOX is implicated in melanocytic development [30,31]. Recent studies showed that SOX5, SOX9, SOX10 and SOX18 are transcriptional factors that affect key melanocytic genes in both regulatory and modulatory functions [30]. However, little is known about the participation of the SOX6 gene in this process.

SOX6 is specifically expressed during the development of the central nervous system, in glioma tissue, in the fetal brain, and in the normal adult brain, as well as in cancer cells [32,33]. SOX6 is expressed in neural stem cells and in gliomas but is not expressed in mature neurons. No significant relationship was observed between SOX6 expression and the grade of malignancy in gliomas. This finding indicates that SOX6 not only promotes proliferation and aberrant expression but also participates in the early stages of oncogenesis in gliomas [32,33].

Research on melanoma has focused on assessing the potential of SOX9 and SOX10 involvement in melanomagenesis; the decreased expression of these genes is correlated with more invasive melanomas [29]. However, the participation of the SOX6 gene in melanoma development and melanoma stem cells, after long-term culture and under special conditions, is not known.

The objective of this work was cultivate melanoma cells over 8 consecutive months; TM1 (less aggressive) and TM5 (more aggressive) lineages were grown on a culture-specific medium containing RPMI, penicillin, streptomycin, factors such as bFGF, EGF, LIF and retinoic acid, without bovine serum. Our hypothesis is that melanoma cultures with an external stimulus can stimulate self-selection of cells with higher adaptive capacities, and that these self-selected cells could be melanoma stem cells.

After 8 months of culture growth, both melanoma lineages presented expression of stem cell markers: c-Kit, Sca-1 and CD133. Stem cell behaviors such as quiescence, formation of adherent and non-adherent spheres and differential plasticity to develop into other cellular types (fibroblast-like, melanocyte-like and neural-like) was observed. We show that melanoma cells after growth in a long-term culture also express transcription factors such as Oct4 and SOX2, inferring a possible cellular reprogramming. We called these subpopulations TM1*dif* and TM5*dif*, respectively.

SOX6 is expressed in melanocytes and in melanomas, but in long-term culture, this transcriptional factor loses its expression. The loss of expression of SOX6 in long-term culture is associated with the presence of melanoma stem cells and poor prognosis and can be used as a specific biomarker for these cells. We identified the subpopulation of the melanoma stem cells (approximately 8% of total population) in both lineages as $Lin^{low}CD133^{+}Sca1^{+}c\text{-}Kit^{+}\ SOX6^{-}$.

Materials and Methods

This study was reviewed and approved by the Ethics Committee on Animal Use in Research Experimentation of the Federal University of Sao Paulo under CEP number 1523/08.

Animals

Thirty C57BL/6 male mice, 2 weeks of age, were obtained from the National Institute of Pharmacology (INFAR), Federal University of Sao Paulo. The animals remained in the vivarium of the Department of Biophysics at the Federal University of Sao Paulo, where the *in vivo* experiments were performed. The animals were given normal food and pure water during all experiments.

Cell culture

The melanoma lineages TM1 and TM5, used in this project, were previously established and described by Oba-Shinjo et al. [34], and the melanocyte lineage melan-a was used as a control. These three lineages were cultured in RPMI (pH 6.9, Cultilab, Campinas, Brazil) supplemented with 5% fetal bovine serum (Gibco, Invitrogen, CA), 200 nM 12-o-tetradecanoylphorbol-13-acetate (PMA, Tocris, Ellisville, MO), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco, Invitrogen, CA).

Cell cultures in serum-free medium

To ensure that the modifications present in the three lineages on specific medium was not due to the absence of the common factors found in fetal bovine serum, we cultured the three lineages without any growth factor. The three lineages were cultured in serum-free medium containing RPMI at pH 6.9 with 100 units/ml penicillin and 100 µg/ml streptomycin for 20-30 days.

Long-term cultures using specific medium

Three lineages were cultured in a specific medium containing RPMI at pH 6.9, basic fibroblast growth factor (bFGF) (45 ng/ml, Sigma, USA), epidermal growth factor (EGF) (35 ng/ml, Sigma, USA), leukemia inhibitory factor (LIF) (25 ng/ml R&D System) and retinoic acid (AR) (1 mM, Sigma, USA) [7]. Both melanoma lineages remained viable for 8 consecutive months in culture. melan-a lineage did not survive the changes within the

microenvironment.

MTT assay and Neubauer chamber

This assay was performed to analyze the mitochondrial activity of melanoma lineages. In this assay, we performed analyses of both melanoma lineages in standard short-term culture and in long-term culture under specific medium at two periods: 4 and 8 months. Two lineages were plated in triplicate in 96-well plates at a density of 10^4 cells/well in 210 µL of RPMI medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and incubated at 37°C for 4 hours. Afterwards, 170 µL of 10% SDS with 0.01 M HCl was added, and the cells were incubated again at 37°C overnight to solubilize the formazan crystals. Absorbance was measured at 570 nm in a Biotek Synergy 2 plate reader (Biotek; Winooski, VT). Using the Neubauer camera, the proliferation of both melanoma lineages was also evaluated in standard medium and in short-term culture within a period of 8 days (counting on alternate days), and under specific medium in long-term culture at two moments: 4 and 8 months in periods of 8 days (counting on alternate days). After trypsinization and centrifugation, 25 µL of cell suspension was mixed well with 25 µL of trypan blue in an Eppendorf tube; from this mixture 10 µL were introduced into the counting Neubauer Chamber. The number of live cells was counted. The total number of cells corresponding to the suspension taken into study was calculated.

Cellular cycle assay

The cellular cycle of the three lineages was analyzed by flow cytometry, at 10 days for short-term culture in standard medium (control cells) and at 8 months for long-term culture in specific medium in long-term culture (*dif* cells). Melan-a *dif* were analyzed after 20 days in culture under specific medium. All lineages were counted at a concentration of 5×10^4 cells/Eppendorf. Cells were suspended and washed with phosphate buffered saline (PBS), incubated for 15 min with 10 µL of RNase (4 mg/ml) at 37°C, washed and incubated for 25 min with 5 µL of propidium iodide (PI, 25 µg/ml, BD, USA) and taken to the flow cytometer.

Morphological analyses of long-term culture

Morphological analyses of melanoma lineages and the melanocyte lineage were performed daily over 8 months by optical microscopy using an adapted video camera (Nikon - Eclipse TS100/TS100-F, Japan).

BrdU and C-kit assay

BrdU is a thymidine analogue, and when used, is incorporated into the DNA of cells in the S phase of the cellular cycle. c-Kit is a protein highly expressed in undifferentiated cells. Both biomarkers were analyzed in *difs* cell cultures after 8 months of growth. TM1*dif* and TM5*dif* were cultured with BrdU (10 µM) overnight. After, 10^6 cells were fixed in 2% paraformaldehyde for 25 min and permeabilized with 0.01% saponin for 25 min. These spheres were incubated for 10 min with 2M HCl and suspended in 0.1 M sodium tetraborate (Na2B4O7) at pH 8.5, incubated with the primary antibody anti-BrdU-Alexa Fluor 488 (0.2 µg/10^6 cell) for 30 min, and then incubated with the secondary antibody Alexa 488 for 1h. These cells were also analyzed simultaneously for their expression of the biomarker; these cells were washed and suspended in PBS and analyzed by flow cytometry using mouse anti-c-Kit-APC (dilution 1:300; BD Biosciences, CA). Here, all cells were prepared for and considered in flow cytometry.

qRT-PCR analysis

The expression of anti-apoptotic gene *BCL2* and transcription of the genes *POU5F1* (Oct4) and *SOX2* were assayed by conventional or real-time (quantitative) RT-PCR. β-actin was used as a housekeeping gene. Total RNA was isolated from *difs* melanoma stem cells (MSCs) using TRIzol reagent (Invitrogen) and then treated with DNase (Promega) to decrease the likelihood of DNA contamination. Single-stranded cDNA was synthesized using an M-MLV reverse transcriptase kit (Promega) and oligo(dT) primers (Promega). For non-quantitative RT-PCR, the resultant cDNA was amplified with a PCR Master Mix kit (Promega) for 35 cycles under the following conditions: 5 min of incubation at 94°C, 30 s of denaturation at 94°C, 30 s of annealing at the primer-specific temperature, and 40 s of extension at 72°C, followed by a 7-min final extension step at 72°C. For qRT-PCR, the cDNA samples were amplified using a Step-One-Plus real-time PCR system (Applied Biosystems) and the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. After amplification, the PCR products were analyzed with ABI Prism sequence detection software (Applied Biosystems). In addition, melting-curve analysis was performed to confirm the authenticity of the PCR products. After electrophoresis, the PCR products were visualized using ethidium bromide staining. The image densities of the PCR products were captured using a video-documentation system (VDS; Pharmacia) with LISCAP software. Primer sequences were designed using Primer3 software (version 0.4.0) and are described in Table 1.

Flow cytometry and confocal microscopy

After the chosen periods, the expression of stem cell biomarkers was analyzed by flow cytometry using three sets of antibodies (mouse anti-c-Kit-APC - dilution 1:300; BD Biosciences, CA), (mouse anti-Sca-1-PE - dilution 1:200, BD Biosciences, CA), (primary antibody rat anti-CD133 - dilution 1:500, Molecular Probes, USA). We also analyzed some neural biomarkers: (mouse anti-βIII-tubulin - 1:500, Molecular Probes, USA) for neurons, (mouse anti-nestin - dilution 1:500, Molecular Probes) for mature neurons, and (primary antibody mouse anti-MAP2 - dilution 1:500, Molecular Probes, USA) for neurons. The expression of SOX6 transcription factor decreased over the course of long-term culture; SOX6 was analyzed by cytometry using mouse anti-SOX6 (dilution 1:300, Abcam USA). In this experiment all cells were considered because these cells stopped proliferation of SOX6. All the primary antibodies were conjugated with the Alexa 488 secondary antibody (dilution 1:400; Molecular Probes, USA). These cells were enzymatically detached by trypsinization (0.1% trypsin/EDTA; Gibco) for 5 min at 37°C and fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 (Gibco), and incubated with the conjugated antibodies for 2h; antibodies that were not conjugated were incubated for more 1 hour with secondary antibody. Cell sorting and analysis were performed with a FACSCalibur flow cytometry system (BD Biosciences, Franklin Lakes, USA) and CellQuest software (BD Biosciences, Franklin Lakes, USA). The results were analyzed using the Windows Multiple Document Interface Flow Cytometry Application software package (WinMDI version 2.9; Microsoft, Redmond, WA, USA).

Table : q-PCR analysis - Sequence of PCR sense primers

Gene	GenBank (gi) code	Primers	PCR (pb)	Annealing temperature
SOX2	NM_011443	5' ACCAGCTCGCAGACCTACAT 3' 5' CCCTCCCAATTCCCTTGTAT 3'	388	58°C
SOX6	NM_001024751	5' CATCAAGCGACCAATGAATG 3' 5' CACCTGTTCCTGTGGTGATG 3'	362	56°C
POU5F1	NM_001009178	5' AGAACCGTGTGAGGTGGAAC 3' 5' CCAGAGCAGTGACAGGAACA 3'	345	57°C
ACTB	NM_031144	5'AACCCTAAGGCCAACCGTGAA3' 5'GCGCGTAACCCTCATAGATG 3'	181	59°C
BCL2	NM_016993	5' CACCCCTGGCATCTTCTCCT 3' 5' GTTGACGCTCCCCACACACA 3'	349	60°C

The same biomarkers were used in confocal microscopy as in flow cytometry except β III-Tubulin. Here, we analyzed melanoma cells and melanoma tissue *in vitro*. During the analysis of tissues, we analyzed the biomarker of proliferation Ki-67 (mouse anti-Ki-67-APC - dilution 1:200; BD Biosciences, CA). The cells were cultured on slides into 24-well plates, and when present, spheres were rinsed with PBS for 5 min and treated with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100, and incubated separately with c-Kit, Sca-1, CD-133 and Ki-67 for 2h in the dark. Nonspecific binding was blocked by incubation with 10% bovine serum albumin (BSA). To visualize cellular nuclei, the specimens were counterstained with DAPI (Sigma–Aldrich). The slides were examined using a confocal microscope (Zeiss Axiovert 100M; Carl Zeiss, Oberkochen, Germany). Melanoma tissue (*difs* and ctr) was removed from each animal and immediately embedded in OCT (Dako) to generate 4 µm-thick cryosections. The freshly frozen melanoma sections were incubated in a light-protected humidified chamber at room temperature with the biomarkers c-Kit, CD133, Sca-1 and Ki-67 (in the same dilutions used in flow cytometry) for 2h and then counterstained with the nuclear tracer DAPI (5 mM; Sigma). After, the slides were analyzed with a computer-based digitizing image system (Zeiss Axiovert 100M; Carl Zeiss, Oberkochen, Germany), connected to a LSM 510 confocal laser scanning system.

Nissl technique

We used the Nissl technique to verify if melanoma cells cultured for long periods showed similar morphology to the cells of the nervous system by testing with cresyl violet staining, a specific stain for cells of the nervous system. This method is used for the detection of Nissl bodies in the cytoplasm of neurons in cultures or in paraffin-embedded tissue sections. All cell cultures were fixed in slides with 2% paraformaldehyde for 25 min, washed with distilled water and incubated with 1% cresyl violet for 10 min, then washed with 95% ethyl alcohol for 15 min and analyzed by optical microscopy adapted with a video camera (Nikon - Eclipse TS100/TS100-F, Japan).

Immunohistochemical and histological evaluation of evolution of tumorigenesis

Tumorigenicity was determined by the subcutaneous injection of 105 spheroid cells (only TM5*dif* spheres) in C57Cl/6 mice. The control group was treated with 105 control melanoma cells via subcutaneous injection. Tumor growth was measured during the study by digital pachymetry. Histological analysis was performed after 25 days of follow-up of tumor progression, ending with biopsy of the paraffin-embedded tumor, 4 µm-thick sections (both treated and control) of which were taken. Histological analyses were performed by hematoxylin/eosin staining (H&E). An immunohistochemistry assay was performed on sections of 4 µm-thick of tumor via staining with HMB45 and S100 (mouse, Abcam, USA).

Statistical analysis

All experiments were repeated five times, with similar results. These analyses were performed using the GraphPad Prism 3.03 software package (GraphPad, San Diego, CA). All data were subjected to Student's two-tailed unpaired *t* tests, and Wilcoxon signed-rank tests were used to compare the measurements of the same groups. Significance was set at $p \leq 0.05$.

Results

The three lineages, melan-a, TM1 control (TM1 ctr) and TM5 control (TM5ctr), were cultured in standard medium during short-term culture and presented normal morphology and growth, (Figure 1a, b, c) respectively.

Analyses of the morphology and proliferation of the three lineages cultured in serum-free medium was presented and the cells of the three lineages died after 1 month in culture (Figure 1d, e, f).

Melanoma lineages TM1 and TM5 were cultured in specific medium containing growth factors as described above; some cells of both lineages died after 4 months, however, some cells that survived presented adherent spheres as seen in TM1 in (Figure 1g, h, i) and TM5 (Figure 1j, k, l). We observed an increase of pigmentation in TM5 spheres. We named these spheres TM1*dif* andTM5*dif* according to their lineage of origin.

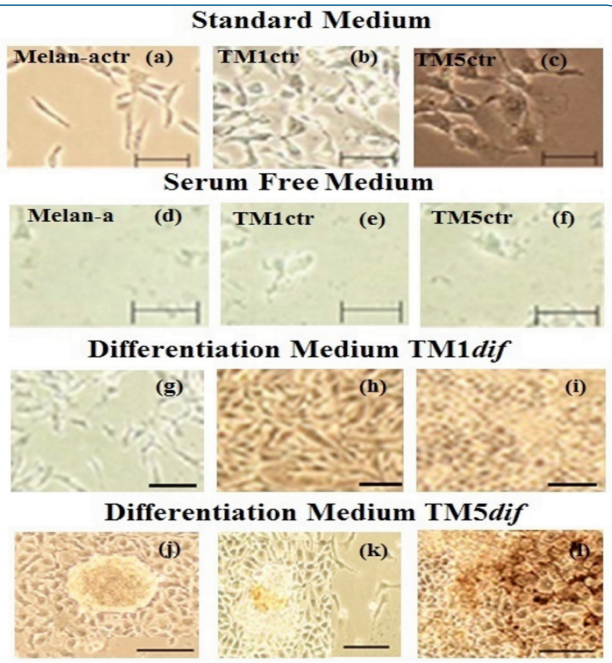

Figure 1: Morphological analysis using optical microscopy of long-term cultures of melan-a, TM1 and TM5 lineages. Control cells cultured in standard medium are shown in (a), (b), (c). When grown for 1 month in serum-free medium, the three lineages show cellular death (d), (e), (f). When grown in differentiation medium during long-term culturing, TM1 and TM5 cells survived, showing a self-selection process and increase of the melanoma spheres, we named these cells TM1*dif* (g), (h), (i) and TM5*dif* (j), (k), (l). TM5*dif* showed increase in pigmentation (l). Scale Bar = 50µm

To analyze the viability and proliferation of the cells that presented capacity for survival, we performed the MTT and Neubauer Chamber counting. TM1ctr and TM5ctr cultured in standard medium for the short-term showed greater viability than TM1*dif* and TM5*dif* cultured in differentiation medium for the short-term (7 days) (Figure 2a). Both TM1*dif* and TM5*dif* demonstrated lower proliferation than TM1ctr and TM5ctr (7.2×10^4 vs 2.38×10^4 and 4.48×10^4 vs 2.1×10^4 cells respectively, $p<0.0001$); all cultures entered this analysis with a starting density of 0.5×10^4 cells (Figure 2b). Melan-a cells showed a complete loss of viability and proliferation cultured in differentiation medium (data not shown). A comparative study of viability of *difs* lineages during months 4-8 did not demonstrate significant difference (Figure 2c), but proliferation of *difs* cells stopped between the period of 4 months - 8 months (Figure 2d). In all these analyses we observed that TM5 (the more aggressive lineage) showed more capacity to adapt to changes in the microenvironment.

To evaluate the cell cycle properties of the TM1*dif* and TM5*dif* and melan-a*dif* cells, we performed a flow cytometry assay with propidium iodide. The standard characteristic curve of the cellular cycle by flow cytometry is presented in Figure 3a.

During the subG0/G1 phase, the melanocyte cells (melan-a) analyzed under standard conditions represented 23% of proliferating cells, but after culturing in differentiation medium, melan-a presented 98% of dead cells ($p<0.0001$). TM1*dif* represented a greater number of the dead cells than TM1ctr ($p<0.05$) and TM5*dif*; TM5ctr did not represent a significant number of cells in this phase (Figure 3b).

In the G0/G1 phase, melan-actr represented a greater number of cells than melan-a*dif* (26% vs 7.8% respectively, $p<0.0001$).

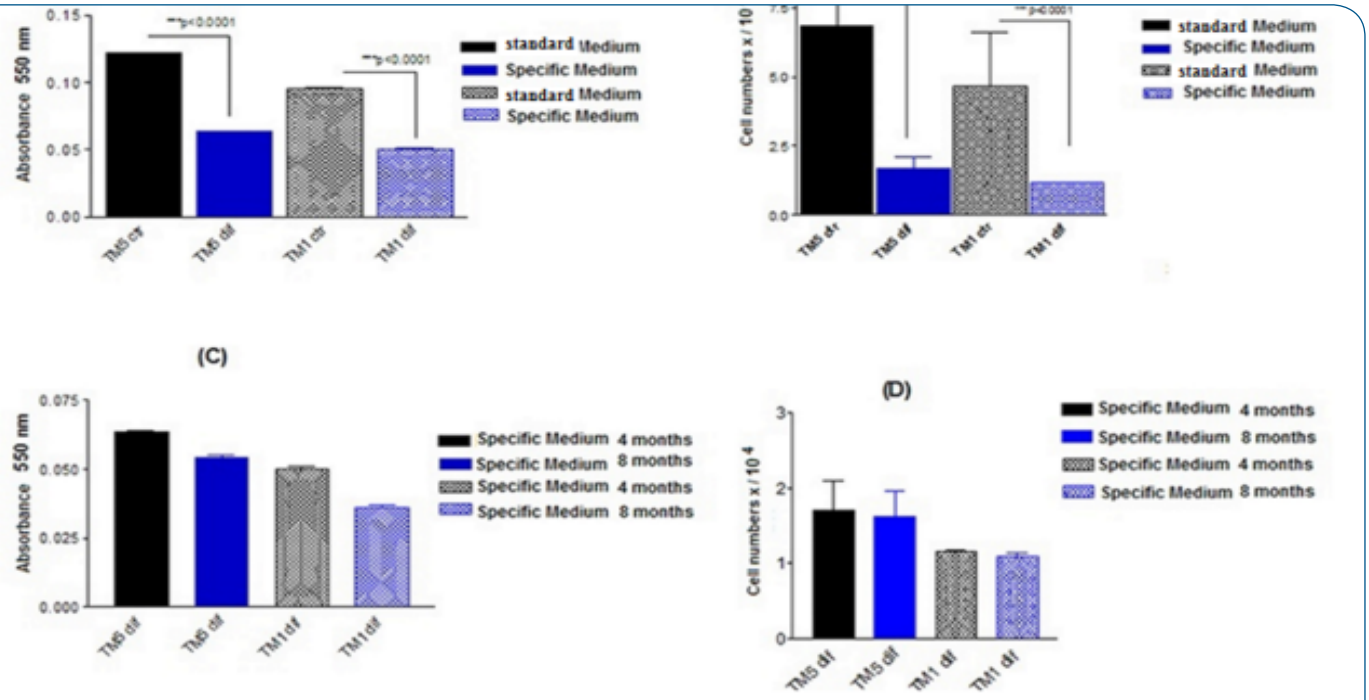

Figure 2: MTT assays and Neubauer Chamber. The long-term proliferation (Neubauer Chamber) and viability (MTT assay) of melanoma cells cultured in standard medium and in differentiation medium were analyzed. We observed that melanoma cells in standard medium presented viability and normal proliferation (TM1ctr and TM5ctr) (a) and (b). However, in short-term exposure to differentiation medium, the difs cultures displayed lowered cell viability and proliferation (a) and (b). The introduction and exposure of TM1 and TM5 into differentiation medium over the long-term (4-8 months) caused decreased proliferation in both lineages, but a subpopulation of cells survived in this microenvironment (TM1dif and TM5dif) (c) and (d)

TM1*dif* represented a higher proportion than TM1ctr (56.6% vs 25.2% respectively, $p<0.0001$,), showing a significant number of quiescent TM1*dif* cells. TM5ctr and TM5*dif* presented (24.3% vs 97.4% respectively, $p<0.0001$) showing a significant number of quiescent TM5*dif* cells (Figure 3c).

In the S/G2/M phase, melan-actr showed normal proliferation and melan-a*dif* did not show proliferation because a significant percentage of this population died (23.6% vs 2.1% respectively, $p<0.0001$). TM1ctr showed greater proliferation than TM1*dif* (48.3% vs 24.9% respectively, $p<0.001$) and TM5ctr showed greater proliferation than TM5*dif* (73.4% vs 13.3% respectively, $p<0,001$) showing that TM1*dif* and TM5*dif* display little proliferation (Figure 3d).

We also analyzed the expression of BrdU by flow cytometry in TM1*dif* spheres and TM5*dif*. Figure 4a shows the flow cytometry of both control lineages. TM5*dif* displayed 33.8% positivity to BrdU, and 8.7% of TM1*dif* cells also presented positivity to BrdU (Figure 4b). With double labeling in both lineages using c-Kit and BrdU, we observed that the percentage of BrdU positive cells decreased to 0.4% BrdU$^+$c-Kit$^+$ in TM1*dif* cells and 7.8% BrdU$^+$c-Kit$^+$ in TM5*dif* cells (Figure 4c). These results show that a small population of the *difs* cells expresses the two markers after long-

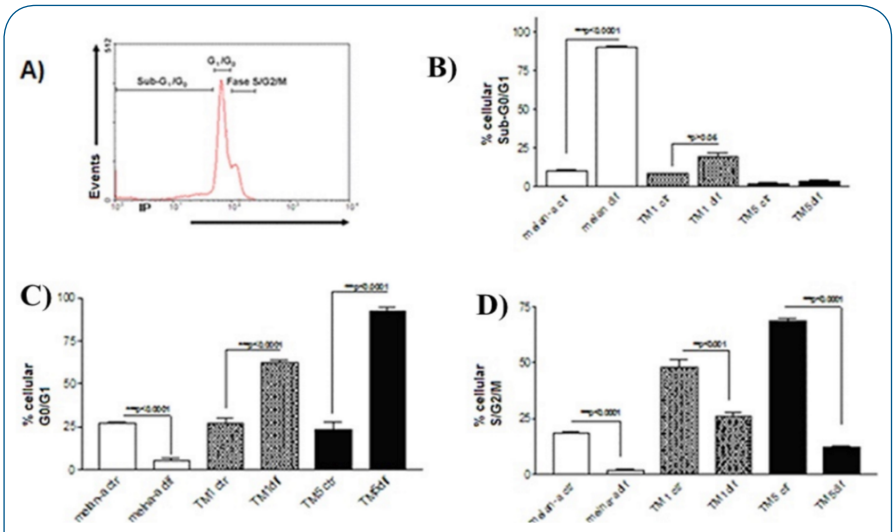

Figure 3: Cellular cycle. (a) Normal curve of the cellular cycle. The cellular cycle of melanoma spheres and melanocyte cells were measured by flow cytometry following propidium iodide staining. More than 80% of the adherent and non-adherent spheres from the TM5*dif* lineage were in G0/G1 of the cellular cycle, and more than 60% of the TM1*dif* lineage were in G0/G1 phase, demonstrating that the cells were in a quiescent state (c). Cellular death was observed in melan-a in differentiation medium and TM1 and TM5 they survived this stage, showing few cells at this stage (b). All lineages under standard medium had populations in S/G2/M (d). The melanocyte lineage demonstrated 79% cell death when exposed to the differentiation medium

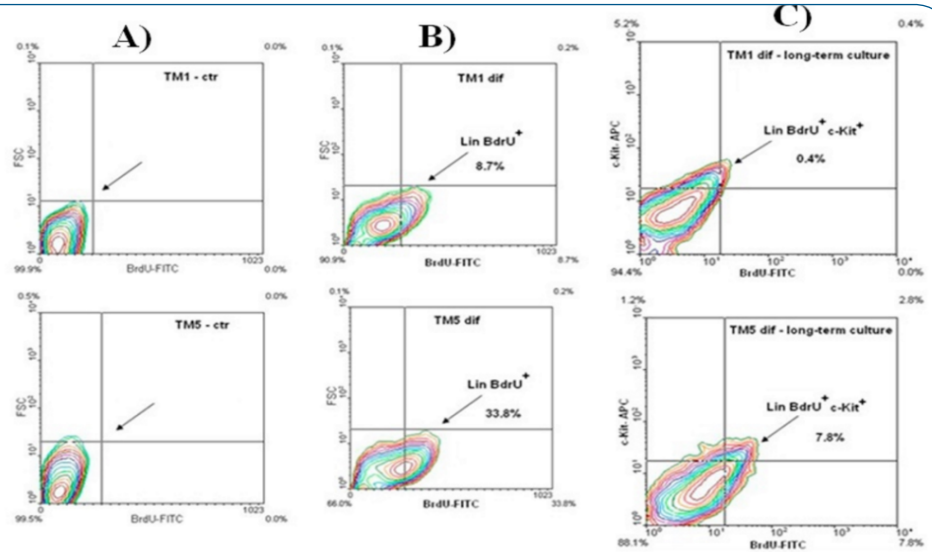

Figure 4: c-Kit and Brd-U assay. Expression in TM1*dif* and TM5*dif* was analyzed by flow cytometry testing for Brd-U and c-Kit. In this experiment, melanoma spheres showed positivity to Brd-U at a rate of 8.7% (TM1*dif*) and 33.8% (TM5*dif*) (b). We performed a double labeling for c-Kit and BrdU on both lineages and we found that only a 7.8% subpopulation of TM5*dif* expressed both c-Kit and BrdU, while in TM1*dif* a subpopulation of only 0.4% expressed both markers (c). This shows that TM5*dif* cells have greater expression of the two biomarkers than TM1*dif*

term culturing, and that only some cells have undifferentiated characteristics.

The expression of stem cell markers in TM1*dif* and TM5*dif* was evaluated by flow cytometry after 4 months and 8 months of culturing. TM1*dif* and TM5*dif* were positive for the c-Kit and Sca-1 markers that are expressed in stem cells (Figure 5a, b), and the expression of these markers increased during long-term culturing. When we simultaneously used c-Kit and Sca-1 to localize and identify a subpopulation of undifferentiated cells, we observed that a small percentage of this population expresses both markers (Figure 5c) while CD133 was expressed only in the TM5*dif* cells (Figure 5d). The TM5*dif* cells express a higher percentage of all the markers than the TM1*dif* cells, thus demonstrating that the presence of a subpopulation of "melanoma tumor stem cells" is directly related to malignancy.

One of the effects of microenvironmental changes *in vitro* is the appearance of cell spheres similar to embryonic bodies (as described in Figure 1j, above). The expression of stem cell markers by qRT-PCR following 8 months of continuous culturing was analyzed. Melanoma spheres overexpress Oct4 and SOX2 (in accordance with the results obtained by [37] (Figure 6a, b). Moreover, melanoma spheres overexpress the anti-apoptotic gene *BCL2*, indicating that this gene may be responsible for the increased survival capability of the spheres in long-term cultures [35,36] (Figure 6c). TM5*dif* shows a significant increase in the expression of stem cell markers than TM1*dif*, which demonstrates the increased number of cancer stem cells in TM5*dif* and their close association with malignancy.

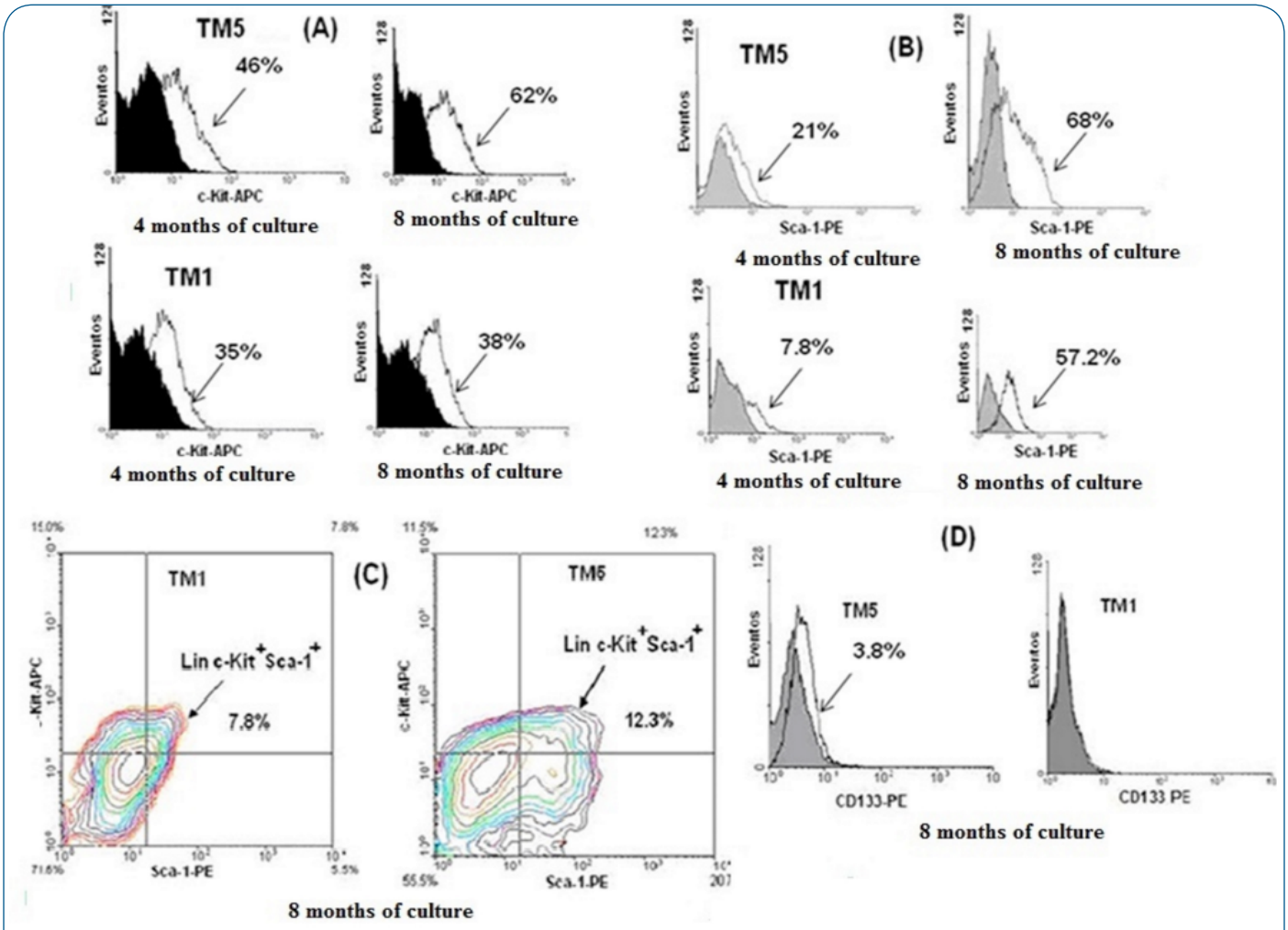

Figure 5: Expression of the stem cell markers by flow cytometry. Some stem cell markers were analyzed in both TM1*dif* and TM5*dif* using anti-c-Kit (a), anti-Sca-1 (b) and anti-CD133 (d). We performed a double labeling against c-Kit and Sca-1 and the percentages of the subpopulation that expressed both markers were 7.8% and 12.3% for TM1*dif* and TM5*dif*, respectively. The histograms showed that only a small subpopulation expressed both markers (c). In fact, the subpopulation with "stem" properties is small in both lineages

Melanocytes do not express SOX6, but recent studies showed that SOX5, SOX9, SOX10 and SOX18 are key melanocytic genes in both regulatory and modulatory phases and that SOX8, SOX9, SOX10, SOX4, SOX11, SOX2 and SOX5 participate in neural crest development, the site of origin of melanocytes [31]. Little is known about the transcriptional factor SOX6 and its roles in this process.

Using qRT-PCR, we found that SOX6 shows low levels of gene expression in melanocytes cultured under standard conditions. In melanocytes before malignant transformation into melanoma cell, the expression of SOX6 increases gradually, particularly in the more aggressive lineage (TM5 – TM5*dif*) (Figure 7a, b). Both TM1*dif* and TM5*dif* after 8 months in culture significantly decreased in expression of SOX6, showing a similar behavior

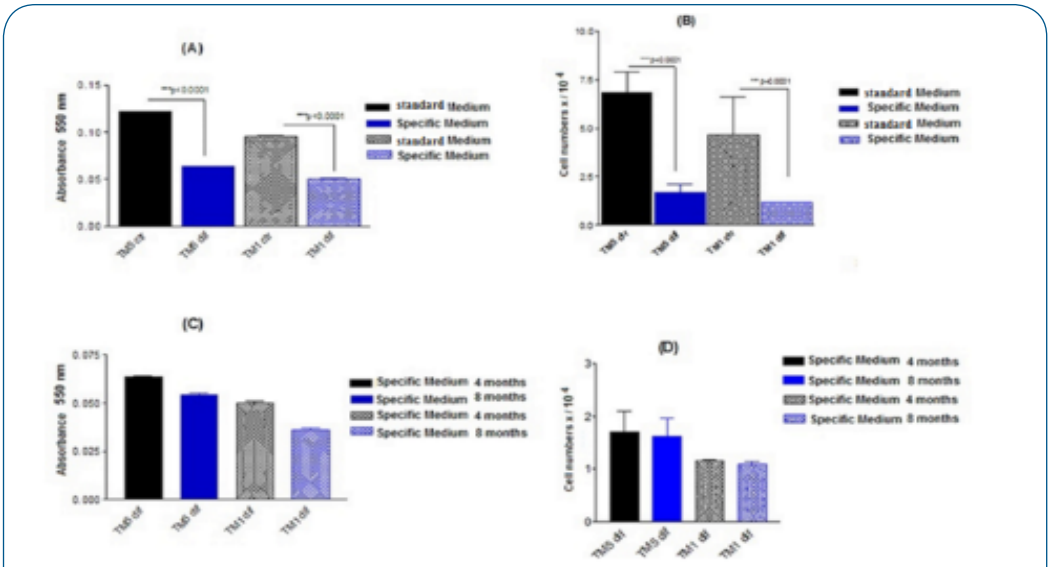

Figure 6: qRT-PCR analysis of markers of embryogenesis phase and anti-apoptotic protein. The formation of embryonic bodies was observed in long-term cultures. Oct4 and SOX2 genes were quantified. The high capacity of survival of these spheres expressing anti-apoptotic protein Bcl-2 was also analyzed by qRT-PCR. It was observed that melanoma spheres express embryonic genes such as POU5F1 (b) and SOX2 (c), and anti-apoptotic proteins such as Bcl-2 (a), unlike control melanoma cells, demonstrating that the melanoma spheres adopted different morphological and genotypic characteristics than their original cells in a reprogramming process due to epigenetic effects

to melanocytic cells (Figure 7c). Flow cytometry confirmed the findings of the SOX6 protein in the lineages studied. Melan-a, TM1*dif* and TM5*dif* cells were analyzed, and analysis showed that the presence of SOX6 decreased gradually and that the TM1*dif* and TM5*dif* lineages did not express SOX6, similar to the melanocytes (Figure 7d).

Stem cells have the capacity to transdifferentiate according to the kinetic model proposed by Rasheed et al. [11]. TM5*dif* cells presented important morphological changes (adherent and non-adherent spheres) (Figure 8a). TM5*dif* cells have presented the potential to transdifferentiate into melanocyte-like, fibroblast-like and neural-like cells (Figure 8a). The identification of the

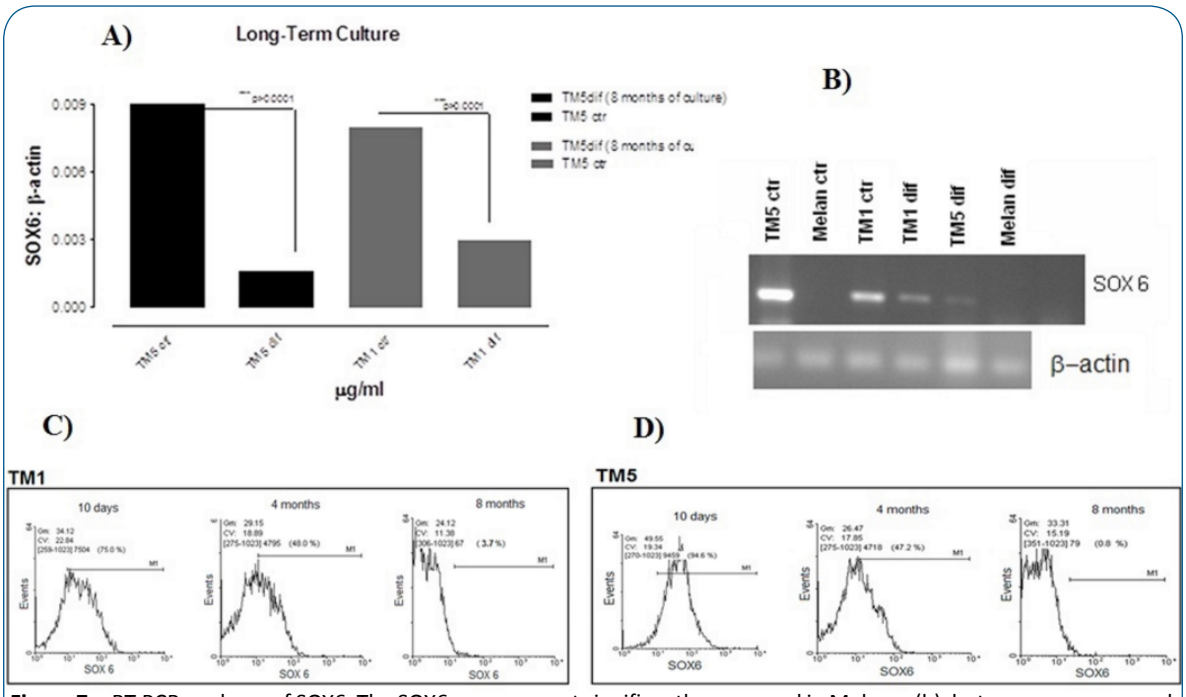

Figure 7: qRT-PCR analyses of SOX6. The SOX6 gene was not significantly expressed in Melan-a (b), but was overexpressed in TM1ctr and TM5ctr lineages under standard conditions (standard medium and short-term culture) (a). These results were confirmed by agarose gel (b). After 8 months of culture in differentiation medium, TM1*dif* decreased expression of SOX6 (c), and similar results occurred in TM5*dif* lineages (d), showing that the long-term culture is important to observe stable changes in both lineages, and that SOX6 can be considered a specific biomarker. N=3 and ± SEM, $p>0.05$. TM5*dif* totally lost expression of SOX6

transdifferentiation process into neural-like cells was reinforced by analysis using the Nissl technique and a stain specific to cells of the central nervous system (Figure 8a). TM5*dif* cells were analyzed by confocal microscopy and flow cytometry for the expression of the neural markers Nestin, CD133, MAP2, and these spheres were positive for all the neural markers (Figure 8b). Analysis by flow cytometry for these neural markers also showed positivity including β III Tubulin, a neural marker (Figure 8c), suggesting that cells that are highly malignant *in vitro* behave similarly to neural stem cells in melanomas.

Subcutaneous injection of TM5*dif* melanoma spheres in mice (C57Bl-6) caused tumors significantly larger than control tumors (Figure 9a, b). In addition, the TM5*dif* tumors presented more pigmentation and ulcerations (Figure 9a, c).

Histological analysis of the subcutaneous tumors after H&E staining demonstrated that morphology of the tumors (control and TM5*dif*) differed and that TM5 tumors possessed a higher number of cells/mm^2 (Figure 9d). The immunohistochemistry conducted on the same tumor tissues demonstrated positive

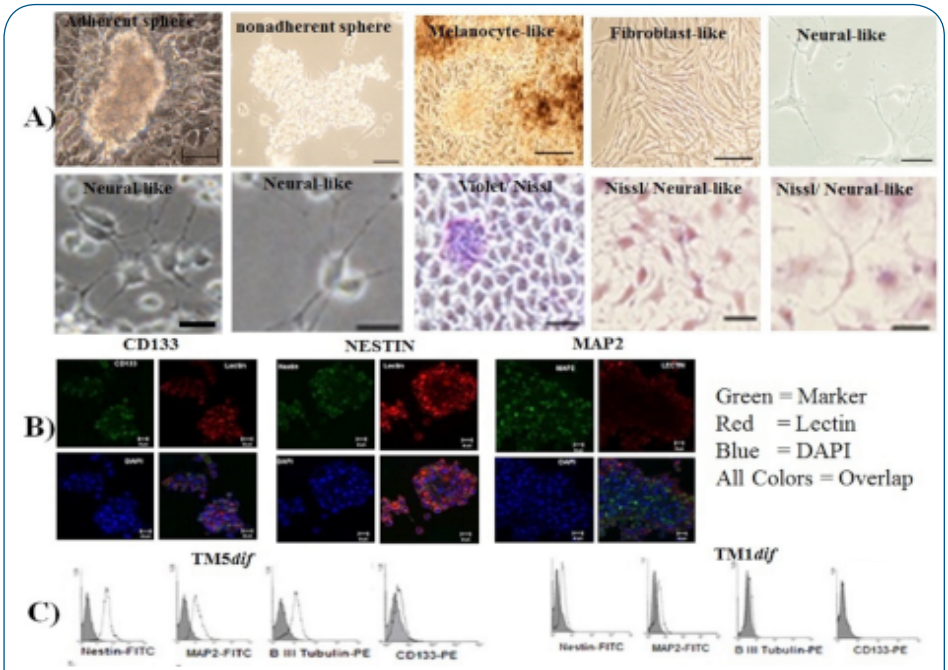

Figure 8: Dedifferentiation or transdifferentiation of TM5*dif* to other cellular types. After 8 months of culture, the cells presented adherents or non-adherent spheres (similar to embryoid bodies), and similar morphology to fibroblast-like, melanocyte-like with increased pigmentation, and neural-like cells (a). Analyses were performed using confocal microscopy and TM5*dif* was positive for neural markers such as CD133, Nestin, MAP2 (b). Flow cytometry also identified positivity to these markers and βIII-tubulin (c). TM1*dif* did not present equal positivity to TM5*dif* (d). Scale Bar = 50µm

staining for S100 and HMB-45, typical markers of melanoma (Figure 9e, f) showing that both control melanomas and spherical melanomas express markers typical of this cancer. Tumor tissues generated following the subcutaneous injection of TM5*dif* demonstrated positive staining for the stem cell markers c-Kit, Sca-1, CD133 and Ki-67 using confocal microscopy (Figure 9g). It was possible to show a vessel involving a tumor (TM5*dif*) with these cells tied into circulation, demonstrating that these cells can be responsible for metastasis in melanoma (Figure 9h). These results strongly suggest that melanoma stem cells should be considered important players in the development and malignancy of melanoma.

Discussion

The therapeutic resistance and malignancy of melanoma is well known. It has also been suggested that these characteristics of melanoma are correlated with the presence of a subpopulation of tumor-initiating cells called cancer stem cells. Recent studies have identified a subpopulation with the same characteristics of stem cells in human cancer and fresh tumors [38,39].

These studies have demonstrated that this subpopulation participates in the formation, growth and maintenance of tumors [40]. However, these studies do not take into consideration the role of long-term culturing in possible morphological and genotypical modifications, because it is difficult to observe stable changes in culture with short-term cultures. Cancer cells display strong biological instabilities *in vitro* in short-term cultures. Therefore, to identify possible properties and new aspects of these types of cells, the study of long-term cultures is important [5,7].

A study revealed that a reciprocal interaction providing a robust self-organizing property in tissues exists between stem cells and committed cells, as the ability of differentiated cells to acquire stem cell properties seems to be inversely proportional and to the same degree that stem cells possess [41].

A model to establish and to characterize a subpopulation of the cancer stem cell in glioblastoma was proposed. The protocol included the use of neural factors and long-term culture (over 2 years), and a subpopulation of the cancer stem cell was identified. These results represent a valuable tool for the study of brain tumorigenesis [5]. A study by Fang et al. [7] utilized

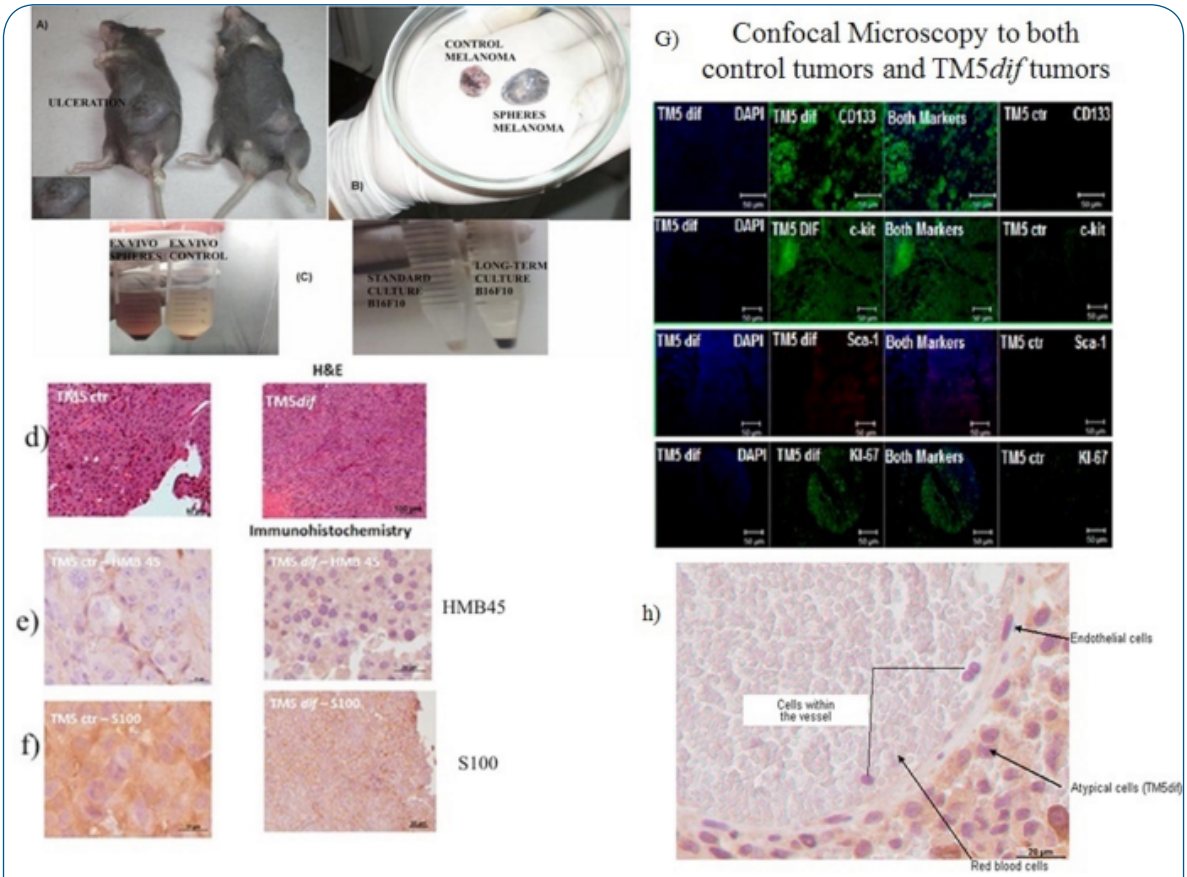

Figure 9: Histology and immunohistochemistry analysis of tumors formed from TM5*dif* (melanoma stem cell) and TM5ctr (control melanoma). The tumor progression of melanoma spheres by subcutaneous injection of B16F10 cells into C57Bl/6 mice was observed. Tumors formed with TM5*dif* showed greater growth, ulceration and pigmentation than control tumors (a), (b) and (c). H&E analysis was performed with sections of tissue of melanoma; we observed that tumors formed from TM5*dif* presented more necrosis, cellular division and vessels than control tumors (d). Immunohistochemistry was performed for HMB45 and S100 (melanoma-specific markers), and we observed positivity for each (e) and (f). Confocal microscopy was performed for markers c-Kit, Sca-1, CD133 and Ki-67. These showed that the TM5*dif* spheres express stem cell markers *in vivo* similar to findings observed *in vitro* (g). Additionally, TM5*dif* cells were observed entering vessels in all tumors formed from TM5*dif* (h)

8-months of culturing (with continuous passage), and succeeded in isolating a subpopulation of 5-10% melanoma cells with stem cell characteristics [7], a similar percentage to our results. Based on other studies, we observed that the isolation of stem cells from the dermis of mammalian skin also requires long-term cultures of over 14 months [27,28]. Thus, these studies corroborate our theory that long-term culture provides a Darwinian selection process where cells with greater adaptive capacity survive, and that changes in expression and morphological characteristics are more constant in long-term culture, as noted in our results [21, 22].

The hypothesis of the present work that was confirmed with our experiments is that under long-term culture, and using differentiation medium containing bFGF, EGF, LIF and retinoic acid, a subpopulation with some stem cell characteristics was cultivated. Moreover, these characteristics tend to be more robust over time, allowing for the identification of patterns correlating with malignancy.

In this study, we observed that after one month in serum-free medium, melanoma cells showed more resistance than melanocyte cells that died after 20 days; however, the melanoma cells also died after 30 days in these conditions (Figure 1d, e, f). Moreover, when grown in a differentiation medium (bFGF, EGF, LIF and retinoic acid), melan-a cells died, and only a small percentage of the melanoma cells of both *dif* lineages survived under these conditions (Figure 1g-l).

Our results suggest that under a selection pressure, persisting cells are those with an increased capacity for survival and quiescence (Figure 2). In fact, after 8 months of culture with continuous passage [7], although many cells died, the cells that survived of both lineages (TM1 and TM5) presented an increased number of cells in the G0/G1 phase. We noted that these cells showed an increase in survival and stopped cellular proliferation – showing quiescence in culture. This quiescence may be viewed as a new robust property of some transformed cells, which becomes increasingly visible if we consider the effects of long-term culture (Figure 3). We named these spheres TM1*dif* and TM5*dif*.

The spheres TM1*dif* and TM5*dif* were observed with BrdU and c-Kit assays, as the simultaneous expression of these two molecules is characteristic of stem cells and cells expressing these molecules usually represent a small percentage of the overall population. TM1*dif* and TM5*dif* cells showed an increased expression of BrdU, mainly in TM5*dif* (Figure 4b), but considering the simultaneous staining with BrdU and c-Kit, the percentage of the

relevant population decreased significantly, demonstrating that only a small subpopulation expresses c-Kit$^+$BrdU$^+$ (Figure 4c).

The expression of the stem cell biomarkers involved in murine embryogenesis, c-Kit, Sca-1 and CD133, was analyzed. The analyses were conducted after 4 and 8 months of culturing, and we observed that the expression of these proteins increased over time, indicating the presence of a subpopulation of cancer stem cells (melanoma stem cells), most likely as a result of the selection pressures. This shows that solid tumors, particularly melanoma, have time as an ally, as the first characteristics of stem cells in the melanoma cell lineages studied were observed after 4 months in culture. We suggest that due to the many mutations that occur over time, which favors the activation of what we call "cellular memory," there is "stem" protein re-expression that is silenced in the embryonic stage via Oct4 and SOX2. The time leading up to increased aggressiveness in melanoma is a great ally, hence the importance of early diagnosis for this disease. Reinforcing these findings, we observed the formation of niches similar to embryoid bodies (Figure 8a). qRT-PCR assays were conducted to verify the expression of the embryonic stem cell markers Oct4 and SOX2 [37].

During long-term culturing, these spheres showed protection against apoptosis. So, the expression of *BCL2* (anti-apoptotic protein) was analyzed; positive results demonstrate that *TM1dif* and TM5*dif* expressed this anti-apoptotic protein, which explains the increased survival of these cells in culture.

We do not know for certain the role played by the SOX6 transcription factor; however, melanocytes do not express this factor. After malignancy and conversion, melanomas strongly express this factor. This factor may be involved with malignancy in melanoma. In long-term cultures, expression of SOX6 is lost (Figure 7); this loss may be considered a specific marker for melanoma cells with "stem" characteristics, because even without expression of this factor, melanoma spheres generate more aggressive tumors than control tumors.

TM1*dif* and TM5*dif* subpopulations can be characterized as *Lin^{low}CD133$^+$c-Kit$^+$Sca$^+$SOX6$^-$*, and the expression of these proteins was more evident in TM5*dif* cells (Figure 5).

Morphological analysis in long-term culture with TM5*dif* cells indicates plasticity to transdifferentiation into fibroblast-like, melanocyte-like and neural-like cells (Figure 8a). After 8 months of culture, we observed stabilization to only the neural-like phenotype. The predominance of the neural-like phenotype was demonstrated by flow cytometry and confocal microscopy using the markers CD133 (neural precursor), Nestin (neuronal marker), MAP2 (glial cells marker) and βIII-tubulin (glial cells markers) (Figure 8b, c) [42,43].

Transdifferentiation into the three cell types mentioned above is intimately related to the differentiation process of the neural crest cells and only the neural-like phenotype survived in long-term culture [42,43]. We suggest that this occurs due to "cellular memory" that is silenced during the embryogenesis process and that after malignization of these cells, activation of this memory occurs, leading to cellular expression of proteins from the embryogenesis period again.

Experiments *in vivo* were performed only with TM5*dif*. TM5*dif* spheres were analyzed according to their tumorigenic potential. Cells from the subpopulation Lin^{low}CD133$^+$c-Kit$^+$Sca-1$^+$ SOX6$^-$ were subcutaneously injected into the treatment group, and the control group was injected with melanoma cells cultivated in standard medium over the short-term. After 25 days, treated tumors and control tumors were extracted and analyzed by histologic and immunohistochemical assays (Figure 9).

TM5*dif* tumors demonstrated greater tumor growth, ulceration and pigmentation than control tumors (Figure 9a). Histological analysis of TM5*dif* tumors revealed cells with irregular chromosomes, larger necrotic regions, and angiogenesis-producing characteristic (Figure 9b). Tumors obtained from the control cells did not display these characteristics.

Confocal microscopy of TM5*dif* tumors has shown that even *in vivo* the tissues display the same expression that *in vitro* TM5*dif* tumors do, namely expression of CD133, c-Kit, Sca1 and Ki-67 (proliferation marker) (Figure 9g). The expression of Ki-67 protein shows that *in vitro* melanoma cells adopt quiescence; however, *in vivo*, the same cells express these same "stem" markers but present an increase in proliferation that gives the cancer great aggressiveness. We believe that this occurs because of the microenvironment *in vivo*, where cellular interactions should favor the proliferation of melanoma stem cells. On the order hand, the control melanoma tumors were not positive for these markers.

Based on these results, we can conclude that the tumors from the TM5*dif* spheres is the same subpopulation that was classified *in vitro* as Lin^{low}c-Kit$^+$Sca-1$^+$CD133$^+$, and that *in vitro* these spheres could be localized specifically using SOX6 as a negative biomarker.

Immunohistochemistry for HMB-45 and S-100 was performed on both TM5*dif* tumors and control tumors; these markers are specific to melanoma (Figure 9e, f).

With respect to *in vivo* findings, we stress the importance of continuing these studies in pre-clinical phase and clinical phase studies, to compare with findings in this animal model developed in this study. This is due to differences between animal and human cells, blood composition, and other factors [44, 45].

In *in vitro* conditions, it is possible to analyze the plasticity of the spheres formed in long-term culture, and to observe their capacity for transdifferentiation and quiescence. These spheres were identified as a subpopulation of melanoma stem cells. The tumorigenic potential of the spheres was demonstrated *in vivo*, through increased tumor growth, ulceration, and expression of proteins specific to stem cells of skin tissue. Our results strongly indicate that the melanoma stem cell subpopulation increases over time, and comprises between 8%-10% of the total population of the tumor. Similar percentages were found for melanoma human cells as shown by Inagaki et al. [5].

Finally, we want to stress the importance of studies about the relevance of cancer stem cells and their relationship with the malignancy; such studies have not been clearly demonstrated previously. Experiments in long-term culture made possible the observation of modifications in both lineages that led to characterization of a subpopulation of the melanoma stem cells, in addition to the discovery of a specific biomarker to these cells. The model we have developed is reproducible, and we strongly

believe it to be a useful tool to answer new questions on the study of tumor development in the context of cancer stem cells in melanomas.

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