Universidade Federal de São Paulo

Escola Paulista de Medicina

Programa de Pós-Graduação em Biologia Estrutural e Funcional

MODELOS DE ESTUDO DE MELANOMA MURINO: REPROGRAMAÇÃO EM ESTADO PLURIPOTENTE INDUZIDO E A IDENTIFICAÇÃO DE CÉLULAS COM FENÓTIPO TRONCO

DIANA APARECIDA DIAS CÂMARA

SÃO PAULO

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Dedicatória

Primeiramente dedico a Deus, o autor da vida.

Dedico esse trabalho aos meus pais, Ismael e Cida. Independe de não entenderem bem o que eu faço, sempre tiveram orgulho e me apoiaram.

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“Deus sábio, nós reconhecemos
que a nossa ciência e a nossa arte
e todo o conhecimento humano
da verdade toda é pequena parte.
Jaz muito além da especulação
insondável a nós, o profundo;
ali guardas o plano da criação
e o pulso de vida do mundo.”

Thomas Troeger

Este trabalho deu origem às seguintes publicações:

1. Advances and challenges on cancer cells reprogramming using induced pluripotent stem cells Technologies. Journal of Cancer. 2016.

2. Murine melanoma cells incomplete reprogramming using non-viral vector. Cell Proliferation. 2017.

3. Identification of very small cancer stem cell expressing hallmarks of pluripotency in B16F10 melanoma cells and their reoccurrence in B16F10 derived clones. Submetido em Journal of Oncology. 2019.

4. Prêmio: SBBq Award de melhor apresentação de poster – Preliminary study of reprogramming murine melanoma cells by transcription factors into embryonic state. 2015.

5. Artigo para o site ScienceTrends da Universidade de Duke sobre o trabalho de reprogramação. 2017. Site: https://sciencetrends.com/scientists-just-reprogrammed-malignant-melanoma-cancer-cells-less-aggressive-cancer-cells/

6. Matérias no Estadão, Band e BandNews sobre o artigo de reprogramação. 2018.

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LISTA DE SIGLAS E ABREVIAÇÕES

ALDH - Aldeído deidrogenase

BSA – "Bovine sérum albumin"

CSC – Inglês cancer stem cell

CD – Grupo de diferenciação. É um Sistema de classificação para anticorpos monoclonais contra moléculas de superfície celular.

CO_2 - Dióxido de carbono

CT – Célula tronco

CTA – Célula tronco adulta

CTE – Célula tronco embrionária

c-Myc - Fator de trasncrição envolvido na progressão tumoral, denominado de protooncogene

DAPI – 4,6 diamidino-2 fenilindole

DNA - Ácido desoxirribonucleico

DMSO – Dimetilsulfoxido

EDTA - Ácido etileno diamino tetraacético

EMT – Inglês Epithelium-mesenchimal transition

ESC – Ingês Embryonic Stem Cell

FITC – Inglês Fluorescein isothiocyanate

INCA – Instituto Nacional de Câncer

iPSC – Inglês Induced Pluripotent Stem Cell

Klf-4 "Krueppel-likefactor 4"

mg – Miligrama

ml – mililitro

M – molar

mES – Inglês murine stem embryonic cell

MEF – Inglês murine embryonic fibroblast

MMRC – Inglês Murine Melanoma Reprogrammed Cells

Nanog- *Nanog Homeobo x*- Aminoácido localizado no núcleo celular e é um fator chave na manutenção da pluripotência.

Oct-4 – Inglês octamer-binding transcription factor 4

OMS – Organização Mundial da Saúde

PBS – Inglês Phosphate Buffer Solution (Tampão de fosfato salino)

PCR – Inglês Polimerase chain reaction

PI - Iodeto de Propideo

RNA– Ácido ribonucleico

rpm – Rotação por minuto

SDS - Sodiododecil sulfato

SFB - Soro Fetal Bovino

SOX2 "SRY (sex determining region Y)-box 2"

TAE - Tris base, acidoacetico e EDTA

µg – micrograma

µl – microlitro

µm – micrômetro

VSCSC – Ingês Very Small Cancer Stem Cell

RESUMO

O melanoma maligno é um tumor heterogêneo, originado a partir de melanócitos que sofreram alterações genéticas e epigenéticas. Muitos estudos indicam a existência de uma pequena subpopulação celular que seria responsável pela manutenção e crescimento do tumor. Tais células são conhecidas como células tronco tumorais (CSCs), pois além de dar origem a células fenotipicamente diferentes, elas ainda expressam marcadores típicos de células troncos convencionais. Além disso, estas células sofrem alterações (transição epitélio-mesênquima) e adquirem a capacidade de migrar para sítios distantes do foco tumoral, ao mesmo tempo em que se tornam resistentes à quimioterapia. Deste modo, a identificação destas células pode ser de grande interesse em oncologia, visto que as terapias poderiam ser direcionadas para estas células. Portanto, este trabalho visa produzir, identificar, isolar e caracterizar populações de células com características de células tronco, com o intuito de avaliar a capacidade de originar populações heterogêneas e suas associações as fatores relacionados à pluripotência.

Palavras-chave: Melanoma; Reprogramação; Heterogeneidade; Células tronco tumorais; Fatores de pluripotência.

ABSTRACT

Malignant melanoma is a heterogeneous tumor, originating from melanocytes that undergo genetic and epigenetic changes. Many studies indicate the existence of a cellular subpopulation that would be responsible for tumor growth and maintenance. Such cells are tumor stem cells (CSCs), because in addition to giving rise to phenotypically different cells, they still express the characteristic stem cells. In addition, the cells undergo changes and may migrate to disorders of tumor focus, at the same time as they become resistant to chemotherapy. This article, the diagnosis the cells can be of great interest in oncology, which whirling therapies have been directed to these cells. Therefore, this work aims to produce, identify, isolate and characterize cell populations with characteristics of stem cells, in order to evaluate the ability to originate heterogeneous populations and their associations factors related to pluripotency.

Key words: Melanoma; Reprogramming; Heterogeneity; Cancer stem cell; Pluripotency factors.

Sumário

1. Introdução

O câncer é visto como um processo micro evolutivo (1,2), no qual células portadoras de alterações genéticas e epigenéticas adquirem capacidade proliferativa gerando uma progênie de diversos fenótipos que podem se infiltrar nos tecidos adjacentes normais ou mesmo colonizar sítios distantes (3).

Para que uma alteração em uma célula gere um câncer, a mudança deve resultar em uma única célula que tenha o potencial de se dividir e que não seja eliminada durante a renovação tecidual normal. Portanto, para que um câncer se desenvolva, é necessário que a célula neoplásica possua mecanismos que possibilitem que estas células escapem do efeito citotóxico apresentado pelo sistema imune.

O tumor apresenta uma constituição celular bastante heterogênea compreendendo células tumorais diferenciadas, células não diferenciadas e células não tumorais, tais como fibroblastos, células endoteliais e células inflamatórias, as quais cooperam no crescimento do tumor (4).

Hanahan e Weinberg sugerem que os vários genótipos das células tumorais derivam de dez alterações essenciais na fisiologia celular que determinam coletivamente crescimento maligno: autossuficiência em sinais de crescimento, insensibilidade a sinais inibidores do crescimento (anticrescimento), evasão da morte celular programada (apoptose), potencial replicativo ilimitado, angiogênese sustentada, invasão de tecido e metástase, desregulação celular energética, instabilidade do genoma e mutação, resistência à resposta imune e promoção de inflamação (5,6).

Apesar dos avanços nas pesquisas e do desenvolvimento de novas estratégias no combate ao câncer, esta doença mantém-se como um desafio à saúde pública nacional e mundial. No Brasil, a estimativa para o biênio 2018-2019 aponta a ocorrência de aproximadamente 640 mil novos casos de câncer (INCA, 2019), desses novos casos, 6.260 são de melanoma. Segundo a Organização Mundial de Sáude (OMS), a incidência de melanoma vem aumentando gradativamente nas últimas quatro décadas, cerca de 200 mil novos casos de melanoma são registrados por ano em todo o mundo.

O melanoma é o mais letal e mais agressivo dentre todos os tumores cutâneos, responsável por 90% dos óbitos por câncer de pele (7). Enquanto os demais cânceres de pele apresentam baixa possibilidade de proporcionarem migração, invasão e metástases, o melanoma apresenta uma frequência muito maior dessas ocorrências. Esse fato é relacionado a diversos fatores, tais como, diagnóstico tardio, características intrínsecas

que favorecem a infiltração dos tecidos e resistência aos tratamentos convencionais (8).

O melanoma se origina de melanócitos que sofreram danos no DNA, causando a ativação de oncogenes (c-MYC, BRAF, KIT, NRAS) e/ou inativação de genes supressores de tumor (p53, p21, p16), que culmina na proliferação celular descontrolada (8,9). Nos estágios finais, o melanoma é extremamente resistente à quimioterapia e frequentemente recidiva após a ressecção cirúrgica, levando a um mau prognóstico (11). Além disso, sabe-se que o melanoma possui uma subpopulação de células que demonstram propriedades comuns de células tronco (12), tais como, autorrenovação, potencial de repopulação clonal e plasticidade pela transição reversível entre estados de células tronco e não-tronco (12,13,14). Em decorrência dessas características essa células presentes em diversos cânceres, incluindo o melanoma são denominadas células tronco tumorais (CSCs) (16).

A heterogeneidade é uma característica dos melanomas, sendo originada em decorrência de alterações genéticas e epigenéticas que por sua vez estão relacionadas com o surgimento das células iniciadoras de tumor ou células tronco tumorais (CSCs). Estas CSCs possuem a capacidade de gerar células filhas idênticas e também células diferenciadas, cuja capacidade proliferativa é variável. Além disso, essas CSCs são comprovadamente resistentes a agentes quimioterápicos (17).

O microambiente apresenta um papel crucial na biologia das CSCs (18). Por exemplo, sob condições não estressantes, a CSC é mantida num estado de repouso que reflete a baixa proliferação (ciclo-fase G0), como as células tronco em seus nichos. No entanto, condições de estresse, tais como hipóxia, baixo pH ou exposição a fármacos, contribuem para a mudança da célula para um estado proliferativo elevado, favorecendo o surgimento de CSC (19).

Não somente as características de células tronco adultas (CTA) são compartilhadas com as células tumorais, mas também as características de células tronco embrionárias (CTE), principalmente em relação a pluripotência. A expressão de fatores de pluripotência na tumorigênese ganha atenção especial (20), uma vez que foi demonstrado que a introdução dos fatores de pluripotência principais (Nanog, Oct4 e Sox2) em células somáticas permite sua reversão em estado embrionário, essas células foram denominadas células tronco pluripotentes induzidas (iPSCs) (20,21). Embora esses fatores de transcrição sejam importantes na iniciação e manutenção em CSCs (23),

seu papel na carcinogênese e metástase, e suas implicações na resistência a drogas e na heterogeneidade de células tumorais ainda são pouco compreendidas.

Portanto, a presença dessas células está relacionada à manutenção, progressão e também ao surgimento de metástases. Assim, o entendimento da biologia das CSCs, é fundamental para a compreensão dos mecanismos que levam ao desenvolvimento do melanoma, da heterogeneidade, da colonização de sítios distantes, e da resistência a quimioterápicos. Assim como a modulação que o microambiente embrionário pode exercer e inibir o desenvolvimento tumoral. Por isso focamos nossos trabalhos no desenvolvimento de modelos que pudessem ser utilizados para estudar essas relações no melanoma.

2. Objetivos

Estabelecer modelos de estudos para o melanoma através da reprogramação em estado pluripotente induzido e da avaliação de marcadores de pluripotência em células com característica tronco.

2.1 Objetivos específicos

1. Reprogramar as células tumorais de melanoma, para células pluripotentes induzidas.
2. Avaliar a expressão de marcadores relacionados a pluripotência em células de melanoma.
3. Avaliar os possíveis mecanismos envolvidos na heterogeneidade em associação à divisão simétrica e assimétrica das células de melanoma.
4. Estabelecer um modelo *in vitro* que possibilite correlacionar a pluripotência em células tumorais com a tumorigênese e as CSCs.

3. ARTIGOS

3.1. Advances and Challenges on Cancer Cells Reprogramming Using Induced Pluripotent Stem Cells Technologies. Câmara DA, Mambelli LI, Porcacchia AS, Kerkis I. J Cancer. 2016 Nov 25;7(15):2296-2303. eCollection 2016. Review.

3.2. Murine melanoma cells incomplete reprogramming using non-viral vector. Câmara DAD, Porcacchia AS, Costa AS, Azevedo RA, Kerkis I.Cell Prolif. 2017 Aug;50(4). doi: 10.1111/cpr.12352. Epub 2017 Jun 15.

3.3. Identification of very small cancer stem cells expressing hallmarks of pluripotency in B16F10 melanoma cells and their reoccurrence in B16F10 derived clones. Diana Aparecida Dias Câmara[1,2*#], Paulo Luiz de Sá Junior[3*#], Ricardo Alexandre de Azevedo[4], Carlos Rogério Figueiredo[5], Rodrigo Pinheiro Araldi[6], Debora Levy[7], Dener Madeiro de Souza[1], Irina Kerkis[1#]. Submetido em Journal of Oncology 2019.

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Review

Advances and Challenges on Cancer Cells Reprogramming Using Induced Pluripotent Stem Cells Technologies

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Abstract

Cancer cells transformation into a normal state or into a cancer cell population which is less tumorigenic than the initial one is a challenge that has been discussed during last decades and it is still far to be solved. Due to the highly heterogeneous nature of cancer cells, such transformation involves many genetic and epigenetic factors which are specific for each type of tumor. Different methods of cancer cells reprogramming have been established and can represent a possibility to obtain less tumorigenic or even normal cells. These methods are quite complex, thus a simple and efficient method of reprogramming is still required. As soon as induced pluripotent stem cells (iPSC) technology, which allowed to reprogram terminally differentiated cells into embryonic stem cells (ESC)-like, was developed, the method strongly attracted the attention of researches, opening new perspectives for stem cell (SC) personalized therapies and offering a powerful *in vitro* model for drug screening. This technology is also used to reprogram cancer cells, thus providing a modern platform to study cancer-related genes and the interaction between these genes and the cell environment before and after reprogramming, in order to elucidate the mechanisms of cancer initiation and progression. The present review summarizes recent advances on cancer cells reprogramming using iPSC technology and shows the progress achieved in such field.

Key words: Reprogramming, Yamanaka`s factors, Cancer cells, Induced pluripotent stem cells.

Introduction

The term pluripotency refers to the ability of a stem cell (SC) to differentiate into all derivatives of the three germ layers: endoderm, mesoderm or ectoderm. Such SC population should express *in vitro* markers of pluripotent cells, form cystic embryoid bodies, which are *in vitro* three-dimensional model of early embryo, and produce teratomas *in vivo*. When reintroduced into early embryos, pluripotent cells are able to contribute partially or completely to the new organism development and to transmit pluripotent cells phenotype to the next generation [1]. Nevertheless, SC potency varies from the pluripotent state such as embryonic stem (ES) cells to incompletely or partially potent state as adult stem cells (ASC). ASC may produce differentiated cells derived from the three germ layers, however may not exhibit all aforementioned characteristics of pluripotent SC [2][3].

A huge effort has been done in many research centers around the world in order to develop, test and implement protocols which could be used in medicine. Such protocols could be therapeutically effective to treat or even cure many human diseases, such as diabetes, Parkinson and others, including

different injuries that are today considered incurable [4][5]. The growing quest for an ideal cell with a high differentiation potential in the target tissue has raised a vast number of questions and several problems that must be faced by scientists, such as immunohistocompatibility between donor and recipient, as well as, bioethics issues.

In 2006, Takahashi and colleagues [6] suggested a manner to solve the issue of bioethics, immunohistocompatibility and pluripotency, using a protocol quite ingenious. The group reprogrammed somatic cells turning them similar to pluripotent ESC-like using retroviral vectors containing genes expressed during early developmental stage. These reprogrammed SC were called induced pluripotent stem cells (iPSC).

Cancer research also acquired a new turn due to iPSC technology. The reprogramming of cancer cells is an interest approach to study cancer-related genes and the interaction between these genes and cell environment before and after reprogramming, in order to elucidate the mechanisms of different stages of cancer development. Additionally, reprogramming cancer cells is one of the ways for discovering novel cancer treatments. Cancer cells may also be reverted into an immature state and, therefore, be able to differentiate into derivatives of the three germ layers. Moreover, using iPSC technology is possible to transform cancer cells into highly immunogenic tumor antigen-presenting dendritic cells, which represent a promising approach for cancer immunotherapy [7][8]. While the reprogramming of normal somatic cells is abundantly highlighted in scientific literature [9][10][11][12][13], reprogramming of cancer cells received less attention [14][15][16]. After a short introduction in reprogramming technology of somatic cells, the present review will be focused on cancer cells reprogramming using iPSC technologies.

iPSC Technology

As soon as pluripotent cells were discovered, many scientists have addressed their studies to better understand the molecular mechanisms of pluripotency. They demonstrated that pluripotent cells express a unique set of transcription factors (TF), which do not serve only as markers, but also are functionally important for the pluripotency maintenance [17]. Takahashi and Yamanaka [6] proposed the use of such TF for the induction of a pluripotent state in somatic cells. More than 20 TF were tested individually or in combination by the group to induce pluripotency in terminally differentiated somatic cells and, finally, the main candidates were selected: octamer 4 (Oct4), SRY box-containing gene 2 (Sox2), Kruppel-like factor 4 (Klf4) and the oncogene c-Myc. These factors together (OSKM) were named as Yamanaka´s factors and they were crucial for the generation of iPSC from mouse embryonic fibroblasts (MEF). Yamanaka´s factors were also introduced into adult tail-tip fibroblasts derived from C57/BL6-129 mice of different ages and they were also able to reverse these cells into a pluripotent state. Later, the same method of reprogramming was successfully tested in human somatic cells [9].

In spite of being an innovative method, iPSC methodology had low efficiency of reprogramming, once the best efficiency reported in scientific literature for human fibroblasts was 0.1%, which turns the method laborious and time consuming [18]. Different groups looked for improving reprogramming technology and many efforts have been done aiming at: (1) increasing the efficiency of reprogramming; (2) obtaining a pluripotent SC population capable of differentiating into the three germ layers-derived cell types *in vitro* and *in vivo* and (3) eliminating the use of viral plasmid transfection. In order to achieve such purposes, different somatic cells, such as: cord blood [19], peripheral blood T e B lymphocytes [20][21], keratinocytes [22], pancreatic β cells [23], amnion-derived cells [24], adipose SC [25], neural SC [26], astrocytes [27], dental pulp SC [28] and others have been reprogrammed. All these cell types have been reported to generate iPSC but also showed variable reprogramming efficiencies and kinetics [19][28]. Besides OSKM, other key TF have been considered for reprogramming, such as Nanog and Lin-28, which also were efficient for iPSC generation [29].

Numerous studies have shown that reprogramming TF can be introduced in cells by using adenovirus or lentivirus. However, the use of retrovirus remains the most used method since higher efficiency levels were obtained. Recently, non-integrating reprogramming methods have been developed to deliver factors into the cells in a safer manner, than using viral methods. The most widely techniques used for generating integration-free human iPSC are: Sendai-virus (SeV), episomal (Epi) and mRNA [30]. Interesting that these three non-integrating techniques were used in parallel with two integrating (retro- and lentiviral vectors) methods to compare their reprogramming efficiencies, which were: mRNA = 2.7%, SeV = 0.077%, Epi = 0.013%, Lenti/Retro = 0.27%. All methods produced good-quality iPSC, however significant differences were registered in aneuploidy rates, reprogramming efficiencies, reliability and time consuming [30].

Cancer and Reprogramming

The idea of cancer cells reprogramming is not new and other methods, such as nuclear reprogramming of somatic cells by the injection of tumor cells – embryonic carcinoma into normal blastocyst [31], by *in vitro* hybridization of cancer cells with ESCs [32] and somatic cell nuclear transfer (SCNT) technique, which implants an enucleated oocyte in a donor nucleus from a cancer cell [33] were already used to suppress the tumorigenic phenotype [34]. Several cancer cell lines have already been reprogrammed using one of the aforementioned methods. It was possible to reprogram embryonic carcinoma cells into an almost normal state by transferring a tumor cell nucleus to an enucleated mouse oocyte, taking an advantage from the embryonic microenvironment. Resulting blastocysts showed the ability to develop, but they had the same tumorigenic potential as the donor cells [35]. Other study used SCNT technology to reprogram melanoma cells. In this study, ESC-like were produced and were able, when re-introduced into recipient early embryo, to complete the normal development and to produce healthy offspring [35]. Recent reports provided additional evidences that the malignant phenotype of cancer cells could be suppressed in embryonic niche, accompanied by alternative expression of miRNA and by epigenetic regulation, such as DNA methylation [36][14]. Taken together, these data confirm the importance of genetic changes in the tumor development and raise the possibility that in certain tumor types, epigenetic changes may play a predominant role. Although epigenetic changes contribute to tumorigenesis, it is still poorly understood how it occurs and also whether it is reversible [37].

The classical view of carcinogenesis mechanisms has considered the tissue de- differentiation during the malignant process [38]. The most modern version is based on the hypothesis that cancer stem cells (CSC) arise from SC of a primitive tissue or from a specific population of progenitor cells that can assume self-renewal and unlimited growth properties [39]. The iPSC technology is based on the reprogramming of somatic cells into ESC-like by ectopic expression of different TF. When this reprogramming occurs, epigenetics markers are also re-established. All of these premises lead to the conclusion that the iPSC technology can be useful for cancer cells reprogramming, which in some cases may lose or achieve a less tumorigenic state. However, these reprogrammed cells vary in reprogramming status at a large extent and, in turn, may accomplish a partial or a complete pluripotent state.

Cancer Cell Lines Reprogramming Using iPSC Technologies

Different cancer cell lines were used in reprogramming experiments [40][41]. One important study employed R545-melanoma cell line, which is trisomic for chromosomes 8 and 11 and conditionally express the oncogene H-Ras [42]. In H-Ras transgenic animals, which also carry deletion of the ink4a/Arf (tumor suppressor locus), *RAS* transgene is activated specifically in melanocytes after the administration of doxycycline, resulting in melanoma formation [42]. R545-melanoma cell line was a smart choice to test reprogramming by TF, once the same group had already showed reprogramming of R545-genome by SCNT [35]. R545-melanoma cells were infected by lentiviral vectors expressing Oct4, Klf4 and c-Myc (OKM). ESC-like were obtained after 14 days of reprogramming and further analysis demonstrated that viral gene expression was silenced following reprogramming. Demethylation of Oct4 and Nanog promoters also occurred and R545-derived iPSC were able to form teratomas, as well as to give rise to chimeras.

Carette and co-workers [43] reprogrammed KBM7 cell line previously obtained from a carrier of a chronic myelogenous leukemia (CML). KBM7 cell line possesses BCR-ABL mutation and shows karyotypic instability in culture [44]. Retrovirus containing OSKM were used to infect KBM7 cells. ESC-like colonies appeared at day 21 after reprogramming and expressed the pluripotent cell markers Oct4, Nanog and Sox2, while the expression of hematopoietic markers CD43 and CD45 was lost. The expression levels of Nanog and Sox2 was similar to human ESC. After reprogramming, the percentage of methylated cytosine-phosphate-guanosine (CpG) sites of Oct4 and Nanog promoter regions was significantly lower than in KBM7 cell line and was comparable to human ESC. Also, KBM7-iPSC still presented abnormal karyotype and maintained the expression of BCR-ABL oncogene. The authors tested weather the removal of one or more TF would be crucial for KBM7 cells reprogramming. Interesting that removal of c-Myc induced cell death, while the exclusion of Oct4, Sox2 or Klf4 from reprogramming mixture decreased iPSC phenotype and colonies formation. These incompletely reprogrammed colonies also maintained the expression of CD43, typical of parental cell line. ESC-like colonies of KBM7-iPSC were able to differentiate *in vitro* into hematopoietic-like cells that were positive for CD34, CD43 and CD45 and into neuronal-like cells. Also, these ESC-like colonies of KBM7-iPSC formed teratomas in NOD-SCID mice. In contrast to original KBM7 cells, which are imatinib-sensitive (drug used to treat different types

of leukemia), reprogrammed cells and their non-hematopoietic derivatives were shown to be completely resistant to this drug [43]. This study raises interesting questions: (i) Does reprogramming method with removal of c-Myc may be used as a tool to combat CML? (ii) Does KBM7-iPSC may be used as an *in vitro* model to study imatinib-sensitivity?

Other authors [45] used eight different gastrointestinal cancer cell (GCC) lines to obtain iPSC-like cells. Several retroviral and lentiviral plasmids containing OSKM were tested and the selected plasmid was introduced into cancer cells by using lipofectamine; iPSC-like colonies were formed at day 31 after reprogramming. Such GCC-derived induced pluripotent cancer cells (GCC-iPCC) expressed endogenous Oct3/4, Sox2, Klf4, c-Myc and oncogenes, such as BCL2 and KRAS, besides the tumor suppressor genes TP53, P16, PTEN, FHIT and RB1. Nanog expression increased significantly after reprogramming and achieved an expression level comparable to the pluripotent teratocarcinoma cell line used as positive control. Similar to KBM7-iPSC, these GCC-iPCC showed epigenetic modifications which occurred in CpG sites in the Nanog promoter, confirming the immature status of this gene in comparison to its status in parental cell line and in *in vitro* differentiated GCC-iPCC derivatives. These reprogrammed cells also showed the capacity to form embryoid-like bodies, which adhered to plastic, producing attached cells named by authors as PostiPC cells. PostiPC cells were capable to differentiate *in vitro* into derivatives of the three germ layers. Tumorigenic properties of PostiPC cells in compassion to parental GCC were tested in NOD/SCID mice. The results revealed the reduction of tumorigenesis in "spontaneously differentiated" PostiPC cells. However, this study did not provide any information about teratoma formation or tumorigenic potential of GCC-iPCC. Further, the authors used fluoropyrimidine 5-fluorouracil (5-FU) in parental GCC and PostiPC cells and demonstrated that PostiPC cells were significantly more sensitive to this drug when compared to the parental cell line, suggesting that PostiPC cells could be more sensitive to therapeutic agents [45].

Osteosarcoma cell lines (Saos-2, MG-63, G-292 and U-2 OS) were also reprogrammed into a pluripotent state using OSKM retroviral transduction method. After reprogramming, these cells showed morphology resembling ESC colonies and expressed alkaline phosphatase, besides pluripotent markers: Oct4, SSEA4, TRA-1-60 and TRA-1-81. Reprogrammed sarcoma cells showed capacity to differentiate into adipocytes and osteocytes. They showed variable responses of reprogramming in respect of efficiency and long term culture effect [46].

Different from aforementioned cell lines, human skin cancer cells were reprogrammed using mir-302 microRNA (miRNA), which is expressed in slow-growing human ESC. The mir-302 – transfected cells expressed markers of ESC and showed a highly demethylated genome. These mirPS cells were able to differentiate into neuron-, chondrocyte-, fibroblast-, and spermatogonia-like primordial cells. The use of intronic mir-302 transfection represents a new and promise tool for the generation of pluripotent stem cells derived from cancerous cells [47].

Table 1 summarizes current knowledge which used iPSC technology to harvest cancer-derived reprogrammed cells. All features mentioned in the table refer to a characteristic necessary to classify a pluripotent cell. Considering the efficiency of reprogramming methods or the combination of TF used during the process, there is no completely reprogrammed population of cancer cells.

Cancer Cell Lines Reprogramming Using iPSC Technologies and Hypoxia

It is of common knowledge that hypoxia helps to maintain undifferentiated state of normal cells and CSC, as well as it also effects proliferation and cell-fate commitment [48][49]. In order to test the effect of hypoxia on cancer cells reprogramming, human lung adenocarcinoma A549 epithelial cell line was infected first by retrovirus expressing hypoxia-inducible factor (HIF) and next by lentivirus expressing Oct4, Sox2, Nanog and Lin28 (OSLN). ESC-like were collected at day 12 after reprogramming and the expression levels of endogenous Nanog and Oct4 in A549 iPS-like cells were lower than in normal human ESC, used as control. The promoter of Oct4 was only partially unmethylated, thus suggesting that these cells were not fully reprogrammed. The tumorigenic capacity of these partially reprogrammed A549 iPSC-like colonies was assessed *in vivo*. The colonies were injected into the femoral muscle of immune compromised mice and the cells rapidly produced highly aggressive tumors [50]. The results obtained by Mathieu and colleagues [50] suggest that hypoxia targets are crucial for maintaining the stemness in malignant cells.

Hypoxia was also used for reprogramming HCT116 colorectal cancer cells and mutant TP53-deficient HCT116 cells, which showed increased iPCC generation efficiency. Additionally, both of these cells showed reduced proliferation, invasive and tumorigenic capacities after reprogramming. Transplantation of iPCC derived from TP53-deficient HCT116 cells into NOD/SCID mice resulted in more rapidly tumor formation, when compared to the same cells without the mutation [51] (Table 1).

Table 1. Studies relating cancer-derived cells reprogrammed by iPSC technology.

CANCER CELL LINES	Karyotype	Reprogramming methods	Viral gene expression	Epigenetic Modification	In vitro Differentiation capacity	Teratomas/ Tumors formation	Chimeras	Drug sensitivity	Authors
Mice Melanoma R545 cell line Ras-induction	Trisomy Chromosomes 8 and 11	Lentiviral OKM[1]	Silenced	Demethylation Oct4 and Nanog Promoters	Unknown	Yes	Yes	No tumors in the absence of DOX[2]	Utikal et al., 2009
Human Leukemia KBM7 CML	Tetraploid, chromossomes 9 and 22 Ph(+)	a. Retrovirus OSKM[3]	Unknown	Partly demethylated Oct4 and Nanog Incomplete	Neuronal- and hemat - like cells	Yes	Not applied	Non-hemat. Derivatives are imatinib resistant – Cell type specific drug sensitivity	Carette et al., 2010
		b. Retrovirus OSK[4] Incomplete Reprogramming	Unknown	Unknown	—				
Human gastrointestinal cancer cells	Abnormal	Retrovirus and Lentivirus + Lipofectamine + OSKM	Silenced	Demethylation of Nanog promoter; Histone modification	Derivatives of three germ line	GCC[5] - Tumor GCC - iPSC Unknown PotsiPC cells - Tumor	Not applied	PotsiPC cells -more sensitive to 5-FU[6] and Differentiation Inducing drugs	Miyoshi et al., 2010
Human gastrointestinal cancer cells	Abnormal	Retrovirus and Lentivirus + Lipofectamine + OSKM	Long term culturing - down regulation of endogenous OCT4, SOX2, KLF4 and c-MYC up regulation and high tumorigenic ability						Nagai et al., 2010
Human Osteosarcoma and liposarcoma	Abnormal	Lentivirus OSKM + Nanog +Lin28	Silenced	Unknown	Ectoderm and endoderm derivatives; Mesoderm with less efficiency	Tumors less aggressive than parental line. No tumor after injection into mice of differentiated Cancer derived iPC[7] cells	Not applied	Unknown	Zhang et al., 2013
Human lung adenocarcinoma A549 epithelial cell line	Abnormal	Lentivirus + OSLN[8] + hypoxia	Unknown	Partly demethylated Oct4	Unknown	Highly aggressive tumors	Not applied	Unknown	Mathieu et al., 2011
HCT116 colorectal cancer cells	Abnormal	Lentivirus + OSLN + hypoxia	Unknown	Unknown	Unknown	Reduced Tumor Formation	Not applied	Unknown	Hoshino et al., 2012
HCT116 colorectal cancer cells + TR53-deficient	Abnormal	Lentivirus + OSLN + hypoxia Increased efficiency	Unknown	Unknown	Unknown	Highly aggressive tumors	Not applied	Unknown	Hoshino et al., 2012
Solid primary human cancer - pancreatic ductal adenocarcinoma (PDAC)	Aberrant karyotype ~20 chromosomal aberrations	Lentiviral (dox)-regulated vector + OSKM	Unknown	Demethylation Oct4 and Nanog Promoters	Unknown	Yes Restricted Mostly endodermal Generates pancreatic intraepithelial neoplasia	Not applied	Unknown	Kim et al., 2013

Abbreviations: [1]Oct4, Klf4 and c-Myc; [2]Doxiciclin; [3]Oct4, Sox2, Klf4 and c-Myc; [4]Oct4, Sox2 and c-Myc; [5]Gastrointestinal cancer cell; [6]5-fluorouracil; [7]induced pluripotent cancer; [8]Oct4, Sox2, Lin-28 and Nanog.

Reprogramming of Solid Primary Human Cancer

According to current knowledge, only one study reported the reprogramming of solid primary human cancer - pancreatic ductal adenocarcinoma (PDAC) - using iPSC technology. Usually, PDAC is detected at advanced stage thus having a poor prognosis to the patient, with less than 5% survival rate. PDAC is initiated by a mutation at KRAS (proto-oncogene that encodes an ~21 kDa small GTPase), which is present in more than 90% of PDAC cases [52]. There are no *in vitro* models in scientific literature which allow study early stages of PDAC. Therefore, reprogramming of PDAC cells may be a tool for studying the progress of the disease. Kim and colleagues [53] obtained biopsies from nine patients and isolated epithelial cancer cells. These cells were infected with doxycycline (dox)-regulated lentiviral vector expressing OSKM, while the margin cells of the biopsies were used as control. Four ESC-like lines from nine tumors were obtained. They harbored the same KRAS G12D mutation observed in the initial tumor epithelial population, besides possesses aberrant karyotype

(~20 chromosomal aberrations). Reprogrammed cancer cells expressed pluripotent cell makers, such as: Oct4 and Nanog (original tumors were negative for such markers). Demethylation was observed in several sites of Nanog and Oct4 promoters of reprogrammed cancer cells, which showed similar demethylation pattern as human ESC. Reprogrammed cancer cells formed embryoid bodies *in vitro* and teratomas *in vivo*. These teratomas generated mostly endodermal structures in contrast to human ESC used as control, which produced mainly neuronal cell lines. Only one reprogrammed PDAC cell line, when injected into immunodeficient mice, generated pancreatic intraepithelial neoplasia (PanIN), precursor of PDAC. The cells isolated from PanIN-like structure secreted proteins that are expressed in human PDAC during cancer progression. The authors used iPSC technology and succeeded to generate partially reprogrammed cells from PDAC and also provided a human cell model of early pancreatic cancer [53] (Table 1).

Reprogramming of Cancer Cells into a Less Tumorigenic State and Derivation of Potentially Malignant Cells from iPSC

Cells reprogramming may have different objectives and one of them consist on the establishment of *in vitro* models to study the mechanisms of cancer cells transformation into a less tumorigenic state, as well as the transformation of normal cells into a malignant state. Both of these models, especially in combination, are of great interest once it may help to understand the mechanisms of malignant transformation and answer the question of how normal cells may become cancer cells. Although Utikal and co-authors [41] already demonstrated the reprogramming of melanoma cells into almost typical iPSC, they did not show if this state is reversible. Also, it was not clear whether normal iPSC in a tumor microenvironment will have a risk of malignant transformation. This is important as the main issue of iPSC transplantation into patients is about safety concerns, once these cells tend to form teratomas. Recently, it has been shown that mouse iPSC cultured in conditioned media by cancer cell lines showed tumorigenic capacity [54][55]. After transplantation into nude mice, all iPSC lines showed formation of malignant tumors and one iPSC line enhanced angiogenesis formation. This work demonstrated that iPSC is an interesting model for the study of malignant cell transformation and also alerts us about possible malignant transformation of normal iPSC and their derivatives.

Li-Fraumeni syndrome (LFS) is a rare autosomal dominant disease, related to mutations at the TP53 gene and characterized by the occurrence of multiple tumors in the same person. Lee and collaborators [56] obtained iPSC from skin fibroblasts of LFS patient with osteosarcoma. Mesenchymal stem cells (MSC) derived from these iPSC upon differentiation were able to differentiate into osteoblasts, which in turn, generated osteosarcoma after their injection into nude mice. This study demonstrated that malignant transformation depends, at least in this case, on cell specification, once iPSC-derived MSC were not able to form any type of tumor. It is still interesting to derive iPSC from primary tumor cells of LFS patients. The generation of such pairs of cell lines obtained from normal and malignant cells of the same patient is a model of interest for studying the mechanisms of tumor transformation, tumor progression and the reversion to normal or malignant state.

Why do We Want to Reprogram Cancer Cells?

The reprogramming of cancer cells have several basic aims: (1) to explore the possibility to normalize *in vivo* the malignant phenotype of such cells, as an alternative to conventional therapeutic protocols; (2) to yield a larger CSC population, which would be available for experimental manipulation and for exploration of their biological properties to better understand resistant tumors and reduce relapses; (3) to produce less tumorigenic iPSC derived from cancer cells that may be differentiated into a specific cell type e.g. dendritic cells or cytotoxic T cells, in order to prepare specific cancer vaccines; (4) to use cancer-derived iPSC for pharmacological screenings [57]; (5) to use reprogrammed cancer cells to create novel therapeutic targets against CSC by combining, for example, small non-coding RNAs with efficient drug delivery systems [58]; and (6) to create a powerful tool for distinguishing epigenetic and genetic alterations that occur during tumor development and progression [40] (Figure 1).

Several factors can enhance the efficiency of iPSC generation, such as cell cycle checkpoints mediated by the cyclin-dependent kinase inhibitor family. The transient inhibition of these proteins may significantly improve iPSC generation [33][59], although the ability of the resultant cells to become non tumorigenic is not completely understood. During reprogramming, cells increase their intolerance to different types of DNA damage. The mechanisms which occur in cancer cells and then undergo an incomplete form of reprogramming, such as the presence of CSC that may increase the heterogeneity of a cancer cell population, remain unclear [58]. It has been proposed that two types of SC coexist in normal and in cancer microenvironment and that these cells populations

are transient and are regulated by epigenetic controls [60][61]. Emerging evidence indicates that quiescent and active SC subpopulations that are in lower metabolic and proliferative states, respectively, may coexist in several tissues [61]. Endogenous expression levels of TF during early embryogenesis and in pluripotent cells could be relevant for tumor cell malignancy and transformation [62].

Conclusions

The investigation of cellular reprogramming and pluripotency encompasses several important decades in the recognition of similarity between normal pluripotent cells and tumor cells. Cellular mechanisms involved in normal pluripotent SC differentiation and in abnormal growth and differentiation of CSC in the neoplasia were also addressed [40][63].

Recent studies demonstrate that Yamanaka`s method [6] can be successfully used for reprogramming cancer cell. Similar to normal cells, cancer cells also showed variable reprogramming efficiencies and kinetics. They respond differentially to each reprogram method when removing or adding TF in the reprogramming vector. It seems that in contrast to normal cells, which after reprogramming and *in vitro* long-term cultivation tend to preserve their pluripotent properties, cancer reprogrammed cells demonstrate down regulation of pluripotent genes and up regulation of the oncogene c-Myc.

These cells also showed epigenetic modifications following reprogramming. Until now, the majority of cancer cells used for reprogramming experiments is from cancer cell lines, which are already highly instable and frequently do not reflect the true scenario of cancer cells populations, when compared to the model of primary cells isolated from tumors. Therefore, the number of studies using primary cancer cells should be increased in order to provide more adequate models for drug screening.

The small number of studies described in this present review already demonstrates the usefulness of this model for cancer studies. Herein, we show that although great advances on reprogramming efficiency of cancer cells have been made, the heterogeneity of such cells remains to be investigated as a novel therapeutic approach. The most striking conclusion is that cancer cells reprogramming results in the ability of the cancer cells to re-engage and terminally execute normal cellular differentiation pathways with consequent reduction of tumorigenic properties [42].

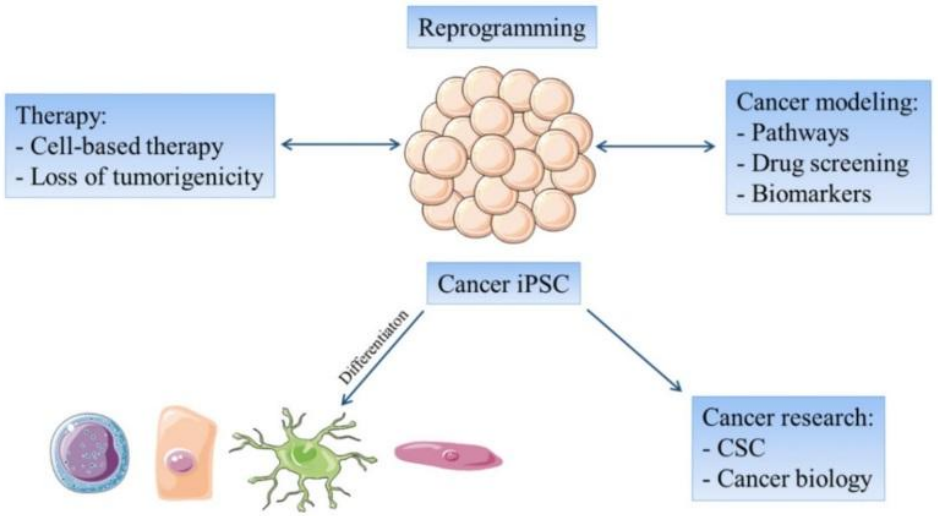

Figure 1. The reprogramming of cancer cells focuses on possible therapeutic use of iPSC-like cancer cells and at cancer remodeling. Cancer- derived iPSC lines can be differentiated into different cell types in order to investigate the features of cancer progression and drug screening or to develop cell-based therapies. On the other hand undifferentiated cancer-derived iPSC lines may be useful for further cancer research.

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Authorship

DC and LM: writing the first draft of manuscript, figure and table production, references organization, and manuscript revision.

AP: participation in the manuscript writing and organization.

IK: research design, writing of the manuscript and critical revision for important intellectual content.

Competing Interest

The authors have declared that no competing interest exists.

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ORIGINAL ARTICLE

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Murine melanoma cells incomplete reprogramming using non-viral vector

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Abstract

Objectives: The reprogramming of cancer cells into induced pluripotent stem cells or less aggressive cancer cells can provide a modern platform to study cancer-related genes and their interactions with cell environment before and after reprogramming. Herein, we aimed to investigate the reprogramming capacity of murine melanoma B16F10 cells.

Materials and methods: The B16F10 was transfected using non-viral circular DNA plasmid containing the genes Sox-2, Oct4, Nanog, Lin28 and green fluorescent protein (GFP). These cells were characterized by immunofluorescence, analysis RT-PCR and cell cycle.

Results: Our results demonstrated for the first time that reprogramming of B16F10 may be induced using non-viral minicircle DNA containing the four reprogramming factors Oct4, Sox2, Lin 28, Nanog (OSLN) and the GFP reporter gene. The resulting clones are composed by epithelioid cells. These cells display characteristics of cancer stem cells, thus expressing pluripotent stem cell markers and dividing asymmetrically and symmetrically. Reprogrammed B16F10 cells did not form teratomas; however, they showed the suppression of tumourigenic abilities characterized by a reduced tumour size, when compared with parental B16F10 cell line. In contrast to parental cell line that showed accumulation of the cells in S phase of cell cycle, the cells of reprogrammed clones are accumulated in G1 phase. Long-term cultivation of reprogrammed B16F10 cells induces regression of their reprogramming.

Conclusions: Our data imply that in result of reprogramming of B16F10 cells less aggressive Murine Melanoma Reprogrammed Cancer Cells may be obtained. These cells represent an interesting model to study mechanism of cells malignancy as well as provide a novel tool for anti-cancer drugs screening.

1 | INTRODUCTION

In recent years, different research groups focused on identification of genetic changes related to carcinogenesis, possible epigenetic mechanisms and chromosomal alterations responsible for cell transformation, tumour initiation and progression.[1,2] Reversion of cancer cells into induced pluripotent stem cells (iPSC) or into a less aggressive cancer cell population is a challenge that has also been discussed during last decades. Due to highly heterogeneous nature of cancer cells, such transformation involves many genetic and epigenetic factors,[3] which are specific for each type of tumour.[4,5] Different methods of cancer cells reprogramming have been established[6,7] and demonstrate a possibility to obtain less aggressive[8] or even normal cells. These methods, however, are quite complex, thus a simpler and efficient method of reprogramming is still required. As soon as iPSC technology, which demonstrated the capacity to reprogram terminally differentiated cells into embryonic stem cells (ESC)-like,[9,10] was developed, it strongly attracted the attention of researches, opening new perspectives for

stem cell personalized therapies and offering a powerful *in vitro* model for drug screening. Currently, it was suggested to be used for cancer cells reprogramming,[11] thus providing a modern platform to study cancer-related genes and the interaction between these genes and cell environment before and after reprogramming, in order to elucidate the mechanisms of cancer occurrence and progression.[7] Using this novel dedifferentiation technique, reprogrammed cancer cells with or without cancer properties can be produced.[12]

Heterogeneity is an intrinsic characteristic of melanoma cells that contribute to the vast phenotypic and genotypic variety of these tumours.[13–16] An interesting way to modulate this phenomenon is the reprogramming of these tumourous cells, followed by check out of what this entails in terms of expression of tumour markers and cancer stem cells (CSC) markers[17–19] as well. Thereby, the tumour cells reprogramming is mostly an interesting strategy to understand which phenomenon leads to heterogeneity.[20]

Commonly retroviral or lentiviral vectors are used to generate iPSC, however such plasmids may integrate into the genome of the host cells.[10,21] This aleatory integration may result in malignant transformations caused by mutagenesis, which can increase the instability in tumoural cells that have already accumulated mutations.[22,23] Moreover, during reprogramming, the cells increase their intolerance to different types of DNA damage that may occur due to different reasons, including viral integration. Therefore, it is of a great importance to test non-viral methods to obtain transgene-free cancer cells-derived iPSC.

Herein, we used non-viral minicircle DNA, which contained the four reprogramming factors Oct4, Sox2, Lin 28, Nanog (OSLN), and the green fluorescent protein (GFP) reporter gene in order to reprogram murine melanoma B16F10 cells, which was previously employed to generate transgene-free iPSC from adult human cells.[24] We also aimed to investigate the reprogramming capacity of these tumour cells in order to establish a model for studying the mechanisms of loss of malignancy through reprogramming of tumour cells into cancer iPSC. This technique is advantageous in translation studies, once it allows verifying the tumoural cell answer after reprogramming in the absence of genomic modification, viral sequences, effectively mitigating safety concerns.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Murine melanoma (B16F10) cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with: 10% foetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (MP Biomedicals, Solon, OH, USA). The cell cultures were maintained in 5% CO_2 at 37°C, in a fully humidified incubator. Primate mES medium combine knockout DMEM, 20% (v/v) ES cell FBS, 0.1 mmol/L non-essential amino acids, and 0.1 mmol/L 2-mercaptoethanol and 10^3 U/mL LIF (ESGRO Merk Millipore, Darmstadt, Germany). The cells were cultivated into feeder-free conditions on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA; diluted 1:100 in DMEM/F12).

2.2 | Reprogramming method

B16F10 cells were cultured under OPTI-MEM medium (Gibco - Life Technologies, Carlsbad, Califórnia, USA) and transfected with non-self-replicating minicircle DNA (Stemcircles™—StemCell Technologies, Vancouver, British Columbia, Canada) containing the four reprogramming factors Oct4, Sox2, Lin 28, Nanog (OSLN), and the GFP reporter gene. Cells were transfected using the reagent Lipofectamine 2000 (Invitrogen). After the transfection, the cells were switched to DMEM/F12 medium (Gibco) supplemented with 20% knockout serum (Gibco) and LIF. GFP+ cells were seen in microscopy 18 hours after transfection. At day 4, the cells were then seeded into feeder-free conditions on Matrigel (BD Biosciences; diluted 1:100 in DMEM/F12) on 6 cm dishes at ~0.5×10^5 cells per well. Culture medium was refreshed every 2-3 days. Colonies with morphologies similar to hESC colonies were clearly visible by 1 week after transfection. At day 12-18 after transfection, GFP-positive cells colonies were individually picked for further expansion and analysis, this expression was transient.

2.3 | Cell cycle analysis

Synchronization of B16F10 and derived cells has been performed through deprivation of serum for 24 hours, which followed by the induction of cell cycle in these cells by serum addition. Next, the cells were harvested by enzymatic digestion and fixed in cold 70% ethanol, and stored at −20°C. For cell cycle analysis, the cells were washed twice in phosphate-buffered saline (PBS) and re-suspended in the same solution following by incubation at 37°C for 45 minutes with 10 mg/mL RNAse. After this, 1 mg/mL propidium iodide (Sigma, St. Louis, MO, USA) was added. Flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Cell DNA content in the different cell cycle phase was determined using ModFit LT software (Verity Software House, Topsham, ME, USA) and Prism 5 (GraphPad Prism Software, CA, USA).

2.4 | Phalloidin staining

The actin cytoskeleton was visualized using fluorescently labelled phalloidin which binds to and stabilizes f-actin.[25] Cells were washed twice with PBS and then fixed using 1 mL of 4% paraformaldehyde (Sigma-Aldrich Chemie GmbH, Munich, Germany). After washing twice with PBS, cells were permeabilized with 1 mL 0.1%-Triton X-100 (Sigma-Aldrich Chemie GmbH) for 10 minutes at room temperature. Besides, again washing twice with PBS and after the cells were incubated with FICT-phalloidin (Sigma-Aldrich) for 1 hour at 37°C. Co-stainings with Hoechst (Invitrogen) were performed as described above. Specimens were embedded in Vectashield and sealed with cover slips.

2.5 | Immunofluorescence

The B16F10 and their derived cells were grown on chamber slides and were fixed in 4% paraformaldehyde for 15-30 minutes at room

TABLE 1 Primers details

Primer name	Sequence (5′ to 3′)	Sense	Fragment size (pb)	Tm (°C)
Oct4	CCTGGGCGTTCTCTTTGGAA	F	123	57.6
	GCTTCCTCCACCCACTTCTC	R		57.7
Nanog	TGGAAGCCACTAGGAAAGC	F	115	57.2
	GCCCAGATGTTGCGTAAGTC	R		56.3
Sox2	TTTGTCCGAGACCGAGAAGC	F	146	57.1
	CTCCGGGAAGCGTGTACTTA	R		56.4
β-actin	GCTCCGGCATGTGCAAAG	F	114	59.8
	CCTTCTGACCCATTCCCACC	R		60.0

temperature for immunofluorescence preparation. Cells were washed with PBS and permeabilized with 0.1% Triton X-100, and after 5% BSA blocked for 40 minutes at room temperature. Slides were then incubated with anti-Oct4 (Abcam, Cambridge, UK) (diluted 1:600), anti-Nanog (Santa Cruz Biotechnology, Dallas, TX, USA) (diluted 1:100) and anti-Sox2 (Abcam) (diluted 1:100) overnight, at 4°C washed in PBS. Appropriate fluorophore labelled secondary antibody was added at a dilution of 1/500 and incubated for 1 hour at room temperature, and after washing in PBS. Cells were mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA, USA) to reveal nuclear DNA. Immunofluorescence was visualized in a Nikon Eclipse Ni (Tokyo, Japan) microscope.

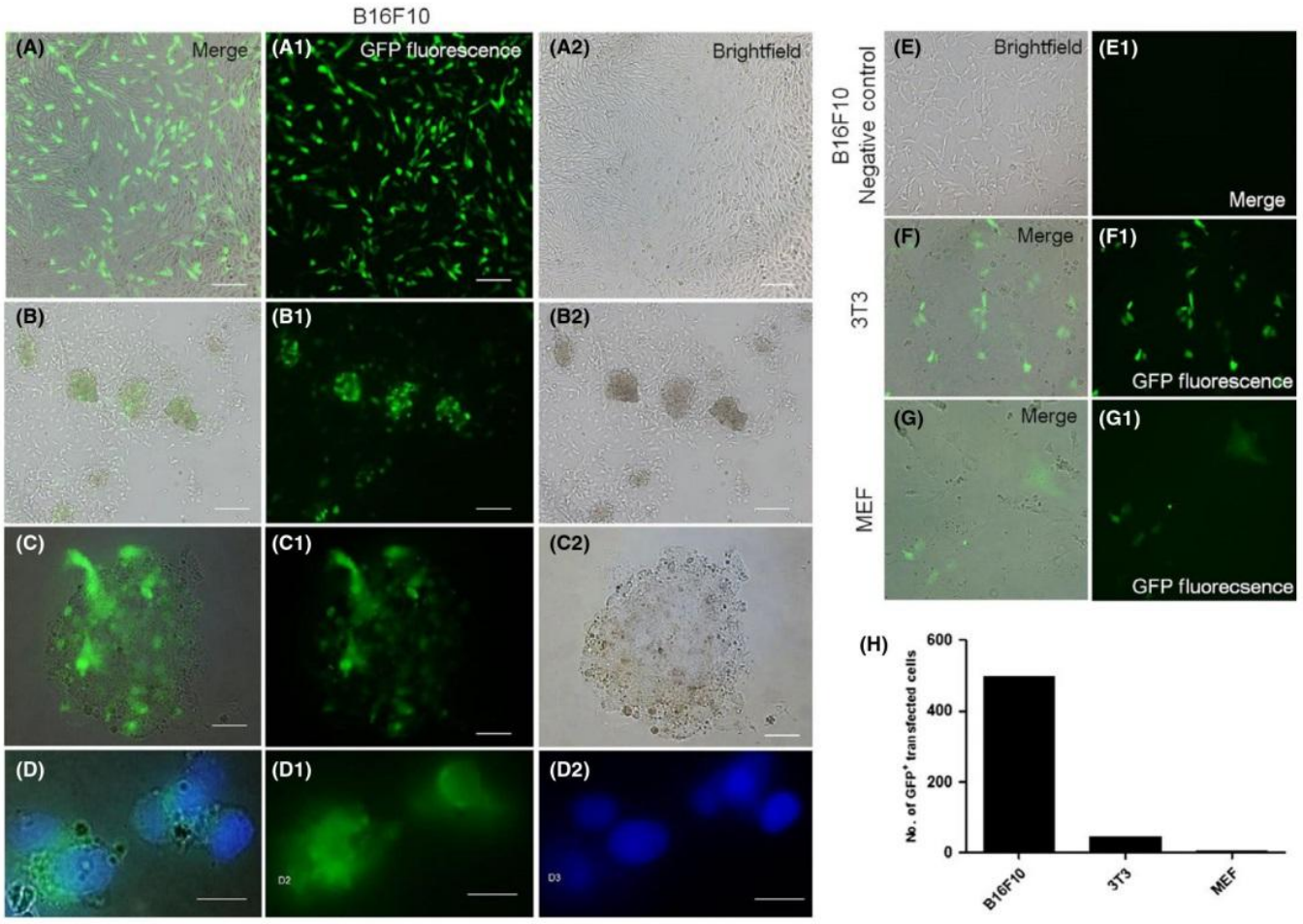

FIGURE 1 Transfection assay. Light microscopy and fluorescent imaging of the B16F10 cell line transfection procedures. A-A2, GFP^+ cells highlighted in green fluorescence 18 h after the transfection. B-B2, It is possible to see GFP^+ colonies formation 72 h after de transfection. C-C2, iPSC-like colony morphology, in which not all cells are GFP^+. D-D2, GFP^+ in the cell nucleus. E-E1, Negative control of B16F10 for GFP^-. Comparison of transfection efficiency between the B16F10 cells and fibroblasts (F and G) 18 h after the transfection, showing the GFP^+ cells. In (H), a comparative bar graph of the number of GFP^+ transfected cells from the B16F10, 3T3 and MEF, calculated by Wimasis Software

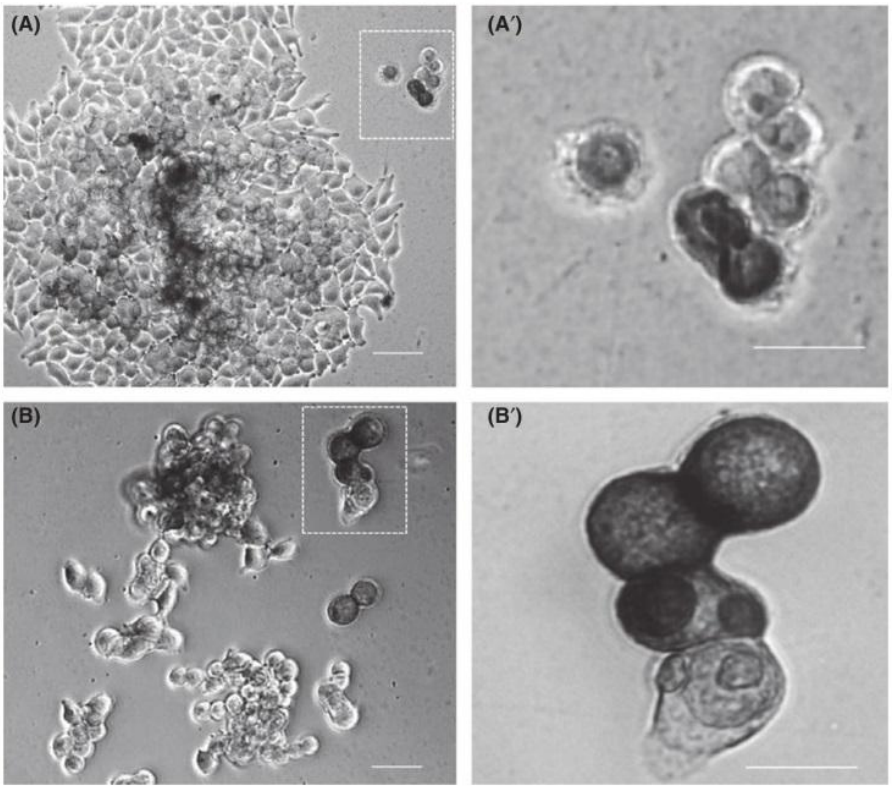

FIGURE 2 Incompletely reprogrammed cells asymmetric division. Light microscopy image of the different colonies morphology during growing on matrigel plate for 1 wk after transfection in (A) and (B). The zoom in (A′) and (B′) showed the asymmetric division between the cells, demonstrating the heterogeneity generated after reprogramming these cells

2.6 | RNA isolation and PCR

Total RNA was extracted from one well of 50-70% confluent six-well plate containing established reprogrammed clones, using the Qiagen RNeasy mini kit following the manufacturer's instructions. Synthesize cDNA with the ImProm-II Reverse Transcription System Kit (Promega, Fitchburg, WI, USA). PCR amplification was performed using GoTaq Green Master Mix (Promega). Primers used in RT-PCR are listed in Table 1. PCR reactions were performed by initially denaturing cDNA at 95°C for 5 minutes followed by 30 cycles of denaturing at 95°C for 30 seconds, annealing at 58-62°C for 1 minute, extension at 72°C for 1 minute, and a final 10 minutes extension at 72°C. PCR products were loaded into 1.2% agarose gels containing 0.6 lg/mL ethidium bromide and run in Tris-acetate-ethylenediaminetetraacetic acid buffer. The Uvitec 2.0 (Cambridge, UK) gel documentation station was used to observe PCR products.

2.7 | Tumour formation and histological analysis

The cells were harvested by triple (Invitrogen) treatment, collected into tubes, and centrifuged, and the pellets were suspended in RPMI, and 5×10^5 cells was injected subcutaneously to dorsal flank of a C57BL/6J mice (Charles River). Twenty days after the injection, tumours were surgically dissected from the mice. Samples were weighed, fixed in PBS containing 10% formaldehyde, and embedded in paraffin. Sections were stained with haematoxylin and eosin. This procedure was approved by Butantan Institute Ethics Committee for Use of Animal Experimentation (CEP 250/06).

3 | RESULTS

3.1 | Transfection assay

We used the minicircles DNA containing GFP in order to induce the pluripotency in B16F10 cells. In the next day after the transfection, multiple cells already showed GFP expression, confirming the presence and expression of minicircles (Figure 1A-A2). At day third, small juxtaposed colonies GFP^+ can be observed (Figure 1B-B2). After 4 days, GFP^+ cells were harvested by trypsinization and plated on Matrigel. These small colonies grew rapidly, achieving iPSC-like morphology (Figure 1C-C2). These reprogrammed cells denominated MMRCs demonstrated GFP^+ expression in the nucleus and cytoplasm (Figure 1D-D2). Accordingly, B16F10 control cells were GFP^-, once they received only lipofectamine without minicircles DNA (Figure 1E,E1). Additionally, murine immortalized fibroblasts (3T3) and mouse embryonic fibroblasts (MEF) were used as controls in transfection assay and both of them demonstrated very limited transfection capacity (Figure 1F,F1, G,G1) compared to B16F10 cells (Figure 1A-A2). Eighteen hours after addition of non-viral vector in B16F10 cells, they present the highest number of GFP^+ cells (~80%) when compared with 3T3 (~10%) and MEF (~1%) that received the same vector (Figure 1H).

After a few passages, the MMRCs green fluorescence gradually disappeared, suggesting minicircles loss.

Approximately, one week after MMRCs were plated on Matrigel, different cells and colonies showing pluripotent-like cells morphologies appeared (Figure 2A). It is of knowledge that stem cells divide asymmetrically, thus producing two daughter cells with different cellular fates: one is a copy of the original stem cell, while second is a daughter programmed to differentiate into a non-stem cell fate.[26] After reprogramming, B16F10 cells demonstrate both asymmetrical and symmetrical division (Figure 2A1,B,B1).

3.2 | Morphology of MMRC clones

Three colonies of different morphologies were selected for further analysis. The morphology of the parental cell line B16F10 and of three MMRC colonies is presented in Figure 3A-D. In order to demonstrate cytoskeleton rearrangement, the cells were additionally stained by phalloidin (Figure 3A1-D1). The Clo1 forms broad colonies (Figure 3B,B1) composed by the cells more similar to parental B16F10 (Figure 3A,A1). The Clo2 and Clo3 form juxtaposed colony (Figure 3C-D1), resembling to colonies of pluripotent cells, such as of ESC and

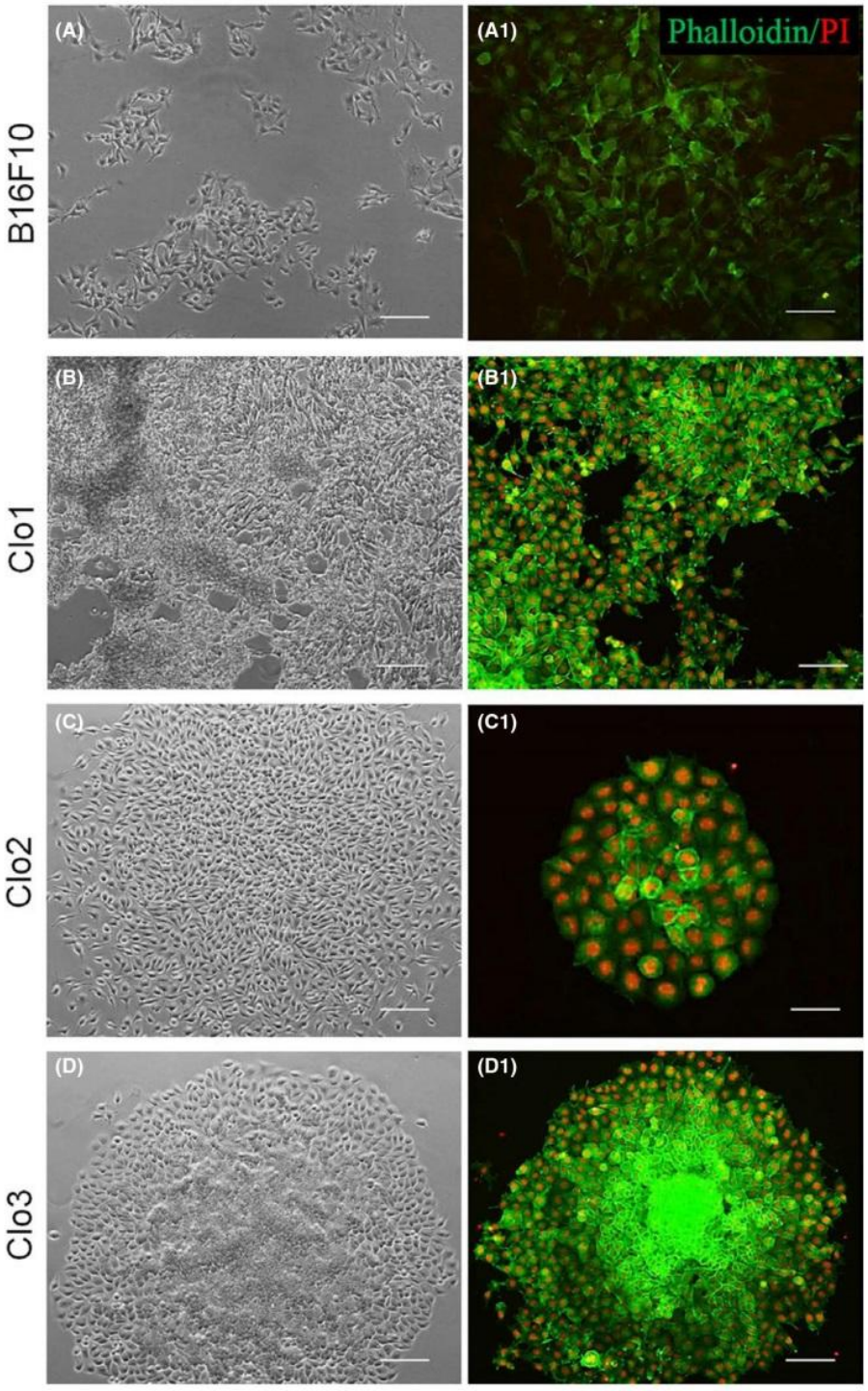

FIGURE 3 Isolated clones morphology. Light microscopy and fluorescent imaging of the clones, with the parental B16F10 cells in (A-D). Phalloidin stain highlights differences in the cytoskeleton morphology (A1-D1). The colonies of Clo1 (B-B1) are composed by separate cells, resembling parental B16F10 (A-A1). Meanwhile, the Clo2 (C-C1) and Clo3 (D-D1) colonies are juxtaposed, being similar to pluripotent cells colonies

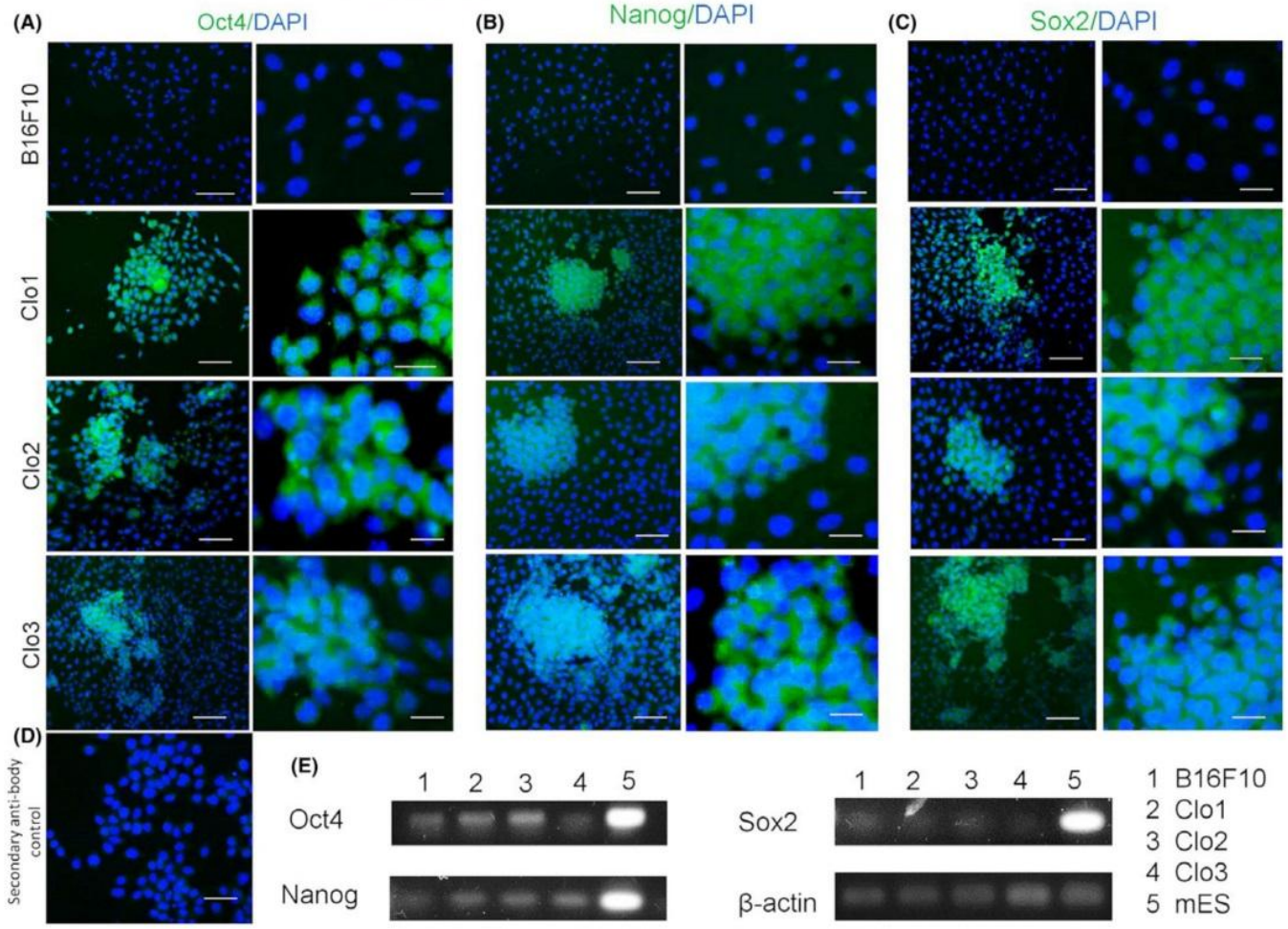

FIGURE 4 Expression of ESC-markers in MMRC clones. ESC markers via immunofluorescence in B16F10 cells and MMRC clones. Reprogrammed cells are positive for ESC markers: Oct4 (A), Nanog (B) and Sox2 (C). (D) Secondary antibody negative control. (E) RT-PCR analysis of ESC-marker genes in B16F10 cell line, MMRC clones and mES positive control. Primers used for Oct3/4, Sox2, Klf4 and c-Myc specifically detect the transcripts of the interest genes

iPSC. Figure 3A1-D1 highlight differences in cytoskeleton organization among parental cells and MMRC clones.

3.3 | Expression pluripotent stem cell markers by MMRC clones

After reprogramming, isolated clones showed expression of the three transcription factors (Figure 4A-C5). However, expression of Oct4, Nanog and Sox2 transcription factors were already observed in a few cells of B16F10 cell line (Figure 4D-D5) before reprogramming. RT-PCR analysis of Oct4, Nanog and Sox2 genes confirm their expression in paternal cell line as well as in all three isolated clones (Figure 4E). Murine ESC was used as a positive control (Figure 4E).

3.4 | *In vivo* pluripotency assay

In order to evaluate in vivo reprogramming of MMRC, the B16F10 cells and the clones were subcutaneously transplanted into dorsal flanks of mice. Twenty days after infection, we observed tumour formation. The tumours were then removed and evaluated in respect of their size and cells composition. In Figure 5, tumours formed by three clones and paternal cell line can be observed. Notable, Clo1 produced tumour of intermediate size and Clo2 and Clo3 generate smaller tumours, while B16F10 cells formed bigger size tumour (Figure 5A). Histological analysis of tumours derived from all clones demonstrated less aggressive tumours formation with reduced tissue necrosis and lower cell heterogeneity, when compared with B16F10 (Figure 5B).

3.5 | Cell cycle of B16F10 and MMRC

Cell cycle of parental cell line and isolated clones was investigated in order to understand *in vivo* suppression of tumourigenic abilities of reprogrammed B16F10 cells. Parental cell line cell cycle demonstrated expressive accumulation of the cells in S phase (Figure 6A,F), while MMRC clones showed a high number of cells in G1 phase and significant reduction in cell number in S phase (Figure 6B-D,F). We also verified whether changes observed in cell cycle occurred in result of reprogramming or

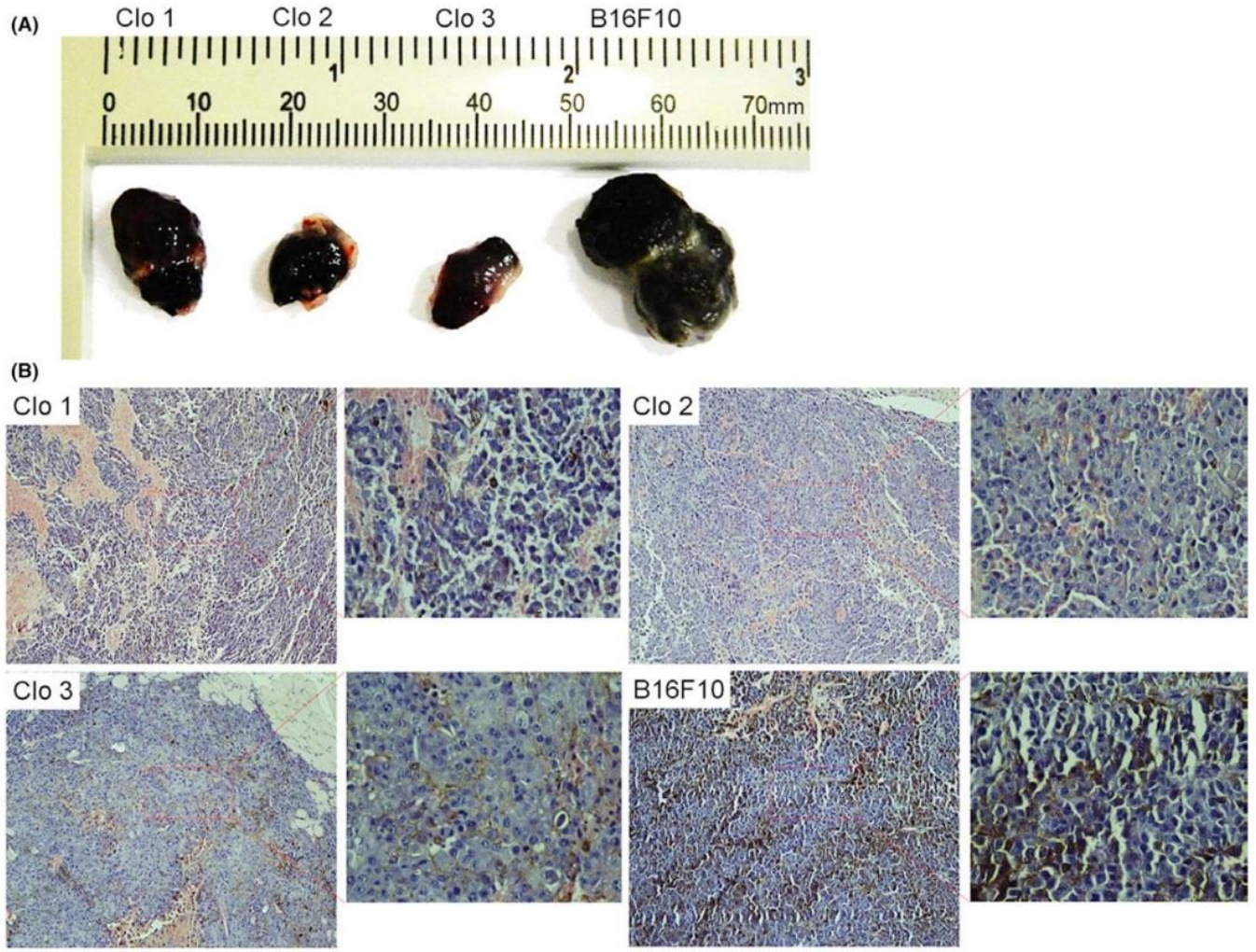

FIGURE 5 Histological tumour-derived analysis. The B16F10 cells and the MMRC were subcutaneously transplanted into dorsal flanks of mice (C57Bl6). After twenty days, the animals were euthanized and the tumours were collected at the same day. In (A) comparison of the size tumours derived from MMRC and B16F10 parental cell line. (B) Haematoxylin and eosin staining of tumour derived from MMRC clones and B16F10 cell line. Compared to B16F10, the MMRC tumour-derived histological analysis suggests lower tumourigenicity, reduced tissue necrosis and decreased cell heterogeneity

clonal selection. Rapidly dividing murine ESC that present great cell number in S phase[27] were used as a control (Figure 6E,F).

4 | DISCUSSION

During reprogramming, cells increase their intolerance to different types of DNA damage,[28] that may occur due to different reasons, including viral integration. Therefore, we supposed that the use of less invasive non-viral vector would help to produce more viable cells, thus increasing reprogramming efficiency. This method was successfully used previously for human adipose tissue stem cells reprogramming and for cancer cells.[29,30] Using non-viral vector, we reprogrammed highly heterogeneous population of melanoma cells into less aggressive Murine Melanoma Reprogrammed Cancer Cells (MMRCC). In fact, eighteen hours after reprogramming, the majority of melanoma cells were alive and 80% of these cells expressed GFP gene reporter. However, overtime the loss of GFP expression was observed, thus indicating loss of minicircles.

Several basic approaches are commonly used to confirm the reprogramming of differentiated cells into less differentiated state, which include: cells morphological changes, expression of pluripotent stem cells markers and teratomas or chimeras formation.[31,32] We showed that MMRCC clones present morphology similar to iPSC and express pluripotent stem cell markers. It is noteworthy that ESC shows a high level of pluripotent markers expression.[33–35] However, this is not a rule for iPSC, because several studies demonstrated that reprogrammed somatic cells, as cancer cells, showed variable and even lower level of pluripotency markers expression, when compared to pluripotent cells. In our study, we also observed that expression of pluripotent stem cell markers in MMRCC was lower than in ESC. Although, endogenous expression levels of pluripotent genes could be relevant to tumour cell malignancy and malignant transformation,[36] it is not clear whether these gene products would be translated into functional proteins.

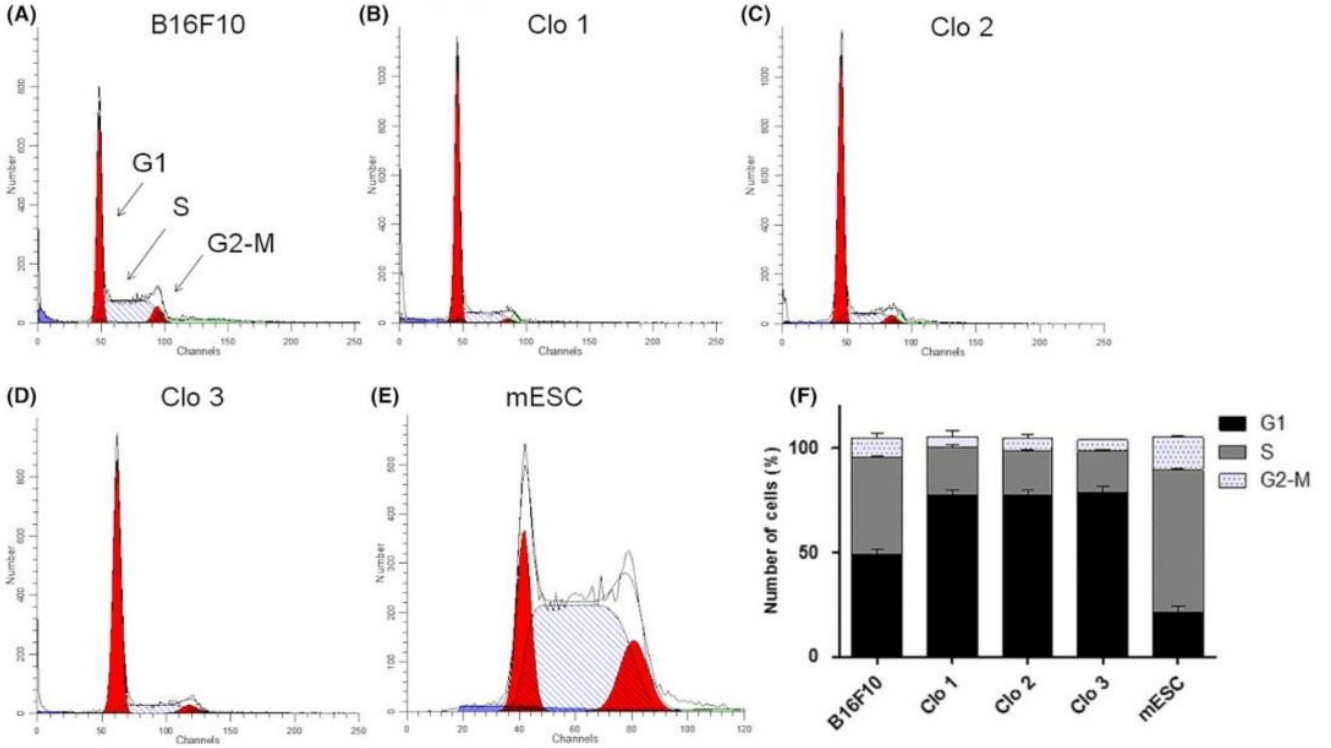

FIGURE 6 Cell cycle analysis. (A-E) Histograms of cell cycle of control cells and MMRC, F representative graph for comparison of the cell cycle phases of MMRC and respective controls. B16F10 parental cell line showed accumulation of cells in S phase (A, F), while MMRC demonstrated a high number of cells in G1 phase, besides the reduction of cell number in S phase (B-D, F). mESC were used as control and shows high number of cells in S phase, due to rapidly cell division (E, F)

However, neither morphology nor expressions of pluripotent stem cell markers are trustable to conclude whether the cells were in fact reprogrammed. Therefore, all three isolated clones, as well as the cells of B16F10 line, were injected subcutaneously into mice dorsal flanks. We did not observe teratomas formation, which is a proof of concept of complete reprogramming. In contrast, MMRCC formed tumours, however, these tumours showed significantly smaller size, when compared with tumours formed by B16F10 cell line. Histological analysis of tumours derived from MMRCC clones demonstrated less necrosis and less tumour cells phenotypic heterogeneity than paternal B16F10 line. Therefore, although the reprogramming was incomplete it leads to less aggressive tumours formation.

In normal cells, cell cycle control is regulated by a complex series of signalling pathways that also include mechanisms that correction DNA damages. In cancer cell, this regulatory process is defective and results in uncontrolled cell proliferation.[37–39] Therefore, we analysed cell cycles of MMRCC clones, of parental cell line and of clones derived from parental cell line. Accumulation of the cells in S phase occurs due to more active cells proliferation or their arrest (eg, because of DNA damage) in the middle of replicating their DNA.[40,41] After reprogramming, the three MMRCC clones showed significantly decrease in number of cells in S-phase, compared to cancer B16F10 cell line. It seems that reprogramming may reduce cancer cell proliferation that in turn may lead to tumour formation of smaller size as compared with B16F10 cells. Some studies demonstrated in their analysis that aggressive types of tumours contain higher percentages of cells in S-phase, while the less aggressive ones have lower percentage of cells in this phase.[42,43]

Recent reports have identified asymmetric cell division in various cancers that were characterized by the presence of a subpopulation of cells that share some stem cell-like properties (CSC), which shows a negative correlation between the frequency of asymmetric division and their proliferative capacity. Based on this, highly proliferative CSC performs more symmetric division than asymmetric.[44–46] Although we did not perform statistic evaluation of symmetric than asymmetric divisions in parental and reprogrammed cells, asymmetric division was mainly observed in reprogrammed clones, which suggest more immature state of these cells.

Our reprogramming was unstable, compared to Zhao et al. (2015),[30] which achieved a complete murine melanoma reprogramming, corroborating with studies that include an inefficient and unstable reprogramming of tumour cells.[47] The multistep repeated transfections Zhao's protocol, followed by longer time and high cell density generated stable C-iPSC, the same way as Kaji et al. (2009)[48] developed a protocol to induce normal somatic adult cells. Our data, otherwise, open new perspectives to study heterogeneity and asymmetric division of tumour cells. They suggest a new intermediate point in the reprogramming process, which can serve as base to future studies of the cancer biology, the association between pluripotency and tumour cells.

The majority of published works did not mention stability of cancer cells reprogramming. Choong and co-workers (2014)[23] reported that

during long-term in vitro culturing, these cells might regress in reprogramming. We also noted the loss of reprogramming in isolated clones over time. We believe that during long-term culture in vitro, asymmetric division may contribute to heterogeneity among cancer cells, thus inducing regression of reprogramming.

AUTHORSHIP

DC, AP: data acquisition, analysis, and interpretation, writing the first draft of manuscript, figure and table production, references organization and manuscript revision. AC, RA: contributed with reagents, materials, analysis and participation in the manuscript organization. IK: research design, analysis, and interpretation of data, writing of the manuscript, figure production, and critical revision for important intellectual content.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Identification of very small cancer stem cells expressing hallmarks of pluripotency in B16F10 melanoma cells and their reoccurrence in B16F10 derived clones.

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Running title: Identification of very small cancer stem cells

Abstract

Melanoma is characterized by high heterogeneity and plasticity, most likely due to the presence of mutated melanocyte stem cells or immature progenitor cells in skin that serves as precursors to melanoma. In the present study, for the first time, we identified rare cells in the murine melanoma B16F10, and human A2058 and SK-MEL-28 cell lines that express pluripotency markers, including Oct4, Nanog, Sox2 and a marker of melanoma cancer cells (ALDH1/2). These cells are very small with round morphology and melanin pigmentation. They grow onto melanoma cells, thereby demonstrating feeder layer dependence similar to that of other pluripotent cells. These cells underwent self-renewal, symmetric and asymmetric division. We called these cells murine very small cancer stem cells (VSCSC). VSCSC were also found in B16F10-derived clones after 3–5 consecutive passages, where they occur as single cells or as small colonies, nevertheless, always using melanoma cells as feeders. These cells formed melanospheres enriched with Oct4- and ALDH1/2-positive cells. We also evaluated the possible effect of VSCSC that presented in the parental cell line (B16F10) and in clones based on their functional characteristics. We found that VCSCS present in the B16F10 cell line reappearing in their clones were required for continuous tumor growth and were responsible for melanoma cell heterogeneity and plasticity rather than directly affecting functional characteristics of melanoma cells. Our data, together with those of previous reports that suggested the existence melanoma-competent melanocyte stem cells, corroborate the hypothesis of the existence of tumor-initiating cells and cancer stem cell hierarchies, at least in melanoma.

Key words: Melanoma; Cancer stem cells; Symmetric and asymmetric division; Pluripotency factors; Tumor initiating cells

Introduction

Melanoma is the most aggressive and deadliest skin cancer, accounting for 90% of skin cancer-related deaths worldwide [1]. In late stages, melanoma is markedly resistant to chemotherapy and frequently relapses after surgical resection, leading to poor prognosis [2].

Cancer stem cells (CSCs) or tumor-initiating cells are a small subpopulation of cancer cells that self-renew and initiate tumors. CSCs show strong plasticity, allowing bidirectional conversion between stem and non-stem cells [3,4,5]. This plasticity may help to explain the tumor heterogeneity observed in solid tumors. CSCs undergo symmetrical division, giving rise to two identical daughter cells that further may pass through an asymmetrical division event to give rise to one daughter CSC and one differentiated progenitor cell. CSCs may use this mechanism to increase the number of CSCs that, in turn, are accompanied by growth and expansion of the tumor. Previously, a sub-population of melanoma cells demonstrating stem-like properties have been described [6,7,8].

The expression of pluripotent stem cell factors during tumorigenesis garner special attention because it has been demonstrated that the introduction of key pluripotency genes, including Nanog (Homeobox protein NANOG), Oct4 (octamer-binding transcription factor 4) and Sox2 (sex-determining region Y HMG-box 2) into terminally differentiated somatic cells allows their reversal into an embryonic-like state. It was suggest that CSCs might present similar features to those of pluripotent and other stem cells [9]. The expression and role of these factors in melanoma has been the focus of several studies. It was shown that Sox2 expression regulated self-renewal and tumorigenicity of melanoma cells and acted as a contributor to oxidative metabolism [10,11,12]. Expression of Oct4 in melanoma cells marked tumor initiating cells, metastasis, and resistance to anticancer therapies [13]. The expression of Nanog and Oct4, in turn, increased motility and transmigration of melanoma cells [14]. Furthermore, ALDH1/2 (aldehyde dehydrogenase 1/2) enzyme, also expressed in ESC, is recognized as a potential marker of melanoma CSCs [15]; elevated expression of ALDH1/2 indicates migration and metastatic potential of these cells [16]. Despite the fact that the expression of each of these factors has been studied individually, it is unknown whether all of these markers are co-expressed in melanoma cells and melanospheres, and if so, how they influence phenotypic and functional features of melanoma cells *in vivo* and *in vitro*.

Therefore, we analyzed the expression of Oct4, Nanog, Sox2 and ALDH1/2 in B16F10 murine melanoma cells and melanospheres as well as in vivo after inoculation of these cells into mice. We also studied whether the expression of these markers would be reestablished in B16F10-derived clones obtained using a limiting dilution assay. Finally, *in vitro* and *in vivo* we studied the principal features of B16F10 cells and their clones that express all the aforementioned markers.

2 Materials and Methods

2.1 Cell culture

Murine melanoma cells (B16F10, ATCC CRL-6475) were cultivated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 IU/ml penicillin and 100 µg/ml streptomycin (MP Biomedicals, Solon, OH). The cells were maintained at 37 ºC and 5% CO_2 using a humidified incubator.

2.2 Single-cell cloning by limiting dilution

B16F10 cells were trypsinized and diluted to 100 cells/ml. Next, 10 µl of cell suspension were seeded per well in 96-well culture plates. A volume of 100 µl of complete medium was added in wells containing only a single cell, previously identified using an inverted binocular microscope. Wells with no cells or with more than one cell were discarded from the experiment. Cells were checked daily and after five days, 100 µl of medium was added in each well with cells. After 10 days, the single-cell derived colonies became visible. Colony morphology was examined and seven colonies, presenting distinct morphology was selected to expand.

2.3 Cell cycle analysis

Synchronization of B16F10 and clones 1 and 4 was performed through deprivation of serum for 24 h, followed by induction of the cell cycle using 10% serum. Next, the cells were trypsinized, fixed in 70% cold ethanol and stored at -20 ºC. For cell cycle analysis, the cells were washed twice in 1x phosphate-buffered saline (PBS), re-suspended in 1X PBS and, incubated at 37 ºC for 45 minutes with 10 mg/ml RNAse

(Invitrogen, Carlsbad, CA). Subsequently, 1 µg/ml propidium iodide (PI) (Sigma, St. Louis, Mo) was added. Flow cytometry analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA). Cell DNA content in the various cell cycle phases was determined using ModFit LT software (Verity Software House, Topsham, ME, 10,000 events analyzed).

2.4 Cell viability using the MTT assay

Aiming to analyze multidrug resistance in B16F10 cells and in respective clones, an MTT assay was performed. Briefly, cells were seeded at 1.0 x 10^3 cells/100 µl in 96-wells plates in triplicate. Next, we added docetaxel (DTX) (Sigma Aldrich, Saint Louis, MO, USA) diluted in complete medium using serial dilutions to obtain concentrations ranging from 100.0 to 15.62 µM. After 24 h of incubation with DTX, cell viability was determined using the MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. For this, 10 µl of MTT reagent (Sigma-Aldrich, St. Louis, USA) were added to each well. The plates were incubated at 37 °C for 4 hours. In order to dissolve the formazan crystals, 100 µl of dimethylsulfoxide were added to each well. The absorbance at 570 nm was determined using a plate reader (SpectraMax 250; Molecular Devices, Sunnyvale, CA)[17], and the value of the IC_{50} dose was calculated using GraphPad Prism 4.0 (GradPad Software, CA).

2.5 Tumorsphere formation assay

For the tumorsphere formation assay, cells were plated at a density of 5,000 cells/well in 24-well with 1% of agarose. Cells were maintained in serum-free media consisting of DMEM/F12 basal media, B27 supplements (Invitrogen), 20 ng/m1 human recombinant epidermal growth factor (EGF) and 10 ng/m basic fibroblastic growth factor (bFGF; Invitrogen), 10 µg/ml insulin, 1 ng/ml heparin and 1% penicillin/streptomycin. Melanospheres that arose within 4 days were recorded.

2.6 Colony forming unit (CFU) assay

The CFU assay was performed with B16F10 cells and respective clones (at passage 6) by plating 1.0 x 10^3 cells into 100 mm^2 culture dishes (in triplicate). The medium was changed every three days. After 10 days of culture, adherent cells were washed twice

with PBS and fixed with 4% paraformaldehyde. Colonies were stained with 1% crystal violet solution (Sigma-Aldrich, USA). CFU colonies were counted using ImageJ v.1.47 software.

2.7 Wound-healing assay

The cells (2.5 x 10^5) were grown to confluence in 12-well plates in triplicate, and placed in medium with 1% serum for 24 h at 37 ºC in an atmosphere of 5% CO_2. Upon reaching confluence, the cell layer was scratched with a sterile plastic tip and then washed twice with culture medium. Next, serum was increased to 5% to facilitate cell migration, the cells were photographed at 3, 6 and 24 h after scratching. Cell migration was recorded using a Nikon TE2000-E microscope system (Nikon Instrument). Migration was calculated using Image J software.

2.8 Immunofluorescence

The B16F10 and clones C1 and C4 were grown on chamber slides and plates. Cells were fixed in 4% paraformaldehyde for 15–30 minutes at room temperature, washed with PBS and permeabilized with 0.1% Triton X-100. Next, cells were treated with 5% BSA blocking solution for 40 minutes at room temperature. Slides were incubated without (experimental control) and with primary antibodies as follows: rabbit anti-Oct4 (diluted 1:400) (Abcam, Burlingame, CA), rabbit anti-Sox2, rabbit anti-Nanog, rabbit anti-ALDH1/2 (diluted 1:100), and rabbit anti-vinculin (diluted 1:50) (Santa Cruz Biotechnology, Dallas, TX) overnight, at 4 °C, washed in PBS. Next, we added the secondary antibody conjugated with FITC (dilution of 1:500) and incubated for 1 h at room temperature. Cells were washed with 1x PBS. For the plates incubated with anti-vinculin antibody, we performed an additional incubation with 3 U/mL FITC-phalloidin (Sigma-Aldrich, St. Louis, Mo) in PBS for 1 h in order to stain the F-actin cytoskeleton. Slides were mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA, USA) to reveal nuclear DNA. Immunofluorescence was visualized in a Nikon Eclipse Ni (Tokyo, Japan) microscope. The plates were then washed twice with

PBS and analyzed using ImageXpress. Nine sites per well and three wells per treatment were acquired as proposed by Levy et al. (2014).

2.9 Cell protein extraction and western blotting

B16F10 cells and the seven clones were seeded in Petri dishes (6 cm^2). Next, 2 × 10^6 cells of each clone and the parental cell line were washed with PBS and lysed with 100 µl of 1X SDS sample buffer (62.5 mM Tris-HCl, pH 6.8 at 25 °C, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). The lysates were transferred to polypropylene tubes. These samples were heated to 95 °C for 5 minutes following by centrifuging at 12,000 *g*, 4 °C for 10 minutes. Total proteins from each cell lysate were separated in SDS gel electrophoresis and western blotting was carried out as described [18]. The following primary antibodies were used: rabbit anti-β-actin, (Cell Signaling Technology, Beverly, MA), rabbit anti-Oct4 (Abcam), rabbit anti-Nanog, rabbit anti-Sox2, rabbit anti-ALDH1/2, (Santa Cruz), with β-actin used as loading control. Secondary antibodies were conjugated with IgG horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) and immunoreactivity was detected using the Amersham ECL Western Blotting Analysis System (GE Healthcare, UK); the chemiluminescence was revealed using Amersham Hyperfilm ECL (GE Healthcare, UK).

2.10 Experimental lung metastasis assay

A total of 5 x 10^5 B16F10 cells and respective clones diluted in 100 µl of PBS were injected into the retro-orbital venous plexus of 8–12-week-old C57BL/6J female mice, in five animals for cells (Charles River). Animal studies were conducted in compliance with the Butantan Institute Ethics Committee for Use of Animal Experimentation (CEP 9411280318). Animals were euthanized using a CO_2 box 14 days after injection, and lungs were excised and fixed in phosphate-buffered 10% formaldehyde. Metastatic foci at lung surfaces were counted by two observers in a blinded fashion.

2.11 Hematoxylin and eosin staining (HE)

Tumor tissues were collected and fixed in 10% formaldehyde and paraffin sections were prepared for HE stains. The tumor tissues were sectioned (3–5 μm), deparaffinized in xylene and dehydrated in a series of decreasing concentrations of ethanol. The sections were stained with hematoxylin and eosin (HE) and the slides were examined under an optical microscope (Nikon DS-Ril, Tokyo, Japan).

2.12 Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded tissue specimens were sliced into 3–5 μm sections that were then deparaffinized and hydrated. An endogenous antigen retrieval procedure was performed using citric acid buffer (10 mmol/L citrate buffer, pH 6.0). The slides were incubated with the same antibodies from immunofluorescence experiments overnight at 4 °C, then with secondary antibodies conjugated with alkaline phosphatase for 1 h at room temperature, followed by treatment with a Liquid Fast-Red Substrate Kit (Abcam). As a control experiment, an identical immunohistochemical procedure was performed with omission of the primary antibody. All slides were examined using an optical microscope (Nikon Eclipse 600, Nikon, Tokyo, Japan).

Results

Expression of pluripotency factors in B16F10 cell line and its clones

We verified the expression of key regulators of pluripotency, including transcriptional factors Oct4, Nanog and Sox2 in B16F10 murine melanoma, as well as expression of aldehyde dehydrogenase (ALDH1/2), considered one of the more important biomarkers for melanoma CSC. Only a few B16F10 cells reacted positively with these markers, showing appropriate nuclear localization (Figure 1A–D).

Next, we verified whether these rare cells that express pluripotency factors and ALDH1/2 would be found in the B16F10 derived clones. We isolated seven clones through using a limiting dilution assay. The expression of Oct4, Nanog, Sox2 and ALDH1/2 was studied when clones and B16F10 achieved cell numbers sufficient for analysis (generally after 3–5 passages). All isolated clones possessed only very rare

cells positive for studied markers, expressed in the similar manner in all clones (Figure 1A–D2) and in the parental cell line (Figure 1A–D, S1). These cells appeared as single cells (Figure 1A3), single closely-placed cells (Figure 1B – B2), or they formed small colonies (Figure 1A, C, D, D1, D2). Figures 1E-G and 1I-K demonstrate at high magnification and using both immunofluorescence and phase contrast the morphology of various single cells (Figure 1F, G) and of small colonies (Figure 1E) formed by Sox2+ cells in C3, C4 and B16F10. Note that all these cells are very small, round, have very small nuclei, and grow onto the other melanoma cells that are large with substantial nuclei, using them as a "feeder layer" (Figure 1E–G; I–K, S2). We also showed at high magnification, for example, the details of Oct4 intracellular localization, which is nuclear and cytoplasmic (Figure 1H). Antibodies against Nanog, Sox2 and ALDH1/2 showed the same nuclear and cytoplasmic localization as that of Oct4 (Figure 1A–G and 1I–K).

Expression of pluripotency factors in human A2058 and SK-MEL-28 cell lines

In order to verify the universal character of occurrence of the cells that express pluripotency markers in cancer cell lines, we verified that expression of Sox2 and Nanog in highly invasive A2058 (Figure S1, G-G3) cell line, as well as Oct3/4 and ALDH1/2 in A2058 (Figure S1, H) and in SK-MEL-28 (Figure S1, I). Both cell lines express aforementioned markers in a very small proportion of the cells (Figure S1, G-I).

Self-renewal, symmetric and asymmetric division

The distribution of Oct4, Nanog, Sox2, and ALDH1/2-positive cells within the clones and B16F10 cells, as well as their close localization suggest that they are clonally reproduced. Figure 1I demonstrates one self-renewing symmetrically dividing Sox2+ cell in C4 that produced two cells of equal size. Figure 1J shows three Sox2+ cells in C5, one of which is very small, while two other cells are larger (however, smaller than other cancer cells). Considering that these three cells were produced clonally by one ancestral cell, we suggest that the very small cell self-replicated symmetrically, producing, however, two cells of different sizes: a very small cell and a larger cell. The larger cell that is also Sox2+ self-replicates symmetrically and produces two larger Sox2+ cells (Figure 1J). In Figure 1J, one symmetric and asymmetric divisions can be

observed. This figure demonstrates two very small Sox2+ cells from C4, obtained probably after self-replication, each of which divided asymmetrically, producing two cells of different size: one very small Sox2+ cell and one larger Sox2– cell that are one stem cell and one differentiated cell. In Figure 2, we demonstrate various types of very small Oct4-, Nanog-, Sox2-, and ALDH1/2-positive cells division observed in B16F10 and their clones. In Figure 2A,B self-renewing symmetrically dividing Oct4+ cells in C4 and ALDH1/2+ cells in C1 at early telophase stage were observed, while Figure 3C shows an ALDH1/2+ cell from C6 at late telophase stage. Figure 2D shows three ALDH1/2+ cells in C2, produced after two cycles of consecutive symmetric division. Interestingly, Figure 2E displays a tetrad-like structure composed by four ALDH1/2+ cells from C2 that are of very small size and larger size. We suggested two possible interpretations for this phenomenon: one very small and one larger ALDH1/2+ cell self-replicate symmetrically producing daughter cells of equal size (Figure 2E, (a)) or they self-replicate symmetrically producing two daughter cells of larger size (Figure 2E (b)).

Another type of symmetric division, where stem cells produce two differentiated cells is shown in Figure 2F. This figure demonstrate one Nanog+ cell from C3. We suggest that this cell already self-replicated symmetrically, producing two equal cells, one of which (showed in the schema in gray) divided symmetrically producing two Nanog– differentiated cells. In turn, Figure 2G demonstrates three consecutive symmetric divisions producing Nanog+ cells in C1, one which (showed in the schema in gray) divided symmetrically, however, producing two differentiated Nanog– cells.

We also observed asymmetric division, which was rarer than symmetric division. Figure 2H demonstrates two ALDH1/2+ cells from C1 that further divided asymmetrically, each stem cell producing one differentiated cell.

Alternate variance between symmetric and asymmetric division was also observed. Figure 2I demonstrates two Sox2+ cells from C3, obtained presumably after one symmetric division (note they are of slightly different sizes), each of which divided asymmetrically, producing differentiated Sox2– cells.

Western blot analysis of Oct4, Nanog and Sox2 and ALDH1/2 expression

We performed western blot analysis to confirm the expression of Oct4, Nanog, Sox2 and ALDH1/2 protein. Figure 3 demonstrates relatively medium levels of expression of Oct4 protein in B16F10, C1 and C4 cells. The expression of Nanog was

equal and of medium level in C1 and C4, but low in B16F10 cells. Nanog was expressed at low levels in comparison with Oct4 in all studied cells. Sox2 expression was very strong in B16F10 cells and very weak in C1 cells; however, it was at medium level in C4. ALDH1/2 expression was high in B16F10 cells, medium in C1 and weak in C4.

Because very small cells were observed in the parental cell line and their clones presented several characteristics of stem cells, including expression of pluripotent markers, feeder layer-dependent growth and use of symmetric/asymmetric division for their reproduction, we called these cells very small cancer stem cells (VSCSC).

Functional characteristics of parental cell line and their clones

We found that that clones (C1–C7) demonstrated morphology very similar to that of B16F10 (Figure 4A). The clones also showed differences in progression through the cell cycle, when we compared parental B16F10 and C1 cells, showing the highest number of cells in S phase, while this number decreased in G2-M (Figure 4 D,E). We performed two-way ANOVA based on the number of cells in each cell cycle phase. This analysis determined significant differences between the parental cell (B16F10) and the two clones (C1 and C4) derived from B16F10 cells ($p < 0.0001$). For this reason, we performed the Bonferroni's *post-hoc* test, which showed that both clones (C1 and C4) presented increased cell percentages in S, while C1 also had a higher cells percentage in G2-M phases (Fig 4 D, E).

Melanin levels are known to correlate with malignancy; these were evaluated in both studied clones (Figure 4 F). Our results demonstrate that melanin pigmentation varied among the clones and the parental cell line: C1 and B16F10 presented similar melanin pigmentation of the pellets, while C4 demonstrated strong melanin pigment.

To evaluate the chemoresistance of clones C1, C4, and B16F10, they were treated with docetaxel at various concentrations. Chemoresistance was evaluated using the MTT assay. Based on mean absorbance, two-way ANOVA showed significant differences between the parental and clonal cell lines ($p = 0.0013$). Bonferroni *post-hoc* analysis showed that clones C1 and C4 exhibited chemoresistance against docetaxel in all concentrations (Figure 3 D), especially at low concentrations (15.62 μM, 31.25 μM

and 62.5 μM, $p < 0.001$, Table). This result was not observed for B16F10 cells (Figure 4 G). No significant difference was detected between clones ($p > 0.01$, Table).

Table 1. Chemotherapeutic resistance against docetaxel.

	B16F10 vs C1		B16F10 vs C4		C1 vs C4	
Concentration (μM)	Differences	p	Differences	p	Differences	p
0.00	**-0.0002500**	**> 0.05**	**0.08400**	**> 0.05**	**0.08425**	**> 0.05**
15.62	**0.2453**	**<0.001**	**0.2174**	**<0.001**	**-0.02790**	**> 0.05**
31.25	**0.2679**	**<0.001**	**0.1918**	**<0.001**	**-0.07615**	**> 0.05**
62.50	**0.1613**	**<0.01**	**0.1544**	**<0.01**	**-0.006900**	**> 0.05**
125.00	**0.06020**	**> 0.05**	**0.1046**	**> 0.05**	**0.04440**	**> 0.05**
250.00	**0.1043**	**> 0.05**	**0.0993**	**> 0.05**	**-0.004950**	**> 0.05**
500.0	**0.0430**	**> 0.05**	**0.0554**	**> 0.05**	**0.0124**	**> 0.05**

Considering that metastatic potential is associated with migratory capability (17), which in turn is related with acquisition of CSC phenotype, we analyzed the migratory potential of parental and clonal cells. As shown on Figure 6, parental cells exhibited an intermediate migration potential with respect to C1 and C4 cells (Figure 4 H). Two-way ANOVA demonstrated significant differences among B16F10, C1 and C4 cells (table). The Bonferroni *post-hoc* test showed statistically significant differences between clones C1 and C4 at 24 hours. C1 presented higher migratory potential than C4 (Table 2, S2). Both clones, when seeded at low density, showed variable clonogenic capacity (Fig 4 I). C4 produced a higher number of sub-clones, similar to that of B16F10, while C1 produced a lower number than B16F10 and C4 (Fig. 4 J)

Table 2. Cell migration analysis

	B16F10 vs C1		**B16F10 vs C4**		**C1 vs C4**	
Time (h)	**Differences**	**p**	**Differences**	**p**	**Differences**	**p**
3	-1.142	> 0.05	-2.279	> 0.05	-1.137	> 0.05
6	-1.271	> 0.05	-10.35	> 0.05	-9.076	> 0.05
24	-11.81	> 0.05	-12.56	> 0.05	-24.38	<0.01

Vinculin–F-actin interaction observed in melanoma cells in the clones

The CSC phenotype is known to be closely related to epithelial-mesenchymal transition (EMT), characterized by cytoarchitectural changes. We investigated the distribution of vinculin, a mesenchymal marker, overexpression of which is a hallmark of EMT, and F-actin (Figure 5A). The actin fluorescent intensity in B16F10 cells was lower than that of C1 and C4 (Figure 5 B) cells. Both clones presented medium level fluorescent intensity, similar to that of actin. Vinculin expression was low in B16F10 cells, strong in C1 and medium in C4 (Fig 5 B) cells. F-actin and vinculin demonstrated intracellular co-localization in all studied cells (Figure 5 A). In figure 5C, filopodia in B16F10 cell can be observed.

Sphere formation

Sphere forming ability is one of the properties of melanoma murine CSCs (16). Melanospheres (melanoma spheroids) are enriched in cells with clonogenic potential, reflecting the self-renewing capacity of CSCs. Therefore, we analyzed the capacity of both clones to form melanospheres (Figure 6A). All studied cells (B16F10, C1 and C4) were able to form melanospheres and various clones produced melanospheres of diverse sizes (Figure 6A). Next, we determined whether melanospheres obtained from parental cell lines and both clones expressed ALDH1/2 and Oct4. Melanospheres showed intracellular co-localization of both of these markers in the B16F10 (Figure 6B), C1 and C4 cells. Interestingly, it can be seen that the cells that begin to migrate from the spheres either lose their intensity or have no marking (Figure 6B).

***In vivo* tumorigenicity and metastasis forming capacity of the B16F10 and their clones**

We also verified the capacity of B16F10, C1 and C4 cells to form lung metastases. After inoculation of studied cells in mice, multiple melanoma nodules were formed, however C1 cells formed more nodules than did the C4 and B16F10 cells (Figure 7 A, B).

The parental cell line and both clones formed melanomas after their subcutaneous inoculation into C57Bl/6 mice. The clones did not show delay of tumor

formation, which commonly occurred approximately 10 days after inoculation. Microscopic images of tumor tissues stained with hematoxylin and eosin (Figure 7 C-E1) demonstrated strong vascularization, necrotic areas and intense nuclear chromatin staining in tumor tissues derived from B16F10 (Figure 7 C, C1) and C1 (Figure 7 D, D1) cells. In turn, tumor tissue derived from C4 cells demonstrated poor differentiation, moderate vessel formation, presence of diffuse melanin and weak nuclear chromatin staining (Figure 7 E, E1). All clones showed particular cell features, demonstrating heterogeneity *in vivo*, consistent with the *in vitro* results.

Expression of pluripotency factors in tumors

We also verified expression of studied markers in the same melanomas that were used to examine tumorigenicity. Very rare expression of pluripotency factor Oct4, Nanog and Sox2 was found (Figure 8). Single Oct4-positive cells were found in tumors generated by B16F10 and C1 (Figure 8A, A1), demonstrating nuclear and cytoplasmic localization, while no Oct4+ cells were observed in tumors generated by C4 (Figure 8A2). Nanog+ cells were observed in all tumors (Figure 8B–B2); however, in C4-derived tumors, Nanog showed only cytoplasmic localization (Figure 8B2). Single Sox2+ cells were also observed within the tumors (Figure C-C2). By contrast, anti-ALDH1/2 antibody reacted positively with single cells (Figure 8D–D2) and with multiple sites of melanoma demonstrating localization in small groups of melanoma cells (Figure 8D-D2). Such groups of ALDH+ cells were primarily observed in B16F10 and C4 (Figure 8E, 8E2), while C1 only presented single ALDH+ cells (Figure 8E1).

DISCUSSION

In the last two decades, prevailing opinion was that tumors are initiated and maintained by a population of rare CSCs [19,20]. Our present study supports this hypothesis.

We demonstrated that murine melanoma B16F10 cells presented rare very small melanin-pigmented cancer stem-like cells that expressed such transcription factors as Oct4, Sox2, Nanog and ALDH1/2. We called these cells VSCSC. In various clones

derived from B16F10 after 3–5 passages, rare VSCSC expressing the aforementioned markers appeared again. Our findings agree with those of a previous report that demonstrated the presence of temporarily distinct small subpopulation of CSCs in melanoma, required for continuous tumor growth [21]. However, in this previous work, the authors did not show that these cells expressed pluripotency markers and ALDH1/2, and they did not describe the morphological features of these cells.

Our data also support the hypothesis that non-stem cell subpopulations may dedifferentiate/reprogram into stem-like cells [22], because it is unlikely that all isolated clones were started from Oct4-, Sox2-, Nanog- and ALDH1/2-positive cells. However, in all seven clones derived from B16F10 line, we observed reappearance of very small melanin-producing cancer stem-like cells.

Melanocytes are small melanin-producing cells, typically 7 μm in length. Their reappearance occurs by a small pool of immature stem cells, melanocyte stem cells (MCSCs) residing in hair follicles. A recent report demonstrated melanoma formation from melanoma-competent MCSCs following extrinsic stimuli [23]. We demonstrated that Oct4-, Sox2-, Nanog- and ALDH1/2-positive cells are small and melanin-pigmented, making them similar to both melanocytes and MCSCs. The role of *in vivo* microenvironment in melanoma cell fate determination and phenotype is currently accepted [24]. When the *in vitro* microenvironment is poor, this appears to be sufficient to influence the reappearance of Oct4-, Sox2-, Nanog- and ALDH1/2-positive cells in B16F10-derived clones (Figure 1 A-C and S1).

In addition to the microenvironment, low seeding density may be a factor that induces dedifferentiation of B16F10 cells and their clones, because it has been shown that in human chondrocytes, dedifferentiation occurs more extensively with low seeding densities and passaging [25]. We believe that B16F10 cells and their clones are good models to study the factors that might induce cancer cell dedifferentiation, as well as plasticity and phenotypic reversibility.

The microenvironment also plays an important role in CSC division. Under non-stressful conditions, CSCs are maintained in a quiescent state, while under stressful conditions, such as hypoxia, low pH or drug exposure, they start to proliferate, favoring the emergence of new CSCs through asymmetric divisions [26]. We reported that VSCSC demonstrated asymmetric division, as evidenced by morphological and immunofluorescence studies. During asymmetric division, a single stem cell produced two differently functional daughter cells: one retained stem cell identity, while the other

became specialized and lost stem cell properties [27]. We often observed two divided and still closely-placed cells, one of which expressed pluripotency factors and presented very small melanin-producing cancer stem-like cell morphology, while the other cell demonstrated a large nucleus and size and was negative for pluripotency factors (Figure 1 A-C, S1). The program of asymmetric division is frequently unregulated in cancer cells [28]. Therefore, our work provides a compelling cancer cell model that will be useful to shed a light on the mechanisms that drive asymmetric division in CSC.

Melanoma cells can recapitulate tumor phenotypic and functional heterogeneity. This capacity is widely shared by many phenotypically diverse melanoma cells [29]. In Table 3, we summarized the principal phenotypic and functional features of B16F10, C1 and C4 cells. This table displays the high level of phenotypic and functional heterogeneity observed between B16F10 C1 and C4 cells. All studied cells showed high expression levels of Oct4 and low levels of Nanog. Expression of Sox 2 was low in B16F10 cells and high in C1 and C4; in turn, ALDH expression was low in C4 and high in B16F10 and C1. Melanin level also varied: B16F10 and C1 cells presented low levels of melanin, while in C4 it was high. With respect of drug resistance, the clones were more resistant than B16F10 cells. Clonogenic capacity was stronger in B16F10 and C4 cells than in C1.

Melanospheres are enriched in cells with clonogenic potential, when compared with adherent monolayer cultures, thereby reflecting the self-renewing capacity of cancer stem-like cells [30] [31]. We demonstrated that B16F10 and their clones formed melanospheres of varying sizes. When B16F10 and C1 form small and medium-sized melanospheres, C4 also formed also large melanospheres, appearing to be the result of fusion of medium and small spheres. These melanospheres were also enriched with Oct4+ and ALDH ½+ cells, suggesting that these cells potentially have the properties of tumor-initiating cells [32].

Vinculin is a cytoplasmic actin-binding protein that plays an important role in modulation of cell adhesion and migration [33]. Previously, differential expression of vinculin in B16F10 cells was reported [34]. The network of actin filaments also influences cell migration, thereby regulating the elasticity of the cell [35]. Actin and vinculin expression was low in B16F10 cells, while C4 expression of these markers was of medium level. In C1 cells, vinculin expression was strong and that of actin was of medium level (Table 2). C1 presented higher migratory potential than C4 and B16F10

cells (Table 2). Vinculin also contributes to formation of lamellipodia, facilitating cell migration [36]. High levels of vinculin in clone C1 can explain the high migratory potential verified in this clone. It is of interest that, although C1 demonstrated less expression of Sox2 protein (Figure 2) than C4 and B16f10 cells, this clone showed a higher cell percentage in S and G2-M phases, high vinculin expression, higher migration capacity and pulmonary nodule formation, than did B16F10 and C4 cells. High vinculin expression and migrating capacity of C1 cells could be explained by high Oct4 and moderate Nanog expression. However, C4 demonstrated the same properties and did not present similar characteristics.

Overall, it is difficult to link the pattern of expression of pluripotency markers in B16F10 and their clones and functional features of cancer cells. We are more inclined to believe that pluripotency marker-positive cells are tumor-initiating cells than can be found at least in melanoma and that they are responsible for tumor reoccurrence and heterogeneity. This hypothesis was supported by our *in vivo* data that showed the presence of pluripotency factor-positive cells in melanomas obtained from the parental line and their clones. These cells are the root of heterogeneity, subsequently reflected in functional features of tumors, including recurrence of tumor, increased metastases and drug resistance.

The histological features of the xenografts generated by the B16F10 parental line and their clones recapitulated the original tumor heterogeneity. B16F10 cells are more heterogeneous than their clones and show multiple vessels and large areas of necrosis. C1 showed more moderate histology than the parental cell line, while C4 presented melanoma with poor differentiation, small vessels and diffuse melanin.

Many reports support the existence of CSC hierarchies in many tumor types; however, some experimental data indicate that not all cancer cells follow the CSC model [37]. For example, in the case of melanoma, the xenotransplantation frequency of melanoma-initiating cells could vary dramatically, depending on the methodology applied, because up to 25% of human melanoma cells could form xenografts [38]. It appears that these tumors do not follow a stem cell hierarchy, or that most cells in advanced-stage melanomas behave as CSCs. The presence and reestablishment of melanoma cells positive for pluripotency markers suggest that these cells are a source CSCs and tumor heterogeneity that depend on the methodology applied, increasing

frequency of melanoma-initiating cells [22]. Therefore, the use of our model may help clarify whether CSCs hierarchies exist in melanoma.

The presence of CSCs is closely related with tumorigenesis and therapeutic drug resistance. Although many efforts have been made to target CSCs, anti-cancer therapies focused on these cells remain in the distant future because of CSC-derived cancer malignancies, tumor recurrence, increased metastases and drug resistance. CSC plasticity and bidirectional conversion existing between stem and non-stem cells has contributed to the tumor heterogeneity observed in solid tumors, making CSCs a target difficult to achieve [39]. A complete understanding of CSC-derived heterogeneity will provide novel insights into the establishment of efficient targeting strategies to eliminate CSCs. Pluripotency transcription factors may provide advantageous targets for the elimination of CSCs. Therefore, it is important to understand how these pluripotency factor-expressing cells are able to maintain tumor heterogeneity, growth, tumor progression and drug resistance.

Our data demonstrate that B16F10 and their clones are a good model for studying how these markers are involved in the progression of melanoma. These markers can be of potential biological and prognostic importance for clinical studies.

Authorship

DC, PL: Data acquisition, analysis, and interpretation, writing the first draft of manuscript, figure and table production, references organization and manuscript revision.

RA, CR, RP, DL, DS: Contributed with reagents, materials, analysis and participation in the manuscript organization.

IK: Research design, analysis, and interpretation of data, writing of the manuscript, figure production, and critical revision for important intellectual content.

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Competing Interests

The authors declare that they have no competing interests.

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Legends

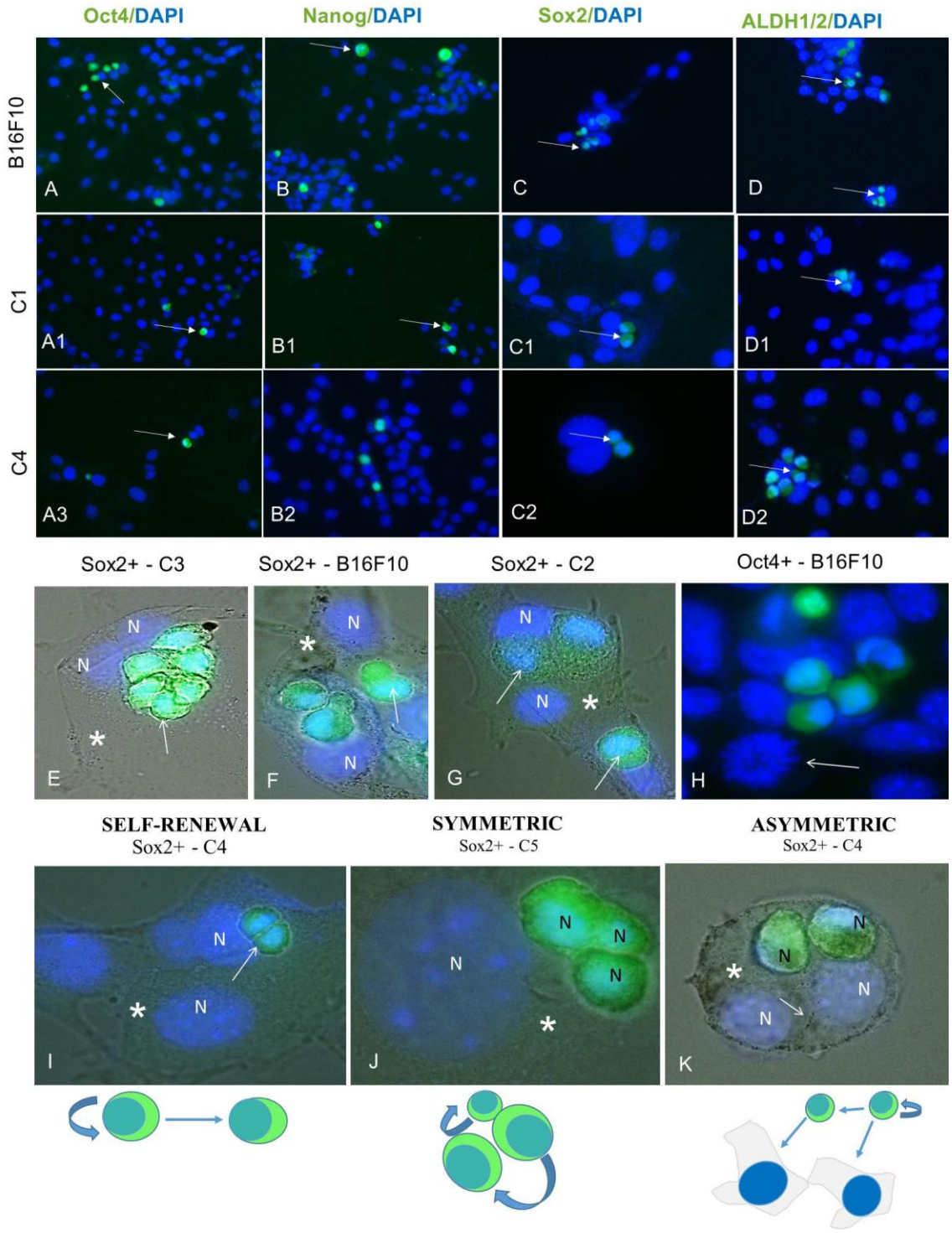

Figure 1: Representative figure shows immunofluorescence staining for pluripotency markers and ALDH1/2 in B16F10 cells and their clones; morphology of very small cancer stem cells and types of division. In **(A-D)** B16F10; in **(A1-D1)** C1; in **(A2-D2)** expression of Oct4, Nanog, Sox2 and ALDH1/2, respectively. Rare melanoma cells are positive for these markers, which show green fluorescence; nuclei

stained with DAPI (blue) – DNA biding dye. In (**A1, A3** and **B**) white arrows indicate single pluripotency markers positive cells. In (**A, D and D2**) white arrows show groups of these cells. (A-D3) Epi-fluorescence (EF). Scale bars: 10 μM. (**E-G**) show the morphology of pluripotency factors positive cells (green) in different clones and the parental lineage: (**E**) small colony of Sox2+ in C3; (**F**) individual Sox2+ cells in B16F10; in (**G**) same as in (F) in C2; in (**H**) Oct4+ cells in B16F10 demonstrating details of nuclear and cytoplasmic localization of this marker. Metaphase of Oct4 negative cell is shown with white arrow. Note that very small cancer cells are grown onto the other cancer cells, using them as feeder (white asterisk) In (**E-G**) Sox2+ cells are indicated by white arrow. DAPI stained nuclei shown by white N. In (**I**) self-renewal of Sox2+ cells (white arrow) in C4 is demonstrated. In (**J**) the result of supposedly symmetric division of Sox2+ cells in C5 is evidenced. In (**K**) the result of supposedly symmetric division of Sox2+ cells in C4 is shown. Graphic presentation of events in (**I-K**) is shown below of the figures. Other designations are the same as on E-G. (**E-G and I-K**) = EF and Phase contrast (PC).

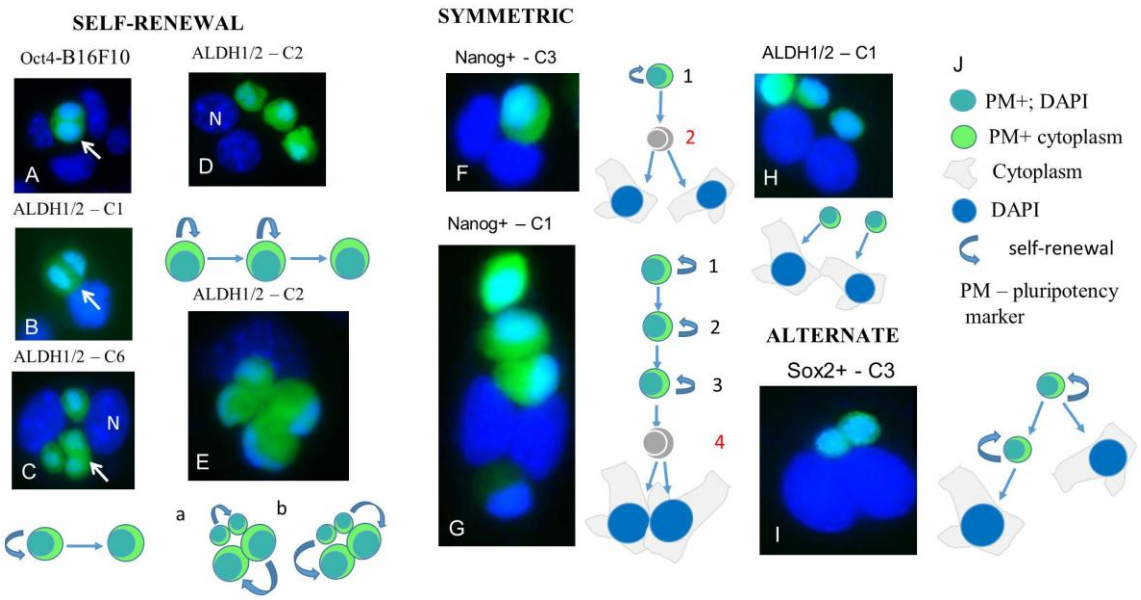

Figure 2. Detail of supposed self-renewal, symmetric, asymmetric and alternate division of very small positive for pluripotency hallmarks cancer cells. (**A-E**) demonstrate self-renewal: (**A**) Oct4+ cell in B16F10; (**B**) ALDH1/2+ cell in C1; (**C**) ALDH1/2+ cell in C6; graphic presentation of events in (**A-C**) is shown below (C). (**D and E**) Consecutive self-renewal events of ALDH1/2+ cells in C2: (**D**) Two

consecutive self-renewal producing three cells (graphic presentation shown below); (**E**) Two different types of self-renewal: (a) producing pluripotency hallmarks cancer cells of equal size and (b) of different size. (**F and G**) Symmetric division observed in (**F**) demonstrates self-renewal of Nanog+ cell in C3, graphic presentation (right) shows (1) self-renewal; (2-red) symmetric division producing two differentiated cells and in (G) demonstrates three consecutive self-renewal of Nanog + cell in C3 (graphic presentation – right (1-3)) and symmetric division producing two differentiated cells (4-red). (**H**) Asymmetric division of two very small ALDH1/2+ cancer cells in C1 each producing one differentiated cell (graphic presentation – right). (**I**) Alternate between self-renewal (symmetric division) and asymmetric division (graphic presentation - right) of Sox 2+ cells in C3. (**J**) Designations used on present figure, other designations same as on Figure 1. (**A-I**) = EF, Scale bars: 10 μM.

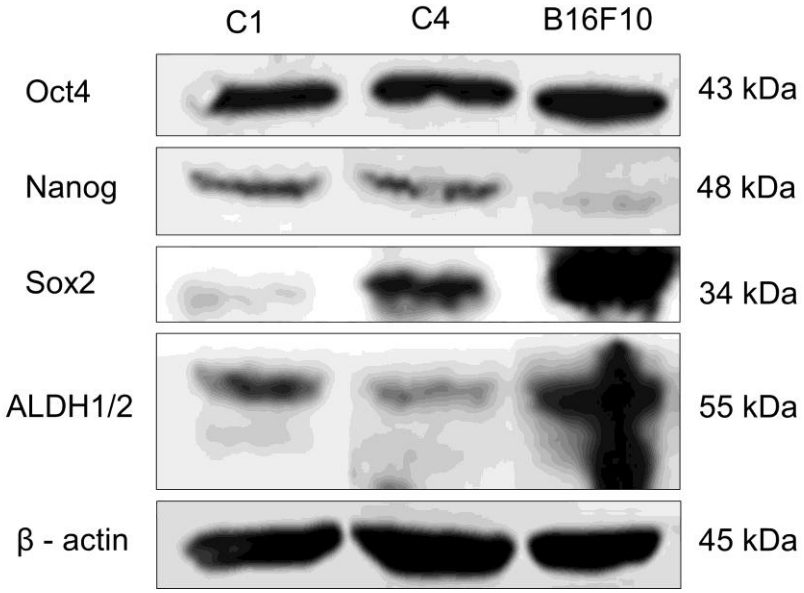

Figure 3: Expression of stem cell-associated markers in B16F10 cells. Western blot analysis of protein levels of the stem cell-associated transcription factors Oct4, Nanog, Sox2 and ALDH1/2 in parental cell line and clones.

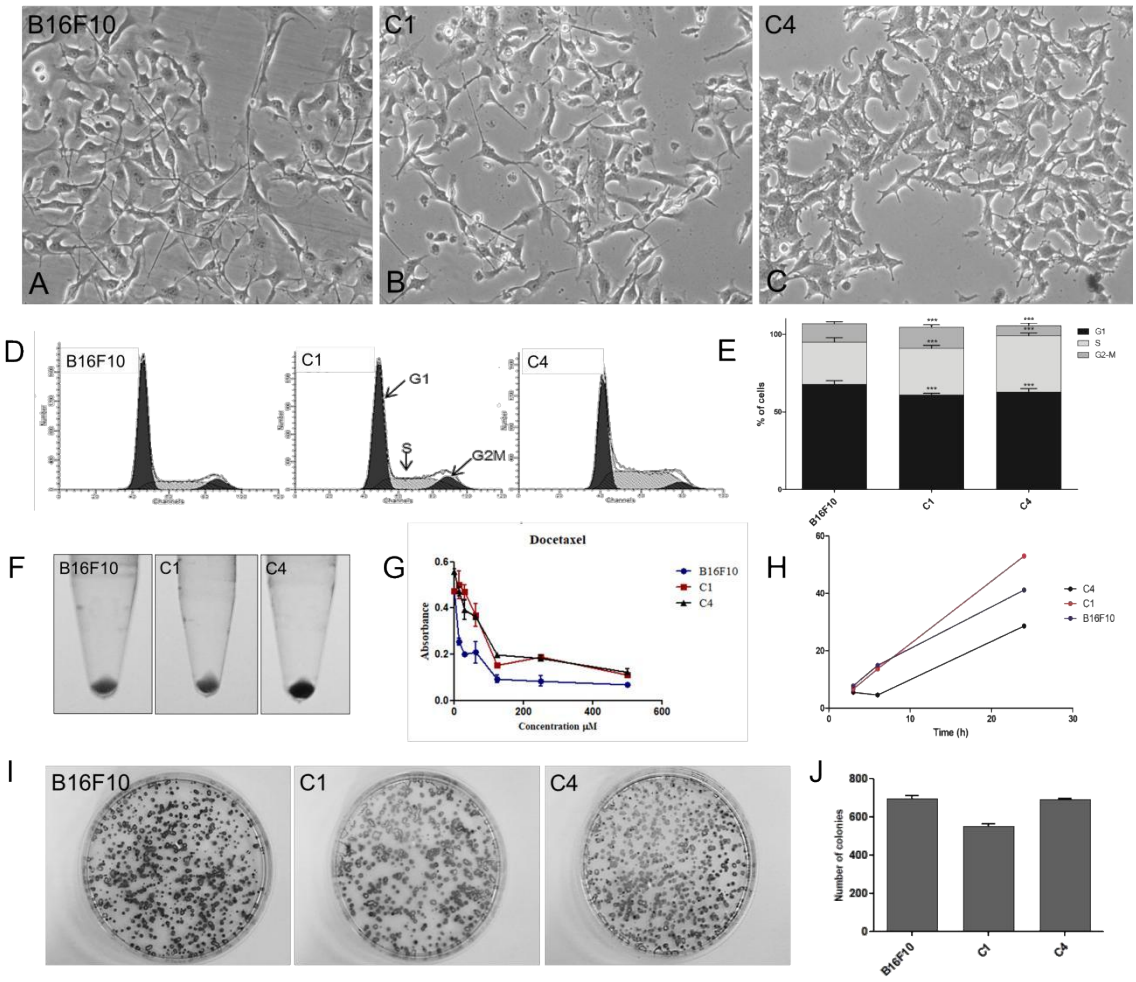

Figure 4: Characterization of B16F10 - derived clones. (**A-C**) Morphology of B16F10 cells and their clones (C1 and C4), respectively. (**D**) Histograms of B16F10, C1 and C4 cell cycle, (**E**) Graphical representation of cell cycle phase proportions for B16F10, C1 and C4 demonstrating the difference between parental cell line and its clones. The cells show difference in the number of cells in S and G2 phases, which is more evident in C4. (**F**) Comparison in melanin production observed through the pellets formation by the B16F10, C1 and C4, apparently C4 shows more pigmentation. (**G**) Cytotoxic analysis using DTX antitumor drug observed between the parental cell and its clones; the clones show more resistance than B16F10. (**H**) Cell migration assay. Cell layers were wounded at 3, 6 and 24 hours. Relative wound closure ability was determined by measuring the grayscale of the wounds. X ± s, n = 3. (**I**) Colony formation assay. B16F10, C1 and C4 cells stained with crystal violet and analyzed for their colony forming capacity. (**J**) Graphical representation of colony numbers formed by B16F10, C1 and C4 cells.

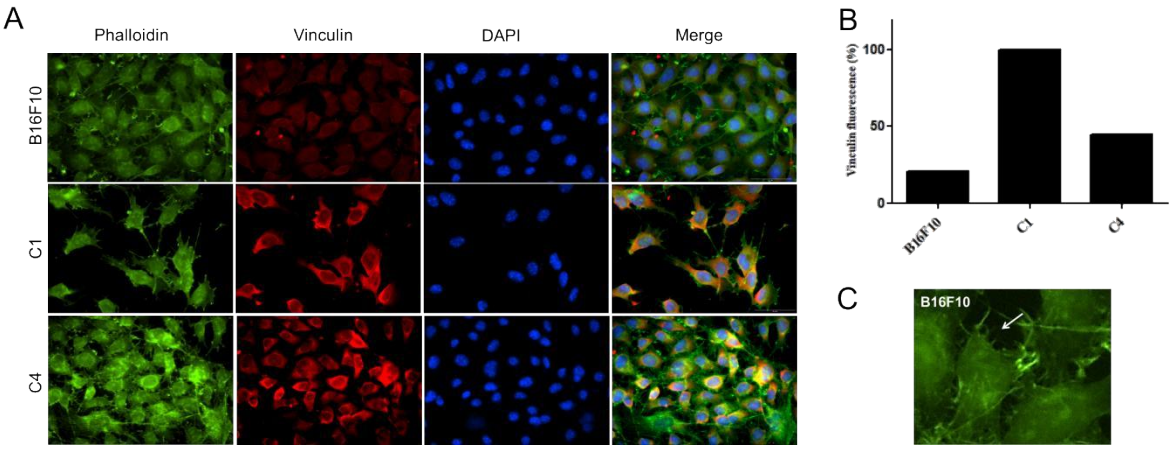

Figure 5: Immunofluorescent staining for vinculin and phalloidin. (**A**) Actin staining by phalloidin (green) is relatively similar in all studied cells. Vinculin (red) demonstrates differential staining in studied cells. In B16F10 -week, C1-intermediate and C4-strong staining. Merged images show the heterogeneity in terms of cytoskeleton protein expression. In B16F10 cells, very weak overlapping between Phalloidin and vinculin is observed. In C1 the cells in merged picture show orange color that evidenced more strong cells reaction to vinculin antibody than phalloidin staining. In C4, the cells demonstrate yellow color that shows relatively equal staining intensity with vinculin and phalloidin. Nuclei stained with DAPI (blue). (**B**) Graphical presentation confirms immunofluorescence analysis showing high fluorescence intensity of vinculin in C1 cells (**C**) Filopodia structures. A, C- Epi-fluorescence. Magnification 400×.

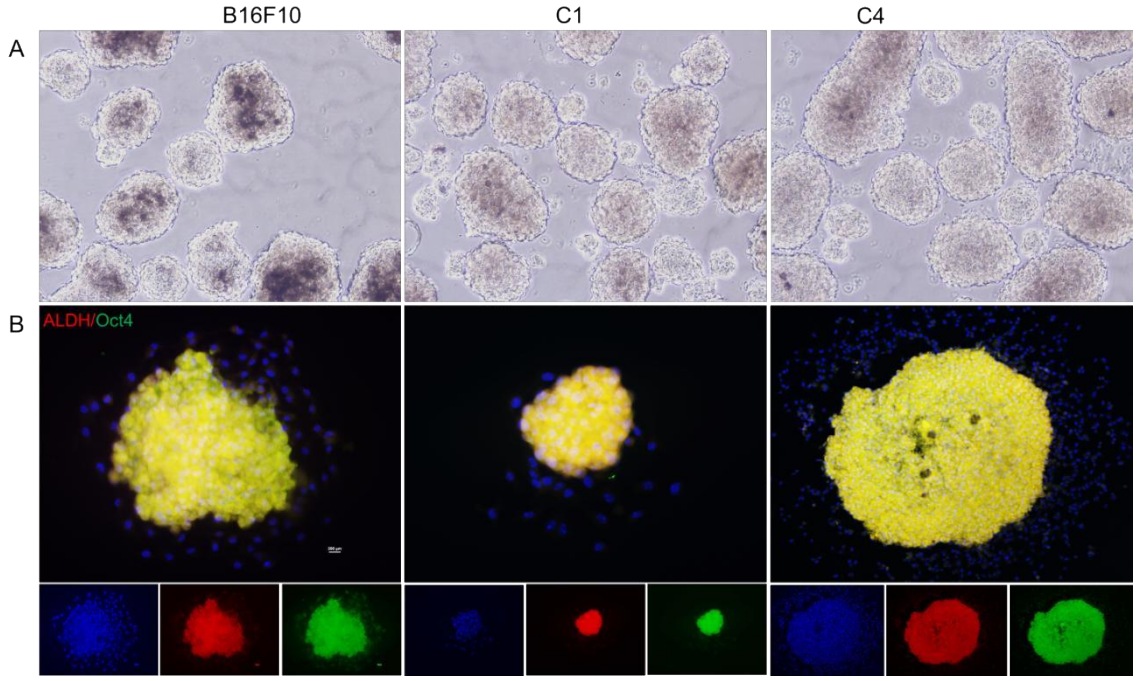

Figure 6: Melanospheres formation by B16F10, C1 and C4, and expression of Oct4 and ALDH1/2. (**A**) Three-dimensional multicellular spheroids obtained from parental B16F10, C1 and C4 cells. (**B**) Merged images show ALDH1/2 and Oct4 positive staining (yellow) in melanospheres. Insets: note, enrichment of melanospheres with cells that express Oct4 (red) and ALDH1/2 (green). Nuclei stained with DAPI (blue), EF. Scale bar 200µm.

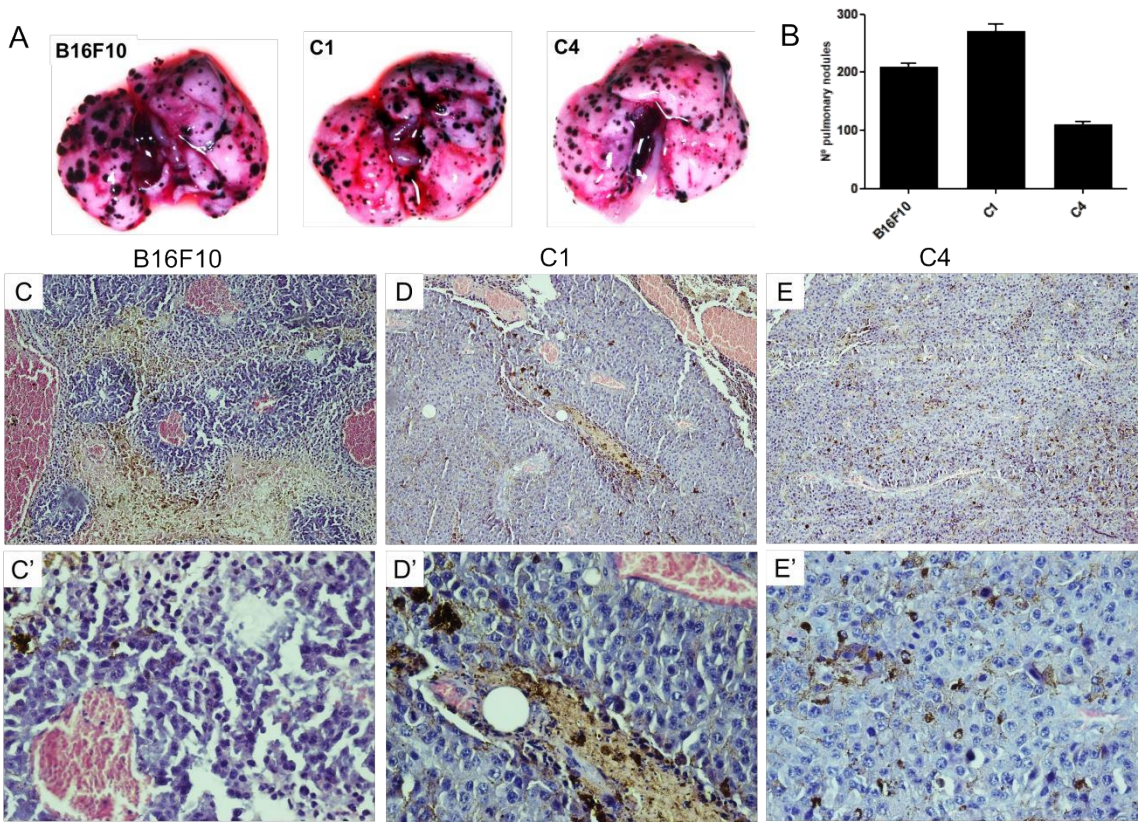

Figure 7: B16F10, C1 and C4 metastatic and tumorigenic capacity. (**A**) Lungs isolated from animals 14 days after intravenous cells implantation. Lung nodules (black) can be clearly observed. (**B**) Quantification of lung nodules demonstrates relatively high number of nodules in B16F10 and low in C4. (**C-E'**) Histological analysis of tumor cuts (5µk) stained with hematoxylin and eosin (HE). The tumors were isolated fifteen days after subcutaneously transplantation of B16F10, C1 and C4 cells into dorsal flanks of mice. Histological analysis demonstrates vascularization, necrosis areas and intense nuclear chromatin staining in tumor tissues; B16F10 cell line, in particular, shows more tissue necrosis, infiltrate and vessels (**C, C'**). C1 (**D, D'**) has less necrosis and infiltrate, cells with biggest nucleus, while C4 (**E, E'**) shows more stroma. **C-E`**- light microscopy. Magnification of 100× and 400×.

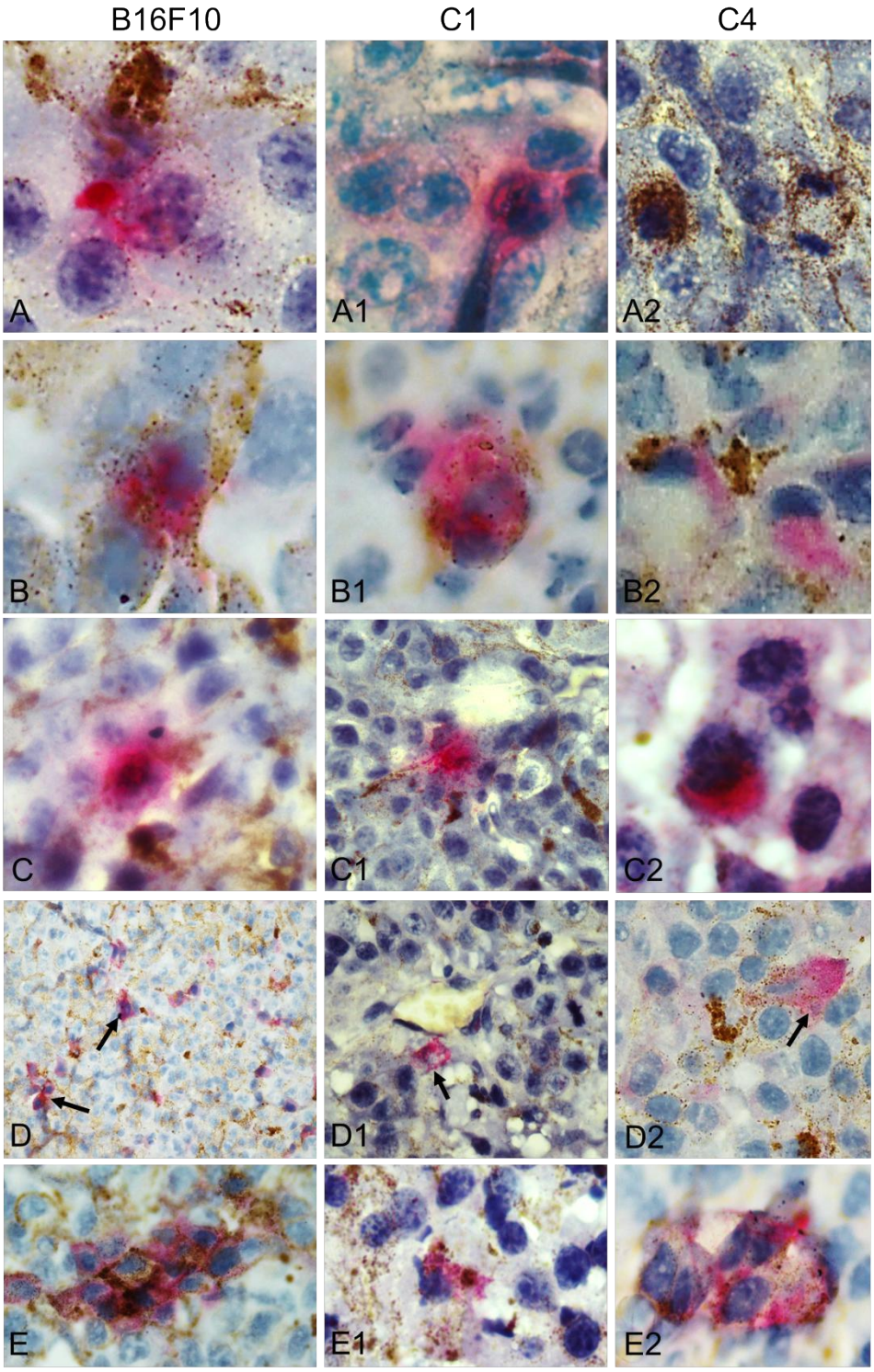

Figure 8: Immunohistochemistry: *in vivo* expression of stem cell-associated transcription factors and ALDH1/2 in tumors obtained after transplantation B16F10, C1 and C4 cells in mice. In (**A, B and C**) Oct4 expression observed in B16F10, C1 and C4 cells, respectively. In (**D**) ALDH1/2 expression observed in B16F10, C1 and C4 cells at low magnification. In (**E**) ALDH1/2 expression observed in

B16F10, C1 and C4 cells at high magnification. Cuts (5µk) stained with HE. Inset in (**D**) shows secondary antibody control: the primary antibody was eliminated. Light microscopy.

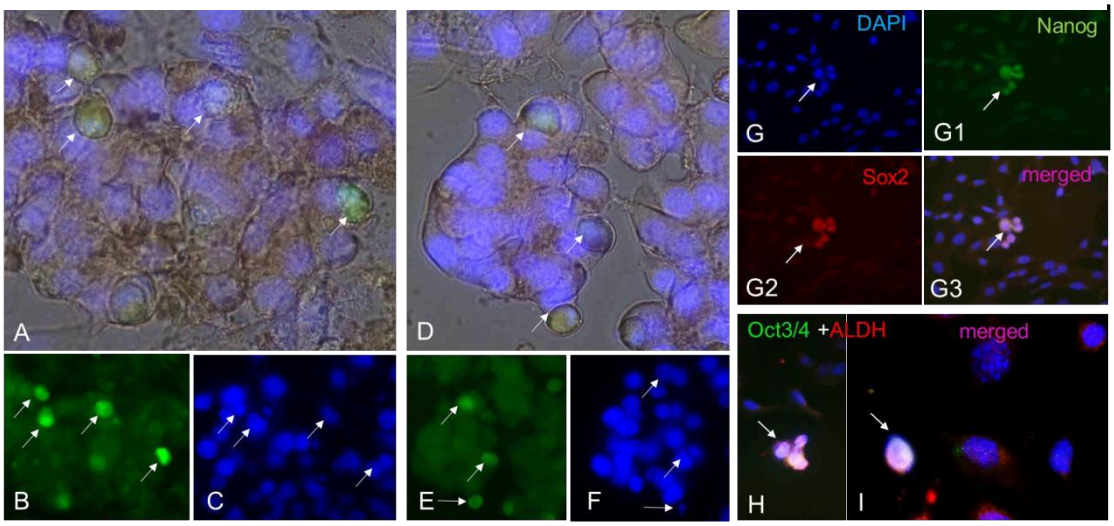

Figure S1: Representative figure shows the growth of pluripotency markers positive cells onto other cancer cells. (**A**) Shows Sox2+ cells in C3, overlapping of EF+PC; in (**B and C**) the same as in **A** (EF). (**D**) Demonstrates Oct4+ positive cells in B16F10, overlapping of EF+PC; in (**E-F**) the same as in D (EF). (**G-G3**) Show expression of Nanog and Sox2 (while arrow) in human melanoma A2058 cell line. Merged image in (**G3**) demonstrates overlapping of Nanog and Sox2 expression. (**H**) Evidences the expression of Oct3/4 and ALDH1/2 (merged) in human melanoma A2058 cell line (while arrow). (**I**) Shows same as in (**H**) in SK-MEL-28 human melanoma cells. Scale bars: 10 µM.

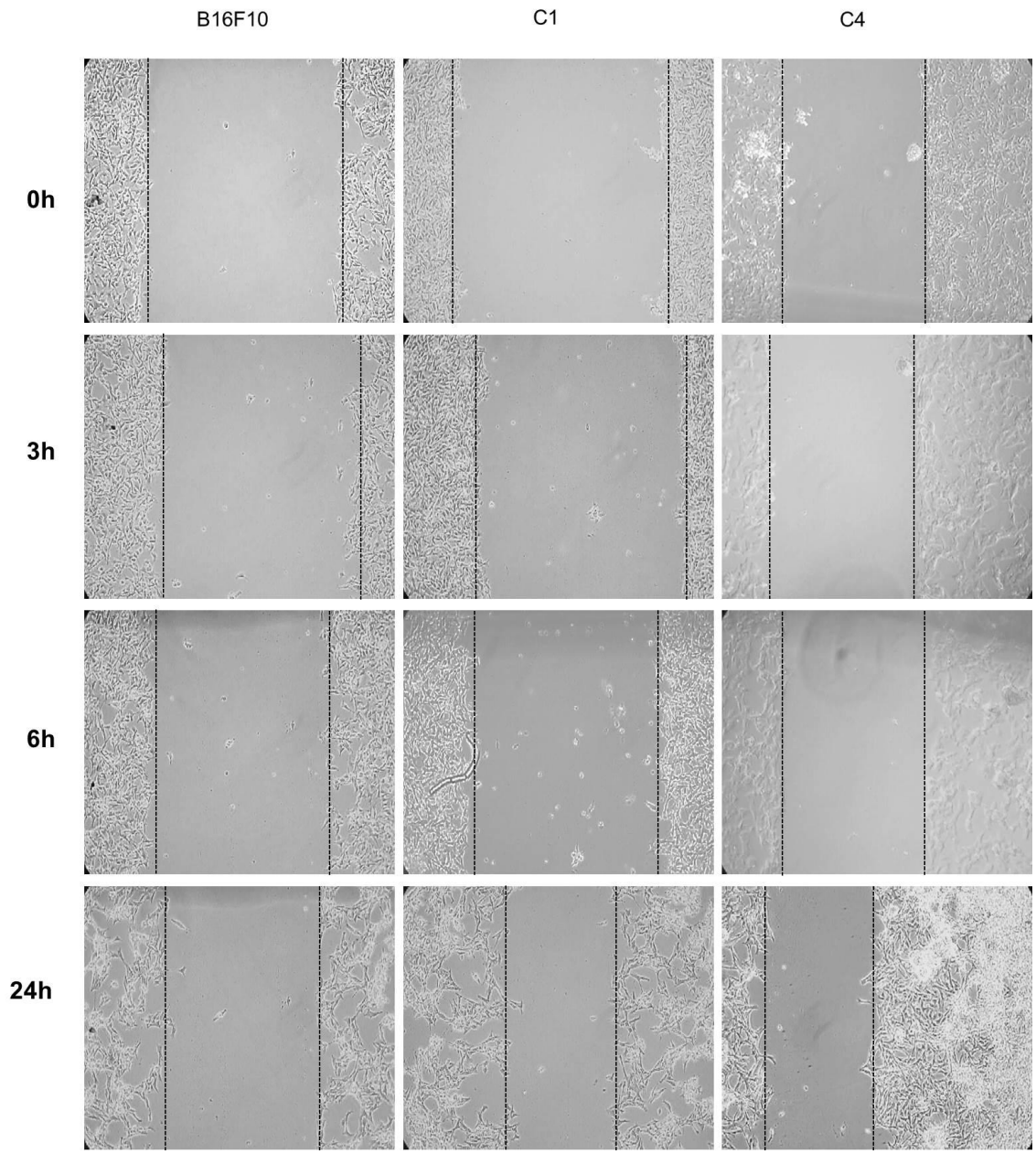

Figure S2: Morphological presentation of cell migration assay. Cell monolayer was wounded at 3 hours, 6 hours and 24 hours. Relative wound closure ability was determined by measuring the gray scale of the wounds. x ± s, n = 3. Magnification, 100×.

Table 3. Resume

FEATURES	**Pluripotency Markers**			**CSC Marker**	**Melanin Level**	**Drug Resistance**	**Clonogenic Capacity**	**Sphere Formation**			**Actin**	**Vinculin**	**Migration**	***In vivo* metastasis**	**Tumorigenicity**	
CELLS	*Oct4*	*Sox2*	*Nanog*	*ALDH*											*Vessels*	*Melanin*
B16F10	S	L	L	S	L	L	S	SM		IM	L	L	L	L	S	S
C1	S	S	L	S	L	S	L	SM		IM	IM	IM	S	S	IM	IM
C4	S	S	L	L	S	S	S	SM	IM	B	S	S	IM	L	L	L

S – strong; L- low; SM – small, IM – intermediate; B – big

4. Discussão

As CSCs e CTEs compartilham muitas propriedades comuns, incluindo metabolismo, inibição da diferenciação, autorrenovação e plasticidade fenotípica. Além disso, as CSCs têm a capacidade de formar tumores de novo e potencial de repopulação (13).

A pluripotencialidade das CTEs é determinada pela ação de vias de sinalização que respondem a estímulos externos, fatores de transcrição intrinsecamente expressos capazes de regular a expressão gênica. Esta rede transcricional das CTEs está centrada na tríade de reguladores da pluripotência Oct4, Sox2 e Nanog (24,20).

Recentemente, a participação de fatores de *stemness* ("tronculares") na tumorigêneses recebeu atenção especial, particularmente depois da demonstração que a introdução de quatro fatores de pluripotência em células somáticas é capaz de reprogramar as células a um estado semelhante às células tronco embrionárias, denominadas células pluripotentes induzidas (iPSCs).

Algumas das CSCs podem expressar fatores típicos de transcrição de células tronco embrionárias indiferenciadas ou células pluripotentes, como Nanog, Oct4 e Sox2. Embora esses fatores de transcrição sejam importantes no início e manutenção do câncer, seu papel na carcinogênese e na metástase e suas implicações na heterogeneidade e resistência aos medicamentos ainda são pouco conhecidos.

O modelo das CSCs argumenta que apenas uma fração de células da massa tumoral é dotada da capacidade de iniciar e sustentar o crescimento tumoral. Acredita-se que estas células comportam-se como suas correspondentes nos tecidos não-tumorais, principalmente em termos de autorrenovação e também originando uma população heterogênea e hierarquicamente organizada (25).

Embora vários eventos possam estar relacionados com a diversidade ou heterogeneidade tumoral, tais como flutuações estocásticas de genes regulatórios, *turnover* proteico, partição dos constituintes celulares, microambiente e divisões celulares assimétricas, os mecanismos que regulam este fenômeno ainda não são bem compreendidos (26).

A reprogramação de células tumorais, sobretudo das CSCs é uma estratégia interessante para compreensão de fenômenos que levam ao desenvolvimento da heterogeneidade. Assim, utilizando técnicas de transfecção não integrativas, nós realizamos a reprogramação de células de melanoma, que embora não tenha apresentado

reprogramação completa para o estado pluripotente, demonstrou que é possível reprogramar estas células.

O microambiente tumoral pode favorecer a reprogramação celular, onde as células assumem novos fenótipos e funções, resultando em alterações moleculares e/ou epigenéticas. Essas alterações estão intimamente relacionadas à transição epitélio-mesenquima (EMT), um processo patológico complexo no qual células epiteliais tumorais reprimem a expressão de genes que codificam proteínas epiteliais associadas à adesão celular (E-caderina, citoqueratina, desmoplaquina, etc.) e ativam a expressão de genes que codificam proteínas mesenquimais (N-caderina, vimentina, fibronectina, metaloproteinases etc.), conferindo perda de polaridade e migração celular (27,28).

O microambiente de células tronco embrionárias, presumivelmente imitando isso no blastocisto, pode contribuir para a supressão do crescimento celular descontrolado no estado pluripotente; isso ajuda a manter o equilíbrio entre a autorrenovação e a diferenciação. Células de melanoma agressivas foram reprogramadas em células semelhantes a melanócitos e a invasividade foi reduzida, pelo menos em parte, por cultivo das células em Matrigel que foi condicionado por CTE humanas, sugerindo sinais supressivos e anti-invasivos associados ao microambiente (29). O melanoma e células de carcinoma de mama expressam Nodal, que é essencial para a pluripotência de CTE humanas, mas esses cânceres não expressam Lefty, um inibidor de Nodal, que é expresso em CTE humanas. A exposição das linhagens de células tumorais a Matrigel condicionado por CTE resultou em uma diminuição na tumorigênese acompanhada de uma redução da clonogenicidade e de um aumento da apoptose, diretamente associada com a secreção de Lefty em CTE humanas células (30). Em conclusão, os primeiros sinais de desenvolvimento regulam naturalmente proto-oncogenes de modo que sua expressão pode ser suprimida até um estágio de desenvolvimento apropriado onde os genes funcionam. Concordantemente, a mudança do ambiente embrionário inicial e a imitação de tais ambientes durante a cultura de CTE pode suprimir fenótipos oncogênicos de células tumorais. Em nosso artigo sobre reprogramação chegamos ao mesmo modelo de inibição do desenvolvimento dos tumores, bem como a diminuição de sua proliferação, gerando células reprogramadas de melanoma murino menos agressivas. Essas células representam um modelo interessante para estudar o mecanismo de malignidade celular, além de fornecer uma nova ferramenta para a triagem de medicamentos contra o câncer.

Enquanto a expressão ectópica coordenada de Oct4, Sox2, Klf-4 e c-Myc (OSKM) induz a reprogramação para a pluripotência de células somáticas (21), a ativação de fatores individuais de pluripotência pode contribuir com a tumorigênese. Por exemplo, a expressão de OCT4 em camundongos iniciou displasia impedindo a diferenciação de linhagens multipotentes (31). A expressão ectópica de OCT4 em células de melanoma humano produziu um câncer mais agressivo, gerando CSCs-like de melanoma (32). Além disso, enquanto a reprogramação à pluripotência em camundongos pode produzir teratomas bem diferenciados (33), a reprogramação parcial produz tumores (34). Esses achados indicam que os fatores de transcrição de pluripotência estão integrados a redes que governam os fenótipos do câncer. Todos esses trabalhos vão de encontro ao nosso artigo de reprogramação, novo ponto intermediário no processo de reprogramação, que pode servir de base para futuros estudos sobre a biologia do câncer, a associação entre pluripotência e células tumorais e com as CSC. A atenuação de tais fatores pode ocorrer de forma transitória durante a reprogramação à pluripotência, e a liberação da pluripotência pode espelhar a progressão para o fenótipo do câncer visto *in vivo*.

Células tumorais são reprogramadas de forma muito ineficiente e apenas um subconjunto dos cânceres são passíveis de reprogramação. Certas mutações como, hipometilações (35), em Brg1 (36) podem tornar as células tumorais imunes à reprogramação. Mas estes tumores podem gerar ou já possuir células com o fenótipo tronco.

Assim, apesar das dificuldades e ressalvas na geração de modelos iPSCs de câncer nossos trabalhos fornecem novos “insights” sobre a progressão e a biologia da doença. Bem como entender a plasticidade das CSCs e sua relação com a pluripotência não somente em melanomas, mas também em outros tipos de canceres humanos.

5. Conclusões

- Foi possível realizar a reprogramação das células tumorais através da transfecção não viral com fatores de transcrição para células pluripotentes induzidas, esses resultados geraram dados interessantes e estes foram publicados.
- Foi possível isolar diferentes clones a partir da linhagem B16F10, os quais demonstraram diferentes padrões de proliferação celular, expressão de marcadores, tumorigenicidade, capacidade metastática e resistência a drogas. Este caráter heterogêneo e divergente entre os clones ocorreu espontaneamente sob as mesmas condições de cultura evidenciando a plasticidade tumoral e o ressurgimento de células com o fenótipo tronco (características de autorrenovação pelo padrão de divisão, crescimento e expressão de fatores de transcrição).
- As linhagens de melanoma possuem células que são positivas para marcadores de células tronco, e estas apresentam algumas características diferenciadas das demais células.
- Estabelecemos dois modelos para estudar a relação da pluripotência em melanomas, tanto de forma induzida como de forma espontânea *in vitro,* onde a pluripotência pode ser um fator de modulação dessas células, sendo uma estratégia para manter ou reverter o *stemness* do câncer.

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