Universidade de Aveiro
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Induced Pluripotent Stem Cells – based cancer immunotherapy

Células estaminais pluripotentes induzidas – imunoterapia antitumoral

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Bruno Bernardes de Jesus, professor auxiliar, e coorientação do Professor Doutor Bruno Neves, professor auxiliar, no Departamento de Ciências Médicas da Universidade de Aveiro.

À memória dos meus avós, José e Arminda.

o júri

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palavras-chave iPSC, ESC, Imunoterapia, Vacina contra a cancro, Antigénios tumorais

resumo

O cancro continua a ser uma das principais causas de morte em todo o mundo, principalmente devido à resistência adquirida aos tratamentos atualmente implementados. Estudos recentes demonstraram que as células estaminais embrionárias (ESC) e as células tumorais partilham algumas características, como antigénios semelhantes ou fatores de crescimento angiogénico. Estas descobertas sugerem que as ESC podem ser utilizadas como fatores de imunização para a promoção de respostas antitumorais.

Neste trabalho foram utilizadas células TNGA, que são uma linha celular de células estaminais embrionárias de ratinho (mESC), que foram sujeitas a diferentes condições de cultura de forma a tentar obter estados ground e primed de pluripotência. Para isso foram utilizados três fatores de pluripotência fundamentais, LIF e dois inibidores, PD e CHIR.

Inicialmente o objetivo foi avaliar a morfologia das células quando submetidas a estímulos diferentes, e verificou-se que, permanecendo em meio com LIF e 2i as células alcançavam um estado ground de pluripotência e, quando se retirava o 2i, as células revertiam para um estado primed de diferenciação. De seguida, foram analisados 7 genes diferentes por RT-qPCR que, em análises de RNA-seq anteriores, demonstraram estar mais expressos quando as células iniciavam processos de diferenciação, podendo, por isso, ter relevância como possíveis novos antigénios tumorais. Os resultados confirmaram que todos os genes têm uma maior expressão nas condições LIF. Foram também realizados ensaios de Western-Blot para avaliar a expressão de *Nanog*, um marcador de pluripotência, e validou-se que a sua expressão é aumentada nas condições de pluripotência, LIF/2i. Para complementar estes resultados, foram feitos ensaios de transfeção de alguns dos genes testados, para perceber se têm, realmente, alguma influência na promoção da diferenciação das células, sendo que, para o silenciamento do gene *c-Myc*, os resultados demonstraram um aumento no número de células que reverteram processos de diferenciação. Fez-se ainda uma análise de proteómica onde se comparou a linha celular de cancro da mama, E0771, às células TNGA em diferentes condições de cultura, apesar dos resultados não terem sido conclusivos, verificou-se uma tendência para a similaridade entre os padrões dos estados primed e da linha tumoral.

Alguns dos resultados obtidos confirmaram a bibliografia existente, sendo promissores para esta área de investigação, contudo é necessário repetir os ensaios e fazer estudos complementares para perceber se estes genes são seguros e se conseguem promover uma resposta imune num organismo.

keywords iPSC, ESC, Imuntherapy, Cancer Vaccine, Tumor antigens

abstract

Cancer is still one of the leading causes of death worldwide, mainly due to its resistance to current treatments. Recent studies have shown that embryonic stem cells (ESC) and tumor cells share some characteristics such as similar antigens, angiogenic growth factors and also lead to cell apoptosis. These findings suggest that ESC can be used as immunizing factors to promote antitumor responses.

In this work, TNGA cells were used, a mouse embryonic stem cell (mESC) cell line, which were subjected to different culture conditions in order to try to obtain ground and primed pluripotency states. For this, three fundamental pluripotency factors, LIF and two inhibitors, PD and CHIR, were used.

Initially, the objective was to evaluate the morphology of cells when subjected to different stimuli, and it was verified that, when subjected to medium with LIF and 2i, the cells reached a ground state of pluripotency and, when the 2i was removed, the cells reverted to a primed state of differentiation. In addition to this evaluation, 7 different genes were analyzed by RT-qPCR which, in previous RNA-seq analyses, showed to be more expressed when cells started differentiating processes and, therefore, may have relevance as possible new tumor antigens. The results confirmed that all genes have a higher expression under LIF conditions. Western-Blot assays were also performed to assess the expression of *Nanog*, a pluripotency marker, in cells under different conditions, verifying that its expression is increased under pluripotency stimuli (LIF/2i). To complement these results, transfection assays were carried out on some of the genes analyzed, to see if they certainly have any influence in promoting cell differentiation, and for the *c-Myc* gene silencing, the results showed an increase in the number of cells that reversed differentiation processes. A proteomics analysis was also performed comparing the breast cancer cell line, E0771, to TNGA cells in different culture conditions, although the results were not conclusive, there was verified a tendency towards the similarity between the patterns of primed TNGA cells and the tumor cell line.

Some of the results obtained confirmed the existing bibliography, being promising for this study area but, however it is necessary to repeat some assays and carry out additional studies to understand if these tested genes are safe and if they can promote an immune response in an organism.

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Abbreviations

2i – Two Inhibitors

AA – Antibiotic Antimycotic

APCs – Antigen-Presenting Cells

ASCs – Adult Stem Cells

B cell – Bone marrow derived cell

BCA – Bicinchoninic acid

CAR – Chimeric Antigen Receptor

CD4 – Cluster of Differentiation 4

CD8 – Cluster of Differentiation 8

cDNA – Complementary DNA

Cldn6 – Claudin 6

CpG – Cytosine-phosphorothioate-guanine

CSCs – Cancer Stem Cells

CTLA-4 – Cytotoxic T Lymphocyte Antigen 4

DC – Dendritic Cell

dLN – Draining lymph nodes

DMEM – Gibco Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic acid

dNPs - Deoxyribonucleotide triphosphate

ECL – Electrochemiluminescence

ESCs – Embryonic Stem Cells

FBS – Fetal Bovine Serum

GFP – Green Fluorescent Protein

GSK3 – Glycogen synthase kinase-3

GSK3β - Glycogen Synthase Kinase-3 Beta

hESCs – human Embryonic Stem Cells

hiPSC – Human induced Pluripotent Stem Cells

HRP – Horseradish peroxidase

IARC – International Agency for Research on Cancer

IFN – Interferon

IFN-γ – interferon-gamma

IL – Interleukin

ILC –Innate Lymphoid Cell

iPSC – Induced Pluripotent Stem Cells

JAK-STAT3 – Janus kinase-signal transducer and activator of transcription 3

KD – Knockdown

Klf4 – Krüppel-like factor 4

KSR – Knockout Serum Replacement

LIF – Leukemia Inhibitory Factor

LNA – Locked Nucleic Acid

lncRNA – long noncoding RNA

MAPK – Mitogen-activated protein kinase

mESCs – mouse Embryonic Stem Cells

MSCs – Mesenchymal Stem Cells

NEAA – Non-essential amino acids

NK – Natural killer

NKG2D – Natural Killer Group 2D

Oct4 – Octamer-binding transcription factor 4

PCA - Principal Component Analysis

PD-1 –Programmed cell death protein 1

pH – Potential of hydrogen

qRT-PCR – Quantitative Real-time PCR

RNA - Bibonucleic acid

RNA-seq – RNA sequencing

ROS – Reactive Oxygen Species

RPE – Retinal Pigment Epithelium

rRNA – Ribosomal RNA

SDS-PAGE – Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

si – Small interference

Sox2 – sex-determining region Y-Box transcription factor 2

SSCs – Somatic Stem Cells

T cell – Thymus derived cell

TCR – T cell receptor

Th1 – T Helper Cell Type 1

Wt1 – Wilms' tumor 1

Wt1os – Wilms tumor 1 homolog, opposite strand

Zeb2 – Zinc finger E- box binding homeobox 2

Zeb2-NAT – Zeb2 antisense RNA 1

1. Introduction

1.1 Framework

This work was part of the second-year internship of my master's degree in Molecular and Cellular Biology of the Department of Biology of University of Aveiro. It had the duration of one year and was held at the Institute of Biomedicine of University of Aveiro under the supervision of Prof. Dr. Bruno de Jesus.

The work was inserted in the use of TNGA cells, a mouse embryonic stem cell (mESC) cell line and understand how their expression and morphology changed with the promotion or elimination of some stimuli in the growth medium, and what was intended to be verified was whether their pluripotency levels were altered by changing the optimal conditions. This cell line has the particularity of having a coding sequence for a green fluorescent protein, GFP, which is inserted after the initiation codon of the *Nanog* gene, which is associated with the degree of pluripotency of the cells. Consequently, through microscopy it was possible to read their levels, by the presence and the intensity of the green marker.

Studies reported that embryonic stem cells, when subjected to differentiation processes, resemble cancer stem cells, especially in sharing some tumor antigens. This discovery led to the study of a potential iPSC-based vaccine with antitumor properties, which led to the formulation of the main and global objective of this work, which is the development of a patient specific iPSC treatment in order to attempt to increase the immune responses to eliminate cancer cells.

As the study is in primary stage, we also needed to analyze some molecular processes because, as there is evidence that iPSCs resemble cancer stem cells when they begin to lose their pluripotency, it was also necessary to realize what type of genes are more and less expressed under these conditions.

Herewith, this report is organized with an introductory part with theoretical concepts that elucidate the importance and the reasons why this study is relevant, understanding the interest in this cell line and its possible applicability in anticancer therapies. This section also includes a chapter of a review article in which I participate as a co-author but has not been submitted yet. The subject is about "Aging barriers in stem cell mobilization and cardiac transdifferentiation" where I spoke specifically about "Aging of stem cells and iPSC". After this introductory part follows the methodology used, the results that were obtained with its consequent discussion and, finally, the general conclusions retained with this work.

1.2 Bibliographic review

According to the International Agency for Research on Cancer (IARC), in 2020, there were an estimated 19.3 million new cancer cases and nearly 10.0 million cancer-related deaths, worldwide. In Portugal, where the total population is just over 10 million habitants, 60 467 new cases of cancer were registered, and 30 168 patients were declared dead (Ferlay et al., 2021)**.**

Cancer is defined by the accumulation of various genetic modifications, with the consequent loss of normal regulatory processes at the cellular level (Taefehshokr, Baradaran, Baghbanzadeh, & Taefehshokr, 2020)**.**

Among all the methods used to treat cancer, the ones that continue to be the most conventional are surgery, chemotherapy, and radiotherapy, which, despite having some positive results for some patients, they are not fully effective in eliminating the tumor cells or the cancer stem cells that support a developing tumor. In addition, another main problem with these therapies is their lack of specificity and the damage they cause to healthy tissues (Sachamitr, Hackett, & Fairchild, 2014). Given its inherent specificity, adaptability and ability to generate a memory response, immunotherapy as a treatment for cancer, promises to be more effective and long-lasting than classic treatment modalities (Riley, June, Langer, & Mitchell, 2019).

1.2.1 Immune responses and cancer therapies

When it comes to immune responses of the human immunologic system, there are two ways in which it is possible to activate an immune response when recognizing the presence of malignant cells, first of all, the cells that belong to the innate responses of the immune system, such as macrophages and neutrophils (Disis, 2010). Innate immunity is present throughout life, and includes physical barriers such as skin, mucous membranes and physiological barriers, for example, the pH variations in different parts of the human body and temperature (Abbott & Ustoyev, 2019). Furthermore, the innate response involves different cell types of the myeloid line, such as dendritic cells (DCs), monocytes and innate lymphoid cells (ILCs) such as natural killer (NK) cells (Demaria et al., 2019).

The adaptive response is developed when antigens are recognized by B (bone marrow-derived) and T (thymus-derived) cells in the presence of co-stimulatory signals, triggering the immune response by the proliferation of antigen-specific lymphocytes

(Adam, Odhav, & Bhoola, 2003). In this response, the main cells mediators are the CD8+ T cells, which are responsible for eliminating tumor cells through the production of cytokines or molecules that induce apoptosis, and the T Helper Cell Type 1 (Th1) cytokine-oriented CD4+ lymphocytes, which amplify the antitumor response by helping CD8+ T cells secreting cytokines such as interferons (IFN) (Giraldo, Becht, Vano, Sautès-fridman, & Fridman, 2015).

These two systems are distinct, but the innate and adaptive responses are often correlated. While T cells, from adaptive responses, recognize tumor cells through specific receptors for their antigens, innate response cells express a set of germline-encoded receptors. However, adaptive immune system cells also express some germline receptors, such the Natural Killer Group 2D (NKG2D) receptor of CD8+ T cells. In addition, sometimes the adaptive response is amplified or even dependent on some innate recognition mechanisms, such as the example of NK cells that induce the maturation of DCs, which can amplify the T cell response (Marcus et al., 2014).

Among all the cellular and molecular changes that exist in the presence of tumor cells, one of the most important, and which opened doors for the implementation of new therapies using the patient's immune system, is the fact that these cells express some foreign antigens to the body, some of them neo-antigens, which make tumor cells detectable by the immune system, directing them to their destruction (Farkona, Diamandis, & Blasutig, 2016). Until today, several clinical studies have been carried out to stimulate the immune system into the recognition of tumor cells, such as the use of antibodies to block cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), and also by chimeric antigen receptor (CAR) T cells (Y. Yang, 2015).

Neo-antigens are derived from somatic mutations and are, in fact, good targets for immunotherapy, because they do not show off-target reactivity, and is expected central T cell tolerance. Although the accumulation of somatic mutations are a marker of tumor progression, only a minority is under positive selection, which means that there is this excessive variability between cancers and patients, which implicates that the spontaneous elimination of these cells, mediated by the immune system, does not occur in most patients (Jitske, Miranda, & Verdegaal, 2018) (Woo, Corrales, & Gajewski, 2015). Consequently, during the last two decades several clinical studies have been trying to address the development of new therapies to combat various types of diseases, including cancer (Hawsawi, Al-zahrani, Mavromatis, Saggu, & Oyouni, 2018).

1.2.2 Embryonic and Adult Stem Cells

Stem cells are undifferentiated cells whose main characteristics are their ability to extensively proliferate *in vitro* (self-renewal), usually come from a single cell (clonality) and differentiate into different types of cells or tissues (potency) (Kolios & Moodley, 2012).

There are two major groups of stem cells, adult or somatic stem cells and embryonic stem cells.

Adult stem cells (ASC) or somatic stem cells (SSC) are present in most tissues and are responsible for their replacement during life cycle, organizing themselves into a specialized structure, called a niche. They provide an expanded population of proliferating tissue that differentiates into mature cells with specific functions and, eventually, cease to proliferate. They are essential for the maintenance of homeostasis of the tissues in which they are present (Dulak, Szade, Szade, Nowak, & Józkowicz, 2015) (Gurusamy, Alsayari, Rajasingh, & Rajasingh, 2018). Currently, there are different known types of ASC with different therapeutic applications. Among them, the most used in clinical studies are mesenchymal stem cells (MSCs), as they exist in several tissues, being first discovered in bone marrow, but later found in other tissues such as the adipose, muscle, peripheral blood, and perinatal tissues. They have the ability to differentiate into different cell types and also have low immunogenicity (Han, Li, Zhang, Han, & Chang, 2019) (Moreira et al., 2018).

Some preclinical studies have shown that MSCs can be potential alternatives for anticancer therapies, due to their properties of migrating to damaged tissues and exerting immunosuppressive, anti-apoptotic, anti-fibrotic, angiogenic and anti-inflammatory functions, due to grafted cells that release bioactive mediators such as growth factors, cytokines, and extracellular vesicles (Lin et al., 2019) (Hmadcha, Martin-montalvo, Gauthier, Soria, & Capilla-Gonzalez, 2020).

Studies in the field of neurodegenerative diseases, have also been carried out, mainly due to the ability of MSCs to differentiate into neurons and glial cells under specific conditions (Lo Furno, Mannino, & Giuffrida, 2017). In Alzheimer's disease, one of the most prevalent neurodegenerative disease, studies have shown that MSCs are able to secrete some neurotrophic factors that, subsequently, exert neuroprotective effects, in addition to modulating neuroinflammation (Zhang, Dong, & Zhang, 2020). Additionally, some clinical trials are also in place using MSCs to treat diseases such as Parkinson's

(Filho et al., 2018) and also for Amyotrophic Lateral Sclerosis (Gugliandolo, Bramanti, & Mazzon, 2019).

Stem cells from the embryonic line, embryonic stem cells (ESCs), were first discovered in mouse models precisely 40 years ago, in 1981, by Evans and Kaufman, where they established the isolation of tissues from pluripotent cell lines by culturing mouse blastocysts. They were able to verify, both *in vitro* and *in vivo* that, after inoculating the cells into a mouse tumor, they are capable to remain in a differentiated state (Chen-Konak, Fine, & Levenberg, 2011)(Kaufman & Evans, 1981). In humans, the first time they were able to successfully create cell lines of ESCs from human blastocysts was in 1998. They used embryos from patients who, after undergoing *in vitro* fertilization, donated them for the study, and from this point they isolated the cells from the inner cell mass of the blastocyst. The group realize that these cells, when placed in culture, were capable to maintain a pluripotent state (Thomson et al., 1998).

The discovery of ESCs led to a great advance in the study of their potential to be applied in cell therapies for the treatment of various types of pathologies, mainly due to their infinite capacity for self-renewal *in vitro* and, with the correct stimuli, it is possible to differentiate them in, theoretically, any cell type of the adult organism (Menendez, Bueno, & Wang, 2006). However, as human ESCs (hESCs) are isolated from human embryos, the only way to isolate these cells is through manipulation of embryos, which raises many ethical problems (Volarevic et al., 2018). In an attempt to overturn this problem, in 2006, K. Takahashi and S. Yamanaka realized that, by inducing four transcription factors, Oct3/4, Klf4, Sox2 and c-Myc in mouse embryonic and adult fibroblasts, the cells began to exhibit the morphology and growth properties of ESCs, demonstrating the same cellular genetic markers. This discovery led to the development of induced pluripotent stem cells (iPSC) (Takahashi & Yamanaka, 2006). The following year, Yamanaka and his group demonstrated that it is possible to differentiate human fibroblasts in iPSC by retroviral transduction of the same factors (Takahashi et al., 2007). At the same time, James Thomson's group confirmed the same results, but inducing different transcription factors, OCT4, SOX2, NANOG and LIN28 (J. Yu et al., 2007).

1.2.3 Aging of stem cells and iPSC

With the evolution in recent years of the quality of life and the progression in medical care, the average standard of living in humans has increased. Data from the World

Health Organization (WHO) estimates that, over the next three decades, the percentage of population over 60 years will increase from 12% to 22%, and nowadays it is already expected that most people will live to, and beyond, that age (Ageing and health, 2018). However, as an organism ages, its susceptibility to acquiring diseases increases (Booth & Brunet, 2016).

Biological aging is characterized by a reduction in the reparative and regenerative potential of organisms at molecular, cellular and tissue levels. These reductions lead to homeostenosis, contributing to decreased physiological functions in response to stress (Khan, Singer, & Vaughan, 2017).

In humans, age is a key factor for the onset of various pathologies, including neurodegenerative and cardiac diseases, diabetes, and cancer. Heart diseases are still one of the main causes of death in people over 65, and can lead to functional changes, such as diastolic dysfunctions in the left ventricle, mainly due to tissue decreased elasticity and the consequent increase in its stiffness, increasing the blood pressure. Changes in electrical functions are also observed, due to a decrease in pacemaker cells in the sinoatrial nodule, which will influence the heart rate (Steenman, 2017) (Wessells & Bodmer, 2007). At the molecular level, mitochondrial functions are one of the most affected processes with age, causing a decrease in ATP production, accumulation of damage in mitochondrial DNA, causing increased amounts of reactive oxygen species to be produced (ROS), telomere dysfunctions and alterations in sirtuin signaling, insulin/insulin-like growth factor-1 (IGF-1) and also in the mammalian target of rapamycin (mTOR) signaling (Steenman, 2017) (Lane, Hilsabeck, & Rea, 2015) (Boyette & Tuan, 2014). As the heart is an organ with a high metabolic rate, these alterations make it particularly vulnerable to damage (Dai, Chen, Johnson, Szeto, & Rabinovitch, 2012).

With advancing age, there's also a decrease in the regenerative ability of adult/somatic stem cells, which leads to a decreased response to tissue damage in the organs in which they operate. Although they are often described as immortal, there is already evidence that somatic stem cells (e.g., hematopoietic stem cells, intestinal stem cells, satellite cells, skin stem cells, and germline stem cells) are also susceptible to accumulation of damage, which can lead to cell death, senescence, and loss of its regeneration potential (Schultz & Sinclair, 2016) (Shufian et al., 2017) (Cianflone et al., 2020). Despite this, there are other groups of stem cells that have been studied, showing great promise for therapies to combat age-related diseases, mainly to replace cells that have been lost or damaged and no longer perform their function (Yabut & Bernstein, 2011).

Nowadays, stem cells are used in cell replacement therapies. Great promises for the treatment of heart diseases are already being studied using different types of stem cells such as embryonic stem cells (ESC) (Müller, Lemcke, & David, 2018). The most important goal is that the application of these cells could be less aggressive than the current implemented therapies, and that it allows a more efficient way of obtaining different cell types for regenerative purposes, such as human cardiomyocytes for myocardial regeneration (Rikhtegar, Pezeshkian, Dolati, Safaie, & Afrasiabi, 2019).

ESCs have two unique properties that make them a good study model. They continuously self-renew *in vitro,* and with the right stimuli, they manage to maintain their undifferentiated state, being able, theoretically, to differentiate into any cell type (Menendez et al., 2006). However, in the case of human ESCs (hESCs), there are many ethical problems associated, as they are isolated from human embryos (Kimbrel, 2020). As such, in 2006, a work by Shinya Yamanaka and Kazutoshi Takahashi discovered that it is possible to reprogram differentiated cells to a pluripotent state. They realized that, by inducing the expression of transcription factors Oct3/4, Sox2, c-Myc and Klf4, under ESC culture conditions, it is possible to convert mouse fibroblasts into a cell type with the same characteristics as ESC, which they called induced pluripotent stem cells (iPSC) (Zakrzewski, Dobrzy, Szymonowicz, & Rybak, 2019) (Takahashi & Yamanaka, 2006). A year later, the same authors were able to differentiate human fibroblasts into iPSC by retroviral transduction of the same factors. Although these two cell lines were able to differentiate into iPSC, it was observed that c-Myc expression in hESC led to cell death and differentiation (Takahashi et al., 2007). In a later study, 14 sets of genes were tested, and *OCT4*, *SOX2*, *NANOG* and *LIN28* demonstrated to be involved in the reprogramming of somatic cells into pluripotent stem cells with ESC characteristics (J. Yu et al., 2007).

In 2016, Shiba et al. found that it is possible for cardiomyocytes derived from allogeneic iPSCs to improve contractile function in a non-human primate myocardial infarction model for, at least, 12 days. However, despite not being lethal, ventricular tachycardia was observed, which can translate into a reduced portion of grafted cardiomyocytes (Shiba et al., 2016). Despite this, in recent years, there have been advances in this field, and there are already some clinical studies in preparation, in Japan and Germany, where iPSC-derived cardiomyocytes will be transplanted in patients with heart failure. More recently, an already registered study (NCT 03763136), with an estimated 5 participants, will use cardiomyocytes derived from allogeneic iPSCs from healthy donors to inject into patients' myocardium at the time of surgery (Desgres & Menasché, 2019).

1.2.4 iPSC and their immunotherapeutic potential

Since its discovery, iPSC has become one of the main and most interesting study models in areas such as regenerative medicine or cell therapies, for the treatment of various pathologies, such as cardiac diseases, neurodegenerative diseases, and even cancer, as it is a cell line with the same characteristics as hESC, but without their technical and ethical complications (Bindhya et al., 2019). In addition, tissues derived through iPSC are less likely to be rejected by the donor organism, mainly because in many therapies using these cells, differentiation is made through the patient's own cells, which makes it a very important and relevant factor in the discovery of disease modeling and for the screening of new drugs (Ye, Swingen, & Zhang, 2013).

As soon as they became public for the scientific community, studies and trials with the use of iPSCs began to be carried out, and one of the first pre-clinical studies was in 2007, a year after its discovery, where they used autologous iPSCs in a mouse model of sickle cell anemia, demonstrating clinical success (Hanna et al., 2007). After that, studies in the area of neurodegenerative diseases began and, through the differentiation of human iPSCs (hiPSC), they managed to arrive at the formation of dopaminergic neurons without signs of neurodegeneration, after transplantation, in mouse models of Parkinson's (Hargus et al., 2010). In a mouse model of spinal cord injury, Satoshi Nori and his group investigated the therapeutic potential of neurosphere-derived hiPSCs and confirmed their differentiation into mature neurons and axonal regrowth leading to significant motor recovery (Nori et al., 2011). In addition to these studies, several experiments have also been carried out for the recovery of mouse models with age-related macular degeneration, where they attempt to convert hESC and hiPSC into photoreceptor cells and retinal pigmented epithelium (RPE), and the cells exhibit important RPE markers and restored some visual functions in the animal models in study (Songstad et al., 2015) (Rowland et al., 2013).

Despite these studies with positive outcomes, there were also several others that demonstrated numerous challenges regarding the use of iPSC, especially when they were trying to differentiate them in cells or tissues for transplantation therapies (Yasuda et al., 2018). Even in the 2007 study by Yamanaka and his group, one of the problems they described is that iPSCs are susceptible to the formation of teratomas and also tumors if transplanted in their undifferentiated pluripotent state *in vivo* (Takahashi et al., 2007). These cells were initially derived by the transduction of four factors and *c-Myc* is one of the most studied oncogenes to date (Albihn, Johnsen, & Arsenian Henriksson,

2010). However, the remaining factors, *Sox2*, *Klf4* and *Oct3/4* have also been found with high expression in some types of tumors (Ben-David & Benvenisty, 2011).

Krüppel-like factor 4 (*Klf4*) is a gene that is both involved in tumor suppression and oncogenicity, depending on the genetic target. It has been shown to be overexpressed in laryngeal squamous cell carcinoma, and they also found that its expression increases in ductal carcinoma of breast cancer, also, when this gene is highly expressed in skin cells, it leads to hyperplasia and dysplasia which can lead to squamous cell carcinoma (Evans & Liu, 2008).

Octamer-binding transcription factor 4 (*Oct4*), a key stemness transcription factor, has also been discovered to be a factor involved in the progression of some tumors, such as lung cancer, by regulating some long noncoding (lnc) RNAs that binds directly to *Oct4* (Jen et al., 2017).

Sex-determining region Y-Box transcription factor 2 (Sox2) is expressed in several types of cancer and can act as a marker for cancer stem cells (CSC), in particular, in osteosarcoma this gene can support a group of tumor cells with the same characteristics as CSC. In this study, after performing the knockdown (KD) of *Sox2*, they were able to verify a regression in the tumorigenic potential of the cells, suppressing their cancerous phenotype (Maurizi, Verma, Gadi, Mansukhani, & Basilico, 2018).

Despite these problems associated with iPSC-based therapies, several improvements have already been made in attempts to decrease the tumorigenic potential of these cells. One of the processes was the generation of iPSC through fibroblasts and mouse liver cells using non-integrated adenoviruses that transiently express the genes involved in iPSC transcription, solving the previous problem because, as the cells were produced through viral expressions, they will integrate into the genome of the cells and, consequently, increase their tumorigenicity (Stadtfeld, Nagaya, Utikal, Weir, & Hochedlinger, 2014). Given the oncogenic characteristics of the *c-Myc* retrovirus, a study was performed where they generated iPSC, also from mouse and human fibroblasts, but using only three transcription factors, *Oct4*, *Sox2* and *Klf4*. Mice derived from these iPSC demonstrated a reduction in the incidence of tumor formation compared to those derived from iPSC generated through the four factors (Nakagawa et al., 2008). Furthermore, efforts were made for the generation of insertion-less or insertion-free iPSCs by using chemical compounds, adenovirus vectors, transposons, plasmids, recombinant proteins, episomal vectors and modified RNA (Okano et al., 2013).

With these improvements in the iPSC-based therapies area, this technology has increased the curiosity in the fields of anticancer therapies. One of the first approaches was the differentiation of iPSCs into immune system cells, such as T cells. They found that iPSCs derived from T lymphocytes maintain the pre-rearranged T cell receptor (TCR) gene, which suggests that these cells could be induced to differentiate into functional T cells, that is, taking advantage of the epigenetic memory it would be possible to reprogram the T cells with some tumor antigens in iPSC and differentiate them again in order to expand before clinical use (Sharkis, Jones, Civin, & Jang, 2012).

Another approach was to use iPSCs to develop a vaccine. Knowing that iPSCs share some characteristics with CSCs, including some oncofetal genes, Yi Li and her group, using a mouse model of colon cancer, hypothesized that the immune response against these antigens would cross-react with the cancer antigens, and this would generate protective immunity against tumors. This, in fact, generated a humoral immune response in the animal models, causing an expansion of interferon-gamma (IFN-γ) producing cells and a reduction in myeloid suppressor cells (Li, Zeng, Xu, Liu, & Li, 2009). Although the results have shown greater success, studies need to be performed as this is an area with great potential for cancer therapies

1.2.5 iPSC based-vaccine for cancer therapy

Given the characteristics of iPSC, it did not take much time for their interest and study in the possible implementation of this cell line in cancer therapies.

Cancer stem cells are identified as cells that are involved in tumor initiation and have the function of maintaining and developing tumors. As main characteristics, these cells are able to self-renew and differentiate into different cellular tissues, which may explain the heterogeneous characteristics of cancer cells within the tumor. In addition, they also have tumorigenic characteristics, just like iPSCs (Osman et al., 2020) (Friedmann-morvinski & Verma, 2014).

One of the main problems in targeted therapies is due to these cells, as another characteristic, they can overcome anoikis, which is a form of programmed cell death that is induced when cells detached from the surrounding extracellular matrix, which, in this case, justifies their resistance to therapies, such chemotherapy (Chao & Chern, 2018).

These overlapping characteristics between iPSCs and CSCs led researchers to examine patterns of genes expression that might be related to these similarities. These findings, as well as the fact that oncogenes are used in somatic cell reprogramming, lead to the hypothesis that common signaling pathways can be used for the acquisition of pluripotency in cells as well as for tumorigenesis (Kim & Orkin, 2011).

These results led to the development of some strategies with promises in the immunization against tumor cells, such as unlocking some immune checkpoints in order to allow tumor recognition and also make a primary recognition for the immune system for the identification of a higher variety of antigens existing in tumor cells (de Jesus, Neves, Ferreira, & Nóbrega-Pereira, 2020). This strategy of reactivating the immune system has made great improvements through the use of CAR. This is based on the creation of a cancer-specific antigen receptor and coupling this to an effector cell, such as T cells. However, the results obtained in some patients demonstrated a new cancer reappearance, possibly due to the nonspecificity of this therapy, mainly because of the large amount of tumor antigens, which raises the need for research in this study area. In order to overcome this problem associated with CAR therapy, a study by Kooreman and his colleagues demonstrated that, through data analysis by RNA sequencing (RNA-seq), there are genes that are upregulated both in iPSC cell lines as in tumor cell lines. This confirmation led to the hypothesis that an iPSC-based vaccine could be created, capable of developing an immune response in several types of cancer by recognizing of several types of tumor antigens (Kooreman et al., 2018).

In that study, in a mouse model of breast cancer, they routinely created one vaccine per week for a total period of 4 weeks with the combination of irradiated autologous iPSCs with a oligodeoxynucleotide adjuvant, cytosine-phosphorothioate-guanine (CpG) (Kooreman et al., 2018). These are unmethylated synthetic oligonucleotides that are similar to microbial DNA and are able to activate immune responses by activating immune factors such as macrophages, dendritic cells, and B cells (Ouyang et al., 2021). The premise of this trial is that the vaccine is properly processed by antigen-presenting cells (APCs) and that this response is transported to draining lymph nodes (dLN), where the antigens that are associated with the tumor are recognized, and the response is activated by of tumor-specific cytotoxic CD8+ T cells. As a result, Kooreman's group verified that this vaccine induced an immune response, both *in vitro* and *in vivo*, mainly by the antitumor responses generated by the $CD4^+$ T helper and $CD8^+$ cytotoxic T cell (Kooreman et al., 2018).

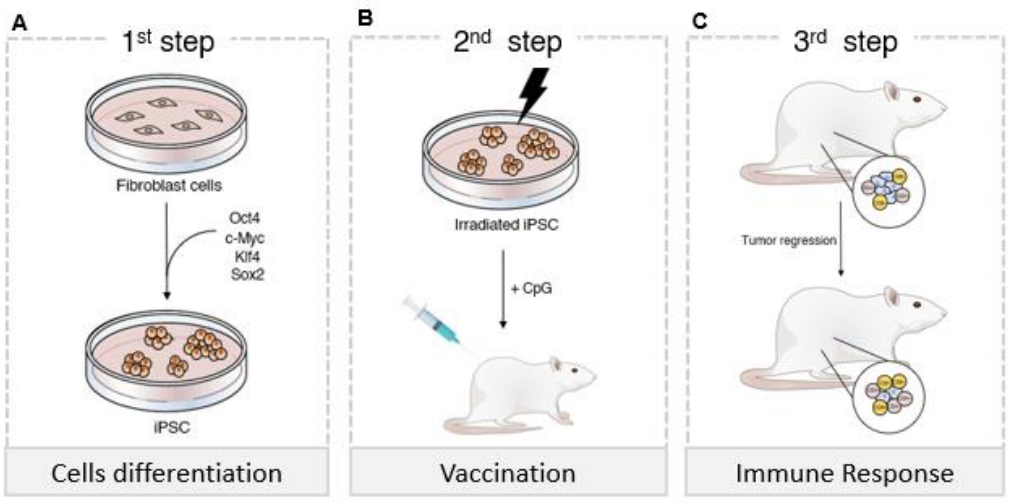

Figure 1 | iPSC-based vaccine for cancer therapy. iPSCs are generated by the reprogramming of fibroblasts by inducing *Oct4*, *c-Myc*, *Klf4* and *Sox2* factors (A). iPSCs share some antigens with tumor cells, so a vaccine based on eradicated miPSC with CpG adjuvant was generated (B). After molecular analysis, it was found that this vaccine triggers the immune system directly to tumor cells by recruiting factors such as CD8+ and CD4+ T cells (Scheme adapted from Goyvaerts & Breckpot, 2018).

Because of the potential of this study, our research group started to be interested whether ESC share the same characteristics with iPSC, regarding the immunotherapeutic potential. Through the analyses of ESC growth under different conditions (RNA-seq - unpublished results) we selected genes being overexpressed in both tumor cells and ESC. The genes studied were *c-Myc*, which is one of the most expressed oncogenes in different types of tumors and is directly related to cell cycle progression, apoptosis and cell transformation (Miller, Thomas, Islam, Muench, & Sedoris, 2013), but is also involved in stem cell proliferation, thus as in its self-renewal and proliferation (Wilson et al., 2004); the *Frat1* gene is also reported to be overexpressed in some cancers and is also a regulator of the Wnt/β-catenin signaling pathway that is involved in stem cell self-renewal (Y. Wang et al., 2006) (Pai et al., 2017); the Claudin 6 (*Cldn6)* gene is a member of the claudin family and an important component of tight junctions, it is also known to be highly expressed in gastric cancer (S. Yu et al., 2019). Studies have also been carried out showing that this gene is expressed in stem cells, more particularly in undifferentiated cells, and its expression decreases when pluripotency markers such as *Oct4*, *Sox2* and *Nanog* are downregulated (Linlin Wang et al., 2012); zinc finger E-box binding homeobox 2 (*Zeb2*) is a transcriptional regulator of DNA binding, and recent studies show that this gene is involved in the complexity of cancer, inducing its proliferation, suppressing

apoptosis and angiogenesis, becoming an important factor in promoting tumor initiation and further development (Fardi, Alivand, Baradaran, Hagh, & Solali, 2019). This factor downregulates E-cadherin and enhances the epithelial-to-mesenchymal transition, which is relevant for its tumorigenic capacity, but also for the fate of stem cells and, in a study where they used mESC, the authors found that this gene is essential for the differentiation capacity of the cells, so when they performed the Zeb2 KD, they verified that the cells maintained the epithelial characteristics when subjected to differentiation (Stryjewska et al., 2017); Zeb2 antisense RNA 1 (*Zeb2-NAT*) is a lncRNA that increases *Zeb2* protein expression by regulating splicing and inhibiting the expression of E-cadherin in mesenchymal cells which, in a way, will promote tumor metastasis (Zhaojian et al., 2012). In addition, studies demonstrated that an higher expression of this gene is directly related to differentiation states of ESCs, that is, they proved that, by blocking the expression of this antisense, the cells remained in a pluripotent state (de Jesus et al., 2018); Wilms' tumor 1 (*Wt1*) is a tumor suppressor gene with high expression in leukemias and solid tumors, being an important regulator in tumor cells proliferation of, as well, as in the vascularization of the tumor microenvironment (Wagner et al., 2014). In some studies, this gene is defined as an oncogene that can be used in anticancer immunotherapy, because in a study to search for new cancer antigens, although none of them has all the established criteria, such as its therapeutic function, immunogenicity, oncogenicity, *Wt1* was at the top of the list (Sugiyama, 2010) (Hecht et al., 2018); Wilms tumor 1 homolog, opposite strand (*WT1os*) is an lncRNA that, so far, has not been much studied, however, as it is an lncRNA and they are involved, both in the development of various types of cancer through the regulation of chromatin organization and in the transcriptional and post-transcriptional processes, may have some relevance in this study and could become a potential cancer antigen (G. Yang, Lu, & Yuan, 2014).

1.2.6 TNGA cells and culture medium conditions

TNGA cells are mouse embryonic stem cells (mESC) with the insertion of a green fluorescent protein (GFP) right after the promoter in one of the *Nanog* alleles (Figure 2B) (Faddah et al., 2013).

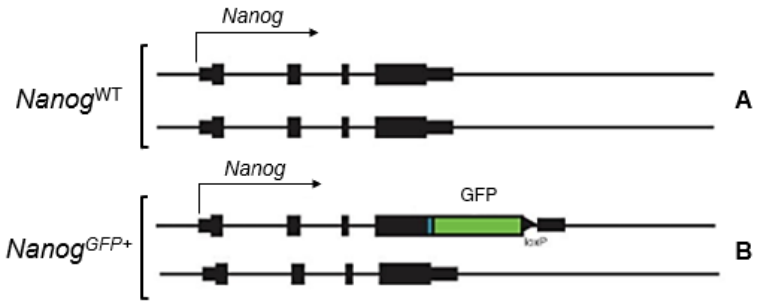

Figure 2 | Representative scheme of the transcriptome of the *Nanog* gene (*Nanog*^{WT}). When it has a copy of the fluorescent protein GFP in only one of its alleles, it represents TNGA cells (*Nanog*$^{GFP+}$). Figure adapted from Faddah et al., 2013.

The presence of *Nanog* is considered a pluripotency marker, both *in vitro* and *in vivo* assays and, consequently, the loss of expression of this gene is an indicator of cells differentiation (Chambers et al., 2007).

Initially, mESCs were maintained in a pluripotent state when cultivated in medium with serum (Fetal Bovine Serum), under a layer of inactive fibroblast cells, the "feeder" cells, which were essential in providing a factor external to the cells, but essential for its maintenance, the leukemia inhibitory factor (LIF). After this discovery there was no need to add the “feeder” cells to the culture dish, but the need for the presence of an extracellular matrix, such as gelatin, remains (Tosolini & Jouneau, 2016).

LIF is one of the most recognized factors as having a fundamental role in the maintenance of pluripotency of mESC *in vitro*. This is a member of the interleukin (IL)-6-type cytokine family, which is primarily involved in maintaining a pluripotent state in embryonic cells by activating the Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) signaling pathway, by inhibiting differentiation and promoting self-renewal of cells (Tang & Tian, 2013).

As mentioned above, the standard medium for mESC culture is a medium that contains serum and LIF. Studies have shown that this culture medium confers heterogeneity to the expression of mESC, pluripotency and regulatory factors such as Nanog, Rex1, Stella, Esrrb and β-catenin (Godwin et al., 2017). However, they observed that the expression patterns of cells in medium with these conditions resulted in subpopulations of cells starting to differentiate. Despite this phenotype being reversible, these results led to the discovery of an optimal medium that allowed the cells to remain, for a longer time, in an undifferentiated state, that is, a medium without serum (although currently the media includes serum – KSR, see materials and methods), with LIF and with two

chemical inhibitors (2i) (Godwin et al., 2017). The two inhibitors, PD0325901 and CHIR99021, are involved in mitogen-activated protein kinase (Mapk) and glycogen synthase kinase-3 (Gsk3) signaling pathways, respectively (Marks et al., 2012). These pathways are involved in the differentiation of ESCs, so one of the ways to keep them in an undifferentiated state is through their inhibition (Geest & Coffer, 2009) (Force & Woodgett, 2009).

Cells cultured under LIF/2i conditions demonstrated a more homogeneous conformation, with a uniform and higher expression of *Nanog*, in a state more similar to pluripotent preimplantation epiblast, however, they found that in cells with low *Nanog*, the 2i lead to an increase cell death, that is, these compounds have two effects on the cells, an inductive effect, and a selective effect (Hastreiter et al., 2018). Under the inductive effect, the cells are considered to be in a ground state, which is when the isolation of authentic ESC with full pluripotency capacity is achieved (Nichols & Smith, 2009). On the contrary, when cells have limited pluripotency, with higher differentiation, they start to have a prime state (Deb, Sarkar, & Ghosh, 2017).

Pluripotent stem cells are able to remain in culture for some passages, depending on their division rate, because the change of the culture medium allows inputs in signaling and transcriptional machinery, making this cell line a good model of cell plasticity, because it is possible for cells that initiate differentiation processes reverse this state under the correct stimuli, and on the contrary, cells in a pluripotent state quickly initiate differentiation processes if these stimuli are removed (Lynch, Bernad, Calvo, & Serrano, 2020).

1.3 Objectives

Given that there are data proving that when ESCs are subjected to differentiation stimuli, they begin to acquire some characteristics of cancer cells, such as the similar expression of some genes and the existence of angiogenic growth factors, it was hypothesized that these cells could function as immunizing agents that can develop antitumor responses in an organism.

The objective of this dissertation was to understand if TNGA cells growing in different media tend to have a more heterogeneous phenotype and gene expression patterns comparable to those of cancer cells. This work is just a preliminary study that is part of

a larger research project that focuses on the development of an iPSC-based vaccine with autologous cells from patients.

2. Methods

2.1 Cell Culture

TNGA cells were provided by the Institute of Molecular Medicine in Lisbon, and previous studies with this cell line had already been carried out by the research group at the Institute of Biomedicine of the University of Aveiro.

Cells were plated onto 0.1% gelatin-coated culture dishes (gelatin from porcine skin, Sigma-Aldrich, G9136-10MG) in the following culture medium: Gibco Dulbecco's Modified Eagle Medium (DMEM) with 1% Antibiotic Antimycotic (AA) and 10% sodium pyruvate; 15% Knockout Serum Replacement, KSR, (Gibco™, 10828028) which has been documented as essential in inducing this type of cells, as well as in their quality, providing a source of amino acids, vitamins and antioxidants, trace elements and even proteins (Liu et al., 2014); β-mercaptoethanol which works as a reducing agent, preventing the formation of reactive oxygen species (ROS); non-essential amino acids (NEAA) because cells need an adequate concentration of these elements; finally, the pluripotency agents, Leukemia Inhibitory Factor, LIF, (ESGRO® Recombinant Mouse LIF Protein, ESG1107, EMD Millipore - 1:10000 dilution) and two inhibitors, 2i, (CHIR99021, #72054, STEMCELL Technologies – 1:1000 dilution; PD0325901, PZ0162-5MG, Sigma-Aldrich – 1:1000 dilution) are needed for the cells to retain a naïve pluripotent state (Chen et al., 2010).

Regardless of the experiment to be carried out, in this study there were four different conditions, with the cells being kept in culture for 24 and 48 hours, with two variables, with cells cultivated in medium only with LIF or in medium with LIF and 2i. With the exception of gene knockdown assays which, in order to try to verify a greater difference, the condition was 72 hour of cell culture (Figure 3).

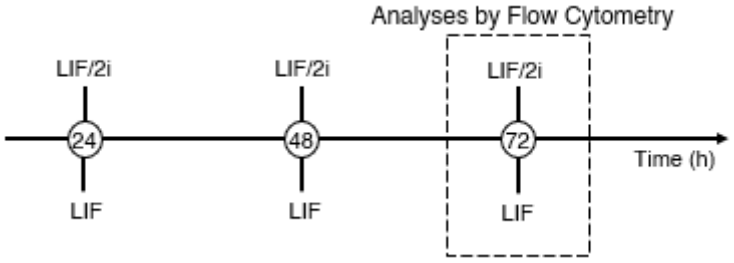

Figure 3 | Scheme representing the different cell culture conditions: 24h LIF/2i, 24h LIF, 48h LIF/2i, 48h LIF and 72h LIF/2i, 72h LIF when the Wt1 transfection was analyzed by Flow Cytometry.

2.2 RNA extraction

Total RNA was extracted from cells with NZYol (NZYtech), according to the manufacturer's instructions. Briefly, NZYol was added directly to the reagent to the culture dish after the removal of the medium. Then, mechanically, it was pipetted several times up and down to homogenize the solutions and to allow the disruption of cell membranes.

To obtain the separation of the different phases, that is, to allow the isolation of RNA from the DNA and proteins, the samples were incubated at room temperature and chloroform was added, the samples were incubated during 3 minutes at room temperature (RT), and centrifuged at 12000xp during 15 minutes at 4°C. Consequently, it is possible to see three different phases, the RNA being in the aqueous phase. This was later passed to a new eppendorf and, to precipitate the RNA, isopropyl alcohol was added. The samples were incubated during 10 minutes at 4°C and centrifuged at 12000xp during 15 minutes at 4°C. The supernatant was discarded and then the RNA was washed with 75% ethanol. Samples were vortexed and centrifuged at 17000xp during 15 minutes at 4°C.

Finally, the tubes were left open in the fume hood, so that the maximum amount of ethanol would evaporate. Subsequently, the pellet was resuspended in RNase free-water and the tubes were left for 5 minutes at a temperature of 65°C. The samples were stored at -20°C for further use.

The RNA samples were quantified using a NanoDrop™ spectrophotometer and the absorbances were measured at 260nm. From here, the RNA concentration in the samples is obtained (ng/µL), the absorbance at 260nm and the ratios between the absorbances of 260nm and 230nm and between 260nm and 280nm that evaluates the degree of purification of the samples.

2.3 cDNA synthesis

Conversion of RNA to complementary DNA (cDNA) was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech).

This kit has four solutions: random hexamers that will allow the binding to random sites of the target RNA, working as primers for DNA synthesis by reverse transcription (RT), and oligo(dT)18 primers that will hybridize with the polyadenine tail at the 3' terminus to make sure this site is tagged to bind the reverse transcription promoter (Nam et al.,

2002). Primers are included in the NZYRT 2× Master Mix which also contains dNTPs, $MgCl_2$ and an optimized RT buffer. Furthermore, it also contains NZYRT Enzyme Mix with components that will not allow RNA degradation due to ribonuclease contamination. It also contains an RNase H solution, which allows the RNA strand to degrade after the reaction that gives rise to the cDNA takes place.

For a final volume of 20µL, all components were added to sterile, nuclease-free microcentrifuge tubes, considering the volumes recommended in the protocol and the consequent amount of RNA measured in the samples. The solutions were gently mixed and incubated at 25°C for 10 minutes. Next, they were incubated at 50°C for 30 minutes. In order to inactivate the reaction, the samples were heated at 85 °C for 5 minutes, and then left on ice.

In the final step, 1µL of NZY RNase H was added, incubating at 37 °C for 20 minutes. The samples were stored at -20°C until further use.

2.4 Quantitative Real-time PCR (qRT-PCR)

In order to analyze the expression of different genes in the previously prepared cDNA samples, a real-time quantitative PCR was performed using the qPCR green master mix (2x), ROX plus kit (NZYtech).

As the guideline is very generic, and given our study conditions, the protocol provided was further optimized. For this, the MIX solutions were made, for each gene, using a volume of 202.5µL of master MIX, which has deoxyribonucleotide triphosphate (dNTP's), DNA polymerase and magnesium that will help its activity, then 10.8µL were added for the forward and reverse primers and, finally, 45.9 µL of nuclease-free water. Lastly, a volume of 10µl of MIX solution and 5µl of cDNA of the different conditions under study were added to the wells of the PCR plates.

Two housekeeping genes were used: 18S ribosomal RNA (rRNA) and Actin, but only 18S rRNA was used to normalize the results.

Relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.5 Protein extraction

Protein extraction was performed using a 6M urea solution in 200mM ammonium bicarbonate. The conditions analyzed were 24h and 48h LIF and LIF/2i and also the breast cancer cell line, E0771.

In cell culture, the medium was first removed, and the cells were washed with PBS. Trypsin was added and inactivated with serum-containing medium. The solutions from the different conditions with the cells were added to different eppendorfs and were centrifuged at a speed of 1000 rpm for 3 minutes. The supernatant was removed, and the cell pellet was washed once in PBS.

The PBS was removed, and the urea solution was added making, vigorously, up and down with the pipette in order to disrupt the cell membrane. The solution was left to rest on ice for approximately 30 minutes. The samples were centrifuged, and the supernatant transferred to new tubes.

Subsequently, protein quantification was performed using the Bicinchoninic acid (BCA) method. This is a chromogenic agent that permits the quantification of the proteins present in the samples. It can be determined by spectrophotometry through the color shift, from blue to purple, which absorbs light at a wavelength of 562nm. The absorbances of the different samples were read using the Tecan infinite M200 software and these are directly proportional to the amount of protein, that is, after reading the absorbances, a concentration/absorbance curve is obtained. From here, a mathematical equation (y=mx+b) is illustrated from which it is possible to calculate the specific concentrations of the different samples under our study conditions.

2.6 Western Blot

To assess how Nanog was expressed in the different study conditions, a Western Blot was performed, by analyzing the proteins of cells subjected to different conditions: LIF/2i and LIF.

15µg of protein were loaded, per lane, in a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) 10% and, subsequently, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a 5% BSA solution in TBS-T (Tris-buffered saline, 25mM Tris, 0,15M NaCl, 0,05% Tween-20, pH7,5) and then incubated, overnight, at 4°C, with the primary antibody: anti-mouse Nanog Purified (Affymetrix), which has a molecular weight of, approximately, 45KDa, with a concentration of 1 µg/ml. After, the membrane was washed with TBS-T, 3 times in 10

minutes periods and then incubated with the secondary antibody, HRP (horseradish peroxidase)-conjugated anti-rat. The membrane was washed again 3 times with TBS-T.

The detection was made through enhanced chemiluminescence (ECL), using ChemiDoc.

2.7 siMyc and siWt1 transfection

In order to understand whether, by silencing specific genes that were previously studied, the cells adjust their pluripotency, a protocol for silencing *Wt1* and *c-Myc* was carried out.

The experiment was divided into 4 days, so that the cells remained 72 hours in culture (for the *c-Myc* gene the conditions were 3 days, 48h). On the first day, the cells were only transferred to new plates, in this case, 12-well plates because there are four different study conditions and two duplicates for each one. There were four different conditions, cells cultured in medium with LIF/2i, cells cultured in medium with LIF only, a negative control of an antisense locked nucleic acid (LNA) gapmer with the respective sequence 5'-+A+A+C ACG TCT ATA +C+G+C-3' (Integrated DNA Technologies – ID:314765610), and the condition of the KD for the genes in analysis. Two knockdowns (KDs) of two different genes were made, one with the *c-Myc* gene, through an interference RNA sequence (Invitrogen – ID:24163043), and the other one with the *Wt1* gene, using a small interference (si) RNA (Ambion – ID:188990).

On the second day, oligonucleotides were transfected using Lipofectamine RNAiMAX Transfection Reagent from Thermo Fisher Scientific. For each transfection well, 2µl of lipofectamine was used for 98µl of Opti-MEM™, Reduced Serum Medium, also from Thermo Fisher Scientific. Then the solution was incubated for 5 minutes. At the same time, in two other tubes, solutions for KD and negative control were made. These solutions were prepared with a concentration of 200nM. The lipofectamine solution was then added to these conditions and remained incubated for 20 minutes to allow the formation of complexes with the oligonucleotides.

On the third day, the medium was changed, adding new culture medium to all wells and the procedure performed on the second day was repeated. And lastly, on the fourth day of cell culture, the results were analyzed by flow cytometry and by RT-qPCR.

As the cells exhibit GFP, detection was performed using the FL-1 channel, on the Accuri™ C6 Flow Cytometer equipment, and analyses was performed on the FlowJo software.

3. Results and Discussion

3.1 TNGA cells morphology and GFP expression

One of the main aspects to consider when studying pluripotent stem cells, in this case TNGA cells, is their morphology. Simply by observing their structures under a microscope it's possible to predict the state of differentiation or pluripotency of these cells.

When cells are subjected to different stimuli, depending on the growth medium and the time they are in culture, they will exhibit different morphologies.

Nanog expression is directly related to the cells phenotype, with cells that have less expression having a more differentiated phenotype and those with high expression tend to self-renew. Treatments with 2i shift *Nanog* expression, showing a high distribution and cells under these conditions have a pluripotent phenotype, however, in medium with LIF alone, cells oscillate between high and low conditions (Cannon, Corrigan, Miermont, Mcdonel, & Chubb, 2015).

In the control conditions there were two timepoints, 24 and 48 hours, with two variables: cells cultured in medium with LIF and 2i and cells left in medium with LIF.

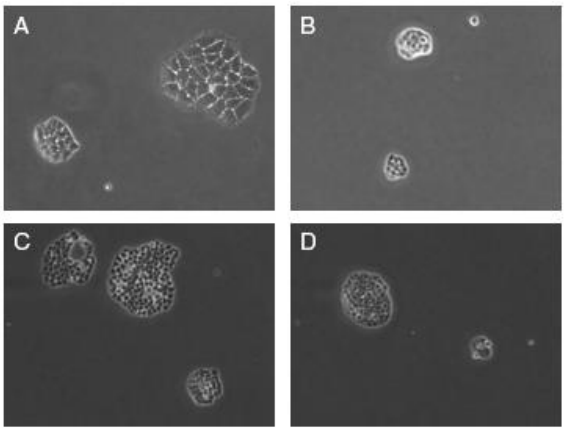

Figure 4 | Representative images of TNGA cells when left in culture for 24 hours with medium with LIF (A), and when exposed to medium with LIF and 2i (B). Another condition is 48 hours of culture with LIF (C), and with LIF and 2i (D).

With pluripotency stimuli, such as those obtained with LIF and 2i, cells reach a ground state, as shown in Figure 4B and 4D. They have a more uniform morphology and are more homogeneous. As they establish strong bonds between them, they tend to form cell clones. When removing some pluripotency stimuli, such as the 2 inhibitors, the cells have a higher LIF consumption and, with a deficit of this element, the cells begin

to adapt and lose their pluripotent characteristics, beginning to change their morphology. At this point, cells reach a primed state, where they present dispersed structures, without a defined shape, and begin to grow in a disorganized way on the culture dish as represented in Figure 4A and 4C.

Although both LIF and 2i are factors that enhance the pluripotency of the cells, several studies have been carried out to understand why cells, when in medium with LIF alone, begin to acquire some differentiated characteristics, radically altering their morphology in short periods of time. Although not much is known about the molecular properties related to the regulation of differentiation and pluripotency processes of stem cells when controlled by *Nanog* expression, one study explained that, in ESCs growing in medium with LIF and serum the *Nanog* expression was only observed in one allele, and also the cells showed asynchronous replication of its locus, while in cells that remained in culture in LIF/2i medium, the expression was biallelic (Miyanari & Torres-Padilla, 2012).

As these cells have, in their genome, a copy of the *Nanog* gene followed by a fluorescent protein, the pluripotent or differentiation levels can be verified, under a microscope (EVOS™ M5000 Imaging System, AMF5000), by the intensity of GFP they exhibit. In other words, when cells are in a ground state, normally under LIF conditions with 2i, the *Nanog* gene will be more expressed and the cells will, consequently, have more intense green levels. When they are in medium with LIF only, the cells start to lose their pluripotent characteristics, shifting to a primed state, were their morphology changes as well as their *Nanog* expression levels.

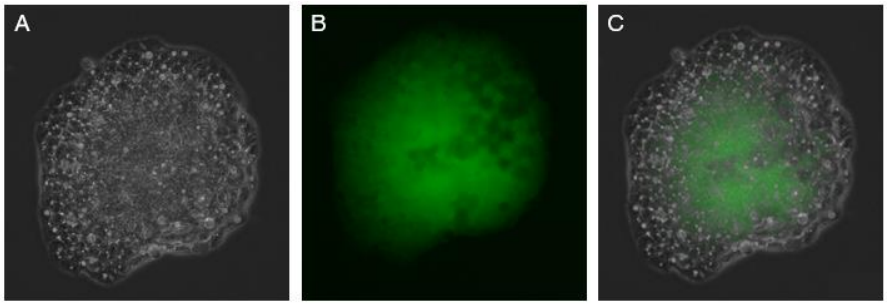

Figure 5 | TNGA cell clones, in LIF/2i medium, with different levels of *Nanog* expression.

In Figure 5 it can be seen that, in the same clone, with the same cells type, they express different levels of *Nanog*. Within the same environment, and subject to the same conditions, there are cells with higher green intensity than others (Figure B).

Nanog gene is considered a pluripotency marker, and by looking at the cells fluorescence it is possible to measure its distribution in a qualitative, but effective, way, that is, it is possible to understand if they are in a stationary state or if they suffered some perturbation. It is believed that the different distribution of Nanog-GFP in individual cells is different due to different steady states of *Nanog* levels, with cells moving between these states stochastically, i.e., each cell's transcriptional system is regulated by distinct genetic activities that are influenced by signaling pathways. This process is also recognized as “noise” and may be the explanation for the fact that there are different levels of *Nanog* and, consequently, GFP expression in cells that are in the same medium, under the same culture conditions (Luo et al., 2012).

3.2 Nanog expression

To confirm that the increase mRNA levels was associated with increase levels of proteins or, on the other hand, whether non-optimal conditions resulted in loss of pluripotency we looked at some proteins through Western Blot.

Nanog is essential for the early stages of embryonic development, becoming a gene that is necessary for the assessment of pluripotency levels in cells (Silva et al., 2009), being a fundamental element when its intended to study its expression in order to understand if there is a high expression in the cells, considering the different study conditions.

As previously explaind, the LIF/2i condition is predictive of a more pluripotent state, and in the LIF condition the cells begin to lose those characteristics. So, to confirm the theory that *Nanog* is more expressed in cells in a ground state, a WB was performed where the cells proteins, subjected to our different study conditions, were run on a gel, and then incubated with specific antibodies for Nanog binding in order to allow the assembly of the protein-antibody complexes.

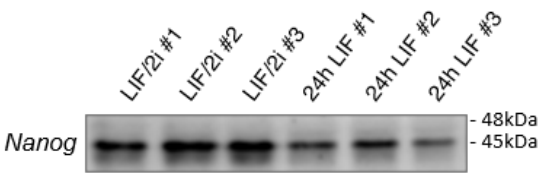

Figure 6 | Nanog expression levels in the different conditions, analyzed by Western Blot (n=2).

Analysing the molecular weight and the subsequent intensity of the marked band on the membrane, it is possible to make a qualitative analysis of the proteins expression under the study conditions, that is, by the result of Figure 6, and knowing that the molecular weight in the Nanog is approximately , 45kDa, we verified that the bands are more intense in the LIF/2i conditions, confirming that these proteins are more expressed when the cells are under pluripotent stimuli.

3.3 Gene expression analyses

Following the RNA-seq analysis we would like to confirm whether non-optimal conditions would increase the expression of cancer-associated genes in mESC (using the TNGA cell line).

Seven different genes, that were previously detected by RNA sequencing, were analyzed in order to validate these results and also to understand how these genes would be expressed in TNGA cells, because many of them are also documented as being highly expressed in some tumor cells.

The RNA-seq results have not been published yet, but, fundamentally, they analyze the expression of all these genes when removing pluripotency stimuli, with results that showed a tendency of cancer-associated genes to be more expressed when cells begin to differentiate, and they also confirmed the principle that *Nanog* is a pluripotency factor.

It was already known that these cells, when subjected to differentiation processes, were, somewhat, similar to cancer cells, so different conditions were evaluated, including 24h LIF/2i, where a lower expression of these genes would be expected since this is a pluripotency condition, and the conditions with LIF where 24h and 48h were included to understand if, with more time in culture, the expression would be increased. Then, in this assay were evaluated three conditions: LIF/2i, 24h LIF and 48h LIF.

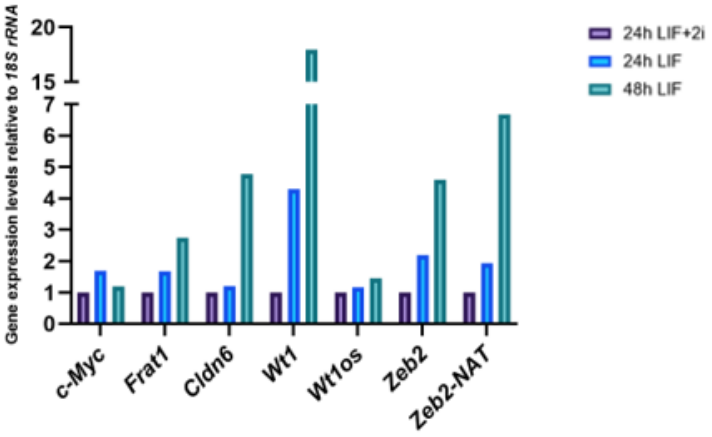

Figure 7 | Results of the evaluation of the expression of different genes by qRT-PCR, normalized with the housekeeping gene 18S rRNA, under the conditions of 24h LIF/2i, 24h LIF and 48h LIF (n=3).

The *c-Myc* gene showed higher expression in the 24h LIF condition and the remaining genes evaluated, *Frat1*, *Cldn6*, *Wt1*, *Wt1os*, *Zeb2* and Zeb2-*NAT* showed higher expression in the 48h LIF condition, emphasizing the *Wt1* gene with a selective expression of RNA in the order of 17.938 (Figure 7).

These were the results that we were expecting, as they confirm those obtained by RNA-seq, however some of these genes had already been studied as being involved in the differentiation of ESC, as it is the case of *Wt1*. This gene is highly expressed during embryogenesis for the development of some tissues and organs such as the liver, the gonads and also the spleen, however, it is also highly expressed in some human acute leukemias (Lee & Haber, 2001). A study that aimed to evaluate the expression of *Wt1* in human endothelium found that the expression of *Wt1* mRNA and proteins was very high when stromal fibroblasts differentiated into decidual cells, which suggests that the expression of this gene is involved in processes of cells differentiation (Makrigiannakis et al., 2001), which can prove the results obtained, including the fact that this was the gene with the highest expression of the whole set of results.

c-Myc, one of the most known oncogenes, is described as being an important and significant factor in homeostasis and in the reprogramming of ESC to a pluripotent state, however it has also been found to be involved in the indirect regulation of genes that participate in differentiation processes (Neri et al., 2012). Some studies explained that these different contributions of *c-Myc* depends on the cell type and microenvironment, that is, they realized that in cells of the intestinal and myeloid epithelium the gene induces a state of pluripotency and self-renewal, but in cells of the

epidermis and hematopoietic stem cells diferentiation processes are induced (Sumi, Tsuneyoshi, Nakatsuji, & Suemori, 2007). Although there is evidence that overrexpression of a dominant negative *c-myc* promotes differentiation into mESC (Smith, Singh, & Dalton, 2010), the mechanism by which it performs in differentiation processes has not been sufficiently studied, but the results obtained in qRT-PCR confirm that it may, in fact, be involved in processes that lead to the primed state of cells.

For *Cldn 6*, studies have shown that its protein levels are downregulated when cells begin to differentiate (Ben-David, Nudel, & Benvenisty, 2013), consequently this gene is more expressed in cells in a ground state. However, in a study that analyzed the expression of several genes after LIF removal, they found that, after 18 and 72 hours, *Cldn6* expression increased from 2.14 to 3.40, respectively (Palmqvist et al., 2005). This proves that this gene is also participating in cells differentiation processes, as it is demonstrated in our qRT-PCR results.

Frat1 gene is related to the maintenance of pluripotency in cells by activating the GSK3β enzyme, which is only achieved by the high activity of the PI3K/Akt signaling pathway (Bechard, Trost, Singh, & Dalton, 2012). However, studies that demonstrate their involvement in the differentiation processes of ESC have not yet been carried out. The interest in studying this gene is that it is a known proto-oncogene (Guo, Liu, Zhong, & Zhang, 2011), making it interesting for studying the discovery of possible new cancer antigens.

In a study to understand the expression of *Zeb2* in colorectal cancer, the group observed, by analyzing the expression of 13 genes and their respective miRNA in the front responses of the invasion, that *Zeb2* was one of the genes with more overexpression, mainly due to its function of E-cadherin repression. (Kahlert et al., 2011). In mESC it was also verified that *Zeb2* inactivation had a negative effect on the cells pluripotency, causing them to start differentiation processes (Di Filippo et al., 2020). Although there are still not many studies to justify that the *Zeb2* gene has any influence in the differentiation of ESC cells, the fact that it is highly expressed in some types of cancer is one of the reasons for the interest of studying this gene, because, in theory, ESC resembles CSC when submited to differentiation stimuli, and the result obtained by qRT-PCR demonstrates that this gene may actually be involved in the conversion of cells to a primed state.

lncRNAs such as *Wt1os* and *Zeb2-NAT*, are already documented as participating in several development and proliferation pathways of several types of tumors, such as

prostate, breast, lung and leukemia (Bhan, Soleimani, & Mandal, 2017). Particularly for the antisense *Zeb2-NAT*, it has been verified that its expression is increased by signs of pluripotency loss (de Jesus et al., 2018), confirming the results obtained. Regarding to the *Wt1os* gene, there are still not many studies on its potential influence of cells differentiation, but according to the results obtained, the analysis demonstrate that its highest expression was in the 48h LIF condition, which is the one that corresponds to the more differentiated cell state.

These data prove that any of these genes can be a potential anti-tumor antigen, however, further studies are needed for their confirmation.

3.4 siMyc and siWt1 transfection efficiency

To explore whether *Myc* and *Wt1* are important to the loss of pluripotency we investigated if reduced levels of these genes would lead to an increase in cells pluripotency.

In order to monitor the pluripotency of TNGA cells, since they have a fluorescent protein in their structure, we used flow citometry.

Through flow cytometry it is possible to create a quantitative phenotype of a large population of cells with higher sensitivity, and it is considered one of the most accurate and objective methods to determine the efficiency of a transfection (Homann et al., 2017).

The genes evaluated was the *c-Myc* because is documented as highly expressed in several types of cancer, and the *WT1* gene because, in the RT-qPCR results, had a very significant shift in the differentiation condition of the cells (48h LIF).

The results of the transfection with the *c-Myc* gene are shown in Figure 8. Here it can be seen that the percentage of cells expressing *Nanog* is higher in the LIF/2i condition (A and B), in the order of 65%. For the negative control (E and F) this expression is lower, being 40%. However, in the condition of the KD of *c-Myc* (C and D), the percentage is 44%, with a slight increase compared to the negative control. Although there is not a big variation, this result is positive because it means that after the KD, a higher percentage of cells were in a pluripotent state. However, the ideal would be a result closer to LIF/2i.

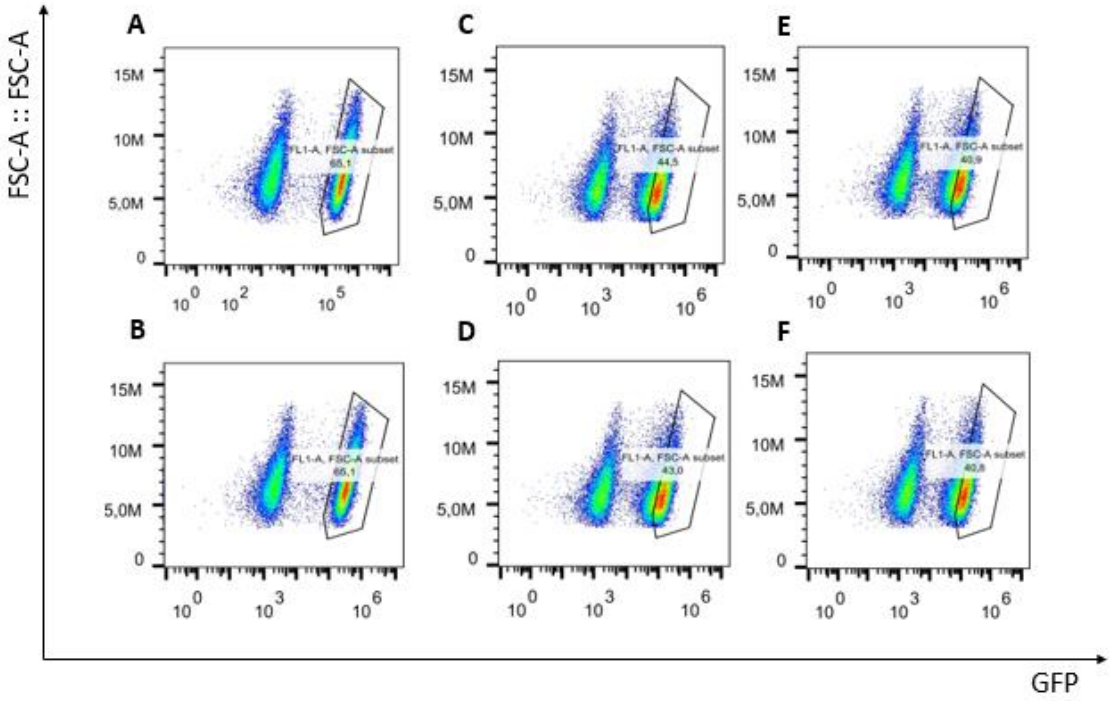

Figure 6 | Results obtained by flow cytometry for KD analysis of *c-Myc* gene using FlowJo software. Duplicates were made for the different conditions, having a percentage of cells expressing the GFP fluorescent protein of 65.1 for the LIF/2i #1 condition (A), 65.1 for the LIF/2i #2 condition (B), 44.5 for *c-Myc* #1 KD (C), 43.0 for *c-Myc* #2 KD (D), 40.9 for negative control #1 (E), and 40.8 for negative control #2 (F) (n=2).

In this assay, the cells remained in culture for 48h and the conditions analyzed were LIF/2i, LIF NC, and LIF KD for *c-Myc*.

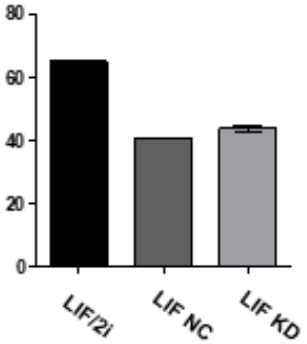

Figure 7 | GFP expression in TNGA cells for evaluation of *c-Myc* gene knockdown, with study conditions LIF/2i, LIF scramble for negative control and LIF KD of *c-Myc* (n=2).

The data shown in Figure 9 demonstrate that there is a significant difference between the negative control condition (LIF NC) and the *Myc* KD condition (LIF KD), with a *p value* less than 0,05. This result confirms the data presented in Figure 8, where there

was also an increase percentage of cells expressing GFP in the KD, compared to the scramble condition.

As the results were positive, but not what we expected, we decided that in the next trial the cells would be in culture for 72 hours to try to get conditions with significant differences. That's how it was done for the *Wt1* silencing experiment. Furthermore, for *c-Myc*, was used a concentration of 100nM of oligonucleotides, and we decided to change it to 200nM in the *Wt1* assay.

Figure 10 shows the results of transfection with the *WT1* gene. Here it is verified that the population of cells expressing the GFP protein is higher in the LIF/2i condition (A and B), with an expression of 95%, which would be expected because this condition keeps the cells in a ground state, since they have a higher expression of *Nanog* and, consequently, of GFP. For the negative control (E and F), which is the condition that represents the differentiation state of the cells, the percentage of GFP expression is 79%. Comparing these two conditions with the *Wt1* KD (C and D), which has a percentage of 77%, the one that resembles the most is the negative control.

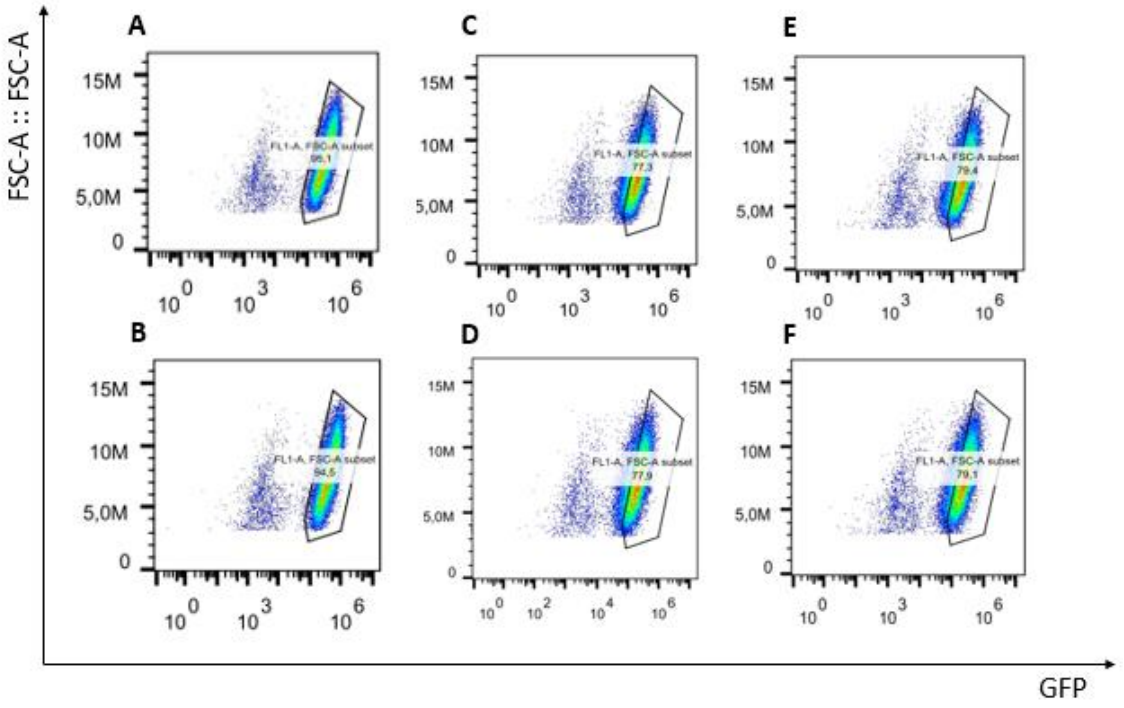

Figure 8 | Results obtained by flow cytometry for the analysis of KD of the *WT1* gene using FlowJo software. Duplicates were made for the different conditions, having a percentage of cells expressing the fluorescent protein GFP of 94.5 for the LIF/2i #1 condition (A), 95.1 for the LIF/2i #2 condition (B), 77.3 for KD of *WT1* #1 (C), 77.9 for KD of *WT1* #2 (D), 79.4 for negative control #1 (E), and 79.1 for negative control #2 (F) (n=2).

This result is not the expected one, as it was estimated that the KD condition would present a slightly higher expression when compared to the negative control. As shown in Figure 11, where there is no significant difference between the conditions LIF NC and LIF KD of *Wt1*, including a decrease in the percentage of expression in GFP cells.

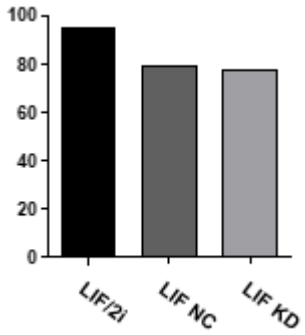

Figure 9 | GFP expression in TNGA cells for evaluation of *Wt1* gene knockdown, with study conditions LIF/2i, LIF scramble for negative control and LIF KD of *Wt1* (n=2).

The time that cells remain in culture and the cells confluence in the dish wells may have some influence on these results. Cells remained in culture for 72h, and the wells were very confluent. Although the percentage of GFP is significant in the LIF/2i condition, the cells could already be starting differentiation processes, so to try to improve the conditions of this assay, it would be necessary to repeat with a lower concentration of cells per dich well.

In order to evaluate if the gene KD in this assay was working as expected, and also to have a deeper understanding of the results obtained by flow cytometry, a qRT-PCR was performed to assess the expression of the *Wt1* and *Nanog* genes under the same study conditions, that is, LIF/2i, LIF, Wt1 KD and LIF scramble (Figure 12).

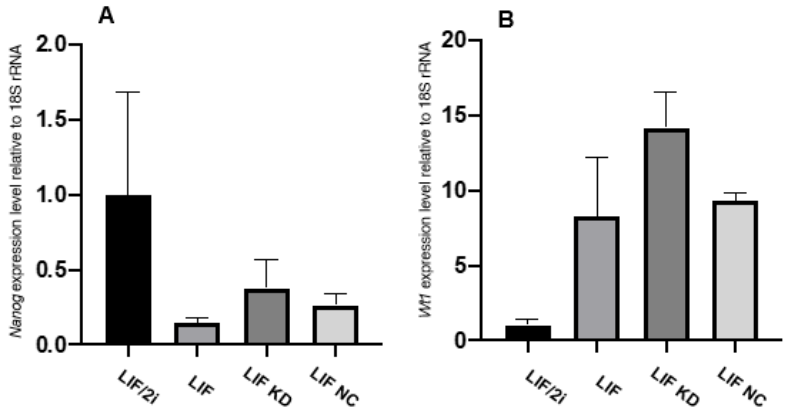

Figure 10 | qRT-PCR results for evaluating the expression of *Nanog* gene (A) and *Wt1* gene (B) under LIF/2i, LIF, LIF KD and LIF NC conditions (n=2).

The results corresponding to the evaluation of the *Nanog gene* (Figure 12A) are positive because they demonstrate an increase in its expression in the condition where the KD of *Wt1* was performed, however, for the evaluation of the *Wt1* gene (Figure 12B), the results were contradictory. In the LIF/2i and LIF conditions, the results are according with the ones obtained previously, with an increased expression in the absence of 2i, but, when observing the LIF KD and LIF NC conditions, the results were not as expected, demonstrating an increased expression in the KD condition. This result explains that, probably, *Wt1* was not efficiently silenced, or the time point used for this assay was longer than it was supposed to be.

For a better evaluation of these results, it is necessary to repeat these tests and try to optimize the protocol, and also, despite showing a tendency, it is necessary to increase de “n” number of our samples in order to perform statistical analyses, such as student t-test, to further understand the significance of our qRT-PCR results.

3.5 Proteome analyses of E0771 and TNGA cells

In collaboration with Eduard Sabidó, Proteomics Facility UPF/CRG, Barcelona, we were able to make a generalized analysis of a breast cancer cell line E0771, and TNGA cells under our different study conditions. For this, a principal component analysis (PCA) was performed, which is a statistical technique that maintains the existing variations in the data set, but reduces its dimension by identifying directions that are common to the data to be evaluated, that is, the samples are grouped

according to their similarities and determines which samples can be grouped (Ringnér, 2008).

The objective of this analysis was to understand if ESCs, in fact, resemble cancer cells when subjected to differentiation stimuli, so this analysis was performed in either duplicate (E0771) and triplicate (48h LIF, 24h LIF and LIF/2i) (Figure 13).

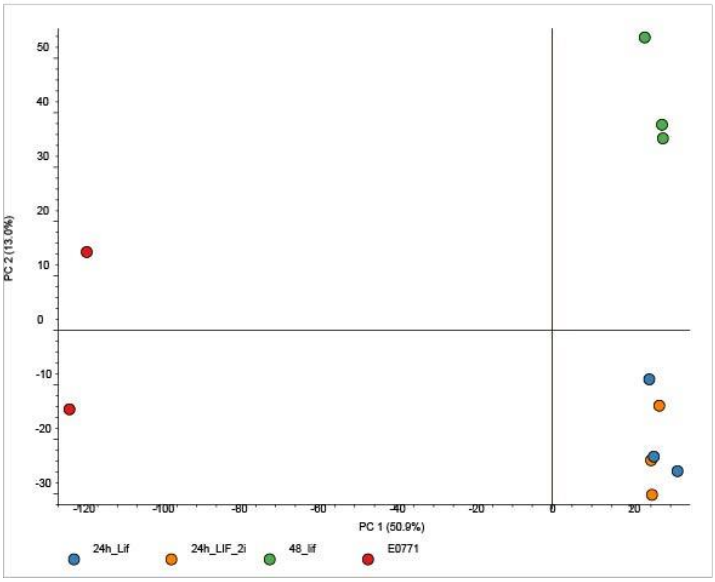

Figure 11 | PCA score plot for E0771 (n=2) and TNGA cells under 24h LIF, 24h LIF/2i and 48h LIF conditions (n=3).

The samples were plotted and organized with their different expression levels, with red dots corresponding to cell line E0771, green dots to 48h LIF condition, yellow dots to LIF/2i condition, and blue dots to 24h LIF condition. By analyzing the results, we can see that the TNGA cells in all three conditions are separated, by PC1, from the breast cancer cell line E0771 (TNGA cells in positive PC1 and E0771 cells in negative PC1). Furthermore, 24h TNGA cells are separated, by PC2, from those from the 48h timepoint (24h LIF/2i and 24h LIF in negative PC2 and 48h LIF in positive PC2). However, we can verify that the E0771 duplicates are in different regions of the score plot (one is placed in positive PC2 and the other in negative PC2), which does not allow us to conclude which TNGA condition (24h LIF/2i, 24h LIF or 48h LIF) is more similar to the cancer cell line.

To supplement these results, we also had access to the expression pattern of the different genes under the same conditions (Figure 14).

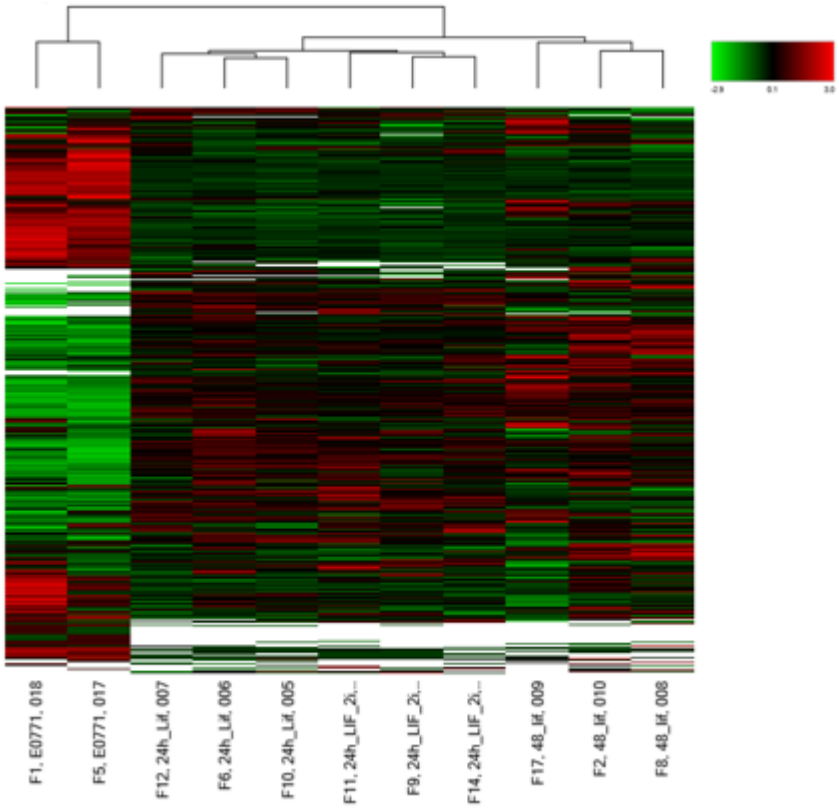

Figure 12 | Protein expression pattern under different study conditions: E0771, 24h LIF, 24h LIF/2i and 48h LIF.

By analyzing the gene expression pattern in the different cell lines and conditions, there is no sequence that shows an identical expression compared to E0771, of all the variables analyzed, the one that demonstrate a higher difference in its expression is one of the duplicates of the 48h LIF condition, which is the one that is expected to have a higher similarity with the cancer cell line.

Although these results are not ideal, they demonstrate that TNGA cells cultured during 48h in medium with LIF have a different pattern from 24h LIF and LIF/2i, and it have a tendency to be more similar to E0771. However, in order to have more significant results, we could try to add a different condition with cells that are for longer periods of time in growth medium with LIF, for example 72h LIF, to see if there was a higher variation in these expression levels because, theoretically, cells are in an exponential level of differentiation.

As the PCA results were also not conclusive, new samples of E0771 have already been sent to evaluation, in order to have a significant number of duplicates and to

really understand which pattern resembles this mammary cancer cell line. These results have not been analyzed yet.

4. Conclusion

The use of iPSC or ESC with an immunological adjuvant to formulate a vaccine with autologous patient cells is one of the great potentials for cancer immunotherapy. This type of approach differs from immunological therapies that are currently used because it allows the recognition of a large variety of cancer antigens, which makes this approach more specific considering tumors heterogeneity and, consequently, the diversity of their antigens.

This approach can bring two clinical advantages, first of all, this therapy can be used as early immunization, that is, patients at high risk of developing cancer, iPSC-based vaccination can activate their immune system to various types of tumor antigens, so that, in the event that tumor cells are formed, the immune response is faster and more efficient. The other approach is for patients who have recently used surgery for localized removal of a tumor, this therapy can help to boost their immune system and also prevent the reappearance of malignant cells (Lin Wang, Pegram, & Wu, 2019).

TNGA cells are a good model for pluripotency studies, because mESCs are easy to maintain in culture, especially after the discovery of LIF that led to the replacement of feeder cells such as mouse embryonic fibroblasts (MEFs) with 0.1% gelatin (Tremml, Singer, & Malavarca, 2008) not requiring several passages to obtain an isolated condition of embryonic cells. Furthermore, the culture medium is simple, with the adjuvant of having LIF when we want the cells to undergo differentiated stimuli or LIF/2i when we need to keep them in a more pluripotent state. One of the main benefits of this cell line in stem cell pluripotency studies is that the different stages of the cells are easily reversed, that is, when cells start to revert to a primed state, it is possible to change them to a ground state by altering the culture medium or passing them to a new culture dish under pluripotency stimuli (LIF/2i). Furthermore, the advantage of TNGA having a fluorescent protein after a pluripotency marker indicates that the evaluation of their different states is simplified by analysis under a microscope with GFP signal.

Comparing the culture protocols for the maintenance of ESC and iPSC, the last ones require more complex factors to obtain their ideal pluripotency conditions. The basis of their development is from ESC, so it’s necessary to start by culture these cells for subsequent reprogramming by the four Yamanaka factors. In addition, other compounds such as human placenta-derived type IV collagen or attachment factors are needed (Chatterjee et al., 2016). For this reason, although the ultimate goal is to use iPSC in the development of a new anticancer therapy, these preliminary studies were carried out with mESC, also due to the great functional similarity between the two

cell lines (Cieślar-Pobuda et al., 2017). Afterwards, after all the confirmatory assays, and when its necessary to start the experimenting with the patient's autologous cells, it will be required to work with iPSC.

According to the results obtained in this work, the *c-Myc*, *Frat1*, *Wt1*, *Wt1os*, *Cldn6*, *Zeb2* and *Zeb2-NAT* genes may be considered as new potential antigens for the creation of this innovative therapy, all of which demonstrated greater expression under conditions where the cells were in a more differentiated state. In this experiment, two housekeeping genes were used: 18S rRNA and Actin, which are fundamental in qRT-PCR evaluations to make the results more reliable because they do not change their expression levels regardless of changes in samples or treatment groups (Turabelidze, Guo, & DiPietro, 2010), however, the results evaluated in this work were only those normalized with 18S rRNA, because there were some expression results with actin that were very different from what was expected.

Transfection results, demonstrate that, especially the *c-Myc* gene, may have some influence on the differentiation potential of cells, because by its evaluation, after silencing this gene, despite not having a significant difference, there was a small shift of cells expressing the GFP protein compared to the negative control, justifying that there were more pluripotent cells in the KD condition. For a better evaluation of these results, it is necessary to review the protocol to understand if the concentration of oligonucleotides had any influence on these results, since that, for *c-Myc* a concentration of 100nM was used compared to 200nM for *Wt1*. Furthermore, it is necessary to improve the conditions for the cells passage so that there are not too many cells per well, because it can also influence the viability of the results.

Although the flow cytometry results were not conclusive for the *Wt1* gene, the evaluation performed by qRT-PCR showed an interesting result when comparing the expression of the *Nanog* gene under our different conditions. However, the evaluation of *Wt1* expression under the same conditions showed contradictory results, observing a higher expression in the KD conditions where, theoretically, was expected to exist a decreased expression of this gene. One possible explanation for these results is that we might not have caught the exact time of the KD, probably because the cells were in extended periods of time in culture, since this essay was followed by a protocol of 72h condition. In order to optimize this protocol, the same experiment must be performed, using different timepoints, probably evaluating the 24h and 48h condition, to try to understand if this problem could be overcome and to realize if the *Wt1* gene is prone to more valid conclusions.

Despite showing a tendency, the mass spectrometry results were not conclusive, requiring further evaluations to be able to understand whether the states of cells differentiation, when pluripotent stimuli are removed, exhibit a pattern similar to the cancer cell line. These results were important to confirm the theory that ESC, when subjected to differentiation processes, share similar patterns with cancer cells.

In conclusion, these results were positive, confirming the bibliography that already existed on this subject, however, in order to reach the final objective results, many procedures are still needed to assess the viability of these possible novel cancer antigens, testing their immunogenicity and oncogenicity and realize if it is possible to generate an immune response in an organism.

As future perspectives, it would be necessary to evaluate how cells behave by silencing the other genes analyzed by qRT-PCR and repeating the assays with the genes that were tested. Furthermore, a trial that is already underway, but has no results yet, is to evaluate, *in vivo*, in a mouse model with breast tumor inoculated with the E0771 cell line, which is very similar to human breast cancer, allowing the investigation of the host and their tumor immunity (Naour, Rossary, & Vasson, 2020), how the cancer cells behave after TNGA cells injections. The aim of this trial is to assess whether mESC inoculation alone can produce an immune response in the animal model in order to reduce the proliferation of these cells and reduce tumor formation.

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