

Integrated expansion and cardiac differentiation of human induced pluripotent stem cells in 2D and 3D conditions

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ABSTRACT: Pluripotent stem cells (PSC) more precisely human induced pluripotent stem cells (hiPSC) turn into reality the establishment of platforms for disease modelling, drug screening or even regenerative medicine. However, to achieve that, there is a need to establish a reliable and prone to scale up system, for their expansion and differentiation. One area of interest is the cardiovascular differentiation, due to the high morbidity and mortality associated with cardiovascular diseases (CVD). This work aimed to expand and differentiate hiPSC into cardiomyocytes using newly developed media in two culture systems, 2D as adherent culture and 3D as suspension aggregates. Cells were expanded in monolayer and as suspension aggregates being their pluripotency confirmed, after the expansion by immunocytochemistry, embryoid bodies formation and flow cytometry. After expansion, the percentage of OCT4 obtained was up to 99.6%, in 2D and up to 97.6%, in 3D. hiPSC were differentiated through the temporal modulation of the canonical Wnt signaling pathway for 12 days. The cells differentiated in 2D started to demonstrate spontaneous contraction at day 8 and in 3D at day 7 of differentiation. The expression of the pluripotency marker (Nanog) gradually decreased over time, while the expression of the cardiac marker (TNNT2) gradually increased. Flow cytometry analysis performed at day 12 revealed up to 42.2% cTnT positive cardiomyocytes in 2D and up to 78.2% in the differentiation performed in 3D. Despite being necessary further optimization, the results suggest that the protocol could be used to the expansion of hiPSC and posterior differentiation into cardiomyocytes.

KEYWORDS: Human induced pluripotent stem cells, expansion, cardiac differentiation, cardiomyocytes

1. INTRODUCTION

Stem cells are a widely discussed topic in biology, that draws attention from researchers and general public, as well. The spotlight in this topic is due to all their possible usages that can go from drug screening to regenerative medicine. Currently, stem cells are functionally defined as cells that have the capacity to self-renew (can divide and maintain the undifferentiated state) and to differentiate originating specialized cells [1]. Stem cells gradually differentiate into more specialized cells, losing their differentiation potential [1]. Consequently, stem cells are classified according to their differentiation potential as: totipotent, pluripotent, multipotent and unipotent. Pluripotent stem cells (PSC) can generate cells from the three embryonic germ layers (endoderm, mesoderm and ectoderm). [1, 2]

One type of PSC widely investigated are the Embryonic Stem Cells (ESC). The first ESC were described in 1981, when were isolated from the murine inner cell mass of the developing blastocyst by Martin Evans and co-workers [3] and Gail Martin [4]. Since that date, ESC had been widely studied and in 1998, a team lead by James Thomson and Jeffrey Jones, obtained, for the first time, human embryonic stem cells (hESC) [12]. The initial hESC lines were derived from “spare” embryos produced by in vitro fertilization. Although, the generation of hESC has arouse some ethical issues, since would imply the use of human embryos for scientific research, making their use very restricted and non-consensual [2, 5]. In 2006, Takahashi and Yamanaka made one of the most promising discoveries of the century by successfully

obtain PSC from mouse embryonic and adult fibroblasts [6]. The induction of PSC was accomplished by means of retrovirus and four transcription factors: OCT3/4, Sox2, c-Myc and KLF4. One year later, the same team obtain PSC from human adult fibroblasts [7]. Also, in 2007, another group of researchers have reported the induction of PSC from human somatic cells using different transcription factors (OCT3/4, Sox2, Nanog and Lin28) and lentiviruses [8]. iPSC had all the key characteristics of ESC, namely having the same pluripotency markers (Stage specific embryonic antigen - SSEA-3/4, tumor rejection antigen (TRA)-1-60 and TRA-1-81), formed teratomas in vivo and had the same morphology [9, 10]. PSC can be cultured in two-dimensional (2D) or three-dimensional (3D) systems. In 2D systems PSC are cultured as a monolayer on top of an adhesion substrate. [11] Currently, a widely used substrate is Matrigel, that is a complex mixture, harvested from Engelberth-Holm-Swarm mouse sarcoma cells and is mainly composed by laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen and growth factors. [12] The 2D culture has the advantage that all the cells in culture receive the same amount of nutrients and growth factors and when in expansion dead cells are easily removed within medium change. Although, the 2D culture does not accurately mimics the 3D environment of cells, misleading data for *in vivo* responses. Besides, cells are morphologically different

from cells in vivo and the production of a large quantity of hPSC in monolayer is very restricted [13].

In the case of 3D culture, the hPSC are expanded as aggregates that can be in a certain type of scaffold/matrix or floating scaffold-free. [13] A popular method for aggregates formation is the random aggregation in suspension culture. This method is very simple and allow the creation of a large number of aggregates, but usually the aggregates present heterogeneity in size and also in shape. [13] The 3D culture sustains many advantages: cells grow in a 3D environment, allowing cells to interact with each other and with the surrounding environment, representing a robust, scalable way to obtain large amounts of hPSC. Suspension culture make possible the regular sampling, allowing a close monitoring of the cells counts, and aggregates proprieties leading to a well-monitored process [13-16]. On the other hand, the main disadvantage is related with different transfer rates of oxygen, nutrients and growth factors through the aggregates [13]. The applications of hiPSC had started to emerge, being already used for some disease modelling and a clinical trial using hiPSC is being performed [17]. This clinical trial uses derived retinal pigment epithelium cells in order to treat macular degeneration.

An organ that have aroused a special interest is the heart. Cardiovascular diseases are the leading cause of death worldwide accounting for more than 17.3 million deaths per year [18]. The need of revolutionary treatments for severe cardiovascular problems has stimulated the research using hiPSC in this area. The three major approaches for the differentiation of hPSC into cardiomyocytes are: aggregates/Embryoid bodies (EB), monolayer culture and inductive co-culture[19]. Independently of the chosen system to the differentiation of hiPSC into cardiomyocytes, the most efficient protocols rely on the basal medium RPMI 1640, supplemented with B27, and in the modulation of the Wnt signaling pathway [20]. RPMI 1640 medium is chemically defined and is a complex mixture of 21 components (with many having animal origin). Despite of that, a new protocol using a chemically defined medium for cardiac differentiation was proposed by Burrige and his co-workers [21]. This medium consists on only three components: the basal medium RPMI 1640, L-ascorbic acid 2-phosphate and, rice-derived recombinant, albumin (an alternative for bovine serum albumin) and has achieved up to 95% Troponin T 2+ (TNNT2) cells, being successful with 11 different linages [21]. Troponin T 2 is the gene that encodes cTnT and it is specific for cardiomyocytes [22] Through the differentiation of hiPSC into cardiomyocytes several markers can be identified, being that the pluripotency markers decrease their relative expression and at the same time the cardiac markers expression increases.

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CM) are without a doubt a promising tool for cardiac cellular therapy and tissue engineering. In consequence, several animal models have been developed throughout the years. Many of the animal models developed and that are used in trials, rely on mice or rats, but the heart of these animals displays several differences from the human heart. These differences concern the size of the heart, the cardiac rate and variations at cellular and molecular level [23]. So, there is a need to support the results obtained in rodent in large animal models more closely resembling the human heart. Chong and Murray [23] developed the first large animal studies using hPSC for cardiac regeneration. Namely, they used hESC derived cardiomyocytes (hESC-CM) in a non-human primate

model of ischemic cardiac injury. The results were very promising since it was obtained an extensive evidence of engraftment, remuscularization and electromechanical synchronization, 2 to 7 weeks after transplantation. However, electrocardiographic analysis revealed some arrhythmias in treated monkeys [23].

The capacity to obtain relatively pure populations of cardiomyocytes is important for multiple applications of these cells. Studies of global gene expression are more reliable if the study is performed in a purified population [19, 24]. Besides, to perform cardiac drug assays, there is a need to generate a purified cardiomyocytes population, to be possible to attribute the measured alterations to direct effects on cardiomyocytes. Also, for hPSC-CM to be a reliable system to obtain cardiomyocytes for cellular therapy, researchers need to ensure that an efficient purification method is developed. The tumorigenic potential of the residual undifferentiated cells raises safety concerns for the application of hPSC-derived cardiovascular cells, due the possible formation of teratomas. Although, it is important to note that the heart is formed by three cellular types, and for some cellular applications it will be beneficial to use a "mixed" population instead of one solely constituted by cardiomyocytes [19, 24]. One of the most used purification method is called the lactate method and it was developed by Tohyama and co-workers [25]. This purification method is based on the marked biochemical differences in glucose and lactate metabolism between cardiomyocytes on non-cardiomyocytes, including undifferentiated cells. Using this method, it was obtained cardiomyocytes up to 95% of purity and with high proliferative capacity, that did not form tumors after transplantation [25].

2. MATERIALS AND METHODS

2.1. Human pluripotent stem cell culture

Cells were routinely maintained in StemFlex™ (ThermoFisher Scientific™) on Matrigel® (Corning®) and passed, as small clumps, using 0.5mM EDTA (Invitogen™) for 5 minutes at room temperature. It was used two pluripotent cell lines in this work: F002.1A.13 (TCLab, Tecnologias Celulares para Aplicação Médica, Unipessoal, Lda) and Gibco® Human Episomal iPSC Line Gibco (Life Technologies – Thermo Fisher Scientific).

2.2. hPSC aggregates formation and expansion

For the hPSC aggregates formation were used cells previously growth in monolayer (as mentioned in **2.1 Human pluripotent stem cell culture**). When cells reached an approximate confluency of 85% were detached from the monolayer using EDTA. Cells were counted to originate aggregates from different seeding densities. The used seeding densities were in a range between 1×10^5 and 2×10^6 cells/mL. Finally, the calculated volume of cell suspension and StemFlex™ supplemented with 1x (v/v) Revitacell™ Supplement (ThermoFisher Scientific™) were both transferred to a 6 well Ultra Low Attachment Multiwell plate (Corning® Costar®) making a total volume of 2 mL/well. The mixture of cells and medium was carefully homogenized, and the newly formed aggregates were incubated in a humidified incubator at 37°C with 5% of CO₂ and 20% of O₂. The aggregates were expanded between 2-7 days and 80% of the culture medium was changed daily. It was performed a cell counting in the last day of expansion in order to calculate the fold increase. The equation used to calculate the fold increase is represented in equation (1).

$$\text{Fold increase} = \frac{\text{Number of cells in the end of expansion}}{\text{Number of cells seeded}} \quad (1)$$

In order to evaluate the efficiency of the aggregation process, aggregates were formed as mentioned before and after 24 hours, the aggregates were selectively separated using a 37 μm Reversible Strainer (Stemcell Technologies™) from the waste and cells that remain in single cell. The aggregates were singularized by incubation with Accutase for 7 minutes. A sample of the dissociated aggregates was collected for cell counting, as well, as a sample from the cells that remained in single cell.

The day by day evolution of the aggregates diameter was calculated. Fiji software for ImageJ [26] was used to measure the aggregates area and Microsoft® Excel to calculate the average diameter.

2.3. hPSC monolayer formation and expansion

The method used was very similar to the method described in **2.3 hPSC Aggregates formation and expansion**. Although, in this case the calculated volume of cell suspension and StemFlex supplemented with Revitacell 100x (v/v) were both transferred to a 12-well tissue culture plate with Matrigel-coated wells, making a total volume of 1 mL/well. It was used seeding densities (cells/cm²) in a range of 1×10^4 - 1×10^5 . The cells were expanded between 1-4 days and the culture medium was changed daily. The fold increase was also calculated with equation (1).

2.4. Embryoid bodies culture

Aggregates were formed as mentioned in **2.3 hPSC aggregates formation and expansion**. Afterwards, to obtain embryoid bodies, the aggregates were differentiated 26 days using Embryoid bodies medium. This medium was composed by KnockOut™ DMEM supplemented with 25% (v/v) Heat Inactivate - Fetal Bovine Serum (Gibco®), 1% (v/v) of MEM-NEAA (Gibco®), Penicillin/Streptomycin (PenStrep, Gibco®) and L-Glutamine 200 mM (Invitrogen™ Gibco®). 80% of the medium was changed every-other-day. At day 26 of differentiation, the aggregates were dissociated using 0.5% Trypsin – EDTA and replated in 24 well tissue culture plates (Falcon®). The wells were previously coated with Poly-ornithine (15 $\mu\text{g}/\text{mL}$ - Sigma Aldrich®) and Laminin (20 $\mu\text{g}/\text{mL}$ – Sigma Aldrich®). Afterwards, the cells were expanded for 7 days in EB medium. The success of the Embryoid bodies differentiation was assessed by intracellular immunocytochemistry, using a confocal Zeiss Laser Scanning Microscope 710.

2.5. Cardiac differentiation of hiPSC

The protocol for cardiac induction used in this work was adapted from the work developed by Burrige and co-workers.[21] It was used a Pluripotent Stem Cells Cardiomyocyte Differentiation Kit (Gibco® ThermoFisher Scientific™). The medium used for the cardiac induction is chemically defined consisting in just three components: the basal medium RPMI 1640, L-ascorbic acid 2-phosphate and rice-derived recombinant human albumin. The three media only differ in two small molecules. Cardiomyocyte Differentiation Medium A (Gibco® ThermoFisher Scientific™) contains 6 μM CHIR99021. On the other hand, Cardiomyocyte Differentiation Medium B (Gibco® ThermoFisher Scientific™) is supplemented with 2 μM Wnt-C59. Finally, Cardiomyocyte Maintenance Medium (Gibco® ThermoFisher Scientific™) only

has the three major components, not being supplemented with small molecules.[21] The approximate confluency (2D culture) and the average aggregate diameter (3D culture) before the cardiac induction were registered. At day 0 of differentiation, medium was changed to Cardiomyocyte Differentiation Medium A. After 2 days, the medium was changed to Cardiomyocyte Differentiation Medium B. At day 4, the medium was changed to Cardiomyocyte Maintenance Medium and 80% of the medium was changed every-other-day until the end of the experiment at day 12 of differentiation. At day 12, cells were replated and collected in order to be qualitatively and quantitatively analyzed. The success of the differentiation was evaluated by the exhibition of spontaneous contraction (registered the first day of spontaneous contraction), by immunocytochemistry (expression of NKX2.5 and cTnT), by flow cytometry (expression of cTnT) and by quantitative real-time polymerase chain reaction analysis (qRT-PCR).

2.6. Immunofluorescence staining

Cells were washed with PBS and fixed with PFA 4% (v/v) for 10 minutes, in the case of hPSC, or for 30 minutes in the case of differentiated cells. After that, cells were incubated with blocking solution (PBS with 10% (v/v) Normal goat serum (NGS) and 1% (v/v) Triton-X100 (Sigma - Aldrich)) for 30-60 minutes at room temperature. Primary antibodies 1:150 OCT4 (Merck Millipore), 1:500 Sox2 (R&D Systems), 1:200 cTnT (ThermoFisher Scientific™), 1:1000 NKX2-5 (ThermoFisher Scientific™), 1:1000 Tuj1 (Covance), 1:1000 α – SMA (Dako) and 1:1000 Sox17 (R&D Systems) were diluted in staining solution (PBS with 5% (v/v) NGS and 0.1% (v/v) Triton-X-100) and the cells were incubated overnight at 4°C. Then, cells were washed with PBS and incubated with secondary antibodies 1:500 Goat anti-mouse IgG (Invitrogen™), 1:250 Donkey-anti-rabbit (ThermoFisher Scientific) diluted in staining solution for 1 hour, in the dark. Cells were washed with PBS and afterwards incubated with 4',6-diamidino-2-phenylindole (DAPI diluted 1:10000 in NaCO₃ – Sigma Aldrich) for 2 minutes at room temperature. Afterwards, cells were washed and left in PBS. Finally, cells were observed under fluorescence optical microscope (Leica Microsystems CMS GmbH, model DMI3000 B) or confocal microscopy (Zeiss Laser Scanning Microscope 710). For hPSC-CM, the Human Cardiomyocyte Immunocytochemistry kit (ThermoFisher Scientific™) was also used according to manufacturer's guidelines.

2.7. Flow cytometry

All samples were collected to a Falcon® tube and singularized due to incubation with Accutase or 0.5% Trypsin -EDTA for up 15 minutes at 37°C and resuspended in 1 mL of PFA 2% in PBS and stored at 4°C.

Intracellular staining of hPSC

Cells stored in PFA 2% were centrifuged for 3 minutes at 1000 rpm and washed twice with 1% normal goat serum (NGS, Sigma Aldrich) in PBS. Eppendorf tubes were previously coated with 1% (v/v) of BSA (Invitrogen) in PBS for 15 minutes. Afterwards, cells were resuspended in 3% NGS and transferred to the previously coated with 1% (v/v) BSA in PBS eppendorfs. Then, cells were centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded, and cells were permeabilized using (1:1) 3%NGS and 1% saponin (Sigma-Aldrich), during 15 minutes at room temperature. Cells were centrifuged for 3

minutes at 1000 rpm. Afterwards, the supernatant was discarded, and cells resuspended in 3% NGS and incubated at room temperature for 15 minutes. Then, cells were washed with 3% NGS and pellets of negative controls were resuspended only in 3% NGS and the others were resuspended with 1:300 OCT4, in 3% NGS and incubated during 90 minutes at room temperature. Afterwards, cells were centrifuged 3 minutes at 1000 rpm and washed twice with 1% NGS and incubated in the dark, for 45 minutes, with 1:1000 Goat anti-mouse IgG Alexa Fluor 488. Afterwards, cells were washed twice with 1% NGS and in the last wash cells were resuspended in PBS, transferred for FACS tubes (Falcon®) and analyzed in FACSibur™ flow cytometer (BS Biosciences®). The results were analyzed using Flowing Software 2.0 (Turku Centre for Biotechnology).

Intracellular staining of cardiomyocytes

Cells stored in PFA 2% were centrifuged for 3 minutes at 1000 rpm and resuspended in 90% (v/v) cold methanol and incubated 15 minutes at 4°C. Following incubation, the suspension was centrifuged for 3 minutes at 1000 rpm, the supernatant was discarded, and cells were washed three times with flow cytometry buffer (FCB) 1 (0.5% BSA in PBS). Afterwards, negative controls were resuspended in FCB 2 (0.5% BSA, 0.1% of Triton X-100 in PBS) and the other cells were resuspended in 1:200 cTnT diluted in FCB2 and incubated during 1 hour at room temperature or at 4°C overnight. Then, cells were washed once with FCB2 and the cell pellet was resuspended 1:1000 Goat anti-mouse IgG Alexa Fluor 488 diluted in FCB2 and left incubate for 30 minutes at room temperature in the dark. Cells were then washed three times with FCB 2 and resuspended in FCB1 and transferred to FACS tubes. Samples were analyzed in FACSibur™ flow cytometer (BS Biosciences®). The results were analyzed using Flowing Software 2.0 (Turku Centre for Biotechnology).

2.8. qRT-PCR

RNA was extracted from the samples using High Pure RNA Isolation Kit (Sigma-Aldrich®). RNA amount for each sample was quantified using NanoVue™ Plus spectrophotometer (GE Healthcare®). Complementary deoxyribonucleic acid (cDNA) was synthesized from 100 ng of RNA through the usage of High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). The qRT-PCR was performed in 384-wells plate, in which cDNA samples were placed with TaqMan® Gene Expression Master Mix (Applied Biosystems) and with the Taqman probes. The housekeeping gene used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the primers were NANOG and TNNT2. The plate was analyzed in ViiA 7™ Real-Time PCR Instrument. Threshold cycles (C_T) were compared with the housekeeping gene C_T originating ΔC_T . Also, the previous mentioned values were normalized using the day 0 C_T resulting in $\Delta\Delta C_T$. Lastly, the final results of gene expression were presented as $2^{-\Delta\Delta C_T}$.

2.9. Statistical analysis

Graphs present the mean value and error bars represent the standard error of the mean (SEM).

3. RESULTS AND DISCUSSION

3.1. Expansion and characterization of hiPSC in StemFlex™ culture medium in adherent culture

Cells in adherent culture were expanded from different initial seeding densities (cells/cm²). The cellular growth was assessed through the fold increase calculation, after 3 days of expansion, for the two cell lines (**Figure 1 A and B**). A fold increase of 2 indicates that after the expansion the amount of hPSC that were collected was two times the seeded hPSC. In all cases the fold increase was higher than 2. This may indicate that the StemFlex medium is appropriate to expand cells maintaining their pluripotent state. The fold increase for TCLab cell line was considerably superior than for Gibco cell line, which may indicate that the proliferative capacity of TCLab is bigger than for Gibco cell line. Also, apart from Gibco cell line with a seeding density of 10.000 cells/cm², lower seeding densities lead to higher fold increases (**Figure 1 A and B**). This may be explained by the fact that the expansion was performed in an adherent system being the space for expansion limited. Also, a previous work in which was evaluated the expansion of hPSC (hiPSC and hESC) in mTeSR™ medium revealed that the cellular growth decreased after reaching a certain cellular density (cells/cm²) leading to slower proliferation [27]. The lower value for the Gibco cell line when seeded at 10.000 cells/cm² could be due to losses of cells in the collection after the expansion or due to a higher cellular death in this condition.

For the Gibco cell line the average value of OCT4 positive cells concerning all the different seeding densities was 97.62% (**Figure 1 C**) and for the TCLab cell line was 96.2% (**Figure 1 D**). Apparently, the different seeding densities do not have a considerable impact in the pluripotent state after three days in expansion in adherent culture. Chen and co-workers [28], evaluated the expression of pluripotency markers of hiPSC after their expansion, in adherent culture on Matrigel, in a culture medium called Essential 8™. In the previous protocol, a high expression of OCT4 was obtained (>90%). The results obtained by Chen and co-workers were very similar to the results obtained in this work. This may indicate that the cells can be expanded in 2D using StemFlex medium and conserve their pluripotency, being able to be posteriorly differentiated.

3.2. Expansion and characterization of hiPSC in StemFlex™ culture medium as suspension aggregates

The hiPSC growth was calculated through the fold increase after hiPSC expansion as 3D aggregates (**Figure 2 A and B**) and in general, with the increase of the number of cell seeded per volume, the fold increase diminishes, indicating that when less cells are originally seeded more growing can occur. This could be due to only be possible to the aggregates to achieve a certain cellular number (cells/aggregate), being that when that number is achieved, the aggregates reduce the overall yield [32]. Although, this did not occur for the seeding density $1.0 \times 10^{+05}$ cells/mL that reveals a yield lower than $1.5 \times 10^{+05}$ and $2.5 \times 10^{+05}$. This could be due to a loss of cells on the collection step or due to an abnormal cellular death during the expansion. In general, the fold increase for the TCLab cell line (except for the seeding density of $1.5 \times 10^{+05}$, **Figure 2 B**) was higher, for the same seeding densities, than for the Gibco cell line (**Figure 2 A**). These results are corroborated by the obtained in the

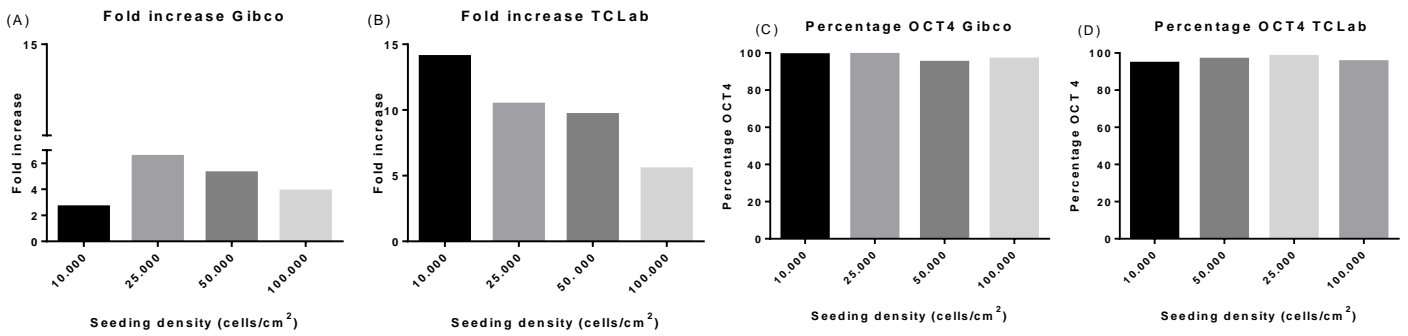


Figure 1 : (A-B): Fold increase in cell number after expansion in adherent culture. Cells were seeded with different densities (10.000, 25.000, 50.000, 100.000 cells/cm²) and expanded in adherent culture for 3 days using StemFlex™ medium. Afterwards, it was calculated the fold increase. It is depicted the fold increase for the different seeding densities for the **Gibco** cell line **(A)** and **TCLab** **(B)** (n=1). **(A-B): Percentage of OCT4 positive cells. (A) Gibco and (B) TCLab** cell line. A flow cytometry analysis was performed, in order to assess pluripotency of the cells (n=1).

expansion as adherent culture, in which the cells from TCLab cell line demonstrate a higher proliferative capacity.

The percentage of OCT4 positive cells after the expansion as aggregates, in StemFlex™, was assessed by flow cytometry. The results for Gibco cell line vary from 86.2% to 97.6% (**Figure 2 C**) and for TCLab from 70.3% to 95.7% (**Figure 2 D**). The values obtained were very similar to the ones obtained by Chen *et al.*, that registered a percentage of OCT4 positive cells of over 90% for cells expanded in aggregates with seeding densities between $3\text{-}3.5 \times 10^{+05}$ [29]. In all the seeding densities, considering both cell lines, the lower value of OCT4 positive cells was associated with the higher seeding densities ($5 \times 10^{+05}$ and $1 \times 10^{+06}$). This may indicate that higher seeding densities in

the aggregates will lead to more spontaneous differentiation. In the higher seeding densities, the number of aggregates that “merge” with each other is more prominent, leading to bigger aggregates with distort shapes. As mentioned by Chen and co-workers, this causes more difficulties for cytokines and nutrients to penetrate the aggregates, which may result in a decreased proliferation and an increase differentiation [30]. A previous expansion of hESC in ultra-low-attachment plates accomplish percentages higher than 90% of OCT4 positive cells after the expansion [31]. The results obtained in this work were similar (>90%) with the exception of the higher seeding densities.

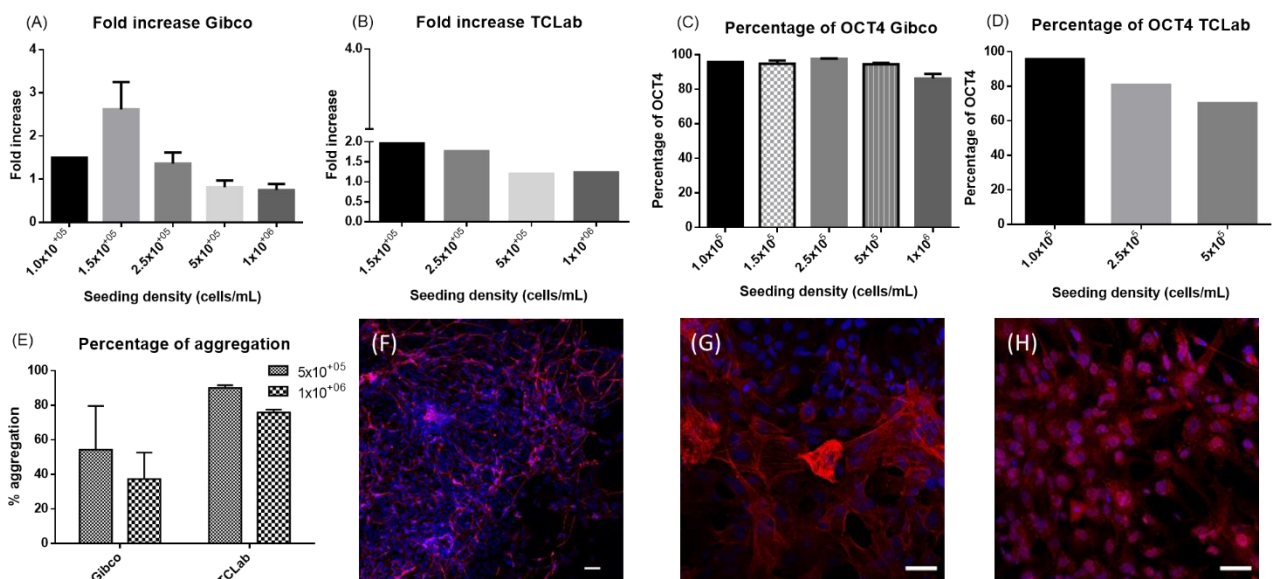


Figure 2: (A-B) Fold increase in cell number after expansion as aggregates in StemFlex medium for (A) Gibco cell line (B) TCLab cell line. Error bars represent the SEM value. **(A)** It was calculated the fold increase after the culture for up to 6 days in Gibco cell line aggregates. It is depicted the fold increase (vertical axis) for the different seeding densities $1 \times 10^{+05}$ (n=1), $1.5 \times 10^{+05}$, $2.5 \times 10^{+05}$, $5 \times 10^{+05}$ and $1 \times 10^{+06}$ (n=2, horizontal axis). **(B)** Aggregates formed with TCLab cell line were expanded for 3 days (n=1). **(C-D) Percentage of OCT4 positive cells. (C) Gibco and (D) TCLab** cell line. Cells were seeded with different densities and expanded as aggregates for up to 6 days in StemFlex™ medium. In the horizontal axis are depicted the different seeding densities used (cells/mL) and in the vertical axis the correspondent percentage of OCT4 positive cells. Error bars represent the SEM value. **(C)** Aggregates Gibco were expanded for up to 6 days (n=1 for $1 \times 10^{+05}$ and n=2 for the other seeding densities). **(D)** Aggregates TCLab for 3 days (n=1). **(E) Efficiency of the aggregation process.** The percentage of aggregation was calculated for the two cell lines, Gibco at left (n=3), and TCLab at right (n=2), for two seeding densities ($5 \times 10^{+05}$ and $1 \times 10^{+06}$ cells/mL). **(F-H) Intracellular staining of the replated EB.** The images were obtained using Zeiss Laser Scanning Microscope 710. The blue staining corresponds to DAPI marker. Scale bars: 50 μ m. This experiment was performed using the Gibco cell line. **(F)** Ectoderm marker (Tuj1) resolution: 2048x2048 **(G)** Mesoderm marker (α -SMA) resolution: 1024x1024 **(H)** Endoderm marker (Sox17) resolution: 1024x1024.

In order to unveil the efficiency of the aggregation process it was performed the cell counting 24 hours after the aggregates formation (**Figure 2 E**). The percentage of aggregation obtained for TCLab cell line was higher than for Gibco cell line and the percentage of aggregation for the seeding density of $5 \times 10^{+05}$ was higher than for $1 \times 10^{+06}$, as well. Therefore, it seems that the aggregation process in StemFlex medium is more efficient for the TCLab cell line, than for Gibco cell line, and that with higher seeding densities, are associated more loss of cells that did not aggregate in the first 24 hours. This could be due to the fact than in that time range only is possible to occur the aggregation of a certain quantity of cells being that all the "extra" cells remain as single cell, being removed within the medium change. This is corroborated by experiments performed by Dang et al. [32], in which with the increase of the of murine ESC density the efficiency of aggregation decreased. To improve the aggregation percentage, cells could be cultured in a rotary system, such as an orbital shaker. Carpenedo et al. [33] compared the efficiency of aggregates formation, using ESC, in static and rotary conditions. They conclude that the efficiency was much higher in rotary system than in static culture.

In addition, a pluripotency assessment through aggregates/embryoid bodies formation was performed. In this test was assessed the capacity of hiPSC to generate cells from the three germ layers (ectoderm, mesoderm and endoderm). [2, 9]. The results represented in **Figure 2 F-H** seem to indicate, once again, that cells cultured in StemFlex medium remain in a pluripotent state and consequently can be differentiated into any cellular type.

3.3. Differentiation of hiPSC in adherent culture

After the expansion in monolayer for up 4 days, the approximated confluency for each seeding density was registered and the differentiation was initiated afterwards. The differentiation was performed for 12 days and the efficiency of the differentiation was assessed through flow cytometry (**Figure 3 A and B**), spontaneous contraction (**Figure 3 C and D**) and immunocytochemistry (**Figure 3 E**).

Gibco and TCLab cells started to contract between day 8 - 12. In previous experiments it was reported that hPSC differentiation into the cardiac lineage as monolayer started to contract spontaneously between day 7 – 9, and here we observe that the start of the spontaneous beating was relatively delayed [21].

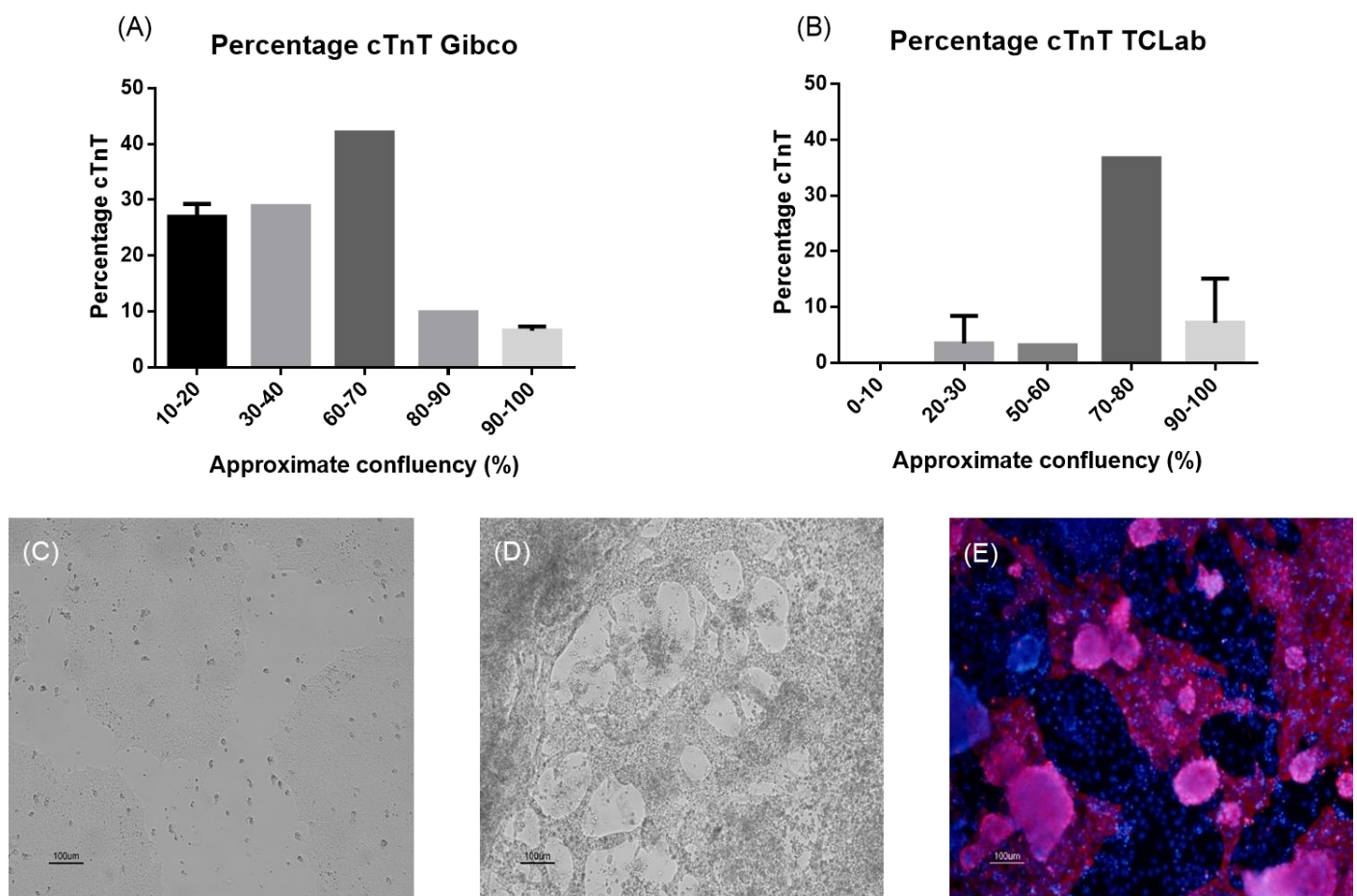


Figure 3: (A-B) Percentage of cTnT positive cells according to the approximate confluency before cardiac induction for Gibco cell line (A) and for TCLab cell line (B). The differentiation process was initiated at different approximate confluences in monolayer and the percentage of cTnT at day 12 of differentiation is represented in function of the initial approximate confluency. For Gibco cell line: 30-40%, 60-70% and 80-90% (n=1), 10-20% and 90-100% (n=2). For TCLab cell line: 50-60% and 70-80% (n=1), 0-10, 20-30% and 90-100% (n=2) **(C) Morphology of cells differentiated in adherent culture at day 0 of differentiation.** It is represented an approximate confluency of 60-70% before cardiac differentiation. Scale bar: 100 μ m. **(D) Morphology of hiPSC-CM in the first day of spontaneous contraction.** In this image is represented Gibco cell line. Scale bar: 100 μ m. **(E) Intracellular staining of hiPSC-CM differentiated in monolayer.** This experiment was performed with Gibco cell line with an approximate confluency (%) of 60-70%. DAPI staining is represented in blue and cTnT in red. Scale bar: 100 μ m

Through the flow cytometric analysis, it was possible to access that, the most suitable approximate confluency for the cardiac differentiation is between 60 and 70% for Gibco (**Figure 3 A**) and between 70 and 80% for TCLab cell line (**Figure 3 B**). For both cell lines higher confluences rates did not lead to a successful differentiation (approximate confluency 90-100%). This fact is corroborated by the work of Burrige *et al.* [21] that highlight that the prevention of over confluence is determinant for a successful cardiac differentiation. At the same time, Lian *et al.* [34] described the need of generate confluent monolayers to perform efficient differentiations. This may indicate that average cell confluence before cardiac induction plays a key role in the efficiency of the differentiation towards the concentration of the paracrine factors. The cTnT positive cells in the two cell lines for the same approximate confluency portrays different percentages, this could be caused by different rates and conditions at which different hiPSC lines differentiate into cardiomyocytes, being difficult to optimize the differentiation conditions for several cell lines [35, 36]. In addition, for the Gibco cell line was possible to obtain higher values of cTnT positive cells for a wider range of approximate confluences than for the TCLab cell line. It is known that hiPSC lines display some epigenetic diversity being more prone to be differentiated in some cellular lineages than others [37]. This can explain the wider range of approximate confluences for the Gibco cell line, in which was possible to obtain higher percentages of cTnT positive cells. Also, the lower percentage of cTnT positive cells that we obtained, comparatively to the article in which our protocol is based, could be explained by the usage of cell lines less prone to cardiac differentiation [21]. In addition, in most of the protocols the differentiation was performed for at least for 15 days [20, 21, 34]. So, if the days of cardiac induction were expanded, probably it will be possible to achieve higher percentages of cTnT. Moreover, an “*off-the-shelf*” culture medium, that has a constant concentration of the molecules to induce and to inhibit the Wnt signaling pathway, was used, and these concentrations could be not adequate to the cell lines/culture systems that were tested. Namely, the concentration of CHIR included in Cardiomyocyte Medium Differentiation A was 6 μM but has been reported that the optimal CHIR concentration for 2D culture is higher than for 3D culture [16]. Namely, for 2D culture the optimal concentration was 10 μM of CHIR and for 3D only 7.5 μM [16]. The previous conclusions were obtained through the usage of an ESC cell line and confirmed with hiPSC, this fact may indicate that for an efficient monolayer differentiation would be necessary a higher CHIR concentration than the used in aggregates [16]. These conclusions were corroborated by posterior works from the same researchers, although the CHIR concentrations that lead to better results were not the previously mentioned [38]. To enhance the cardiomyocytes percentage, it is also possible to use a purification method. Kadari *et al.* [39] tried to develop a robust protocol for the generation of cardiomyocytes through the differentiation in monolayer for several iPSC lines and obtained yields from 33 to 92% of cTnT positive cells, before purification, which once again sustains the high line-to-line variability that leads to very different cardiac efficiencies. Nevertheless, they demonstrate that even the lowest percentages of cTnT positive cells could be optimized up to 74% using the lactate purification method. [25, 39] Moreover, Nguyen [40] *et al.* were able to achieve percentages of ~90%

cTnT from initial populations of 10-40% of cardiomyocytes by generating 3D cardiospheres from adherent cultures of cardiomyocytes, using microwells. Thus, both methods could also be used to improve the cTnT percentage obtained by our protocol under adherent culture conditions.

3.4 Differentiation of hiPSC into cardiomyocytes as suspension aggregates

Previously formed aggregates using StemFlex medium were expanded for up to 7 days before the induction of the cardiac differentiation. The success of the differentiation was assessed through qRT-PCR, flow cytometry (**Figure 4 A-D**), spontaneous contraction (**Figure 4 E**), and immunocytochemistry (**Figure 4 F**). Through qRT-PCR was possible to observe the decreasing of pluripotency marker expression (Nanog) and the increasing of the cardiac marker (TNNT2), gradually over time.

The earliest day that the aggregates demonstrate spontaneous beating was day 7 of differentiation, for both cell lines. Fonoudi *et al.* [41], also performed experiments with the goal of differentiate aggregates of hPSC (both hESC and hiPSC) into cardiomyocytes. They used a more complex protocol, in which besides, modelling the canonical Wnt signaling pathway, they modulated other signaling pathways. However, the earliest spontaneous beating was at day 7 of cardiac differentiation [41]. Using our protocol that only modulates the Wnt pathway was possible to achieve the same results, concerning the start of the spontaneous beating.

Through **Figure 4 A**, is possible to observe that the better results for the Gibco cell line were hosted between the diameters of 155-205 μm with an overall average value of 30.6%. The highest value obtained in one experiment was 78.18% and it was obtained with an average diameter before cardiac induction of 195-205 μm . Also, Gibco cell line aggregates with an average diameter smaller than 135 μm expressed a very low percentage of cTnT. Etoc *et al.* [42] micropatterned hESC colonies that recapitulate the spatial arrangement of the germ layers, in order to unveil the earliest aspects of human embryogenesis. They discover that cells establish their fate by measuring their distance from the edge and that the reduction of the diameter eliminates the mesodermal fates, which can explain the fact that with diameters lower than 135 μm the percentage of cTnT positive cells is below 15%. Also, Fonoudi *et al.* [41], when performed experiments to discover the best aggregate diameter to perform cardiac induction realized that for diameters between 60-120 μm occurred the disruption and the dispersion of the aggregates, after the CHIR treatment. This, once again can explain the low values for cTnT obtained with diameters lower than 135 μm .

On the other hand, in **Figure 4 B**, is possible to observe that the percentage of cTnT-positive cells for the TCLab cell line is very similar for all the diameters. Nevertheless, a slightly higher percentage of cTnT is attained for an average diameter of 205-215 μm and highest result obtained was 23.4%. These results appear to indicate that the optimal hPSC diameter for the initiation of the cardiac induction has line-to-line variability [39].

Fonoudi *et al.* [47], when establishing a protocol for cardiac differentiation in aggregates they identified the better diameters for the beginning of the cardiac differentiation between 150-200 μm for several hESC and hiPSC cell lines

[132]. These results are very similar to the ones obtained in this work: 155–205 μm (considering the two cell lines used).

In **Figure 4 C and D** the percentage of cTnT positive cells is presented accordingly to the seeding density. It seems that the best seedings densities for the cardiac induction are $5.0 \times 10^{+05}$ and $1.0 \times 10^{+06}$ cells/mL for Gibco cells (**Figure 4 C**). The results obtained for TCLab were not very conclusive seeming that the best seeding density is $1.0 \times 10^{+06}$ cells/mL (**Figure 4 D**). Higher percentages of cTnT-positive cells were achieved for a wider range of seeding densities using the Gibco cell line rather than using the TCLab cell line. Kempf *et al.* [40] previously conclude that the initial density was relevant mainly due the concentration of paracrine factors that were released from the cells to the culture medium, appearing to be important to the initial cells survival and a correct aggregate formation.

The percentage of cTnT positive hPSC-CM that was obtained was relatively low, but this percentage could be easily enhanced by cardiomyocyte purification after differentiation. Hemmi *et al.* [36] could obtain a percentage of 99.5% post-purification cardiomyocytes from an initial population of 25.0% using the lactate method. So, after the day 12 of differentiation aggregates can be purified using this method to obtain a higher percentage of cTnT positive cells. As mentioned before in **3.3 Differentiation of hiPSC in adherent culture**, a medium that already had in their composition the molecules to induce and to inhibit the Wnt signaling pathway, was used. Therefore, the used concentrations could be not adequate for the differentiation in this culture system. Also, the aggregates were only differentiated for 12 days, with an increase of the days of

differentiation, probably would be possible to achieve higher percentages of hiPSC-CM [16, 21].

4. CONCLUSIONS

Since the discover of PSC and more specifically the production of hiPSC several researchers tried to establish protocols to ensure their correct expansion in order to be possible to differentiate these cells in almost every cellular type. This holds great promises in the field of disease modelling, drug screening, toxicity assays, personalized medicine and even regenerative medicine. Cardiomyocytes differentiation had attracted special attention, since cardiovascular diseases are the leading cause of death worldwide[18]. So, there is an urge to establish a reliable platform to unveil the mechanisms that are in the origin of these diseases and that at the same time can generate a big quantity of cardiomyocytes and other cardiac cells, that could be used in treatments to ameliorate the quality and quantity of patients. Namely, hiPSC can be considerate an unlimited source of cardiomyocytes [41]. Several differentiation protocols into cardiomyocytes have been developed throughout the years and some obtained impressive percentages of cTnT positive cells. Although, in most of them all the concentrations of small molecules had to be tuned between different cell lines, maybe due to their epigenetic memory [37]. Also, it was not established a reliable protocol that could be both used in adherent differentiation and in suspension aggregates, using different hiPSC lines, as well.

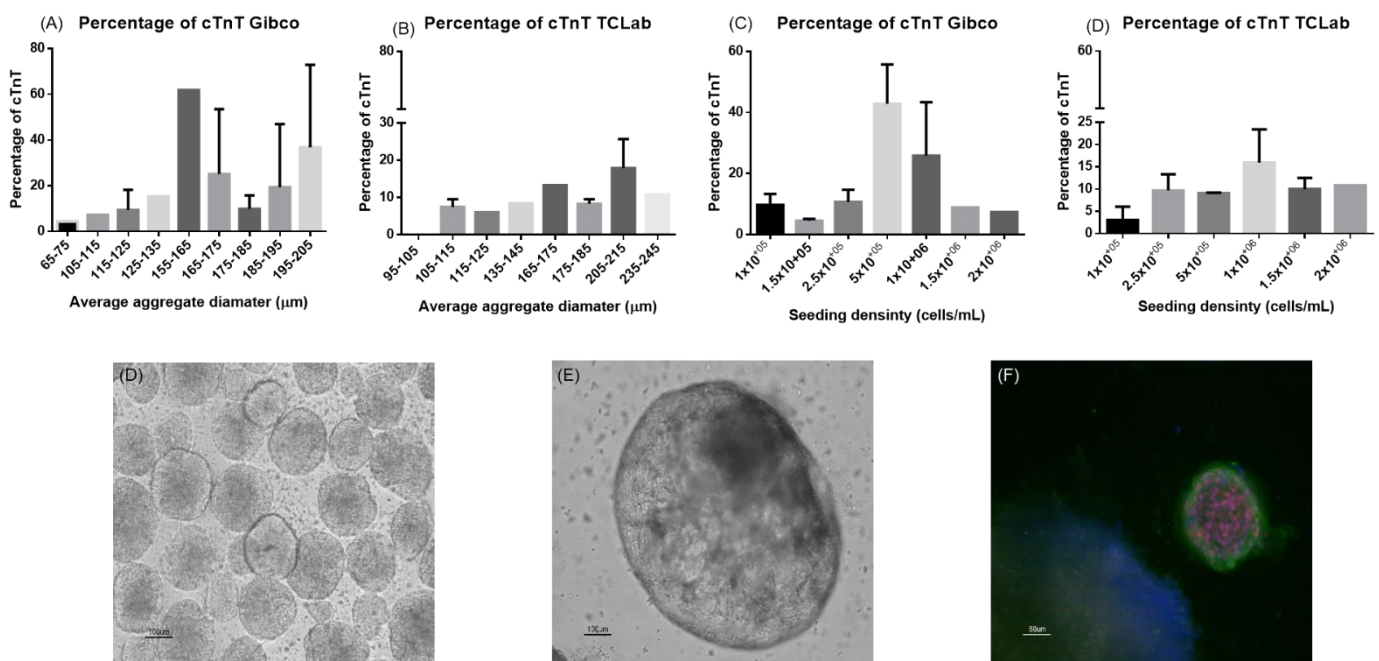


Figure 4: (A) % cTnT positive cells before cardiac induction for Gibco cell line aggregates according to the average aggregate diameter: 65–75 ($n=1$), 105–115 ($n=1$), 115–125 ($n=3$), 125–135 ($n=1$), 155–165 ($n=1$), 165–175 ($n=2$), 175–185 ($n=3$), 185–195 ($n=4$), 195–205 ($n=3$) μm . (B) % cTnT positive cells before cardiac induction for TCLab cell line aggregates according to the average aggregate diameter: 95–105 ($n=1$), 105–115 ($n=2$), 115–125 ($n=1$), 135–145 ($n=1$), 165–175 ($n=1$), 175–185 ($n=2$), 205–215 ($n=2$), 235–245 ($n=1$) μm . (C) % cTnT positive cells for Gibco cell line aggregates, according to the seeding density (cells/mL): $1 \times 10^{+05}$ ($n=3$), $1.5 \times 10^{+05}$ ($n=3$), $2.5 \times 10^{+05}$ ($n=4$), $5 \times 10^{+05}$ ($n=5$), $1 \times 10^{+06}$ ($n=3$), $1.5 \times 10^{+06}$ ($n=1$), $2 \times 10^{+06}$ ($n=1$) cells/mL (D) % cTnT positive cells for TCLab cell line aggregates, according to the seeding density (cells/mL): $1 \times 10^{+05}$ ($n=2$), $2.5 \times 10^{+05}$ ($n=2$), $5 \times 10^{+05}$ ($n=2$), $1 \times 10^{+06}$ ($n=2$), $1.5 \times 10^{+06}$ ($n=2$) and $2 \times 10^{+06}$ ($n=1$) cells/mL (E) Morphology of cells differentiated as suspension aggregates at day 0. It is represented an average diameter of 195–205 μm , obtained with a seeding density of $5.0 \times 10^{+05}$ Scale bar: 100 μm . (F) Morphology of hiPSC-CM in the first day of spontaneous aggregates contraction. Scale bar: 100 μm (G) Intracellular staining of hiPSC-CM differentiated in monolayer. This experiment was performed with Gibco cell line with a seeding density of 1×10^6 cells/mL. DAPI staining is represented in blue, cTnT in green and NKx2.5 in red. Scale bar: 50 μm

The overall objective of this work was to expand hiPSC and generate hiPSC-CM in 2D (monolayer) and 3D (suspension aggregates) systems, using chemically defined media.

Regarding the expansion in 2D and also in 3D, the StemFlex medium seems to achieve good results. The pluripotency assay performed through EB formation confirmed the pluripotent state of the cells. Being the previously mentioned analysis only qualitative, it was performed a quantitative analyses, as well. The fold increase obtained in adherent culture was higher for TCLab cell line than for Gibco cell line. In general, lower seeding densities lead to higher fold increases. Concerning the aggregates, the obtained values were much lower than the ones obtained in monolayer. These results could be due to the aggregates only expand until a certain aggregate density [32]. Through flow cytometry, for cellular culture in monolayer it was always obtained values for up to 99.6% for Gibco cell line and up to 98.2% for TCLab cell line. Concerning the suspension aggregates it was obtained results for up to 97.6% for Gibco aggregates and up to 95.7 % for TCLab aggregates. Although, in aggregates culture, it is possible that higher seeding densities lead to a loss of the pluripotency through the expansion. It was only obtained 80.3% for the highest seeding density ($1 \times 10^{+06}$ cells/mL) for the Gibco cell line and 70.2% for highest seeding density ($5 \times 10^{+05}$) for TCLab cell line. The evaluation of the efficiency of the aggregation process, during the aggregates formation, revealed that with higher seeding densities the efficiency diminishes.

In general, the obtained results seem to indicate that the StemFlex medium could be used with success for the expansion of hiPSC in adherent culture and as suspension aggregates, as well.

The differentiation protocol was considerably heterogenous in the originated results. Concerning the cardiac induction in adherent culture, the best results through cytometric analysis, performed at day 12 of cardiac induction, were obtained using Gibco cell line, with an approximate confluency of 60-70%, being obtained 42.1% of cTnT positive cells. For TCLab cell line, the best result was obtained with an approximate confluency of 70-80% with 36.7 % of cTnT positive cells. For both cell lines the percentage of cTnT positive cells for higher and lower confluences was considerably diminished. These results indicate that cells need to reach a certain level of confluence to result in a good cardiac differentiation, but the over confluence need to be avoided [21, 34]. Considering all the approximate confluences before cardiac induction the percentage of cTnT positive cells obtained for the Gibco was considerably higher than for the TCLab cell line. This could be caused by the different capability than different cell lines hold into differentiate in a specific type of cells. This percentages of cTnT could be explained by the usage of an inadequate concentration of small molecules for the cardiac induction in adherent culture.

The spontaneous contraction of cells was registered from day 8 of cardiac induction. Interestingly, the approximate confluences that display higher cTnT results were the ones that show an earlier spontaneous beating for both cell lines.

Regarding the cardiac differentiation in a 3D system: it was obtained percentage of cTnT positive cells, at day 12 of cardiac induction, up to 78.2% for Gibco and up to 23.4% for TCLab cell line aggregates. The average aggregates diameters that lead to higher percentages of cTnT cells were between 155-205 μm for

Gibco cells. For the TCLab aggregates the percentage of cTnT positive cells was very similar for all the aggregates diameter, being obtained a slightly higher percentage for an average diameter of 205-215 μm . It appears to be possible to achieve higher percentages of cTnT in a wider range of average diameters for the Gibco, than for the TCLab cell line. This may indicate that Gibco cell line is more prone to cardiac induction. Concerning the initial seeding density (cells/mL) the ones that lead to better results for Gibco cell line aggregates were $5 \times 10^{+05}$ and $1 \times 10^{+06}$. For TCLab aggregates was more difficult to define the best seeding density but it appears to be $1 \times 10^{+06}$ cells/mL.

The earliest aggregates to present spontaneous beating for both cell lines was at day 7 of cardiac induction, being that the cardiomyocytes in aggregates demonstrate spontaneous beating before the aggregates cultured in monolayer.

In general, TCLab cell line seem more prone to the expansion in StemFlex and Gibco cell line to the cardiac differentiation. Comparing the differentiation in 2D and 3D, the 3D system was more robust, since in this system the cardiomyocytes started to contract earlier, and it was obtained higher percentages of cTnT positive cells. This could be due to the 3D system that enables cellular interaction with each other and with the surrounding environment. Also, the cells *in vivo* are displayed in a 3D layout being this culture system more similar to the *in vivo* reality.

To validate all the presented results there is a need to replicate most of the experiments, since exists a lack of replicates, that only were possible to perform if there was more time available. Altogether, in this work was assessed the possibility of expand hiPSC, in a recent commercially available medium and posteriorly differentiate the cells into cardiomyocytes using a differentiation kit, in 2D and 3D. The results revealed that the StemFlex medium was appropriate and reliable to expand hPSC cells. It was also possible to obtain through the Cardiomyocyte differentiation kit spontaneously beating cells that through different analyses were identified as cardiomyocytes. Although, the differentiation protocol did not lead to high percentage of cTnT positive cells, it was possible to assess which are the most suitable approximate confluences, in 2D and the most suitable aggregates diameters and seeding densities to induce the cardiac differentiation, in 3D. So, the combination of the expansion in StemFlex medium with the posterior usage of the Cardiomyocyte differentiation kit holds potential, but still requires further optimization in order to be possible to achieve higher percentages of cTnT positive cells.

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