

Studying the Effect of the ROCK Inhibitor on the Proliferation Potential of Human Induced Pluripotent Stem Cells under 2D/3D and Static/Dynamic Culture Environments

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) have unlimited self-renewal properties and the capacity to give rise to cells from the three embryonic germ layers. The clinical potential of these cells has brought to light promising work for future applications, though having fully defined protocols for their maintenance and handling, under good manufacturing practices (GMP), is still a universal challenge.

In this work, two stocks of hiPSCs from the same line (1383D2) were compared, since one of the stocks had displayed significant phenotypical changes. The contribution of the ROCK inhibitor was studied in 2D culture for 24 hours, and in 3D static and dynamic cultivation, for 48 hours and 96 hours. In 2D culture, the growth kinetics for both stocks showed to be similar. However, in 3D dynamic culture, the live cell ratio for the altered cells was not higher than 0.20, when comparing to the other stock (0.67 ± 0.04). Though the apparent specific growth rate was overall much higher in these cells, the highest being $3.57 \times 10^{-2} \text{ h}^{-1}$, achieved in 3D static culture, exposed to the ROCK inhibitor for 96 hours. Naturally, the largest aggregates (assessed by the aggregates' diameter) were obtained in dynamic culture. Furthermore, for the study of the morphology and viability of the aggregates, the expression of E-cadherin and Ki-67 were also analysed, showing that larger aggregates suffered central necrosis as the culture proceeded. Nevertheless, despite the differences, the present results permitted to conclude that these altered cells are possibly viable for future use.

Keywords: Human induced pluripotent stem cells; ROCK inhibitor; Static Culture; Dynamic Culture; Growth kinetics; Aggregate development.

INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) were first created in 2006 by Shinya Yamanaka and his collaborators by retrovirally introducing specific genes encoding four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into mouse embryonic cells, giving rise to a new generation of cells¹. These cells have proved to be similar to human embryonic stem cells (hESCs), regarding their transcription program and chromatin modification profiles², as well as in morphology, surface markers, overall gene expression and age-affected cellular systems, like telomeres and mitochondria³.

Transiting from the laboratory experiments to an industrial scale is still a challenge, not only because of the initial small working number of cells, but also because the success of the process also depends on the perfect

equilibrium between the optimization of the culture parameters and the design of the culture vessel, while maintaining Good Manufacturing Practices (GMP) at all times. The ultimate goal for the applications of stem cells in regenerative medicine and other applications is to guarantee a final and standard protocol for the production of the cells, according to the case they will be used for. In the turn of the millennium, it was realized that human pluripotent stem cells (hPSCs), when present in an environment without adherent conditions, are able to agglomerate into 3D spheroids to seek for support, forming embryoid bodies, which leads to spontaneous differentiation of undifferentiated cells into cells from one of the three primary germ layers⁴. With the discovery of the ROCK inhibitor, it was a great step to explore the

potential of these aggregates to maintain their pluripotency and proliferative capacity⁵, overcoming problems imposed by physical supports, like microcarriers, since there is no requirement of attachment surfaces, adhesion molecules or hydrogels, because the cells simply attach to one another. This fact also allowed to solve problems of downstream processing of their production and cost reduction⁶.

In this work, it was studied the divergence that occurred between two stocks of the same line of hiPSCs, 1383D2, that will affect future experiments. In order to do it, the cells were cultured in 2D static conditions and in 3D static and dynamic ones, using the xeno-free medium, StemFit[®]. Growth kinetics parameters and morphology data were collected to further take conclusions about the behaviour of these cells, comparing to other stock of cells from the same line. An immunostaining technique was also applied to conclude about the robustness of these aggregates. The main goal of this work will revolve around the interest of understanding the viability of this altered stock of cells in regards of the other one with cells from the same line and conclude about the best course of action to use them in future research.

MATERIALS AND METHODS

The hPSC line used, 1383D2, was kindly provided by CiRA (Center for iPS Cell Research and Application, Kyoto University), on behalf of this study. For the present work, it was used the StemFit[®] Basic02 medium (Ajinomoto, AK02N), a defined, xeno-free medium for the culturing of the hiPS cells. Cells were maintained inside the incubator at 37°C and with 5% of CO₂ until passaging, when the initial seeding density was 2.50 x 10³ cells/cm². The plate was washed with 5 mL of DPBS (Sigma-Aldrich[®]) and then discarded again to guarantee the full removal of the wasted medium. Afterwards, 3 mL of 5 mM EDTA (Dojindo Molecular Technologies, Inc.) mixed with 3 µL of 10 µM ROCK inhibitor (Y-27632, FUJIFILM Wako Pure Chemical Corporation, 251-00514) was added into the plate for 10 minutes. After this time, 3 mL of TrypLE[™] 1X (Gibco[®], ThermoFisher Scientific), mixed with 3 µL of ROCK inhibitor, was added into the plate. After 7 minutes,

the detachment of the cells was observable under the phase contrast microscope CKX53 (OLYMPUS[®]). From a previously prepared 10 mL of fresh StemFit[®] mixed with 10 µL ROCK inhibitor, it was drawn 3 mL of the mixture and added to the plate, after complete detachment of the total amount of cells. Then, the total volume of the dish was transferred into a 15 mL Falcon tube (ThermoFisher Scientific[™]), before another 3 mL of the medium with ROCK inhibitor was added to collect the remains of the cell suspension still in the dish. The tube was centrifuged for 3 minutes at a speed of 170 x g. The supernatant was discarded and 1 mL of StemFit[®] with ROCK inhibitor was added once again, cells were rinsed by pipetting before another 2 mL were added. Single cells were counted using the Trypan Blue Exclusion method and using an TC20[™] Automated Cell Counter (Bio-Rad).

To study the cells under 2D static conditions, two 8-well culture plates (Thermo Scientific[™] Nunc[™] Cell-Culture Treated Multidishes) coated with iMatrix-511 (nippi Atrixome), were used. The culture was maintained for a total of 120 hours, and the medium was changed every 24 hours, adding 2.1 mL per well. The ROCK inhibitor was added at $t = 0$, and not added anymore after the first medium change, at $t = 24$ h. Cells were detached and counted to estimate the cell density at $t = 72$ h and at $t = 120$ h.

To study the formation of aggregates in a three-dimensional setting without agitation, 6-well dimple plates (Elplasia[™], Kuraray Co., Ltd.) were used. Cells at the initial density of 1.00 x 10⁵ cells/mL were detached and seeded into one dimple plate with ROCK inhibitor (10 µM) for 48 hours, until when it was performed the first medium change, and the other one with it for 96 hours. The medium change was performed at $t = 48$ h and at $t = 72$ h, using 6 mL of medium per well.

As for study the formation of aggregates in a three-dimensional environment with continuous agitation, at the same initial density of 1.00 x 10⁵ cells/mL, were seeded and then maintained in culture for 96 hours in a 30 mL disposable bioreactor (ABLE Corporation & Biott Co., Ltd). Two of these bioreactors were used, one in which

ROCK inhibitor (10 μM) was present in the culture for 48 hours, before changing the medium for the first time, while in the other one it was maintained for the entire culture time. After seeding, the bioreactors were incubated, with a continuous agitation of 55 rpm. The medium change was performed at $t = 48$ h and at $t = 72$ h.

To rate the viability of the cells, it was possible to calculate the attachment efficiency, in 2D culture systems, ($\alpha(-)$), or the live cell ratio, in 3D culture systems, ($\alpha^*(-)$), using the same expression, as shown by the equation: $\alpha(-) = \alpha^*(-) = X_{24}/X_0$. The increase in cell population during this stage could be defined by the equation: $dX/dt = \mu \cdot X$. When the data was collected twice during the culture time, as for the case of 3D culture conditions, then it is possible to obtain the apparent specific growth rate (μ^{APP}), in h^{-1} , which was given by equation: $\mu^{\text{APP}} = \ln(X_{96}/X_{24})/(t_{96} - t_{24})$. In these equations, X_0 , X_{24} and X_{96} represent the cell density, in cells/mL, at seeding time, 24 hours and 96 hours, and t_{96} and t_{24} the time at $t = 96$ h and $t = 24$ h, respectively. The aggregates' diameter was measured using a bright field system, IN Cell Analyzer 2000 (GE Healthcare Life Sciences) at $t = 24$ h and $t = 96$ h.

Samples of 500 μL collected from the vessels were cryopreserved by carefully being placed in between two layers of Tissue-Tek™ O.C.T. (Optimum Cutting Temperature) Compound (Sakura®), using liquid nitrogen (-196°C) and frozen at -80°C before sectioning. The frozen aggregates were cut with a thickness of 10 μm using the cryostat Leica Biosystems CM1850 in order to proceed with immunostaining later.

Finally, an immunostaining technique was also acquired, using about 200 μL every time. The Tissue-Tek™ O.C.T. compound was first removed by washing the samples three times with PBS, followed by one washing with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) for the fixation of the cells in a period of 10 minutes. After this time, it was discarded, washed twice with PBS and added the solution composed of 50 μL Triton X-100 (Sigma-Aldrich®), 0.5% dilution, and 10 mL Mili-Q® (Mecrk Millipore). After 5 minutes, this

solution was discarded, and the samples were washed using the dilution buffer which was composed of 40 mL from a previously prepared solution, named antibody dilution, containing 2.5 mL of Block Ace (DS Pharma Biomedical Co., Ltd.) and 47.5 mL of Mili-Q®, and 40 μL of Tween® 20 (Sigma-Aldrich®). After discarding, the dilution buffer was added once again for 15 minutes, before it was discarded once again, and the Block Ace added for 90 minutes. During this time, the primary antibody solution could be prepared, containing the working volume of 200 μL of the antibody dilution, 1 μL of E-cadherin and 4 μL of Ki-67. After said time, the Block Ace was discarded, the samples were washed once with TBS (Tris-buffered saline) and the solution of the primary antibodies was added. The samples were left overnight, ideally for 16 hours, in the fridge at 4°C . In the following day, what was left was discarded and then washed once with the dilution buffer, before adding it again for 15 minutes. After this time, it was discarded, and TBS was added, also for 15 minutes. During this period, and in the darkness, the secondary antibody solution was prepared containing 200 μL of the antibody dilution, 1 μL of Alexa Flour® 594 anti-rabbit and 1 μL of Alexa Flour® 488 anti-mouse. This solution was then added for 1 hour after discarding the TBS. After this time, it was discarded, TBS was added again for 20 minutes, discarded, added for 10 minutes, discarded and finally added once again for another 10 minutes. During this sequential process, the DAPI (4',6-diamidino-2-phenylindole) solution was prepared by mixing 200 μL of PBS with 0.6 μL of DAPI (initially with a dilution rate of 1:50, and then diluted again 3 $\mu\text{L}/\text{mL}$). This solution was then added for 20 minutes after discarding the previous one. When done with this time, the DAPI solution was discarded and PBS was added and discarded according to the following sequence: washed twice, added for 10 minutes, discarded and then, right after adding for another 10 minutes, washed and finally discarded. Two or three drops of *SlowFade* (ThermoFisher Scientific), an antifade reagent which extends the primary antibody action, were added before analysing the results using a confocal laser

scanning microscope, the FluoView FV1000 (OLYMPUS®).

RESULTS AND DISCUSSION

In the scope of this work, it was necessary to understand the behaviour of two different stocks of the same line of hiPSC, the 1383D2 line. It was of interest to comprehend and speculate how and why did this culture suffered changes comparing to another from the same hiPSCs line. By studying the behaviour of the cells in 2D and 3D culture conditions, in static and dynamic conditions, and understanding how the permanence of the ROCK inhibitor influenced the culture and could or not contribute to their sustainability and aggregate formation, it was possible to take conclusions about the best course of action to culture these cells in the future. Thus, and to better distinguish the type of cells throughout the present work, to the stock with no issues or modifications of any kind, it was given the name “Type A”, while the cells which were suspected to have undergone changes, were named “Type B”.

For 2D culture conditions, cells were cultured for 120 hours in 8-well plates, using the StemFit® medium with the ROCK inhibitor present in the first 24 hours of the culture. From these results in Table 1, it was possible to observe that the α values from Type B cells, of 1.38 ± 0.09 , is slightly lower than Type A cells', of 1.54 ± 0.00 , even though it is still within a range of 10% discrepancy. As for the μ value, the discrepancy is a bit higher, but not enough to be alarming and compromise the culture in any way. Actually, the variation might be due to technique differences of the operator, since in other data provided by the laboratory members, it is possible to observe that,

for the same cells, namely Type A cells, the specific growth rate value was $(4.91 \pm 0.00) \times 10^{-2} \text{ h}^{-1}$, which is similar to Type B cells'. This might show that the physical stress and aggregate formation are both factors that might have a strong influence in the proliferation of these Type B cells compared to the Type A cells. In the conventional 2D system culture, and as in this case, cells are grown in dishes coated with ECM components, which will aid in their attachment since the moment they are seeded into the vessel. This 2D culture brings a stable environment for the proliferation of Type B cells, although, and as referenced before, it is widely known that 2D culturing limits greatly the scalability of some processes.⁷

Moving onto 3D environment, it was tested if the time of exposure to the ROCK inhibitor would or not be beneficial to these cells, in both 3D static and dynamic culture and finally how the morphology of the aggregates varied regarding the time exposure to the ROCK inhibitor and the type of vessel used.

From the results, the first conclusion that could be observable even to the naked eye was that Type B cells can form lasting aggregates, whose the diameter increased as the culture times proceeded from the 24 hours analysis to the 96 hours analysis. Even though the size of the aggregates is a morphological characteristic, it was also an aspect that was thought to be related to the duration of exposure of the cells to the ROCK inhibitor. Therefore, after one day, the dynamic culture presented, not only a bigger dispersion of diameter results, but also higher values, when comparing to the static culture. However, the higher density of the results revealed to be between 75 and 150 μm in all cases, as shown in Figure 1A.

Table 1 – Growth kinetics parameters, the attachment efficiency (α) and the specific growth rate (μ), for Type A and Type B cells, in 2D static culture. The values represented are the average at $t = 24 \text{ h}$ and $t = 120 \text{ h}$, respectively, for the condition of study of the effect of the ROCK inhibitor in the culture for 24 hours, followed by the standard deviation (\pm). Type B cells' unpublished results were kindly provided by the laboratory members ($n = 1$).

	24 h ROCK Inhibitor			
	$\alpha (-)$		$\mu (10^{-2} \text{ h}^{-1})$	
	Type A Cells	Type B Cells	Type A Cells	Type B Cells
2D Static Culture	1.54 ± 0.00	1.38 ± 0.09	4.24 ± 0.35	5.00 ± 0.00

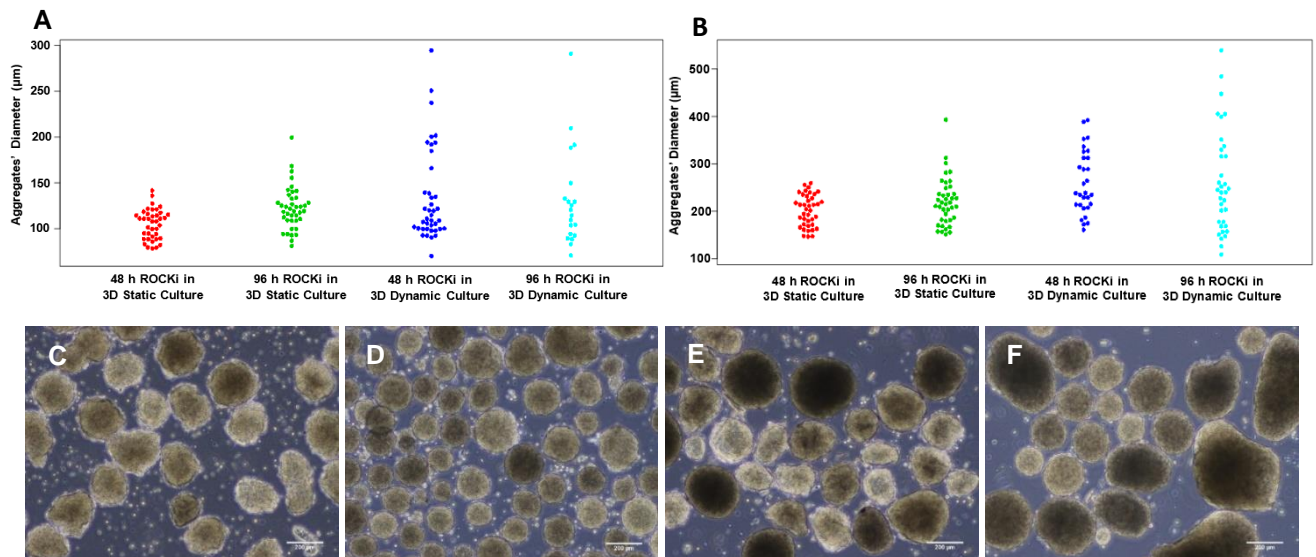


Figure 1 - Variation of the aggregates' diameter and morphology of Type B cells. Data of the aggregates' diameter collected at **(A)** $t = 24$ h and **(B)** $t = 96$ h, in both 3D static and dynamic culture, for the conditions of study of the effect of ROCK inhibitor in the culture for 48 hours and 96 hours ($n = 1$), and phase contrast microscope images taken at $t = 96$ h of aggregates from the dimple plate with the ROCK inhibitor present in the culture for **(C)** 48 hours and **(D)** 96 hours, and from the 30 mL bioreactor, with the ROCK inhibitor present in the culture for **(E)** 48 hours and **(F)** 96 hours ($n = 1$). (Scale bars = 200 μm).

After 96 hours, firstly, and once again, the dynamic culture overcame the static culture in terms of aggregate size (Figure 1B). Even if in 3D static conditions shows to have extensive agglomeration, it is still lesser than the dynamic one, as expected.⁸ This is a valuable aspect to be taken into consideration for future applications, since large numbers of cells are usually required for most of the regenerative medicine or tissue engineering applications.⁹ The formation of larger aggregates in 3D dynamic culture could mainly have been due to the agitation of the bioreactors which promote the collision of the cells more efficiently and, thus their attachment to each other too. Also, surely that the continuous agitation promotes the collision among the cells, but also among the aggregates, as the culture time proceeds. This dynamic between the collapse and coalescence of the aggregates will influence the cells' proliferation rate as the culture develops and the fate of their deviation. This merging of the aggregates also explains the smaller number of aggregates in the vertical plots represented in Figure 1A and 1B. Secondly, it is possible to observe the size discrepancy, regarding day 1 and day 4 analysis, and also the different type of culture systems. After the first 24

hours the aggregates' size were not larger than 300 μm , contrarily to after 96 hours of culture. It is curious to notice that the presence of the ROCK inhibitor for 48 hours or for the entire culture time barely brought any effect to the aggregate size in the case of the static culture, since the higher density of samples is confined between sizes 150 and 300 μm . However, the same was not entirely true in the dynamic culture, since the extended permanence of the ROCK inhibitor lead to a wider range of values and also to a beneficial increment of the diameter of the aggregates, certainly an aspect to consider in future research. This could reinforce the hypothesis that altered cells like the ones from Type B have trouble adapting and adjusting to the dynamic culture and that the ROCK inhibitor might bring an advantage to their culturing.

In order to further understand the variances in the different cultures, it was calculated two growth kinetics parameters, namely the live cell ratio (α^*) and the apparent specific growth rate (μ^{app}), for static and dynamic conditions, with the ROCK inhibitor in the culture for 48 hours and 96 hours, for both cell types (Table 2). Nevertheless, regarding the results for the live cell ratio, if the value varied between 0 and 1, it was possible to

Table 2 - Growth kinetics parameters, the live cell ratio (α^*) and the apparent specific growth rate (μ^{app}), for Type A and Type B cells, in 3D static and dynamic culture. The values represented were collected at $t = 24$ h and $t = 96$ h, for the condition of study of the effect of ROCK inhibitor in the culture for 48 hours and 96 hours. 3D dynamic culture with the ROCK inhibitor for 48 hours results are followed by standard deviation (\pm). Type A cells' unpublished results were kindly provided by the members of the laboratory ($n = 1$).

		α^* (-)		μ^{app} ($10^{-2} h^{-1}$)	
		Type A Cells	Type B Cells	Type A Cells	Type B cells
48 h	3D Static Culture	0.55	0.61	3.00*	2.84
ROCKi	3D Dynamic Culture	0.67 \pm 0.04	0.20	2.30 \pm 0.06	3.22
96 h	3D Static Culture	-	0.47	-	3.57
ROCKi	3D Dynamic Culture	-	0.16	-	3.14

*Calculated value from 24 – 120 h

observe that, the Type B cells' survival was largely compromised in the dynamic culture, since the success rate was only 20% and 16%, when comparing to the static culture, which was 61% and 47%.

When comparing the value to Type A cells, it is possible to conclude once again that there were no drastic changes in the α^* value in the case of the dimple plates, since the value increases from 0.55 to 0.61, but cell apoptosis seemed to have been aggravated in the case of dynamic culture, where Type B cells had a survival ratio of 20%, comparing to Type A cells, which was 67%. This high discrepancy between Type B cells and Type A cells' α^* was the first alarmed evidence of the different state of Type B cells. As quoted before^{10,11}, one reason for this might have to do with the increase of the shear stress caused by the continuous agitation in the bioreactor, compared to the dimple plate, in which there is none. Even if manageable until a certain level, the shear forces still bring damage to the cells, which in this case could be one of the major factors that influence the cells' adaptation and proliferation.

Then, regarding the apparent specific growth rate, it was expected that the μ^{app} would overall decrease in the Type B cells, compared to Type A, right after analysing the results from the first 24 hours of culture, even if the ROCK inhibitor was in the culture for a longer time. However, some aspects could be understood by the presented data.

The first one was that the highest value obtained for the μ^{app} was in static culture, with the presence of the

ROCK inhibitor in the culture for 96 hours. One reason for the high value in this case as to do with what was quoted previously.⁸ Even though different strains of hiPSCs were used in this reference, it was concluded that 3D dynamic culture, compared to 3D static culture, resulted in higher shear stress that leads to the disruption of the cell-synthesized ECM, directly suppressing the activation of the cadherin-catenin signalling. This idea could be reinforced by checking the similarity of values in the 3D static culture with the ROCK inhibitor present for 48 hours, since the values of $2.84 \times 10^{-2} h^{-1}$ has a discrepancy of only about 5.56% from the value of $3.00 \times 10^{-2} h^{-1}$.

Then, when comparing with the Type A cells' value of $2.30 \times 10^{-2} h^{-1}$ with Type B cells' one of $3.22 \times 10^{-2} h^{-1}$, the latter is clearly higher than previously expected. This means that the number of passages of these cells or even the age of the donor might have affected their adaptation to a different vessel, but also proved to have the capacity of making a great recuperation with the progression of the culture time. Moreover, in static environment, it was shown before that the ECM accumulates at the periphery of the aggregates as time goes by. This difference in the distribution of the ECM could have happened in Type B cells, and when grown under dynamic conditions, suggested that mechanical stress induced by the liquid flow could have been prevented because of the ECM covering the surface of the aggregates.^{8,12}

Surprisingly, the time length of ROCK inhibitor in culture barely changed the result in 3D dynamic culture, although it largely contributed to a higher value in 3D static culture. The 96 hours exposure of ROCK inhibitor showed to have increased the μ^{app} value from $2.84 \times 10^{-2} h^{-1}$ to

$3.57 \times 10^{-2} \text{ h}^{-1}$, an increase much more considerable than in dynamic culture, in which the values were very similar, of $3.22 \times 10^{-2} \text{ h}^{-1}$ and $3.14 \times 10^{-2} \text{ h}^{-1}$, with the ROCK inhibitor in the culture for 48 hours and 96 hours respectively. This might be due to the way ECM is developed in both cultures. In the dynamic one, the effect of the mechanical stress from the fluid flow might have prevailed over the effect of ROCK inhibitor in these cells, hence the existence of no difference between the μ^{app} values. On the other hand, the physical stress is much more residual in static culture, which might explain the greater contribution of the ROCK inhibitor when in culture for a longer time.

Furthermore, regarding the aggregates' morphology, images were taken at $t = 24 \text{ h}$ and $t = 96 \text{ h}$, using phase contrast microscopy, and medium was changed at $t = 48 \text{ h}$ and $t = 72 \text{ h}$. The culture's physical characteristics such as turbidity, colour and the presence of any contamination, derived from bacteria or fungus, were also taken into account when analysing the data. At a seeding concentration of $1.00 \times 10^5 \text{ cells/mL}$, the cells grew in both the dimples plates and the bioreactors, and the formation of aggregates in both cases was observable after 24 hours.

Normally, cells from this line generally present round aggregates, especially in the static culture, with smooth surface throughout the entire culture time, increasing the aggregate size considerably until the end of the culture time, as shown before in Figure 1C-F.

Therefore, the first observable aspect has to do with the evident discrepancy in size between the two types of vessels. Regardless of the exposure time of the cells to the ROCK inhibitor, the aggregate size was found to be larger in dynamic conditions than in static conditions. This was expected, since it has been previously stated that the dynamic environment, such as from bioreactors, might be the answer for the scaling-up process in regards to future applications that require a large number of cells.

Another feature that is possible to observe after determining the aggregates diameter after 96 hours (Figure 1B), was that the time exposure to ROCK inhibitor

for both 48 and 96 hours did not affect the size of the aggregates in the case of the dimple plates, although the difference was more noticeable in the case of the bioreactors, as previously stated.

Finally, the permanency of the ROCK inhibitor in the culture might have influenced the shape of the aggregates. It is notorious that the aggregates with higher exposure to the ROCK inhibitor presented a more regular spherical shape and smooth surface, when compared to the 48 hours exposure in both dynamic and static conditions. The same morphological characteristics are also typical of Type A cells, even with higher exposure to the ROCK inhibitor. The ROCK inhibitor is known to play a crucial role on the cytoskeleton of the cells, remodelling it and affecting the actomyosin contractility which is easily affected by the ECM disruption induced by the shear forces of the dynamic flow, hence the change in the aggregates' morphology.¹³

Lastly, after meticulously sectioning several samples of aggregates obtained with a thickness of $10 \mu\text{m}$, an immunohistological analysis was performed in order to elucidate about the state of development of the cells in the aggregates, especially regarding their size. The aggregates used to understand these characteristics were collected from the 30 mL bioreactor, with the ROCK inhibitor for 48 hours and culture time of 96 hours. The markers used targeted Ki-67, a proliferation marker, and E-cadherin, a cell-cell adhesion molecule. DAPI counterstaining was also used to detect the cells' nuclei. Figure 2A-D and 2E-H show the expression of these three markers in two examples of aggregates, in which is visible the variation of expression depending on the diameter of the aggregates.

From the immunostaining results obtained, it is possible to observe the expression of DAPI homogeneously in both aggregates' sections, as seen in Figure 2A-H, staining the cells' nuclei.

The protein Ki-67 is strongly associated with cell proliferation, so it works as an excellent culture growth marker.¹⁴ As it can be seen in Figure 2B, in smaller

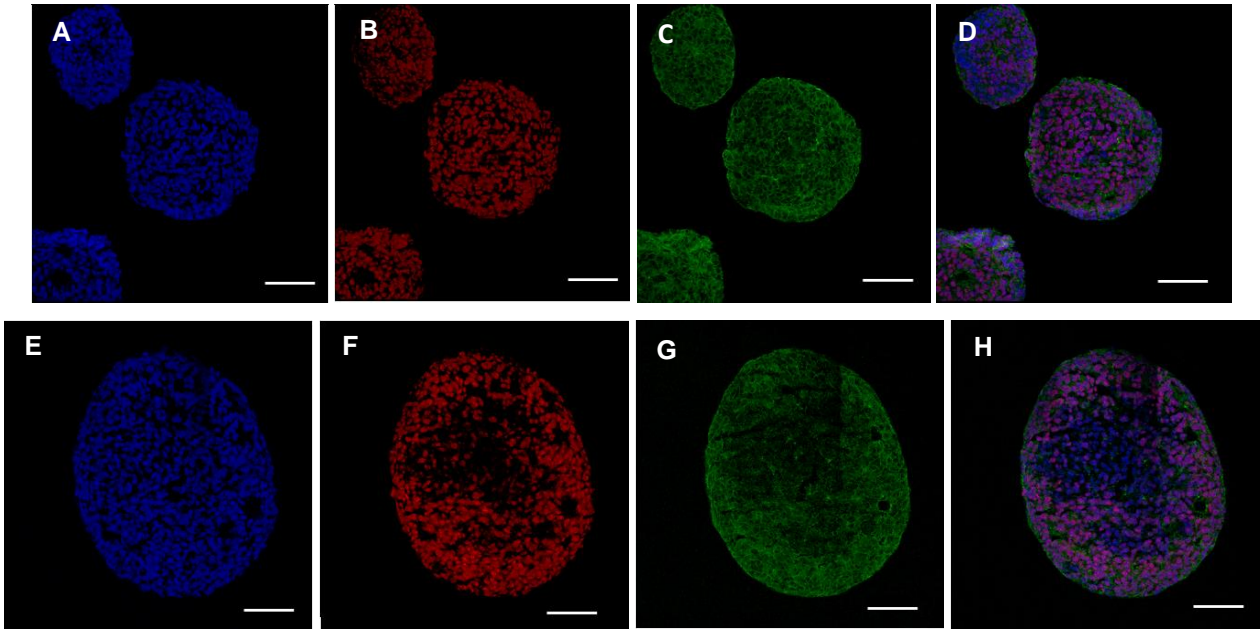


Figure 2 - Immunostaining images of aggregates formed by Type B cells, cultured in a 30 mL bioreactor operating for 96 hours, with the ROCK inhibitor in the culture for 48 hours. The images represent the expression at $t = 96$ hours of (A) DAPI, (B) Ki-67, (C) E-cadherin and (D) the merge of the three late results, for the case of a small-sized aggregate, and the expression of (E) DAPI, (F) Ki-67, (G) E-cadherin and (H) the merge of the three late results, for the case of a large-sized aggregate ($n = 1$). (Scale bars = 100 μm).

aggregates, the expression of Ki-67 is mainly homogenous in all cells of the aggregate's section.

However, in a larger aggregate such as the one from Figure 2F, the centre of the aggregate showed lower expression levels. This suggests that cells in the centre might have started entering the stationary phase of growth sooner than cells on the periphery, due to the reduction of diffusion of components essential for the proliferation of the cells from the medium to the centre of the aggregates. This quiescence state would later lead to the triggering of apoptosis mechanisms, showed by the low or non-existent the expression of Ki-67 in the centre of the aggregates, comparing to the cells in the periphery.¹⁵

As for the E-cadherin, this is a protein that mediates cell-cell cohesion, playing an important role in the cells' survival and self-renewal, which can also be used as an undifferentiation indicator since it is able to tie to the transcription factor circuit that triggers the transcription factor OCT4, a widely used pluripotency marker.¹⁶ By analysing the E-cadherin expression, it is possible to state that the aggregates' size also influences its

expression, since in the one with smaller diameter the E-cadherin expression is uniformly distributed in the periphery of the cells (Figure 1C), but the same is not true for the aggregate in Figure 2G. As central necrosis expanded, the excretion of ECM components would consequently decrease, resulting in a lower protein expression in the immunostaining results.^{15,17} Even so, it is relevant to add that aggregates too small might also result in cell apoptosis due to the low cell-cell contact mediated by E-cadherin, leading to a lower survival rate.

In conclusion, the larger the aggregates, typical from dynamic cultures, the lower is the diffusion rate of components into the middle of the aggregates, leading to the death of the cells in that area. The immunostaining results permitted to conclude that aggregates composed of Type B cells have a Ki-67 and E-cadherin expression similar to other lines of hiPSCs from the literature, according to the size of the aggregate. Furthermore, even if the highest result obtained for the apparent specific growth rate was in 3D static culture in which the obtained aggregates had an overall lower diameter compared to the dynamic cultures. Due to this, the static culture's

aggregates could possibly have generated more uniform aggregates in terms of number of cells in an active phase of development, such as mitosis. Probably, if the culture time proceeded for more than 96 hours, the maintenance of the viability of the cells would have been seriously compromised, leading to a decrease in the proliferation rate of the cells and consequently to a drop in the apparent specific growth rate value, due to the inhibitory competition for the resources in the culture.

Finally, it was possible to conclude from the results in this thesis that the cells from stock B might have undergone modifications at genetical level due to several aspects.

One of them, might be related to the number of passages the Type B culture was subjected to. According to the information provided by the laboratory, Type A culture has suffered not more than 30 passages, while in the case of the stock for Type B cells the number was at least 65 passages. Cells that were kept for a long time and thus suffered several passages might have begun changing their physiological and morphological properties, resulting in differences in their karyotype that affected their phenotype subsequently, when compared to Type A cells, from the same line, as previously tested out.

Even so, this should not be a reason for the difference in characteristics of both groups, since pluripotent stem cells are known for having unlimited capacity for self-renewal, while maintaining their pluripotency. The hypothesis falls on the fact that the maintenance protocol might have not have been followed truthfully and equally every passaging time. Since these cells are extremely sensitive to changes in culture conditions and handling techniques, this fact might have resulted in modifications in Type B cells at the genome level.

Also, and as commented before, the age of the donor is an important factor when it comes to the quality of the cells, since it influences the activity and expression of proteins that regulate the cellular pathways under stress conditions. However, in this case, this factor is unknown and therefore impossible to discuss.

CONCLUSIONS AND FUTURE PERSPECTIVES

Human induced pluripotent stem cells have shown to have clinical potential due to their self-renewal characteristics, capable of taking cell therapies to a new level. The fact that hiPSCs have the advantage of few incompatibility problems with the patient and also overcome some ethical issues that hESCs do not, is highly motivating to continue the research on these cells. Even so, the design of robust culture systems that would allow the scaling-up process with guaranteed reproducibility is still a topic under constant study.

In this thesis, the two stocks from hiPSCs, both from the 1383D2 line, were compared, since one of them have suffered transformations that affected the phenotype of the cells. These altered cells, named Type B cells, were studied and compared to other stock with cells from the same line, named Type A cells, in order to take conclusions about the viability of Type B cells in future works.

Even if with these outcomes, the fact that some of the results are not completely comparable or statistically relevant due to the lack of replicates of certain experiments, mainly due to time restraints, is still an aspect to be noted. Nevertheless, it was imperative for a laboratory specialised in the standardization of the processes regarding stem cells technology to understand the differences found in the stocks from the same line of cells. Comparing the exposure time to the ROCK inhibitor and the 2D with the 3D culture environment, and the static with dynamic conditions, the shear stress was believed to be one of the most crucial factors that would affect the adaptation and further growth of Type B cells. Albeit these cells clearly presented modifications in regards of Type A cells, which were exactly from the same line, the high apparent specific growth rate proved to be a game-changer. Even if with low live cell ratios, Type B cells were able to proliferate more than Type A culture. This is a very motivating reason to use these cells in the future for other experiments, even though further research at genetical level must be done in order to proceed with their research. Moreover, the immunostaining results reinforced the idea that Type B cells are, in fact, prone to be viable to be used

in upcoming research works, since their behaviour was expectable when compared to the literature.

Overall, this work consisted on a first approach to the understanding of the behaviour, quality, morphology and other aspects of these altered cells. As future work, it would be of interest to test how other culture parameters might affect the development of these cells, such as the initial cell density, the concentration of ROCK inhibitor in the culture or the agitation speed, in the case of the bioreactor. Furthermore, testing pluripotency markers such as OCT4, NANOG or SOX2 would support the conclusions taken about the undifferentiated state of these cells. This work would include testing these markers and also the Ki-67 and the E-cadherin ones for aggregates grown in 3D static culture and possibly also in 2D culture.

As later work, certainly that a genome sequencing technique to both types of cells must be considered. Firstly, because it would show the variations that could have happened in the karyotype of Type B cells in regards of Type A cells and conclude if Type B cells are, in fact, safe to be used in future applications. If their karyotype showed to be within the approved standards from stem cell research, then these cells might lead to new and promising outcomes in future works. Moreover, this analysis could also finally help to elucidate which genes were affected that could have explained the change in phenotype and proliferation rate.

In conclusion, this area of study embraces many features that must be carefully taken into account, so there is still further work to be done to wholly optimize the process of production of these cultures at a larger scale. The previous and still on-going investigations have brought encouraging results to continue the exploration in this branch of bioengineering, medicine and computer science, which, with truthful interest from the right institutions, will undoubtedly lead to a ground-breaking impact in the field.

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