Serum-free and Feeder-free Expansion of a patient-specific Fanconi Anemia Human Induced Pluripotent Stem Cell (hiPSC) line corrected by gene editing

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Since the emergence of the Induced Pluripotent Stem Cell (iPSC) technology that one of the greatest strengths of these cells is their source: iPSCs can be obtained from the reprogramming of the somatic cells of any individual. This characteristic opened the world to the possibility of personalized medicine. In fact, when obtained from an individual suffering from a genetic disease, iPSCs can, in theory, be used to recapitulate the effects of that disease at a cellular level, allowing to perform disease modelling and drug screening. On the other hand, cells obtained in such a way could be used for cell therapy of genetic disorders in a patient-specific context upon the correct genetic editing. In this study, we used a hiPSC model obtained from a patient with Fanconi Anemia, a genetic disorder that is defined by a predisposition for cancer and eventually bone marrow failure, after genetic correction in order to restore their proper function. This cell line was obtained in the context of a collaboration with the Division of Hematopoietic Innovative Therapies in Madrid, Spain. Using the experience and knowledge of the Stem Cell Bioengineering Laboratory at Instituto Superior Técnico, it was possible to establish a protocol that allowed the expansion of these cells in a chemically defined medium (mTeSR1) and using the Matrigel™ substrate. After expansion, cells showed the expression of pluripotency surface markers (Tra-1-60, Tra-1-81, SSEA-4) as well as transcription factors (Nanog, Sox-2, Oct-4). It was possible to expand the cells up to six times the original number after eight days of culture. Although this culture procedure cannot give rise to Good Manufacturing Practices (GMP)-grade cells for therapeutic use, it demonstrates that the expansion of these cells is possible and, in the future, the use of GMP-grade materials could be used to produce hiPSC for clinical applications.

Keywords: Induced pluripotent stem cells, Fanconi anemia, cell expansion, cell therapy, disease modelling, good manufacturing practices.

Introduction

Fanconi's Anemia (FA) is an autossomal recessive disease that belongs to a group of diseases associated with chromosomal instability, being collectively called chromosome break-up syndromes or DNA-repair disorders. They are characterized by a susceptibility to chromosomal anomalies, developing a higher frequency of genetic aberrations, spontaneous or induced by exposure to diverse agents that damage DNA [1]. FA however can be distinguished from the other considered disorders by a hypersensitivity to the cytotoxic and clastogenic effects of DNA cross-linking agents such as mitomycin C, diepoxybutane, cisplatin and photoactivated psoralens, for example. The other considered disorders are not sensitive to these same agents and so this characteristic allows for a reliable and sensitive diagnostic of FA [2]–[4].

Due to the characteristics of this disease, the symptoms of FA usually include several easily identifiable disorders such as that do not vary considerably between patients.

Thirteen genes were identified as sources of the FA disorder, all of them belonging to the same cellular pathway and therefore mutations on any of them lead to the same overall symptoms. Since FA causes irreparable genetic damages in the affected cells, these cells tend to accumulate in the G_2 stage of the cell cycle preventing their division [4].

Therefore most of the symptoms of FA revolve around growth defects. Patients affected usually show bone defects and small stature and other complications all related to the accumulation of genetic defects. About 20% of all FA patients develop several tumours, the most common of them all being acute myeloid leukaemia [5].

The most recognizable symptom however is bone marrow failure that develops in all patients of FA. This stems from a progressive loss of all hematopoietic stem cells in the patient's bone

marrow. The disease begins as thrombocytopenia and slowly progresses to pancytopenia from which all fractions of blood produced in the bone marrow are no longer created [5].

The treatment of the disease currently passes through bone marrow or umbilical cord blood transplants. These techniques efficiency varies wildly as FA patients are extremely sensitive to DNA cross-linking agents such as ionizing radiation, commonly used in these therapies. New therapies that take this hypersensitivity into consideration exist such as the usage of Anti-thymocyte globulin as a prophylaxic agent against Graft Versus Host disease and reduced doses of radiation, and obtain more reliable results [6].

With the advent of Stem Cell technology other therapies become available. One such therapy consists on the usage of isolated and characterized stem cell derivatives to treat the disease's bone marrow failure. For example, autologous bone marrow transplants using genetically edited cells or iPSC-derived hematopoietic progenitor cells [7]. These cells would be able to bypass immune reactions from the patient since they were derived from the patient's own cells and would have a healthy phenotype that would allow for the treatment of the pancytopenia in FA.

The cells used in that therapy would be stem cells which are defined as cells with the ability to produce identical clones of themselves, called self-renewal capacity, and the ability to differentiate into other specialized cells [8]. Pluripotent stem cells are a subset of stem cells in that they are capable of differentiating into any of the approximately 200 cell types present in the adult human body [9]. Pluripotent stem cells present other molecular characteristics that allow for their identification. They present a high expression of specific transcriptions factors such as Oct-4, Sox-2 and Nanog, several surface markers like SSEA-3/4, Tra-1-60 and Tra-1-81, high telomerase and alkaline phosphatase activity [9].

Stem cells can be classified according to their source and also to their differentiation potential. Embryonic Stem Cells are cells collected from the inner cell mass of the blastocyst. They are capable of differentiating into any of the cell types existent in the adult human body and are also capable of self-renewal [8]. However, there exist concerns about their use due to possible immune rejection of transplanted tissues generated from them and the fact that to obtain them the destruction of an embryo is necessary and therefore their use implies several ethical and religious qualms [10].

Adult Stem Cells are undifferentiated cells present in all tissues of the adult body. They are capable of self-renewal but are unable to differentiate into all cells of the adult body. Instead they are multipotent stem cells capable of differentiating only to the cells that comprise their tissue of origin. Their quality also depends on the donor's age and health [10].

Induced pluripotent stem cells (iPSCs) are obtained through the reprogramming of normal adult somatic cells. The reprogramming process consists on delivering several genes in order to force their expression on differentiated cells. These genes are commonly expressed in ESCs and their expression causes the reprogrammed cells to dedifferentiate back to a pluripotent state [11], [12]. iPSCs present the exact same generic characteristics as ESCs, actually several studies have shown that both kinds of cells are functionally indistinguishable [13], [14]. However these cells much less problematic when compared to the ethical and religious problems that plague ESCs [11].

However the usage of these cells for any application requires that they first be expanded in culture. Several clinical applications, for example, use numbers of cells in the order of the 10^8 or even 10^9 [15], a number of cells completely impossible to obtain without expansion.

The first attempts to culture these cells *in-vitro* used serum-supplemented media and feeder cell layers to support their expansion [16]. However the variability of these components, and the risk of xenogeneic contamination led these conditions to be discarded in favour of other more defined and xeno-free approaches [16], [17].

From there the scientific community took steps towards the complete definition of both the culture medium and culture substrate. The next discovery was the use of Matrigel™ as a culture substrate. Matrigel™ is composed of extracellular matrix proteins and growth factors derived from Engelbreth-Holm-Swarm mouse tumours [16], [18]. This substrate is capable of support cell adhesion and expansion and this

way it was possible to remove the need for feeder cell layers in culture. However Matrigel™ is still of animal origin and therefore the risk of xenogeneic contamination and the problems with variability still persist [16].

To use any of the cultured cells in a clinical setting it is a requirement to use completely defined culture medium and substrates [9]. To do so the scientific community has identified all necessary biological components from both the serum-supplemented undefined media and undefined substrates like Matrigel™ with the objective to generate their recombinant forms and use them in a completely defined culture system [19].

mTeSR™1 is an example of a completely chemically defined medium, although it still uses bovine serum albumin in its composition [18]. There also exists several substrates composed of several defined biological molecules that have been shown to be able to support stem cell expansion and pluripotency. One such molecule is cadherin or even vitronectin [20]. As a final step, current culture systems can use synthetic proteins that are completely xeno-free, with no risk of contamination and are easily modified to present the correct characteristics for the culture of a specific cell line [9].

Nevertheless, it is necessary to perform the adaptation of culture systems to each specific hiPSC line in order to maximize its growth potential, especially when concerning to genetically modified hiPSCs.

This study follows the derivation of a specific genetically corrected cell line from an FA patient. The research group in the Division of Hematopoietic Innovative Therapies in Madrid, after generating this cell line was incapable of expanding it *in-vitro*. This study attempted to do just that and in doing so provide a proof that this new therapeutic avenue is feasible.

Materials and Methods

Matrigel™ Coating

Aliquots of Matrigel™ were kept at -20ºC. When it was necessary to coat a new culture plate, the aliquot was thawed either overnight at 4ºC or for 2-3 hours at room temperature on ice. Matrigel™ is liquid when kept at 0-4 ºC but solidifies into a gel-like substance, when exposed to room temperature, which renders it

impossible to be used at this temperature. Matrigel™ should not be used directly from the aliquot, instead it was diluted in cold DMEM-F12 medium at a dilution of 1:30. An aliquot of 200µL of Matrigel™ then gave rise to 6mL of coating solution. Each well should then be coated with the solution. The quantities depend on the size of the well to be coated, the only rule that needs to be followed is that the bottom of the well should be completely covered with the solution at all times. For 6-well plates, each well was coated with 1mL of the solution, in 12-well plates with 500µL and in 24-well plates with 250µL. The plates were then kept at room temperature for 2 hours before being used. If they were not to be used immediately they could also be kept at 4ºC for up to a month.

Culture Media Preparation

The culture media used was mTeSR™1 (StemCell Technologies). This medium is one of the most commonly used media for the expansion and maintenance of both hESCs and hiPSCs. It is a complete medium that allows for a feeder-free and serum-free culture. It is completely defined but uses Bovine Serum Albumin and therefore it is not a xeno-free medium. It also contains rh bFGF, rh TGF-β, Lithium Chloride, Pipecolic acid and GABA. It does not require the addition of any growth factors and, due to its pre-screened raw materials, provides consistent cultures that maintain the undifferentiated state of hiPSCs.

The culture medium is provided by the company in 2 parts, mTeSR™1 Basal Medium and mTeSR™1 5X Supplement. The Basal Medium can be stored at 2-8ºC until the expiration date and the 5X Supplement should be stored at -20ºC up to the expiration date.

In order to prepare 500mL of complete mTeSR™1 medium, first 100mL of 5X Supplement should be thawed and mixed thoroughly and then added to 400mL of Basal Medium and again mixed thoroughly. If other quantities must be prepared, adjust the amounts accordingly.

The complete medium can then be stored at 2-8ºC for up to 2 weeks or it can also be aliquoted and stored at -20ºC. In this case the medium was aliquoted in 50mL aliquots and stored at -20ºC. Before being used, the medium should be thawed and used immediately or stored at 2-8ºC for up to 2 weeks. The medium was pre-warmed at 37ºC before being provided to the cells.

hiPSC Line

A single hiPSC line was used. The line was the Cre-Sort hiPSC line obtained from the Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER in Madrid, Spain. The cell line was derived from the skin fibroblasts of a patient with FA. The cell line was obtained in accordance to CIEMAT regulations. Patient's information was encoded to protect their confidentiality and informed consents were obtained from all of them.

The used skin fibroblasts were reprogrammed in Spain by using a polycistronic excisable lentiviral vector with the commonly used reprogramming factors, Oct-4, Sox-2, c-Myc and Klf-4. They were maintained in Dulbecco's modified medium GlutaMAX^{TM} (DMEM; Gibco), supplemented with 20% fetal bovine serum (Biowhitaker) and 1% penicillin/streptomycin (P/S) solution (Gibco) at 37ºC under hypoxic conditions (5% of O_2) and 5% of CO_2, in co-culture with human foreskin fibroblasts as feeder cells [21]. They were also cultured for 109 passages prior to freezing.

Neural Differentiation of hiPSCs

The culture medium used for the neural differentiation of hiPSC was the N2B27 medium. This medium is composed equal parts with N2 medium and B27 medium. N2 medium is composed of DMEM-F12 GlutaMAX^{TM} supplemented with 1x (v/v) N2-supplement, 1,6 g/L D-(+)-glucose, 20 µg/mL insulin and 1% (v/v) P/S. The B27 medium is composed of Neurobasal medium supplemented with 1x (v/v) B27-supplement, 1% (v/v) glutamine and 0,5% (v/v) P/S.

This medium combination was used for the entire 12 days of differentiation with the addition of two small molecules, SB-431542 (SB) (inhibits transforming growth factor-β receptor I (TGF-βRI), activin-like kinase (ALK) 4 and 7 signalling pathways) and LDN-193189 (LDN) (inhibits bone morphogenic protein (BMP) signalling pathways) in a proportion of 1µL each for every 1mL of N2B27 medium used, as based on the dual SMAD inhibition protocol for neural induction [22]. The medium was changed daily and the cells were placed in incubation at 37ºC in normal atmospheric conditions of O_2 and 5% CO_2. At the end of the 12 days of culture, the cells were marked for Pax-6 and Nestin expression and photos were taken.

Intracellular Immunofluorescence Staining

The medium was removed from the wells before washing the cells with PBS. Then, in order to fixate the cells, 1mL of 4% PFA (paraformaldehyde) diluted in PBS was added to each well and left there for 30 minutes. Due to its characteristics, the cells could be left on PFA for at most 2 weeks without having their characteristics changed or lost. When ready to continue the procedure, PFA was removed and cells were washed twice with PBS. Afterwards, cells were incubated with blocking solution composed of 10% Normal Goat Serum (NGS, Sigma®) 0,1% of Triton-X (Sigma®) diluted in PBS for 30-60 minutes at room temperature. At the same time, the primary antibodies were diluted in staining buffer solution composed by 5% NGS and 0,1% Triton-X diluted in PBS.

After the incubation with blocking solution is completed, the primary antibodies were added to each well and left to incubate overnight at room temperature. After this new incubation was completed, the cells were washed twice with PBS and the secondary antibodies were prepared by diluting them into staining buffer solution and added to each well. The cells were left to incubate for one hour in a dark location at room temperature.

Finally the cells were washed three more times and a DAPI solution was added and the cells left to incubate for 2-3 minutes. The DAPI solution was composed of 15µL of stock solution in a concentration of 14,3mM (5mg/mL) that was then diluted in PBS to give rise to a 300µM intermediate solution. This solution was then diluted in PBS at a rate of 1:1000 giving rise to a final solution of 300nM. After this final incubation, cells were washed again in PBS three times after which the cells were left in PBS and they were ready for immunofluorescent microscopy analysis.

Extracellular Immunofluorescence Staining

The medium was removed from the wells and the cells were then washed with PBS. After this, the cells were incubated for 30 minutes at 37ºC in the presence of the primary antibodies diluted in staining solution. After this, the cells were washed with PBS three times and the secondary antibodies were added diluted in staining solution buffer. The cells were then incubated again for 30 minutes at 37ºC in the dark. Finally, after three more washing steps

with PBS the cells were examined under a fluorescence microscope.

Flow Cytometry Analysis

The eppendorfs used for this protocol should be first coated with BSA (Invitrogen™). For that purpose, 100-200µL of BSA were added per eppendorf, just enough to cover the walls of the tube. The excess of BSA should be removed before any cells are added to the tube for centrifuging.

For intracellular staining the cells were first fixated in 2% PFA for at most 2 weeks before being analysed. When the cells were analysed they were washed twice with 1% NGS and then centrifuged in 1000 rpm for 3 minutes. The supernatant was removed and the pellet was resuspended on NGS 3% with 1% saponin for 15 minutes. The saponin is a detergent and is added to destabilize the cellular membrane and allows the antibodies to enter the cell. The cells were then centrifuged again at 1000 rpm for 3 minutes, the supernatant was removed and pellet again resuspended in NGS 3% for 15 minutes. After that, cells were again centrifuged at 1000 rpm for 3 minutes, the supernatant was removed and the cells resuspended on NGS 3% with the primary antibody or simply in NGS 3% for the negative controls. The cells were left to incubate for one hour and 30 minutes at room temperature. The antibodies were diluted at a ratio of 1:150 for Anti-Sox2 and Anti-Oct-4 and 1:2000 for Anti-Nanog. The antibodies used were the same already described in the immunofluorescent protocols.

After the incubation the cells were washed twice using 3% NGS. After each washing, the cells were again centrifuged and the supernatant removed and the cells were resuspended on 3% NGS. This way all unattached antibodies were removed from the cell solution. After all the washing cycles are complete, the secondary antibodies diluted in 3% NGS were added and the cell pellets resuspended on them. The secondary antibodies were also diluted on 3% NGS and added to the appropriate vials even to the negative control vials, one for each secondary antibody. The cells were then left to incubate for 45 minutes in a dark area at room temperature. After the incubation was completed, the cells were washed twice with 1% NGS and after all the washing cycles, 300µL of PBS were added and the cells were analysed using the FACSCalibur flow cytometer

(BDBiosciences) and the FlowJO software (Tri Star).

The protocol for flow cytometry analysis of surface markers is similar to the protocol for intracellular staining with a few differences. The cells were not incubated for 15 minutes with saponin. Instead, the cells were washed with 3% NGS for 15 minutes after which the primary antibodies were added (Anti-SSEA-4, Anti-Tra-1-60) and the cells left to incubate in the dark for 30-45 minutes. After the incubation was complete, the cells were washed twice with 1% NGS. Before analysis, 300µL of PBS were added to the cells. Finally, the cells were ready for flow cytometry analysis.

Results and Discussion

The Cre-Sort model cell line that was used in this work was previously cultured on feeder layers of mouse embryonic fibroblasts and serum-containing medium. The cell line was then adapted to expansion in a specific chemically-defined and feeder-free culture protocol established at the SCBL lab. The growth kinetics of these cells was characterized in order to determine their expansion potential under these new culture conditions. Specific intracellular and surface marker expression was determined using immunofluorescence and flow cytometry against the pluripotency markers Nanog, Sox2, Oct4, SSEA-4, Tra-1-60 and Tra-1-81. The cells were also differentiated into Neural Progenitor Cells using the N2B27 medium and a protocol based on a dual SMAD inhibition method [22] and the activity of specific proteins was measured in the differentiated cells (Nestin and Pax6 were both tested) in order to evaluate the neural commitment potential of the expanded hiPSCs.

hiPSCs Expansion

The model cell line was expanded using mTeSR™1 as the culture medium and Matrigel™ as the culture substrate. After reaching ~80% confluence, cells were passaged at a rate of 1:4 wells. The cells were passaged using ReleSR™ and the newly seeded cells formed colonies after a few days of culture. As can be seen in Figure 1, the cells took several days to recover from the passaging

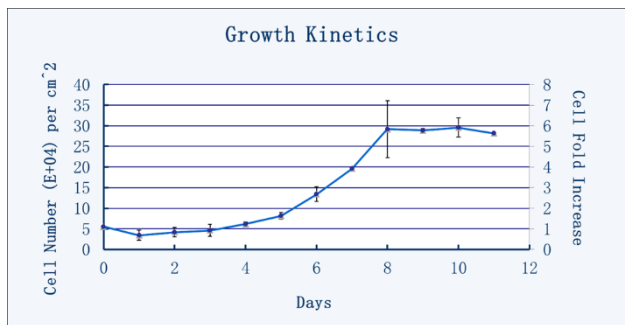procedure and only after 4 days They initiated the exponential growth phase.

Figure 1. Growth kinetics of the Cre-Sort hiPSC line.

After 8 days of culture, the cells would reach a 6-fold expansion and then a new passage could occur.

As Figure 2 shows, the cultured cells appeared to be able to form hiPSC colonies with well defined borders and little signs of spontaneous differentiation. However, as image B of Figure 2 also shows, cases of spontaneous differentiation did arise with colonies of undefined shape and cells of different morphology most commonly found on the periphery of these colonies. Spontaneously differentiated colonies could easily be identified after only two days of expansion. The usage of ReleSR™ in the passaging protocol instead of EDTA is partially to avoid the migration of these differentiated cells from culture to culture after the passaging protocol.
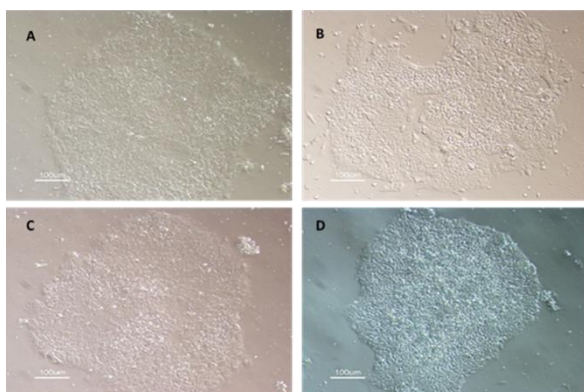

Figure 2. Bright field photographs taken from the cultured Cre-Sort hiPSC line. Image B shows a cell colony with differentiated cells in its periphery.

hiPSC Characterisation

The cells obtained from expanding the Cre-Sort cell line were characterised using flow cytometry and immunofluorescent microscopy.
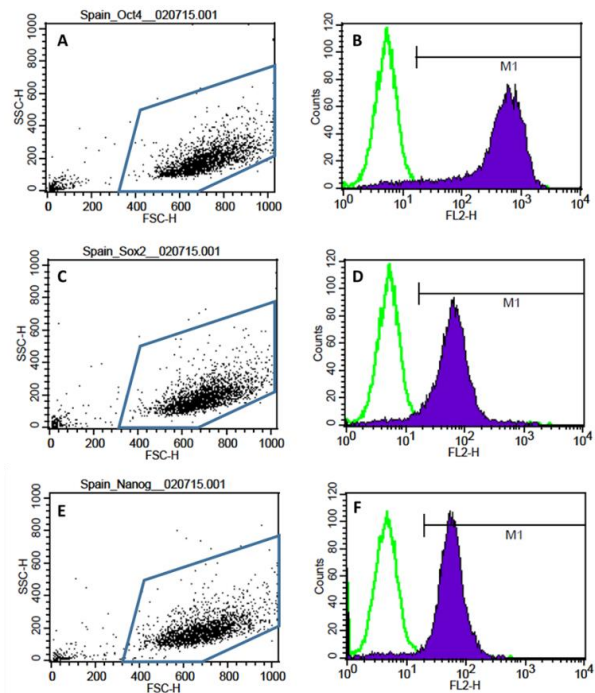

Figure 3: Flow cytometry results from the intracelular staining of the Cre-Sort hiPSC line.

Several cellular markers were tested, all of which related to pluripotency. The percentage of cells expressing the Oct4, Sox2 and Nanog transcription factors was quantified by flow cytometry. As Figure 3 shows, about 94% ± 3% of the detected cells were positive for the presence of Oct-4, at the same time about 93% ± 3% of the detected cells were also positive for Sox-2 and similarly 93% ± 3% of the detected cells were positive for Nanog expression. These results, for the intracellular staining, indicate that these cells retained their pluripotency characteristics. Besides these three intracellular markers, two more surface markers were analysed, both SSEA-4 and Tra-1-60. The results can be found on Figure 4 where we can
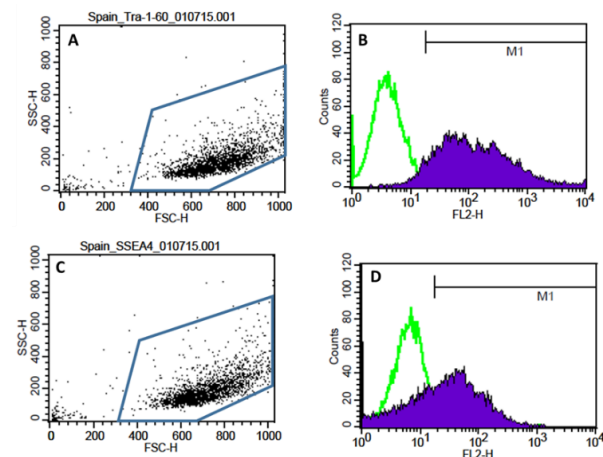

Figure 4. Flow cytometry results from the extracellular staining of the Cre-Sort hiPSC line.

see that about 93% ± 1% of all detected cells were found to stain positively for Tra-1-60. However, only 60% ± 3% of the detected cells were found to be positive for SSEA-4. These results seem to indicate again that the cultured cells maintained in their vast majority their pluripotent phenotype in culture, except for the SSEA-4 surface marker that only seems to be expressed in 60% of the cultured cells. This final result is believed to stem from a line of ineffective antibodies, instead of a loss of pluripotent capabilities of these cells.

The results obtained from flow cytometry analysis allow us to clearly see that a vast majority of all cultured cells seem to maintain
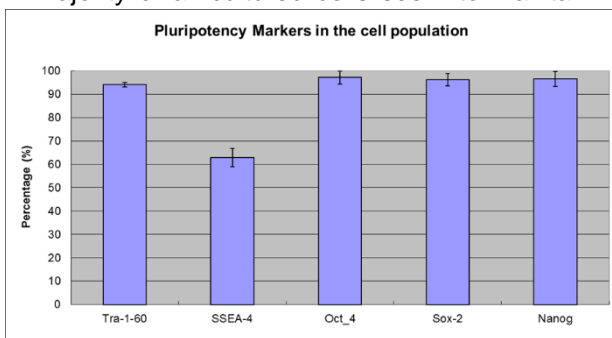

Figure 5. Complete compilation of the results from the flow cytometry assays.

their pluripotent phenotype. About 93% of the considered cells maintained the expression of most of the considered markers (Oct4, Sox2,

Nanog and Tra-1-60). Although these results are not enough to claim that the cultured cells are truly pluripotent they are a strong indicator. Figure 5 comprises all the information found in the flow cytometry assays.

Immunofluorescent microscopy was also used to characterise these cells and to further complement the results obtained from the flow cytometry assay. Both intracellular and surface markers were stained and pictures were taken from the cells while in culture. The cells were stained for the expression of Nanog, Sox2 and Oct4 as intracellular markers and they were also stained for DAPI which allows visual observation of the cell nuclei. The results are shown on Figure 6. We can see on the second column of pictures that all stained cells correspond to cells stained for DAPI, and that the only cells not stained with the pluripotency markers are situated on the periphery of the cell colonies. The Oct4 staining is the clearer example of this. On the surface markers side we can compare the staining pictures to those taken in bright field conditions. Again, only a few cells found on the periphery of the colonies do not show the expression of the considered surface antigens. Another point of note is the staining for SSEA-4. The images of this staining clearly show a comparable percentage of positively stained cells to the other images, showing that the results obtained from flow cytometry that claimed that only 60% of the
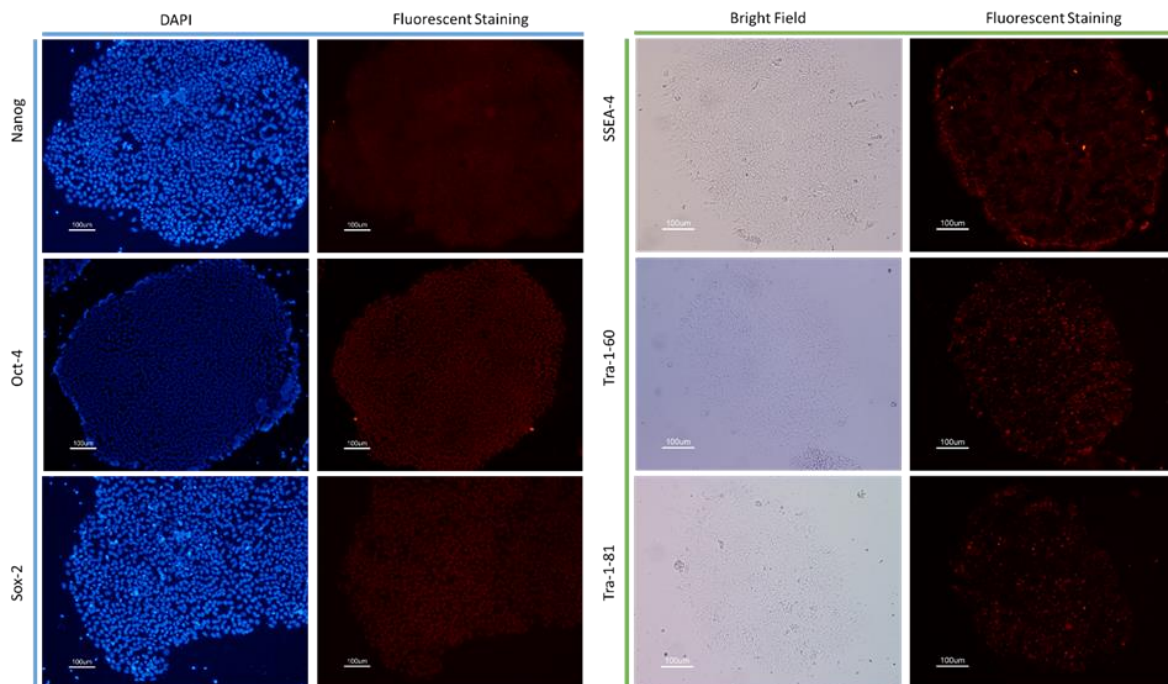

Figure 6. Immunofluorescence microscopy images. Intracellular markers are shown against DAPI staining of those same cells while surface markers are shown against bright field images of those same cells.

considered cells showed SSEA-4 expression are most likely false.

hiPSC differentiation assay

Although the results obtained from both the flow cytometry analysis and the immunofluorescent microscopy assays seem to indicate that the cultured cells maintained their pluripotent phenotype, it is necessary to prove so by differentiating the considered cells. By doing so it can be proven without a doubt that the Cre-Sort hiPSC line maintains its pluripotency potential even after being expanded in the developed culture protocol for months.

To proceed with this assay, the cultured cells were allowed to reach 100% confluence before being cultured in N2B27 medium supplemented with both SB-431542 and LDN-193189. This protocol stems from an adapted dual-SMAD inhibition differentiation protocol and has been proven to steer hiPSCs into neural differentiation. The cells thus committed were then stained for both Nestin and Pax6, both of these are standard markers that neural stem cells expressed and are commonly used to identify them in culture. Using immunofluorescent microscopy techniques, these cells could then be analysed for their expression of these markers. The staining of these cells can be seen on Figure 7, DAPI staining is also provided to more clearly analyse the results. As the pictures show, the vast majority of the considered cells show positive staining for Nestin. This protein is an intermediant filament protein found in the cytoplasm of multipotent neural stem cells, and it is essential for the cell's self-renewal and survival [23]. The staining for Pax6 also shows a ubiquitous expression of this transcription factor with some zones with a higher intensity of staining in the culture. Pax6 is essential to regulate several aspects of cell self-renewal, proliferation and survival *in-vitro*. Several studies show that the expression of Pax6 influences the expression of Nestin in NSCs and the differentiation of PSCs into neurons and oligodendrocytes [21].

These results show that the cultured cells were able to consistently differentiate into the considered cell type. The vast majority showed expression of both markers, Pax6 and Nestin, DAPI staining confirms that the stained cells are the vast majority of the cultured cells and the last images show a detail of the differentiation results where it is more easily seen the combined expression of Pax6 and Nestin by these cells.

Conclusion and Future Work

In this work, it was developed a chemically defined serum and feeder-free expansion protocol for the Cre-Sort hiPSC line of cells. These cells were obtained from an individual suffering from FA [21] and were subjected to several steps of genetic editing to, first correct the genetic disorder in these cells and to then reprogram them into hiPSCs. These procedures can render cell lines difficult to expand which was the case of this specific cell line. Although the considered cell line took longer than most to expand and grow, and showed a tendency to spontaneously differentiate. The developed protocol was able to achieve clear results, being
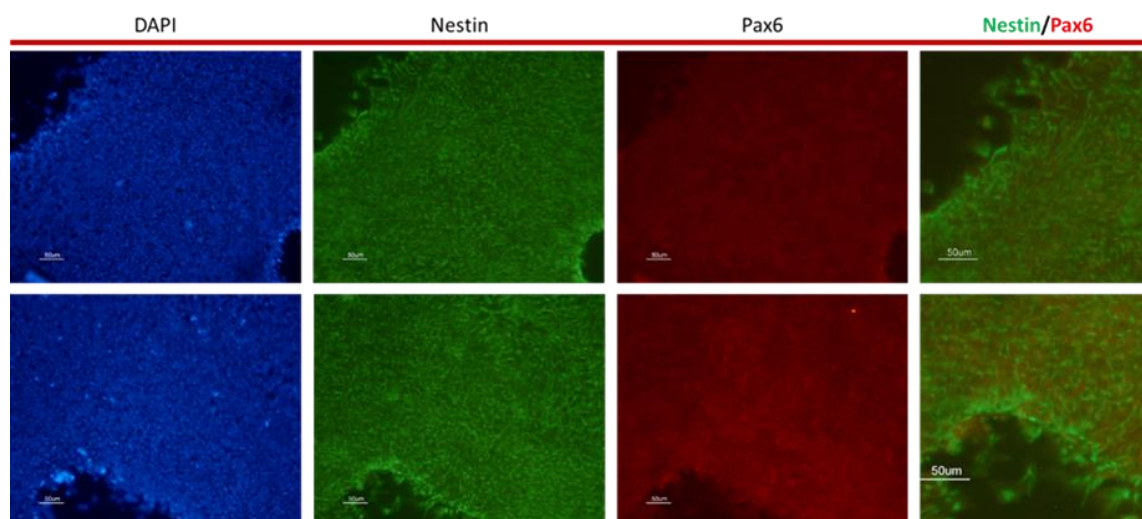

Figure 7. Nestin and Pax6 staining of Cre-Sort cell line cells differentited into neural stem cells. The pictures to the right show a superposition of both stainings.

able of expanding the considered line to a 6-fold expansion after 8 days in culture and maintaining several of the indicators of its pluripotency. From intracellular markers, such as Oct4, Sox2 and Nanog, to surface markers, such as SSEA4, Tra-1-60 and Tra-1-81, a vast majority showed maintenance of these markers after months of expansion in the developed culture protocol. The cells also showed the ability to differentiate into one of the three germ layers when directed to by using a differentiation protocol for PSCs. After 12 days of culture with N2B27 medium supplemented with SB and LDN, the cells showed ubiquitous expression of both Nestin and Pax6, both markers of a neural progenitor cell. Although all of these results are not enough to prove these cell's pluripotency their evidence points clearly towards it.

This study, therefore, proved that it was possible to expand these cells in a satisfactory way opening the door to future applications of these cells into clinical practices. The usage of a culture system based in feeder and serum-free components facilitates this translation as the risks of xenogeneic infection carried by those components would render these cells unusable in such a way.

Still much can be done from this foundation. The complete characterisation of this cell line is still lacking, a wider array of markers can still be used as well as different assays to confirm their pluripotent phenotype. The differentiation of this cell line into the three germ layers is still incomplete with both the mesoderm and the endoderm differentiation assays left to perform. Only after all these tests were successfully conducted and the teratoma generation assay would we be able to claim that these cells maintained their pluripotency in these culture conditions. Still after all this is completed even more can be done, the slow growth of these cells and their tendency to spontaneously differentiate in culture are problems that still need to be addressed and can probably be resolved by optimizing the culture medium, modifying the developed protocol to better suit this cell line specific needs.

After both the optimization of this culture procedure and the characterisation of this cell line in those conditions are complete, this cell line can be used for other applications in a clinical setting, such as cell therapy. This cell line was created with the objective of being used to someday treat FA's symptom of complete bone marrow failure [21]. With an optimized protocol behind this cell line, that is capable of delivering a reliable and robust expansion of these without the loss of their characteristics will it finally be able to launch them into clinical trials and into a therapeutic application.

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