



**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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Hope is important because it can make the present moment less difficult to bear.

If we believe that tomorrow will be better, we can bear a hardship today.

○ Thich Nhat Hanh

Abstract

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 find a protocol that allowed the expansion of these cells in a chemically defined media (mTeSR1) and using the Matrigel™ substrate. It was possible to expand the cells up to six times the original number after eight days of culture. After expansion, these 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). Moreover, the cells were also capable of expressing several neural markers (Pax6 and Nestin) after being differentiated into the neural lineage.

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

Resumo

As células estaminais pluripotentes induzidas humanas (hiPSC) apresentam a enorme vantagem de poderem ser obtidas a partir de células somáticas de qualquer indivíduo. Esta característica abriu ao mundo a possibilidade da medicina personalizada. De facto, quando obtidas a partir de um indivíduo afectado por uma doença genética, as iPSCs podem, em teoria, ser usadas para recapitular os efeitos dessa mesma doença ao nível celular permitindo a modelação *in-vitro* dessa mesma doença bem como a pesquisa de novos fármacos. Por outro lado, as células obtidas desta forma podem ser usadas para terapia celular após a devida correcção genética.

Neste estudo, foram usadas como modelo células hiPSC obtidas por reprogramação a partir de um doente com Anemia de Fanconi, tendo sido depois corrigidas geneticamente por forma a restaurar a sua função normal. Esta doença genética é definida por uma marcada predisposição para o cancro e eventualmente falência da medula óssea. Estas células foram obtidas a partir de uma colaboração com a Divisão de Terapias Hematopoiéticas Inovadoras em Madrid, Espanha.

Usando a experiência e o conhecimento do Laboratório de Bioengenharia de Células Estaminais do Instituto Superior Técnico, foi possível definir um protocolo que permitiu a expansão desta linha celular num meio de cultura quimicamente definido (mTeSR1™) e usando Matrigel™ como substrato de adesão. Graças ao protocolo desenvolvido, foi possível expandir as células até cerca de seis vezes o seu valor inicial durante oito dias de cultura. Após expansão nestas condições, as células mostraram uma expressão normal dos marcadores de superfície (Tra-1-60, Tra-1-81, SSEA-4) bem como dos factores de transcrição (Nanog, Sox-2, Oct-4) associados à pluripotência. Para além disso as células mostram expressão normal de marcadores de células neurais (Pax6 e Nestin) após serem submetidas a um protocolo de diferenciação em linhagem neural.

Apesar deste procedimento não ter sido ainda realizado em condições “Good Manufacturing Practices” (GMP), foi possível demonstrar que a expansão da linha celular modelo é possível e, no futuro, recorrendo ao uso de materiais de grau GMP, poderão então ser usadas para aplicações clínicas.

Palavras-chave: Células estaminais induzidas, Anemia de Fanconi, expansão celular, terapia celular, modelação de doenças

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List of Abbreviations

ASC – Adult Stem Cells

DMSO – Dimethyl Sulfoxide

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

ESCs – Embryonic Stem Cells

FACS – Fluorescence Activated Cell Sorting

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

GMP – Good Manufacturing Practices

HSC – Hematopoietic Stem Cells

ICM – Inner Cell Mass

iPSCs – Induced Pluripotent Stem Cells

Klf4 – Kruppel-like factor 4

DMEM – Dulbecco's modified Eagle's Medium

LDN – LDN-193189

MEFs – Mouse embryonic fibroblasts

NGS – Normal Goat Serum

Oct4 – Octamer-binding transcription factor 4

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

P/S – PenStrep

PSCs – Pluripotent Stem Cells

Rpm – Rotations per minute

SB – SB-431542

SCBL – Stem Cell Bioengineering Laboratory

Sox2 – Sex determining region Y-box 2

SSEA-4 – Stage-specific embryonic antigen 4

I. Introduction

I.1. Fanconi's Anemia: Disease Information

I.1.1 Disease Definition and Classification

Fanconi's Anemia (FA) is an autosomal 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].

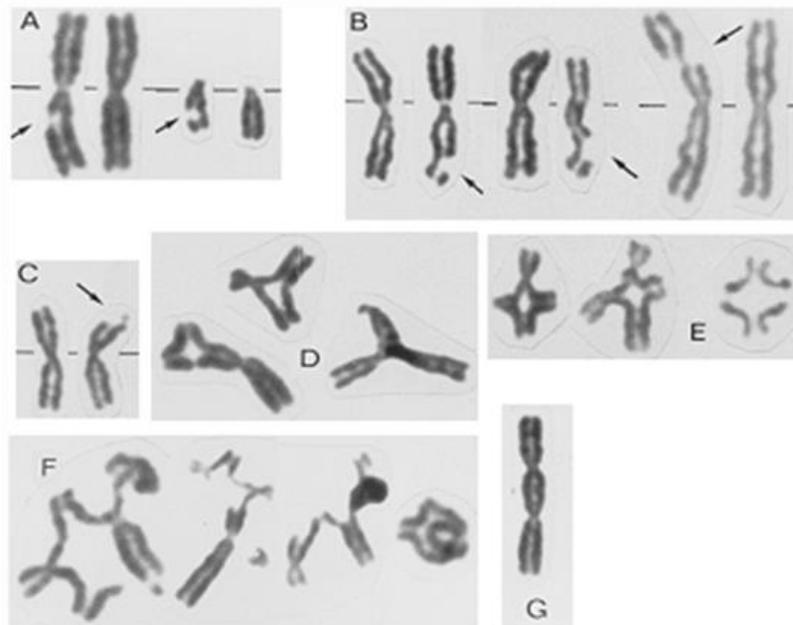


Figure I.1: Several kinds of genetic aberrations prevalent in FA patients: A: gaps, B: breaks, C: deletion, D: triradials, E: quadriradials, F: complex figures, G: dicentric chromosome. Pictures obtained by Giemsa staining. [5]

I.1.2 Pathophysiology of Fanconi Anemia

Currently, 13 genes were identified as being part of the FA disorder, and since patients with FA have a characteristic clinical and cellular phenotype, it was speculated that the 13 proteins cooperate in a common DNA-repair pathway. Indeed FA proteins work in concert to control the monoubiquitinated state of the FANCD2 and FANCI proteins as well as the downstream functions of the pathway.

Monoubiquitination is activated upon DNA damage and entrance into the S stage of the cell cycle, therefore DNA repair is impaired and cells tend to accumulate on the G2 phase of the cell cycle [4].

Cells in FA have, however, a reduced ability to acquire point mutations in response to DNA cross-linking events [6], instead they display extensive chromosomal insertions and deletions. The FA pathway therefore probably facilitates an error-prone repair process that causes point mutation and protects cells from gross chromosomal rearrangements [7].

This characteristic also opens an interesting opportunity for cancer treatment. The mutations found on FA patients can also appear in several cancers on regular healthy individuals. Therefore cancer cells that present a mutation in any of the FA genes are more sensitive to DNA cross-linking agents presenting a new avenue of treatment without expensive and possibly life-threatening procedures such as chemotherapy. Also, in the case of resistant cancer cells, inhibiting the considered pathway, using specially designed drugs, may allow us to induce sensitivity to the same chemicals into otherwise insensitive cancer cells [8].

I.1.3 Diagnosis and Symptoms of Fanconi Anemia

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

- Bone marrow failure with progressive pancytopenia, starting with thrombocytopenia and eventually progressive aplastic anemia;
- Congenital malformations such as skeletal malformations, for example asymmetrical absence of thumbs, microphthalmia and a small face;
- Hyperpigmentation;
- Pre and postnatal growth retardation;
- Urogenital, renal and cardiac anomalies;
- Propensity to develop acute myeloid leukaemia among other cancers;
- High insulin/glucose ratio;
- Hypogonadism, sometimes associated with infertility;
- Cellular hypersensitivity to DNA cross-linking agents [9].

Cancers also develop in about 20% of the FA patients [10] of which acute myeloid leukaemia is the most common. Other tumours include squamous-cell carcinomas of the head and neck [11], gynaecologic squamous-cell carcinoma, esophageal carcinoma and tumours of the liver, brain, skin and kidney.

Several of the genes that are inactive on FA patients are also inactive in a variety of other cancers in the general population [12]–[14]. For example, one of the proteins that can be found inactive in several cases of FA (FANCD1) is the same protein known to confer susceptibility to breast and ovarian cancer (BRCA2) [15].

Diagnosis is done by using the hypersensitivity of the cells in order to separate it from other DNA repair impairing diseases. The DEB-induced chromosome-breakage assay (DEB test) is a diagnostic tool for FA. By exposing cells from patients to DNA chromosome-breaking drugs, such as diepoxybutane (DEB), it increases genetic anomalies, namely chromosome cross-linking events, and accumulates cells in the G2 phase of the cell cycle. The level at which this happens is then compared to that in cells from known FA patients and from control subjects [4].

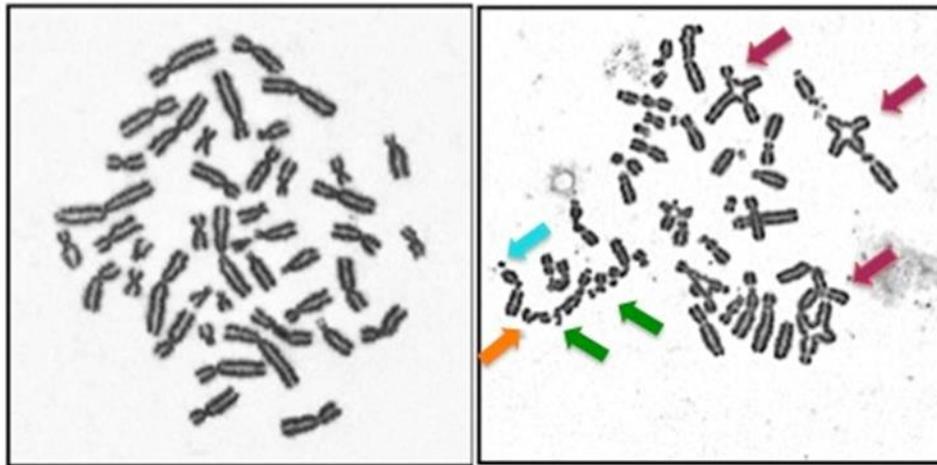


Figure 1.2: Results obtained from a DEB test. The results of a patient without FA on the left and with FA on the right. Genetic abnormalities are marked with arrows [16].

It is possible to analyse the sensitivity to DNA cross-linking of amniotic cells taken from heterozygous mothers and therefore determine if the unborn child will be afflicted with FA upon birth [9].

The haematological disorders from bone marrow failure usually appear early in life at around 7 years old [17], however they can arise earlier at about birth or even more rarely at or after 40 years of age. Approximately 33% of FA cases show no congenital malformations and only progressive bone marrow failure.

I.1.4 Epidemiology of Fanconi Anemia

FA can be found in all ethnical groups with a frequency of 1/350000 births. It also has a higher frequency in two specific ethnical groups: the Ashkenazi Jews and the Afrikaans population of South Africa. In these groups the incidence of homozygous forms is 1/22000 births with an allelic frequency of 1/77 versus a frequency of 1/300 in the general population [9].

I.1.5 Treatment of Fanconi Anemia

The main treatment for the bone marrow failure associated with FA are bone marrow or umbilical cord blood transplants. These treatments are relatively effective in curing this symptom although some complications may arise. Androgens and hematopoietic growth factors although effective at treating bone marrow failure in FA patients usually only achieve transitory improvements and carry a heavy risk of hepatic toxicity and malignant transformation [9].

Due to FA patients being extremely sensitive to DNA cross-linking agents, such as ionizing radiation and cyclophosphamide, the results of traditional bone marrow transplants can vary wildly. Nowadays new therapeutic techniques that take into account this hypersensitivity are able to significantly improve those results [18]. Although cellular therapies concerning bone marrow transplants are use other cellular therapies with isolated and characterized stem cells are becoming a promising therapeutic approach. For example, autologous bone marrow transplants using genetically edited cells or iPSC-derived hematopoietic progenitor cells may become options [19]. Gene therapy has also been the object of several trials and research. The usage of retrovirus and other gene targeting vectors carrying the corrected genes of interest is still being studied for effectiveness and usefulness.

The most important part after resolving the bone marrow failure symptoms is the control, surveillance, prevention and treatment of solid tumours [20].

I.2 Stem Cells

I.2.1 Definition and Classification of Stem Cells

A stem cell is defined as a cell with the ability to produce either identical clones of itself (self-renewal) or to differentiate into other specialized cells [21]. Pluripotent stem cells can therefore maintain themselves virtually indefinitely *in-vitro*, and can also differentiate into any of the approximately 200 specialized types of cells in the adult human body. This makes them prime candidates to be used as sources of cells for drug screening, cell therapy and disease modelling [22].

Embryonic stem cells (ESCs) are stem cells capable of indefinite self-renewal and pluripotency, having the ability to differentiate into any cell present in the adult body (Figure I.3) [21]. They are also capable of teratoma formation and are difficult to control, often leading to spontaneous differentiation. There also exist concerns about immune rejection of the transplanted tissues. Their usage is plagued by religious and ethical qualms because to obtain them it is necessary to destroy an embryo [23] and therefore the death of a potential human life, since they are harvested from the inner cell mass of that embryo.

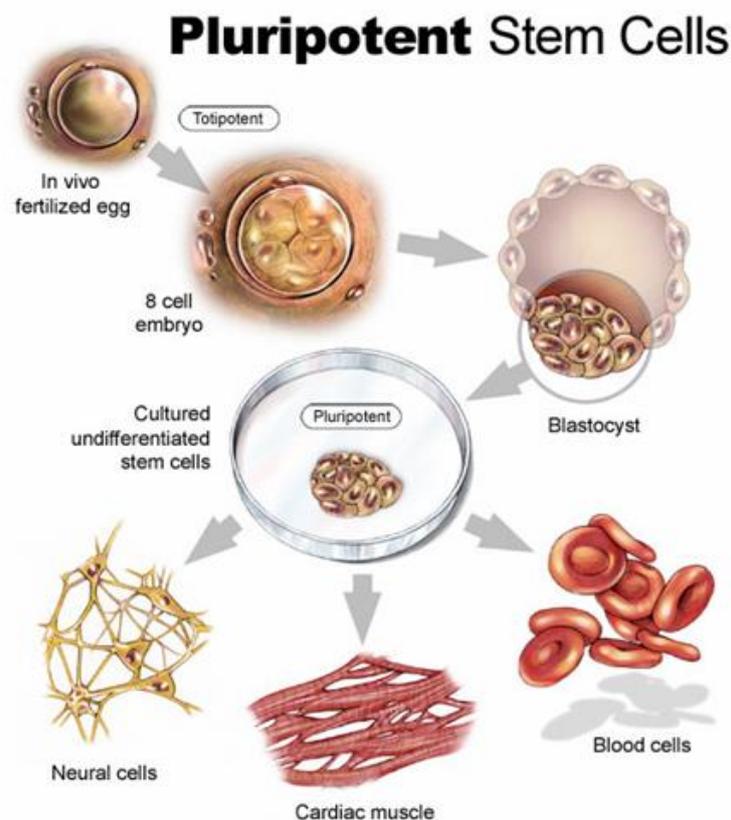


Figure I.3: Embryonic Stem Cells are obtained from the inner cell mass of a blastocyst, they have pluripotent characteristics being capable of differentiating into any of the three germ layers. [24]

Adult stem cells, are undifferentiated cells present in all tissues of the adult body. They have the function to produce cells to regenerate that specific tissue in case of damage and to replace dying cells. They do not give rise to any ethical concerns since they are obtained directly from a donor or the patient and can be obtained easily or with a great difficulty dependent on their source tissue. However they vary in quality depending on the age and health of the donor and their differentiation potential is usually limited to cells found in the same tissue from where they were obtained. These cells therefore are only multipotent cells versus pluripotent cells such as ESCs. [23]

I.2.1.1 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are another kind of stem cell commonly studied alongside ESCs and adult stem cells. They are obtained from the reprogramming of somatic cells, or differentiated cells, found in the adult human body. The reprogramming process consists of delivering several genes that encode transcription factors normally found active in ESCs. These factors, such as Nanog and Sox-2, when expressed in those somatic cells force them to adopt a less differentiated phenotype, eventually becoming pluripotent stem cells with the same generic properties of ESCs. Several types of human somatic cells have been reprogrammed. Out of all of them, dermal fibroblasts are the most common choice mainly due to their ease of accessibility and availability [25], [26].

iPSCs present exactly the same characteristics as ESCs. Several studies have shown that these cells are functionally indistinguishable [27], [28]. Upon using non-integrative reprogramming methods, the gene expression of both iPSCs and ESCs was extremely similar, only differing in a very small number of genes, being this difference attributed to cell line variability [29]. iPSCs display the same expression of transcription markers as ESCs, like Oct-4, Sox-2 and Nanog, several surface markers, like stage specific embryonic antigens (SSEA) 3 and 4 and keratin sulphate-related antigens (TRA-1-60 and TRA-1-81), and they both also have high telomerase and alkaline phosphatase activity [22]. These specific markers are of extreme importance for the identification of pluripotent stem cells since Oct-4 is an essential factor for the initial formation of the pluripotent cell population in the embryo formation and in the maintenance of pluripotency. When a cell loses that characteristic it is usually accompanied by a downregulation of this transcription factor [30]. Sox-2 is also another essential factor for the maintenance of pluripotency in stem cells, however it is also expressed in neural stem cells and therefore it alone does not define a pluripotent stem cell [31]. Nanog, on the other hand is absolutely essential for the establishment of pluripotency both *in-vivo* and *in-vitro*. *In-vivo*, without Nanog expression, the cells of the embryo never develop pluripotency and instead the inner cell mass remains in a pre-pluripotent nonviable state. *In-vitro*, Nanog is believed to provide a similar function as *in-vivo* by managing the dedifferentiated intermediate cells transit into a ground state of pluripotency [32]. These three markers are universally expressed in pluripotent stem cells and therefore are good indicators of pluripotency.

Also the fact that iPSCs are capable of generating teratomas in animal models shows that those cells are very similar to ESCs [33], presenting the same development potential and pluripotent capabilities. iPSCs however, since they are obtained from the forced expression of key transcription factors in adult somatic cells, do not have the same ethical concerns as ESCs, although they still have ethical concerns considering they are obtained from human sources [25].

Another advantage of iPSC line technology is its simplicity. iPSC lines can be obtained on any laboratory using standard techniques and equipment. Every experiment with iPSC lines generates several clones, although they still need to be selected to ensure a quality cell line [26]. However, several points must be taken into consideration when reprogramming cells to iPSCs. Reprogramming potential is inversely proportional to the stage of differentiation of the considered somatic cells. Hematopoietic progenitor cells, for example, have a bigger efficiency when being reprogrammed than fully differentiated B or T lymphocytes [34]. Also, the cell age and number of cell passages in culture, has the same inverse correlation to the efficiency of reprogramming of those cells [19].

iPSCs can be used for patient-specific cell therapy. In that setting, their utility is mainly attributed to their immunological compatibility. These same reasons have lead scientists to explore new methods and possibilities to generate stem cells from somatic differentiated cells instead of using ESCs to reach the same goal [35].

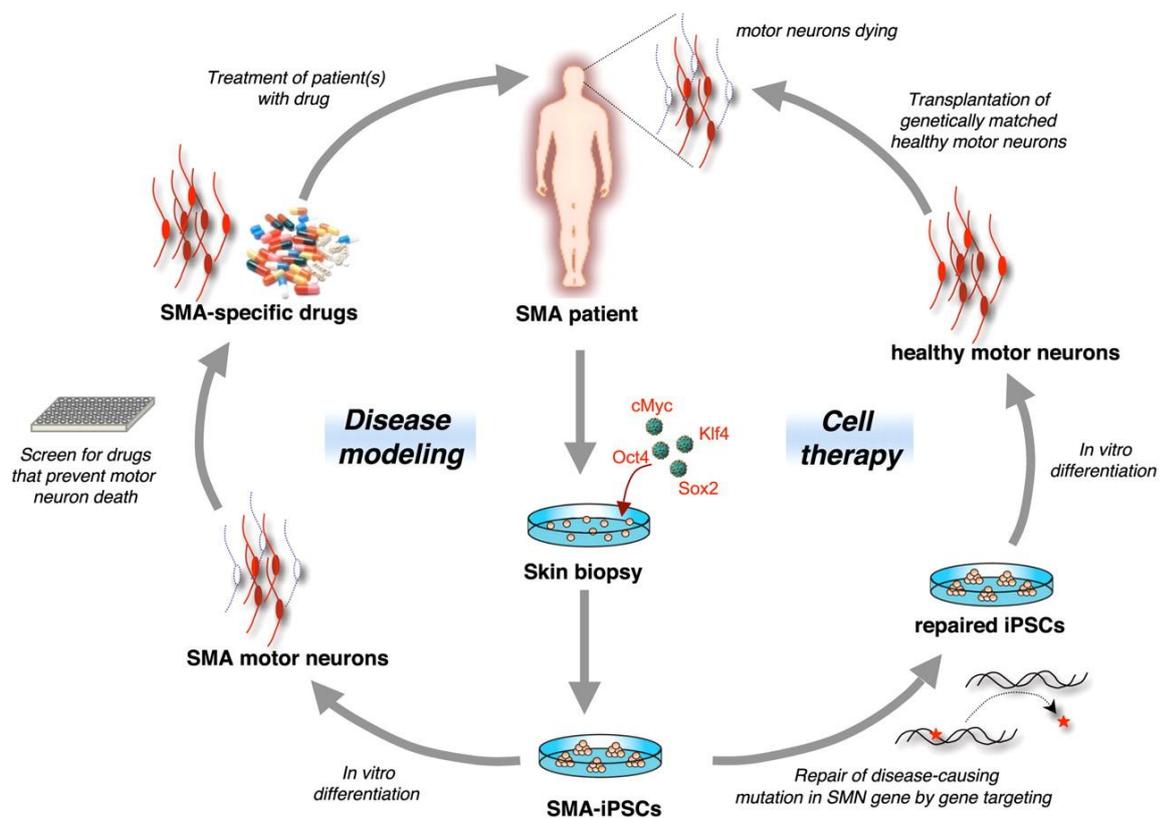


Figure 1.4: Possible applications of iPSCs using a patient with spinal muscular atrophy (SMA) as an example. iPSCs after being reprogrammed can be used to model the disease afflicting the patient or, if corrected, can be transplanted to heal the patient in cell therapy methods [36].

A word of warning, although ESCs and iPSCs can be theoretically kept in culture indefinitely they still suffer the danger of developing karyotypic abnormalities, such as numerical and structural chromosomal abnormalities, point mutations, variations of telomere length, and epigenetic instability [37]. These abnormalities are believed to reflect the cells adaptation to their culture conditions allowing for a bigger capacity to proliferate coupled with an increase in tumorigenicity [38]. This then imposes a theoretical limit to the amount of time any stem cell line may be kept in culture.

I.2.1.2 History of iPSCs

iPSCs were first discovered in 2006 by a scientist named Shinya Yamanaka in Japan, by co-transduction of mouse embryonic or adult fibroblasts using several retroviruses carrying a combination of 24 different transcription factors [39]. However it became clear in later experiments that only four of those factors were needed for the reprogramming to occur, they were Oct-3/4, Sox-2, Klf-4 and c-Myc [39], Oct-3/4 being the most important from them all since its expression is very specific to pluripotent stem cells while the other factors can also be expressed in other cell types (Sox-2 in neural stem and progenitor cells, Klf-4 in skin, stomach, intestine and skeletal muscle and c-Myc is ubiquitously expressed).

Also, Oct-3/4 is absolutely necessary for the maintenance of pluripotency in reprogrammed cells [30] and it has also been proven to be possible to generate iPSCs from the forced expression of Oct-3/4 alone in certain cell types like neural stem cells (NSCs) that already express some of the other factors [40]. There have been discovered several small molecules capable of compensating for one or more of the transcription factors used in the reprogramming process, such as kenpaullone, capable of compensating for the absence of Klf-4 [41] or a combination of BIX-02194 and BayK8644, that compensate for the absence of Sox-2 [42].

The successful cloning of animals, such as Dolly the sheep, lead to the creation of the concept of therapeutic cloning, where the nucleus of a somatic cell is transplanted into an oocyte, ready to give rise to ESCs obtained from the resulting inner cell mass of the formed blastocyst [43]–[45]. The resulting ESCs are therefore tailored for the genetic makeup of the target patient. This approach however presents several social and logistical hurdles that make it unattractive. By contrast, iPSCs generation by forced expression of several transcription factors dodges most of these limitations and is logistically simple without the need for embryonic material or oocytes.

There are several ways to reprogram somatic cells into iPSCs. The first protocols to generate iPSCs used viral vectors to force the expression of the four transgenes, which would then integrate into the genome of the somatic cells to be reprogrammed. Nowadays, however, it is possible to generate iPSCs without integrative methods. These methods are obviously safer since they prevent the integration of other unintended transgenes from the viral vectors used [46].

The vectors can vary from viral vectors, with the use of integrating retrovirus, lentivirus and the non-integrating adenovirus [47], to non-viral means, such as DNA plasmids [48] and recombinant protein delivery [46]. The reason why the scientific community has been looking for new vectors for reprogramming is because the integrative methods create iPSC lines unsuitable for clinical use. The integrated viral transgenes, essential for the use of those vectors, and insertional mutations that these vectors may cause, can give rise to changes in the reprogrammed cells, for example oncogenic events [49], [50]. Therefore, if iPSCs are ever to be used in a clinical settings these new non-integrative methods will have to be explored and used instead of the traditional integrative methodologies.

However the greatest problem needed to be overcome for the application of iPSCs into a therapeutic setting is the lack of knowledge about the nature of these cells as well as the correct amount of expertise in order to translate these cells from the bench scale method to GMP standard processes of stem cell therapeutic product manufacturing [35].

I.2.1.3 Applications of iPSCs

I.2.1.3.1 Cell Therapy

Several somatic cells obtained from iPSCs can be potentially used in regenerative medicine to heal damaged tissue by disease or injury [26]. ESCs are already being used in therapeutic settings with a FDA approved clinical trial for the treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium in 2015, with very satisfying results [51]. In fact, ESCs have been shown to have a discernible therapeutic effect in animal models for several different conditions [52]–[54].

iPSCs are similar to ESCs and actually solve several of the problems that currently plague ESC application, such as immune rejection after transplantation. A big problem present in the use of any of the two kinds of cells is the formation of teratomas. Even a small number of undifferentiated cells is capable of generating teratomas in the patient after transplantation. To avoid this problem, terminal differentiation should be induced in the cells prior to implantation [55]. However not all applications allow for this, some uses of hiPSCs may require the implantation of rather less differentiated cells in order to enhance their effect on the patient. Other unique hurdles for the usage of iPSCs are related to the reprogramming process, since it is possible that during the reprogramming process some of the reprogrammed cells suffer from aberrant reprogramming or abnormal expression of several different pluripotency genes, which may cause the considered cells to not be able to differentiate, contributing to a larger chance of developing teratomas upon implantation of the hiPSC derivatives [56]. Also, when using retrovirus as vectors for reprogramming iPSCs, some viral transgenes can be integrated along with the genes of interest, leading to unforeseen immune responses, aberrant reprogramming or even to a higher chance of tumorigenesis [57].

It has been shown in animal models that the usage of HLA matching iPSCs obtained, for example, from the patient itself and transplanted after differentiation back into the same patient presents no discernible signs of immune responses both in short and long term time frames while the use of mismatched or even just related HLA iPSCs will lead to immune responses ranging from acute immune responses for mismatched cells to chronic responses for related cells [58]. iPSCs are considered to be alternatives to ESCs because they bypass some of the ethical concerns about its use and have diminished probability of immune rejection than ESC-derived tissues [59]. However, partial reprogramming and genetic instability may lead to immune responses in tissues transplanted with iPSC-derivatives [60]. Another possible source of immune rejection is the abnormal gene expression of some of the cells differentiated from iPSCs since this phenomenon can cause T-cell dependent immune reactions to transplanted tissues [61].

The possibility of creating iPSCs tailored for each patient would mean the opportunity to realise cell replacement therapy with no need for immunosuppressants since the autologous transplantation of genetically identical cells or even tissues and organs would circumvent all the dangers of immune rejection.

iPSCs have been already differentiated into several different disease relevant cell types and they have also been shown to have a therapeutic effect on animal models, after genetic correction [62]. These cells presented a healthy phenotype *in-vitro* and were identical to ESCs and iPSCs derived from healthy donors. Therefore, iPSC technology can be used for cell therapy applications by generating patient-specific genetically corrected and phenotypically normal cells that could then be transplanted [19].

Still, there are several challenges to this therapeutic approach. First of all, the implanted cells have to be fully differentiated prior to implantation, since any undifferentiated cell may give rise to tumours *in-vivo*. Also, the purification and selection of the cells to transplant is extremely important in order to guarantee the engraftment of a sufficient number of therapeutic cells in the target site, which is essential for their hematopoietic therapeutic effect to be felt. Nevertheless, reliable and robust methods to solve both of these problems still need to be researched and developed [19].

The usage of hiPSC derivatives for clinical applications demands the development of scalable and robust methodologies for the production of large amounts of these cells under GMP conditions. An efficient GMP culture method requires a GMP optimized culture medium for human stem cells and a robust scalable culture system that provides optimized environment conditions. This system would then allow for the production of highly pure hiPSCs and their derivatives with a high enough number for therapeutic applications [35].

With time, the development of more advanced technologies, like cellular arrays and bioprinting techniques have allowed us to develop cell derivation media, identifying for example cellular pathways responsible for cell fate (self-renewal, differentiation, quiescence or apoptosis), identifying small molecules that act upon those pathways finding ways to produce them in a completely xeno-free manner and determining optimum concentrations for those molecules [35].

Doses for cell therapy applications are reported to be about 10^8 cells, depending on the specific condition to be treated. It was also estimated that even larger doses of 10^9 cells might be needed for other more common conditions such as liver disease or myocardial infarction repair [63]. It has been shown that traditional planar technologies may struggle to produce the amount of cells required for those applications [64]. However this does not mean that planar technologies will eventually stop being used, especially in personalized medicine, since it involves autologous applications of cells from specific patients. This approach requires a scale-out method and therefore planar technologies are still useful in those conditions. The number of needed cells is smaller and the cost of using planar technologies much lower than to transition into a 3D application to achieve the same results [65].

In the case of FA, although there are currently no clinical trials involving iPSCs derivatives, several important steps have been made towards the establishment of those same trials through the derivation of genetically corrected iPSCs and their differentiation into healthy hematopoietic stem cells to transplant into patients in a possible clinical trial [9], [19], [20].

I.2.1.3.2 Disease Modelling

Current data shows that drug therapies for many major diseases only treat 50-70% of the affected individuals and often with several unwanted side-effects [66]. It is therefore a necessity to better understand the molecular problems underlying these same diseases and their progression processes. This can only be done by analysing more relevant models in which to simulate these disorders. iPSCs can be used to allow for the study of these diseases in a controlled environment.

In order to study the molecular pathways associated to certain disorders, the best strategy is to study the cells affected by the disorder itself. Traditionally, those cells were obtained either through the use of animal models or post-mortem tissue samples, for more invasive tissue collecting. However, both of these avenues present their own problems. Post-mortem tissue samples only allow us to study the late stages of a given illness if it was the reason of the subject's demise and also may present a few ethical concerns. Animal models, on the other hand, present several advantages such as allowing for a more complete following of the disease progression over time, including several behavioural changes, accounting for the effects of *in-vivo* cellular interactions and also permitting the immediate testing of candidate drugs once the disease is established. They, however, also have their own set of important hurdles. Animal models have in many studies been shown to have species-specific differences to human systems [67], going as far as showing completely different gene activation patterns before the same

studied stimuli [68] with the corresponding candidate drugs having completely different effects on the human clinical trials [69]. Also breeding and generating transgenic animals in order to reproduce certain disorders is expensive and time-consuming.

On the other hand, using hiPSCs is much faster, iPSCs are easily accessible and capable of self-renewal and large quantities of them can be produced relatively quickly. After differentiation, they would allow for the building of large-scale genetic and chemical screens for any disease phenotype in study. Since these cells are also capable of, in theory, differentiate into any cell type, they would also allow the study of the effects of a certain disease in any of the cells commonly affected by it and at any stage of development. Disease progression can also be studied in these models [70]. As it is obvious, these models suffer from no species-specific differences to human models since they are obtained from human derived iPSCs, supposedly.

Since these cells can also be easily cultured in small numbers, or even as single cells, it becomes easier to identify the cellular phenotypes associated with the molecular causes of the disease. These phenotypes could easily be missed when analysing a more complex tissue where other homeostatic mechanisms may act in a way to prevent the deleterious effects of the genetic disease from showing. For example, phenotypes such as neuron degeneration, that usually take decades to manifest any visible symptoms *in-vivo*, were observed *in-vitro* in a matter of weeks. [71]

Using iPSCs in this manner does not come without its challenges. In fact, compared to the great amount of different cell types in the human body, only a few kinds have been differentiated from iPSCs so far. There are two ways in which we can differentiate iPSCs. To differentiate them the cells are exposed to certain small molecules at certain times and in certain concentrations that stimulate or inhibit cellular pathways present during the process of differentiation into the target cell type *in-vivo*. Other types of stimulation are also needed such as soluble factors, extracellular matrix (ECM) molecules and other physical factors (substrate stiffness, etc.), making the entire process extremely complex and sometimes difficult to perform. The discovery of new efficient differentiation protocols remains a limiting step in this process and a resource and time consuming one [72]. In “direct programming”, the IPSC stage can be ignored completely, the cell is differentiated through the forced expression of determinant genes, usually transcription factors or microRNA, which forces the cell to convert from one cell type into another [73]. However, it is not currently known if these cells can be used for disease modelling, since these changes in their expression patterns can make them too different from the normal patterns of their *in-vivo* counterparts [74].

However, even if the hurdles of the process to obtain these cells were overcome, other problems still remain. Most of the time cells obtained through iPSCs differentiation are immature and do not show all the characteristics that those cells would when developed *in-vivo* [75]. This makes them less optimal targets for disease modelling since currently it is not clear for several diseases whether cell-type-specific mechanisms, such as insulin production in pancreatic cells, are present in immature cells or if they are only triggered later in their maturation cycle. This makes it difficult to estimate to what extent the obtained differentiated cells must be matured *in-vitro* to be used for modelling diseases [76].

However, most of the times the obtained cells are capable of recapitulating the same phenotypes present in their mature *in-vivo* counterparts, even late-onset phenotypes such as neurodegeneration from Parkinson's or Alzheimer's disease [71], [77].

iPSCs can be obtained from patients with several disorders [78]–[81] and these cells are then capable, after differentiation, of simulating the pathology that the original patient suffered from [79]. This way those same disorders could be studied *in-vitro*, no longer needing human patients to suffer it for study, and in a controlled environment. Also, using cells from a specific patient would allow for the use of personalized medicine. In fact, when testing drug candidates in this model, whatever results that came, the drugs would be tailored for that specific disease subtype and perfectly tailored to that patient. It might even be possible to identify specific disease pathways and counteract those minimal side-effects, something that using standard medicine would be completely unthinkable.

For example, disease models made from iPSCs have been developed for several disorders such as ALS [78] where motor neuron cells were obtained by differentiating iPSCs from patients with ALS and provided a unique insight into the death of motor neurons in ALS disease. Parkinson's and juvenile diabetes have also been modelled from iPSCs [81] and even SMA [79]. Of course we could not go by without mentioning an example significant to this project, FA itself has also been modelled using iPSCs derived from patients of the disease. Modelled cells showed an abnormal sensitivity to DNA cross-linking agents as well as an abnormal amount of cells arrested at the G₂ phase of the cell cycle. One of the marks of a functional FA pathway the monoubiquitination of FANCD2 protein was also severely reduced in the considered cell line [82].

I.2.1.3.3 Drug Screening

Liver cells generated from iPSCs and even beating cardiac cells generated from iPSCs could be used to test the possible toxic effects of new drugs in those organs of a certain person or in people with a certain disorder recapitulated by those cells [26].

During the last years, the drug discovery industry has been using high throughput screening techniques to identify new biologically active small molecule drugs as new drug candidates. These small molecules are selected using a plethora of molecular targets obtained from the sequencing of the human genome and advances in combinatorial chemistry. To achieve this objective, the drug discovery industry usually uses isolated catalytic protein domains or cell lines overexpressing the recombinant target protein through genetic modification as targets in their screenings. They evaluate if these new drug candidates are then able to interact and affect these target proteins in hopes of finding a drug capable of combating the specified disease. This allows for a simplification of the process of data analysis and it also helps to drive down the costs of production [83].

However, these techniques are unable to account for the complex interactions between pathways and proteins in the native cell environment. For example, the usage of isolated catalytic protein

domains may indicate the presence of several molecule binding locations that are unavailable in the natural cell environment, either because the full protein changes these domains or because the interactions between the protein and other endogenous binding partners in the cell. Several studies have shown that the results obtained when using these techniques differ substantially from those obtained by using enzyme extracts from *in-vivo* sources [84].

Also, overexpressing isolated proteins may lead to a change in the cell's signalling pathways and interactions between them [85]. This results in a poor relationship between the results of the screening and the results observed on the patients [86].

Therefore, using cells obtained from patients seems to be the best option for a reliable tool for drug screening. These cells are capable of recapitulating the symptoms of the considered diseases *in-vitro* and to react in an appropriate manner to the presented drug candidates as those cells would *in-vivo* [87], [88]. As an example, a study used hiPSC-derived cardiomyocytes from patients suffering from several hereditary cardiac disorders and was able to replicate drug induced cardiotoxicities and differing susceptibilities among the different patient-derived cells, showing that different pathologies will give rise to different drug susceptibilities in different patients and therefore the standard practice of drug identification is ineffective and even dangerous to certain patients [89]. This shows that we need better *in-vitro* models that mimic the complex environment found *in-vivo*, accounting, for example, for the full length proteins interacting with their normal partners in their corresponding pathways and co-cultures of interacting cell types.

Still an argument can be made that using iPSCs to simulate the *in-vivo* conditions is not a valid model that the best strategy would be to harvest cells directly from the source and test on them without reprogramming and genetic engineering. These cells seem to be the best model since their interactions and the expression of their proteins were only shaped by the conditions *in-vivo* from where they were harvested, however they show several limitations that do not affect iPSCs [90].

Most terminally differentiated cells, which would be the case in most if not all of the harvested cells from patients for use in drug screening, do not proliferate, therefore limiting the amount of cells available for testing. Also, the large numbers of cells needed for those tests may raise ethical and logistical concerns [91]. Upon isolation, these cells usually display a dedifferentiation phenotype which makes data analysis difficult and limits the amount of time available for testing [92]. Finally, some cells are unavailable for collecting from human sources and would then need to be substituted by animal cells which, due to the differences between human and animal physiology, are unable to provide robust and reliable results for the testing of the effects of drugs in human patients [67].

Therefore, human induced pluripotent stem cells are the answer and future for this application. They are capable of unlimited proliferation and are genetically stable during their undifferentiated phase. Under the right stimulus they are able to differentiate into any of the terminally differentiated cells in the human body and during that process they recapitulate aspects of the normal development of those target cells allowing for the study of several developmental and degenerative diseases. They also bypass many of the ethical concerns present in both ESCs and somatic cells harvested from humans.

The fact that they can be obtained from any individual allows for the creation of a wide array of cell lines displaying characteristics of specific population segments or disorders. This in turn allows for the creation and application of personalized medicine with drugs and therapies custom fitted to patients and to specific types of illness [25].

However, it becomes painfully apparent that in order for these cells to become viable solutions for drug discovery we must first develop and put in place protocols for the expansion and maintenance of these iPSCs lines, especially from patients suffering from specific genetic disorders that may disrupt their expansion and require special care, such as FA.

I.3 HiPSCs Expansion in Static Culture Conditions

I.3.1 Evolution of Culture Conditions

I.3.1.1 *Early days of hESC culture: undefined Media and feeder Cells*

The first culture conditions used for the expansion of hESCs used mouse feeder cell layers and the medium contained fetal bovine serum. At this time, little was known about what kind of soluble factors were needed to maintain these cells. Therefore, using serum and feeder layers allowed for the easy maintenance of those cells (Figure I.5). All this combined provided a rich extracellular matrix environment that allowed for good cell adhesion and several soluble growth factors that increased cell self-renewal [21]. Feeder cells, for example, supported the self-renewal of the cultured stem cells by secreting essential growth factors, cytokines and extracellular matrix proteins, such as TGF β , activin A, laminin-511 and vitronectin [93].

However, the risks associated with this approach did not allow for a clinical translation of this process. By using materials of animal origin and with an unknown chemical composition the risk of possible contamination with known and unknown xeno-pathogens that could then be transmitted to patients cannot be discarded. Also, the use of serum can lead to variability in the results as the chemical composition of the serum can change from batch to batch due to the fact that the animals from which the serum is taken also change and produce different chemical combinations of soluble factors [93], [94].

In an attempt to prevent contact between the cells and xenogeneic pathogens, scientists attempted to culture stem cells with feeder cells of human origin combined with Dulbecco's modified Eagle's medium, supplemented with knockout serum replacement and basic fibroblast growth factor [94].

Other culture media that are used in labs around the world for the culture of stem cells also include conditioned medium from fibroblast feeder cells, a medium collected after being used in a culture with feeder cells. This medium contains the soluble factors produced by the feeder cells in which it was cultured before and deliver them directly to the expanding stem cells without direct contact with the feeder layers. Although this approach eliminates the need for co-culture with a feeder layer it does not eliminate the associated lot-to-lot variability of the medium, since the used feeder cells secrete their signalling factors differentially, or the danger of infection by contaminants from the feeder cells [22], [35].

This variability makes it impossible to study stem cells and their interactions with their extracellular environment, especially how this environment influences cell fate [22], [95]. However this kind of media formulation was still chemically undefined and although the cells would no longer be exposed to any xenogeneic pathogens they still incurred the risk of infection by viral and non-viral infectious agents from human sources and the effects are just as dangerous. [93]

Nevertheless, these cells, could be considered for clinical applications only if the feeder cells themselves were obtained in GMP conditions, free of possible xenogeneic infections, and more importantly of human origin [21]. Recently, several feeder cell lines have been obtained in those GMP compliant culture conditions mainly due to several advances in developing less complex xeno-free media and defined culture conditions [22].

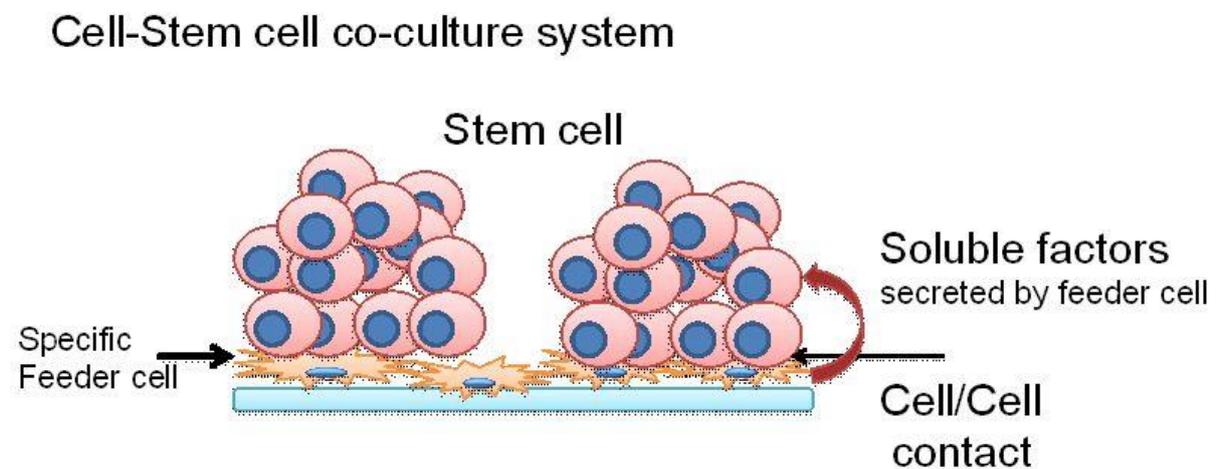


Figure 1.5: Feeder cell co-culture system. Feeder cells are irradiated to prevent their expansion. They provide cell/cell contact to the cultured cells, facilitate cell adhesion and also provide several soluble factors essential to maintain stem cell culture [96].

I.3.1.2 Undefined Media and Matrigel™

The usage of feeder cells presents obvious disadvantages, including the risk of xenogeneic infection and batch-to-batch variability. However in order to lose the usage of feeder cells something else would be needed to help the cultured cells to adhere, something to substitute the rich extracellular matrix and the soluble factors produced by them. In order to fill that void, matrigel was used. Matrigel is a complex mixture of matrix proteins derived from Engelbreth-Holm-Swarm mouse tumours [93], [97]. Although it allows for the complete removal of feeder cell layers for cell adhesion it is still of animal origin and chemically undefined. Therefore, it displays lot-to-lot variability and can present unwanted xenogeneic infectious agents to the culture [93]. Because of this, the use of matrigel, and the cells cultured on it, are not considered GMP conditions and cannot be used for the derivation of products for human usage in a clinical setting.

I.3.1.3 Defined Media and Biological Substrates

In order to generate cells for cell therapy and any other applications in a clinical setting, it is necessary to use chemically-defined media and biological substrates free of xenogeneic substances [22]. To move away from undefined substrates for stem cell self-renewal, attachment and maintenance of pluripotency, the scientific community is researching what proteins of the undefined ECM have a pivotal role in those situations with the objective to then generate their recombinant forms. This way, the risk of xenogeneic contamination could be eliminated and cultures could be maintained for a prolonged time period and robustly [35].

Several proteins have already been identified and obtained for this purpose, such as vitronectin [98] and laminin-511 [99]. In both cases, hPSC were able to expand in monolayer and when implanted *in-vivo* were capable to give rise to teratomas composed by differentiated cells of all three germ layers, showing that the use of recombinant ECM as substrate supports their pluripotent nature and self-renewal capabilities. These substrates can be very useful in order to obtain cells for clinical applications [98], [99].

E-cadherin (Figure I.6), was shown to stimulate stem cell colony formation and self-renewal, and so it has also been used as a substrate for long term hPSC culture with positive results as well [100].

In reality, this is the first instance of completely defined conditions from all the considered culture systems. In 2011, the E8 medium was discovered, eliminating human serum albumin from the culture media and allowing the expansion of hPSC in a completely defined culture system, with both a defined medium (E8) and defined substrates (Vitronectin). In these conditions, the considered hPSC were capable of self-renewal and differentiation into the three germ layers [97]. This new medium, E8, also

reduces the normal cost of media since it eliminates the need to use of expensive animal or human derived components and batch-to-batch variability. Being completely defined, it also presents a cleaner background to analyse specific pathways in the cell and it also improves reprogramming efficiency [97]. More importantly than all that, a defined medium coupled with a defined substrate will help to simplify and accelerate the transfer of stem cell research from the laboratory bench to the clinic where it can be used for several promising therapeutic goals.

However, the use of biological substrates obtained by extraction and purification of the selected ECM proteins from both human and animal sources show several limitations including batch-to-batch variability, limited scalability, difficulty to isolate, expensive costs and the need for a pathogen-free environment during the entire process. To address those concerns, synthetic substrates capable of supporting stem cell expansion were created. Being synthetic, these substrates can be easily modified to present any kind of characteristics necessary for the expansion of any stem cell line [22], [101].

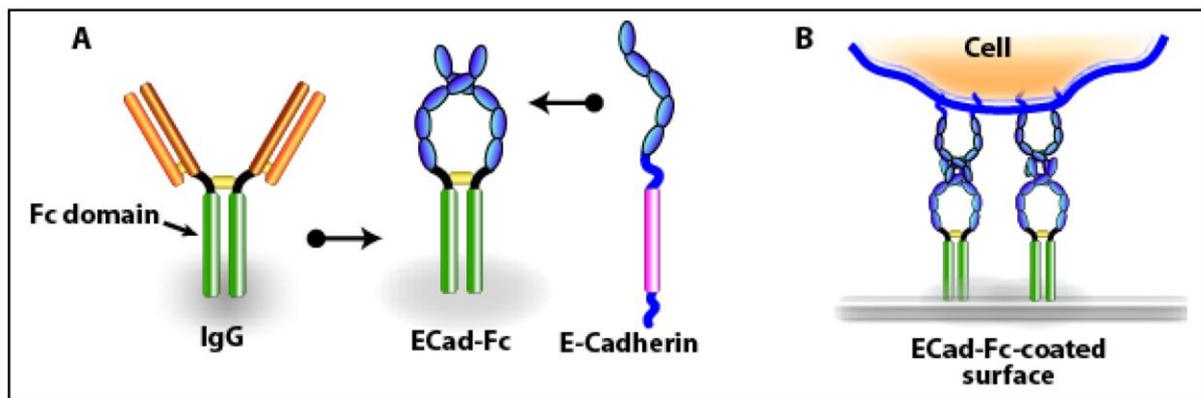


Figure 1.6: E-cadherin coated plates. E-cadherin has been shown to support iPSCs *in-vitro* facilitating adhesion and supporting self-renewal. iPSCs expanded in E-cadherin coated plates have also been shown to maintain pluripotency [102].

I.3.1.4 Defined Media and Synthetic Substrates

The first instance of these new synthetic polymer surface substrates were reported by Mei et al. in 2010. In his work Mei and his team use high throughput screening techniques to quantify the optimal characteristics that a substrate material must have to allow for a good expansion of hPSC in culture while maintaining their pluripotency [101]. The team analysed several biomaterial properties such as wettability, surface topography, surface chemistry and indentation elastic modulus and that way they managed to obtain a correlation between material types and their biological performance [101]. The biggest advantage of these new chemically defined, xeno and feeder-free synthetic substrates is the facilitation of the derivation of stem cells in GMP conditions and their maintenance in an easy and affordable manner. [35]

In order to be used for the expansion of hPSC, these substrates must be able to allow cell adhesion, spreading, self-renewal and colony formation. Besides all that, the substrate must allow the cultured cells to retain their pluripotency and differentiation potential during the entire time of culture. However, that same substrate could not stimulate the arising of genetic anomalies on the cultured cells, that even though it could impart to them a selective advantage, stimulating survival and self-renewal, it also posed the risk of tumorigenesis [22].

Synthetic substrates that contain carboxyl and sulfonyl groups seem to be especially able of supporting hiPSCs self-renewal and pluripotency. It is believed that it is so for they mimic the functional characteristics of heparin [22].

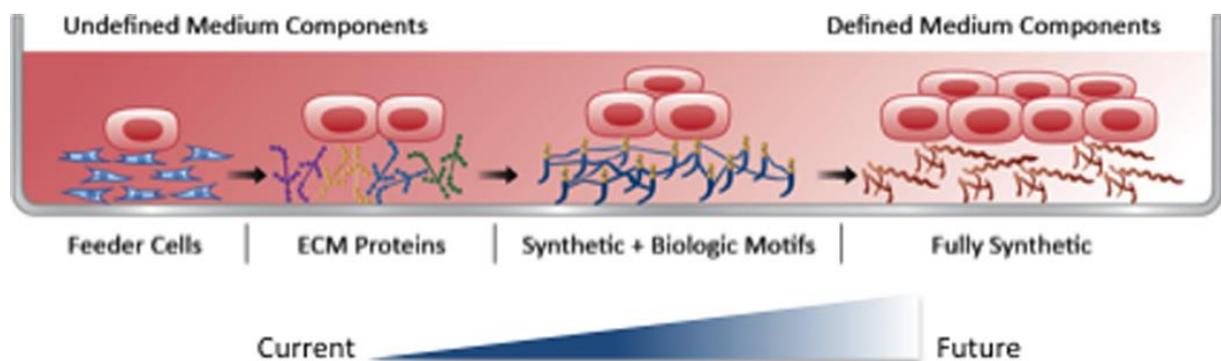


Figure 1.7: Evolution of stem cells culture conditions. Fully synthetic coatings are extremely useful and can be easily changed to control their characteristics, such as wettability, in order to optimize stem cell expansion and cell survivability [22].

Even considering all these evidences and advantages, most groups opt for a hybrid approach using a base of synthetic polymers and then modifying the surface of them with biomolecules more suitable for stem cell expansion, for example Synthemax™ from the Corning™ company that uses an acrylate polymer modified with amino-coating peptides. [5]

II. Motivation and Aims

With the emergence of the Induced Pluripotent Stem Cell (iPSC) technology, the world opened to a great number of new possibilities. One of which is the possibility of personalized medicine. iPSCs are obtained from the genetic reprogramming of adult somatic cells. By forcing those cells to express several transcription factors commonly expressed in ESCs, we can lead those cells into dedifferentiating back into a ESC-like state [27], [28]. Cells in this state show the remarkable ability of both maintaining themselves, giving rise to copies of themselves (self-renewal), and to differentiate into any of the approximately 200 types of cells present in the adult body [21], [22]. They present the same characteristics as ESCs without the ethical concerns that they have. The hiPSC technology also presents the major advantage of allowing the derivation of patient-specific hiPSC, leading to the possibility of performing disease modelling and drug screening in a patient-specific context.

In fact, iPSCs obtained from a patient present the same characteristics of other cells found in that patient, they share the same genetic code after all [35]. These cells are then capable of recapitulating any disease that a patient already has from a genetic origin. This allows for a way to both study these diseases and devise ways to treat them in this specific patient. Every patient has different needs, with cells capable of differentiating into any affected tissue taken directly from a specific patient, scientists are capable of testing and adapting therapies to that specific patient. This is personalized medicine.

The cells themselves can also be a therapy [26]. They are capable of avoiding any immune reaction when implanted back into a patient [58]. If the disease can be cured or at least lessened with new cells, healthy cells, these cells can be obtained from the patient. iPSC technology allows us to obtain any cell from any somatic cell in the patient, without problems of immune rejection, these cells can even be treated by genetic editing giving rise to a healthy phenotype even when the patient suffers from some widespread genetic disease, such as FA [19].

However for any of this to be feasible it is necessary to first establish the basics of this technology. We will only be able to use these cells if we first devise a way to expand them and to support them [63], and even to differentiate them [64]. Every cell-line, like any individual, is different and with different needs, they all require different levels of components to optimally expand and differentiate. Without studies in this area of iPSC technology none of the other applications of this cell type can be accomplished. That is the reason for this study in particular.

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 [19].

Using the experience and knowledge of the Stem Cell Bioengineering Laboratory at Instituto Superior Técnico, it is the aim of this study to establish and optimize a culture protocol capable of expanding these cells while maintaining their pluripotency capabilities. This expansion protocol would have to be serum and feeder-free since using any of them would mean possible variability into the results and a risk of contamination by xenogeneic agents. Even if the contamination never occurred, the existence of the risk would render these cells unable to be used in any kind of clinical setting defeating the overall goal.

This study is only a first step in the creation of the optimized protocol for this specific cell line, a proof-of-concept. This study has the objective of applying a standard culture protocol for iPSC and to gauge the response of this cell line to that culture method. This way future work will have a baseline from where to begin and any modification to the protocol could be measured in what impact it caused the cells expand in this way. This work is extremely important as the basis for many other optimization future works and, if those works are indeed completed successfully, to the usage of these cells in a possible future clinical application.

III. Materials and Methods

III.1. hiPSCs expansion

III.1.1 Matrigel™ coating

Aliquots of Matrigel™ (Corning®) were kept at -20°C. When it was necessary to coat a new culture plate, the aliquot would be thawed either overnight at 4°C in ice or for 2-3 hours at room temperature in ice. Matrigel™ is liquid when kept at 0-4 °C but solidifies when exposed to room temperature into a gel-like substance that 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™ would then give 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 500µL and in 24-well plates 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 4°C for up to a month.

III.1.2 Culture Media Preparation

The culture media used was mTeSR™1 (StemCell Technologies). This medium is one of the most common media used for the expansion and maintenance of both ESCs and iPSCs. 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, Pipelicolic acid and GABA. It does not require the addition of any growth factors and, due to its per-screened raw materials, provides consistent cultures that maintain the stem cells undifferentiated phenotypes.

The culture media is provided by the company in 2 parts, mTeSR™1 Basal Medium and mTeSR™1 5X Supplement. The Basal Medium can be stored at 2-8°C until the expiration date and the 5X Supplement should be stored at -20°C 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 in 2-8°C for up to 2 weeks or it can also be aliquoted and stored at -20°C. In this case the medium was aliquoted in 50mL aliquots and stored at -20°C. Before being used, the medium should be thawed and used immediately or stored at 2-8°C for up to 2 weeks. The medium was pre-warmed at 37°C before being provided the cells.

III.1.3 hiPSC Line

A single hiPSC line was used, the line was the Cre-Sort 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 and maintained in a co-culture with mouse embryonic fibroblasts as feeder cells and using a serum supplemented culture medium. 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™ (DMEM; Gibco), supplemented with 20% fetal bovine serum (Biowhitaker) and 1% penicillin/streptomycin solution (Gibco) at 37°C under hypoxic conditions (5% of O₂) and 5% of CO₂, in co-culture with human foreskin fibroblasts as feeder cells[103]. They were also cultured for 109 passages prior to freezing.

III.1.4 Cryopreservation of hiPSCs

Cells were preserved at certain chosen times in order to create a working cell bank for future experiments. The cells were harvested to be cryopreserved after they had achieved about 80% confluence in the culture plate.

The cells were washed with 1mL of EDTA per well and then 1mL of EDTA was added again per well and the cells were left to incubate for 3 minutes at room temperature. EDTA was then removed from the plates and the cells were scraped from the well by using a pipette with culture medium and using the tip of the pipette to mechanically separate the cells from the well and into suspension. The media with the cells was then centrifuged at 100rpm for 3 minutes and the supernatant was discarded.

The obtained cell pellet was then suspended in freezing medium composed of 90% KO-Serum replacement (StemCell Technologies) and 10% DMSO (Gibco). The cells were then stored in cryovials, 250µL per vial which were then stored in -80°C in an isopropanol-freezing container (Mr. Frosty, Thermo Scientific™ Nalgene™) overnight after which they were transferred into a liquid nitrogen container for prolonged storage (-196°C).

III.1.5 Thawing of hiPSCs

The cryovials containing hiPSCs were removed from liquid nitrogen storage and quickly heated in a 37°C water bath. After that, culture media was added to the contents of the vial, carefully one drop at a time from a pipette containing 1mL of culture media (mTeSR™1). As the cells unfreeze, both the media and the unfrozen cells were collected and transferred to a conical tube. The above steps were repeated as many times as needed until the entire content of the cryovial is unfrozen. The conical tube was then centrifuged at 1000 rpm for 3 minutes, the supernatant was removed and the pellet was gently suspended on culture medium (mTeSR™1). The suspension was then transferred to pre-coated Matrigel™ wells trying to maintain a homogenous cell density over all seeded wells.

III.1.6 hiPSC expansion protocol

III.1.6.1 Culture Conditions

Cells were cultured at 37°C in normal atmospheric levels of O₂ and 5% CO₂. They were plated under static culture conditions on Matrigel™ pre-coated wells. The media was composed of mTeSR™1 supplemented with 0,5% of streptomycin/penicillin solution. The media on each well was changed daily and before changing was pre-heated to 37°C in order to match the temperature at which the cells were incubated.

III.1.6.2 Passaging Method

Two passaging methods were used, at first the cells were passaged using EDTA (Invitrogen™) passaging buffer and then switched to ReLeSR™ (StemCell Technologies).

EDTA is part of an enzyme-free method for passaging cells. The EDTA molecule forms stable complexes with several ions, including Calcium, therefore denying this calcium to the adhesion proteins of the cells and facilitating their release from the plate. Cadherins, one of the proteins essential for cell adhesion, require Calcium in order to allow for cell adhesion, by denying them the Calcium ions they require for adhesion their function is disrupted and it becomes easier to harvest cells. The protocol is simple and fast.

The culture medium was first removed from the plate and the cells were washed with EDTA 0,5mM using 1,5mL per well in a six-well plate. Afterwards 1mL per well of EDTA was added to the cells

and left to incubate at room temperature for about 3 minutes. Then the EDTA was removed and 1mL of culture medium per well was added. Then the cells were gently scrapped from the well using a pippete tip and transferred to a collection tube. From there they were directly seeded in new Matrigel™ pre-coated wells usually in a ratio of 1:3 or 1:4. Meaning that from the cells obtained from one well usually 3 to 4 wells were seeded. The newly seeded wells were then placed in CO₂ incubators at 37°C to continue culture.

Due to complications with spontaneous differentiation of hiPSC the passaging method was changed to use ReLeSR™ instead of the standard EDTA passaging method. ReLeSR™ is an enzymatic-free compound that allows for cell detachment and harvesting, much like EDTA. It differs in the fact that it is specific to iPSCs, unlike EDTA. ReLeSR™ uses the principle that differentiated cells are consistently more robustly attached to the plate than undifferentiated cells, therefore by using ReLeSR™ and a special protocol only those undifferentiated cells should be released.

The culture medium must be removed before washing the cells in a calcium and magnesium-free phosphate-buffered saline (PBS) solution. Without selecting the undifferentiated colonies 1mL of ReLeSR™ was added per well and removed within one minute so the cells became exposed to only a thin layer of liquid. The cells were then incubated for 5-7 minutes at 37°C. After incubation, 1mL per well of culture medium (mTeSR™1) was added. The colonies were then detached by firmly tapping the side of the plate for 30-60 seconds. This way only the colonies more weakly adherent were be released. This way only the undifferentiated cells were collected once the medium was removed from the wells. The medium was then transferred to the pre-coated wells in whatever density was desired and then the plates were placed at 37°C for incubation [104].

III.1.7 Cell Counting

In several steps of the expansion process it is necessary to know the amount of cells that are currently in culture. Be it for seeding new plates or building a growth kinetics graphic. The protocol used in this thesis for counting cells is as follows.

After collecting the detached cells obtained from following the passaging method protocol, the vial was centrifuged at 1000rpm for 3 minutes. The supernatant was then removed and the pellet was suspended in 1mL of culture medium (mTeSR™1). The solution was mixed well and 10µL of it were removed and placed in one of the wells in a 96 well-plate. To this well it was added 10µL of Trypan Blue dye (Gibco®) and both solutions were mixed thoroughly. Then, 10µL of the mixed solution were taken and placed in a Neubauer chamber under an optic microscope in order to count the cells. Trypan Blue is a colorant unable to enter living cells when their membrane is intact. Thus, only dead cells were stained blue and those cells were not counted as viable cells when found in the Neubauer chamber.

III.1.8 Growth Kinetics

Cells were seeded at a known density in 22 wells of a 24 well-plate. They were maintained in mTeSR™1 culture medium, that was changed daily, and every day two wells were harvested using the cell passaging protocol and the cells from each well were counted twice using the cell counting protocol after which the cells were discarded.

The obtained values were also used to calculate the fold increase of the cell line. The formula used divided the initial cell number by the considered viable cell number for that culture day as shown below. The variable X represents the considered culture day, in this case X goes from 0 to 11.

$$\text{Cell Fold Increase} = \frac{\text{Viable Cell Number of Day X}}{\text{Initial Cell Number (Day 0)}} \quad (\text{Eq. III.1})$$

III.2 Neural Differentiation of hiPSCs

III.2.1 Culture Medium

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™ supplemented with 1x (v/v) N2-supplement, 1,6 g/L D-(+)-glucose, 20 µg/mL insulin and 1% (v/v) PS. The B27 medium is composed of Neurobasal medium supplemented with 1x (v/v) B27-supplement, 1% (v/v) glutamine and 0,5% (v/v) PS.

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 [105]. The medium was changed daily and the cells were placed in incubation at 37°C in normal atmospheric conditions of O₂ and 5% CO₂. At the end of the 12 days of culture, the cells were marked for Pax-6 and Nestin expression and photos were taken.

III.2.2 Characterization of hiPSCs and hiPSC-derived cells

The hiPSCs were characterized using immunofluorescence microscopy and flow cytometry in order to characterize their pluripotency profile. The cells obtained after the 12 days of the neural differentiation protocol were characterized only by immunofluorescence microscopy.

III.2.2.1 Immunocytochemistry

III.2.2.1.1 Analysis of Intracellular markers

The medium was removed from the cell 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®) and 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.

The primary antibodies used in this work and their characteristics can be seen on Table III.1.

Table III.1. Table with information on the primary antibodies used for intracellular staining in Immunocytochemistry of hiPSCs and their derivatives.

Primary Antibodies	Isotype	Dilution	Supplier
Anti-Nanog	Rabbit IgG	1:5000	Millipore
Anti-Nestin	Mouse IgG	1:400	R&D Systems
Anti-Oct-4	Mouse IgG	1:400	Millipore
Anti-Pax6	Rabbit IgG	1:400	Covance
Anti-Sox2	Mouse IgG	1:100	R&D Systems

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.

The secondary antibodies used in this work and their characteristics can be found on Table III.2 below.

Table III.2. Table with information on the secondary antibodies used for extracellular staining in Immunocytochemistry of hiPSCs and their derivatives.

Secondary Antibodies	Isotype	Dilution	Supplier
Alexa Fluor 488	Goat anti-mouse IgG	1:500	Invitrogen
Alexa Fluor 488	Goat anti-rabbit IgG	1:500	Invitrogen
Alexa Fluor 546	Goat anti-rabbit IgG	1:500	Invitrogen

After this final incubation, 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 15 μ L of stock solution in a concentration of 14,3mM (5mg/mL) that was then diluted in PBS to give rise to a 300 μ M intermediate solution. This solution was then diluted in PBS at a rate of 1:1000 giving rise to a final solution of 300nM, which was the one used for this assay. 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.

III.2.2.1.2 Analysis of surface Markers

The medium was removed from the cell wells and the cells were then washed with PBS. After this the cells were incubated for 30 minutes at 37°C in the presence of the primary antibodies diluted in staining solution. The cells were not fixed in PFA, unlike those for used for intracellular staining, and neither were they incubated with saponin in order to permeate the cellular membranes. 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 37°C in the dark. Finally, after three more washing steps with PBS the cells were examined under a fluorescence microscope.

The primary antibodies used for surface marking of hiPSCs and their characteristics can be seen in Table III.3 below.

Table III.3. Table with information on the primary antibodies used for surface marker analysis in Immunocytochemistry of hiPSCs.

Primary Antibodies	Isotype	Dilution	Supplier
Anti-SSEA-4	Mouse IgG	1:100	Stem Gent
Anti-Tra-1-81	Mouse IgM	1:100	Stem Gent
Anti-Tra-1-60	Mouse IgM	1:100	Stem Gent

The secondary antibodies used in this work for surface marking and their characteristics can be found in Table III.4 below.

Table III.4. Table with information on the secondary antibodies used for extracellular staining in Immunocytochemistry of hiPSCs.

Secondary Antibodies	Isotype	Dilution	Supplier
Alexa Fluor 546	Goat Anti-Mouse IgG	1:500	Invitrogen
Alexa Fluor 546	Goat Anti-Mouse IgM	1:500	Invitrogen

After this final incubation, cells were again washed three times and used for immunofluorescent microscopy analysis. In the case of the analysis of surface marker expression, cells were not subjected to DAPI staining, unlike the cells stained for intracellular markers.

III.2.2.2 Flow Cytometry Analysis of hiPSCs

III.2.2.2.1 Intracellular Markers

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 Eppendorf. The excess of BSA should be removed before any cells are added to the Eppendorf for centrifuging.

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).

III.2.2.2.2 Surface Markers

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.

IV. Results and Discussion

IV.1 Expansion of hiPSCs in Static Conditions

IV.1.1 Protocol for the Expansion of hiPSCs

The used Cre-Sort hiPSC line was obtained from a patient with FA from the Division of Hematopoietic Innovative Therapies in Madrid, Spain. In Spain, this cell line was cultured on a feeder layer of human foreskin fibroblasts and with serum-containing medium. This cell line was then adapted in the SCBL lab to expansion using a standard serum and feeder-free iPSC expansion method. The objective of this approach was to gauge the expansion potential of these cells in a standard known and studied protocol, in a way to see the cell's response against the usual response from unmodified iPSCs.

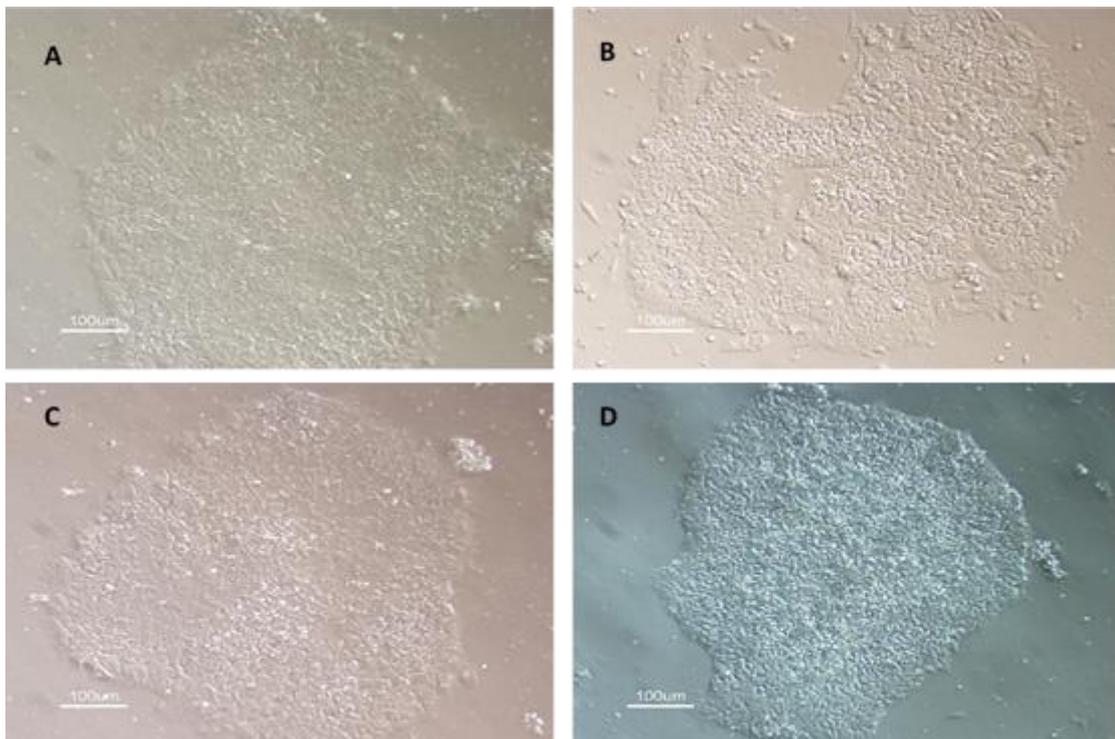


Figure IV.1: Bright field images of the Cre-Sort hiPSC line under feeder-free and serum-free expansion conditions. As we can see in B most clearly the colonies could sometimes show an irregular shape and spontaneous differentiation.

The cells were kept on static conditions on wells of, usually, 9.5 cm² in a 6-well plate of growth space pre-coated with Matrigel™ and in the presence of mTeSR™1 medium (Figure IV.1). Upon the beginning of the study, the cells were thawed and had already been passed by over 100 passages. As the cells reached 80% confluence, they were passaged with a 1:4 ratio of wells by using the EDTA passaging protocol. However, it was observed that only a few two days after passaging, spontaneously

differentiated cells appeared in the culture, as can be observed in Figure IV.1 B. In an attempt to diminish this phenomenon, the original passaging protocol was modified to use ReLeSR™ instead of EDTA. This way the number of colonies of differentiated cells could be reduced and all further differentiated colonies would supposedly come from spontaneous differentiation and not from the culture of the previous passaging.

Although differentiated colonies still appeared regularly on the culture, their number was reduced after the protocol was adapted. In the rare occasion when spontaneous differentiation caused the culture to be unfeasible and the possible results to become unreliable, the pluripotent cells were harvested using the ReLeSR™ passaging protocol and then reseeded at a dilution of 1:8. The objective was to minimize the amount of cells in each seeded well to allow for at least one of the wells to have no differentiated cells on it at the beginning of the new culture.

IV.1.2 Growth Kinetics of the Cultured hiPSCs

The cell's growth potential was accessed by running a growth kinetics experiment, the protocol of which can be found in the materials and methods section of this report. The growth kinetics experiment was run for 11 days in which the cells were allowed to grow in the same culture conditions as the ones used for all the other cultures. The cells were seeded in several wells and two wells per day were harvested and the numbers counted for each well and the fold increase values calculated as depicted in the Materials and Methods section (Eq. III.1). The results were presented in Figure IV.2.

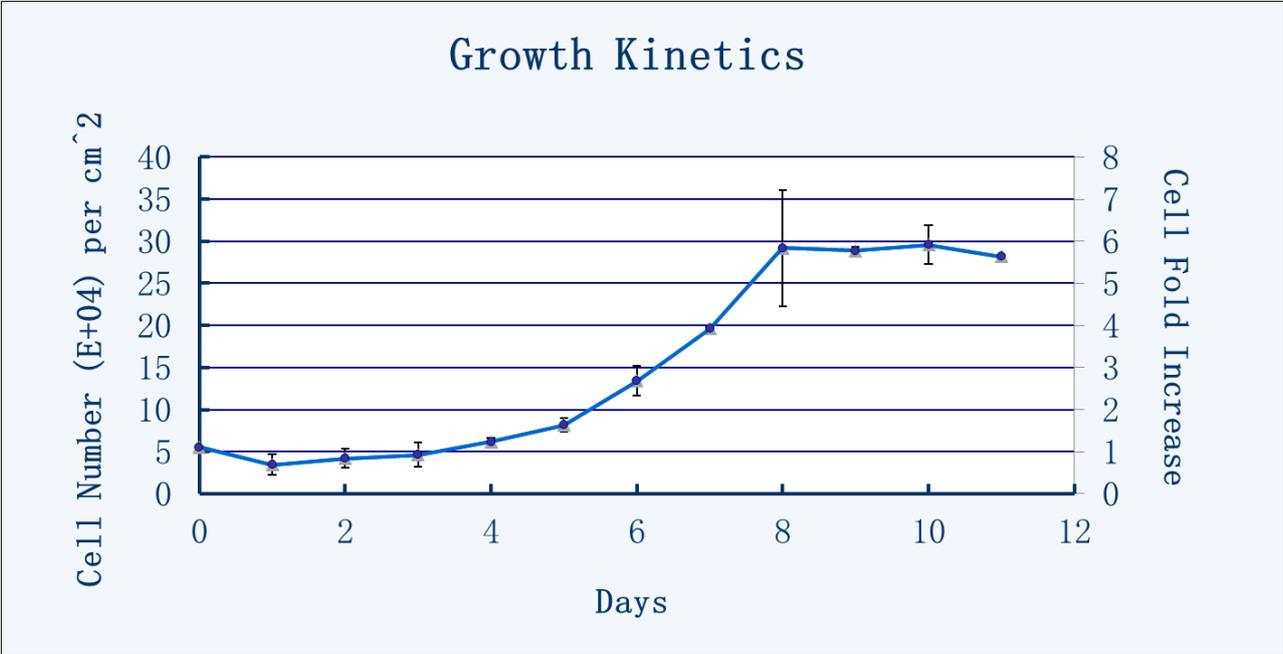


Figure IV.2: Growth kinetics of the Cre-Sort hiPSC line. The values were obtained by counting two wells per day for 11 days, all wells were seeded simultaneously with the same cell density. The error bars were obtained from the standard error deviation obtained by the two countings of each day. The Cell Fold increase line in the graph is coincident with the cell number line.

The graph shows that the used protocol was able to expand the initial population of cells into 6 fold the original cell number in a space of 8 days after the beginning of the culture. Although it is difficult to compare these numbers with those found from other cell lines there are a few points that can be stressed. In most cultures the seeded cells reach confluence and are passaged 3 to 4 days after seeding. If we refer to Figure IV.2, we can see that 3 to 4 days after seeding is not enough for this cell line to grow. At this point in time this cell line is still in its lag phase, showing no considerable growth from the initial cell numbers. Exactly one week after seeding (6 days) this cell line reaches approximately 3 fold the initial number, again still below the value typically obtained at the SCBL. From day 6 to day 8 we can clearly see that the cell line reached its exponential phase rising from 3 fold the initial value to 6 fold that amount. From there on out the cells stabilize and show no more growth all the way to day 11 as can be seen in figure IV.2.

In conclusion, it was observed that the cell line shows a characteristic and normal growth profile, which clearly shows a lag phase (Days 0-4), the exponential phase (Days 5-8), the stationary phase (Days 9-11) and even the death stage on day 11. This cell line however seems to show a slower cell growth speed than other hiPSC lines

IV.1.3 Cre-Sort Cell Line Characterization

IV.1.3.1 Flow Cytometry of the Cre-Sort Cell Line

In order to be used as an expansion method for iPSCs, a culture protocol must guarantee two aspects, the cells subjected to it are capable of self-renewal and therefore expanding their initial numbers and that they do not lose their pluripotent characteristics. Having shown by the growth kinetics experiment that the culture allows for hiPSC expansion, the next step was to determine if the expanded cells maintained their pluripotent phenotype. To do so the first measure checked was the expression of several transcription factors and cell markers that are characteristic of stem cells.

IV.1.3.1.1 Intracellular Markers

Three of the tested markers of hPSC, Oct-4, Sox-2 and Nanog, are transcription factors found within the nucleus of the cell. Their expression can indicate pluripotency on a cell.

The results of the flow cytometry analysis to detect all three of these markers are shown in Figure IV.3. The graphs to the left of both Figure IV.3 and Figure IV.4 represented the detected cells, each point in the graphic is a cell and only the cells that fell inside the considered gate (blue shape) were counted for the elaboration and analysis of all graphs to the right in both figures. These graphs to the left are made from the results of Forward Scattering (FSC-H) and Side Scattering (SSC-H) shown by each of those cells. The gate used only allows cells with a low forward scattering and therefore we avoid counting cell aggregates in the final graph. It also allows for cells with a high side scattering of the beam and this way we only count intact cells and not cell debris that are smaller in size and with simple internal complexity.

As it can be seen from Figure IV.3, about $94\% \pm 3\%$ of the detected cells were positive for the presence of Oct-4, at the same time about $93\% \pm 3\%$ of the detected cells were also positive for Sox-2 and similarly $93\% \pm 3\%$ of the detected cells were positive to Nanog expression.

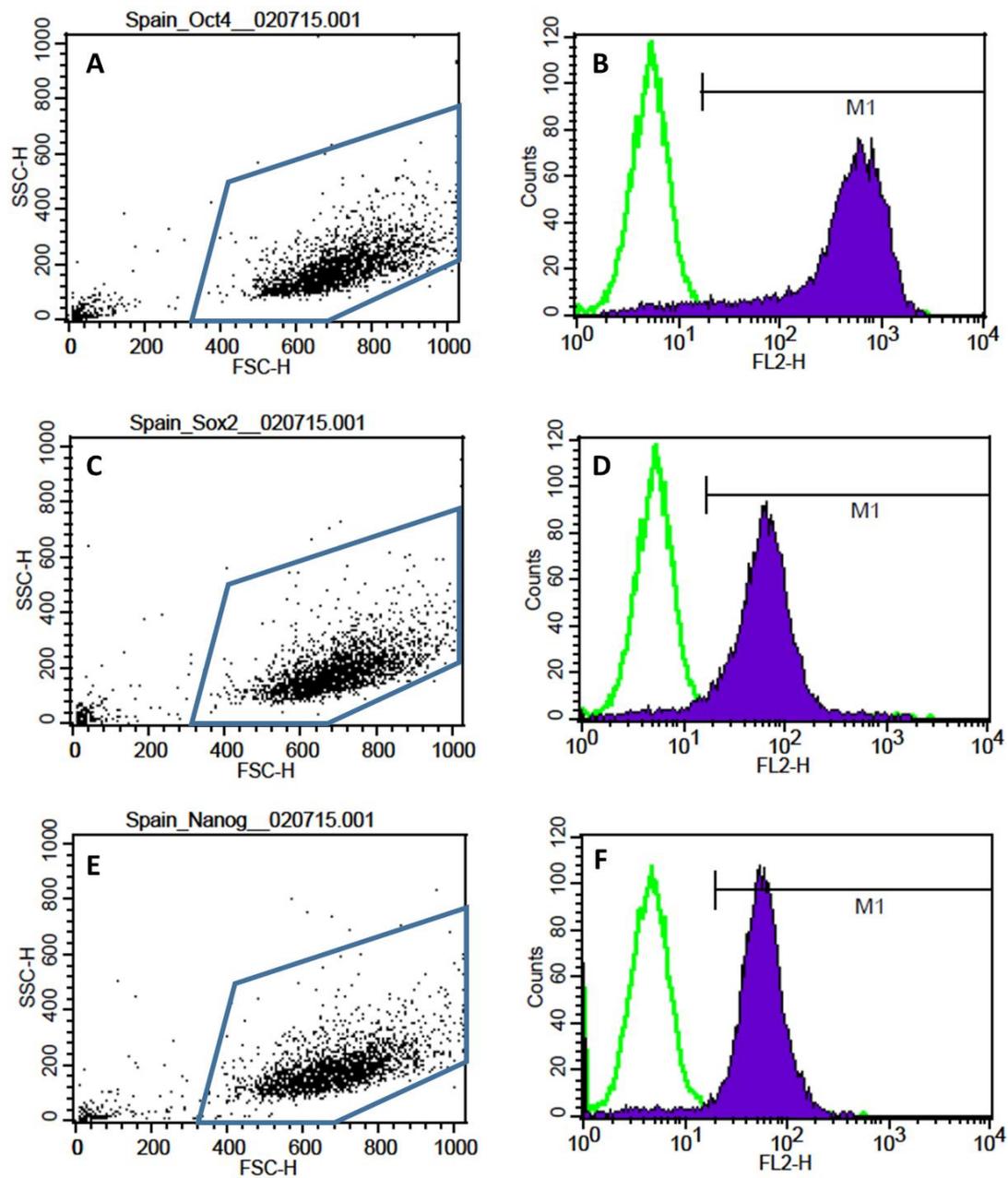


Figure IV.3: Flow cytometry results for the detection of the expression of Sox-2, Oct-4 and Nanog in the cultured Cre-Sort cell line. In graph B, D and E we can see two curves, the green curve is the false positives curve and all signals detected under it were ignored for this study. On the left we can find the characteristics of considered cells. All cells inside the gate (blue shape) were counted for the creation of the graphs to the right.

IV.1.3.1.2 Extracellular Markers

Besides Oct-4, Sox-2 and Nanog other cellular markers were tested, all of them considered to be staple signs of pluripotency. The other markers were extracellular signalling molecules namely Tra-1-60 and SSEA-4. Both Tra-1-60 and Tra-1-81 are surface antigens that can be found in several different cancers as well as in every human pluripotent stem cell. They are therefore commonly used to identify undifferentiated pluripotent stem cells [106]. In this study, it was only tested Tra-1-60 for logistical reasons. SSEA-4 is a commonly used marker to identify pluripotent stem cells since it is present in all pluripotent stem cells [107], [108].

The results of the flow cytometry for both Tra-1-60 and SSEA-4 can be found in Figure IV.4.

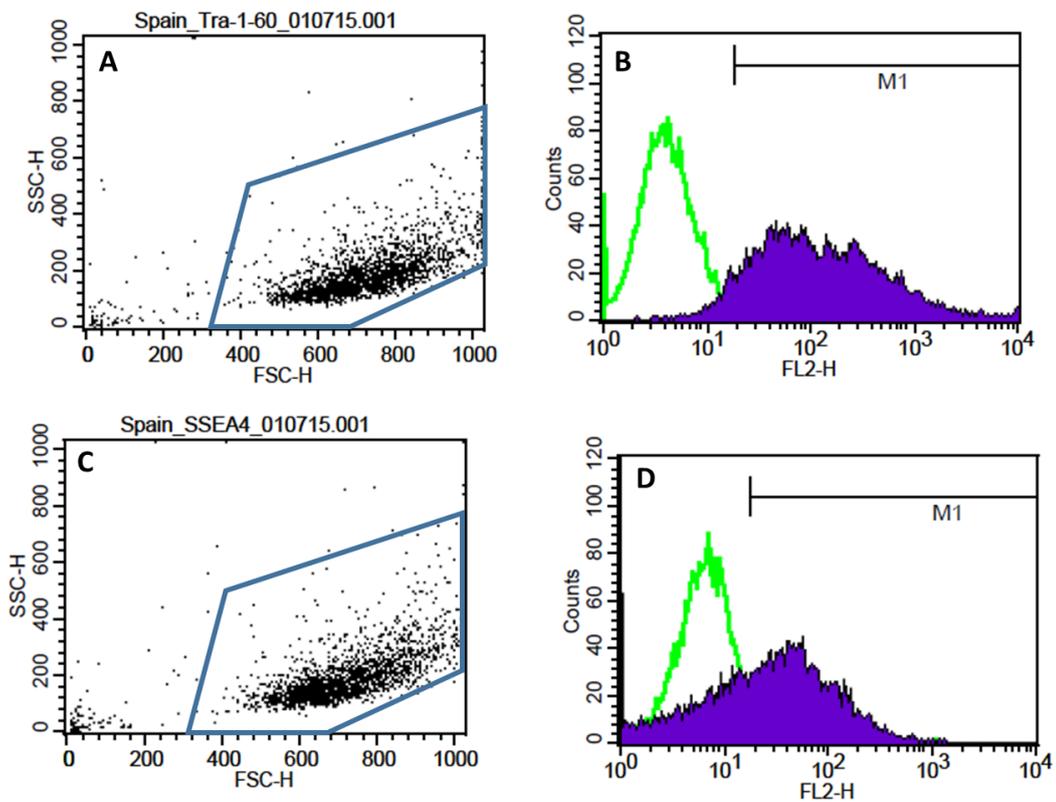


Figure IV.4: Flow cytometry results for the detection of the expression of Tra-1-60 and SSEA-4 in the cultured Cre-Sort cell line. In graph B and D we can see two curves, the green curve is the false positives curve and, as before, all signals detected under it were ignored for this study. On the left we can find the characteristics of considered cells, all cells inside the gate (blue shape) were counted for the creation of the graphs to the right.

As we can see on the graphs of figure 4, about 93% \pm 1% of all detected cells were found marked positive for Tra-1-60, however only 60% \pm 3% of the detected cells were found to be positive for SSEA-4.

IV.1.3.1.3 Flow Cytometry: Final Remarks

According to these flow cytometry results (Figure IV.5) it is clear that a vast majority of the cultured cells seems to maintain the expression of the pluripotency markers. Although the mere expression of these markers is not enough to claim that the considered cells are truly pluripotent, they still represent a strong indication that that is indeed the case. In order to fully characterise the pluripotency of this cell line and therefore claim that these cells are indeed pluripotent we would need to use a wider array of cellular markers, to differentiate these cells into all three germ layers and prove that they indeed managed to do so by analysing these cells new marker expression and to perform a teratoma formation assay in immunocompromised mice. About 93% of the considered cells maintain the expression of all the considered markers except for SSEA-4. This final marker deviated from the other results having only been detected in 60% of the considered cells. It is believed that this result does not mean that the cells do not express SSEA-4 instead the antibodies used were most likely ineffective.

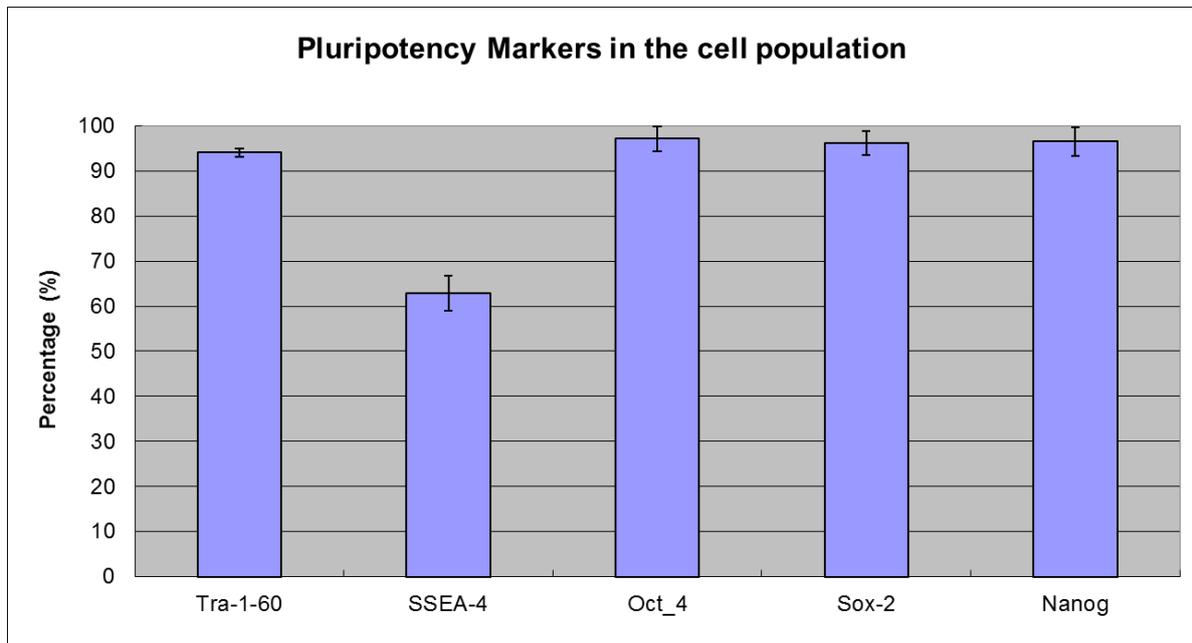


Figure IV.5: Complete set of Flow Cytometry results for the expansion of the Cre-Sort iPSC line after 10 passages and about 2 months of culture. The cell line was on passage 120 when the cells were harvested for the test.

IV.1.3.2 Fluorescent Microscopy of the Cre-Sort Cell Line

Besides using flow cytometry to characterize the cultured cells, it was also used fluorescence microscopy. This technique allowed for a visual confirmation of the results obtained from flow cytometry and also allowed for an easy identifier of the cell characteristics of differentiated and undifferentiated cells of the Cre-Sort cell line. The same molecular markers used for Flow Cytometry were used for Fluorescence Microscopy, with the exception of Tra-1-81 that was used only for Fluorescence Microscopy (Figure IV.6 and IV.7).

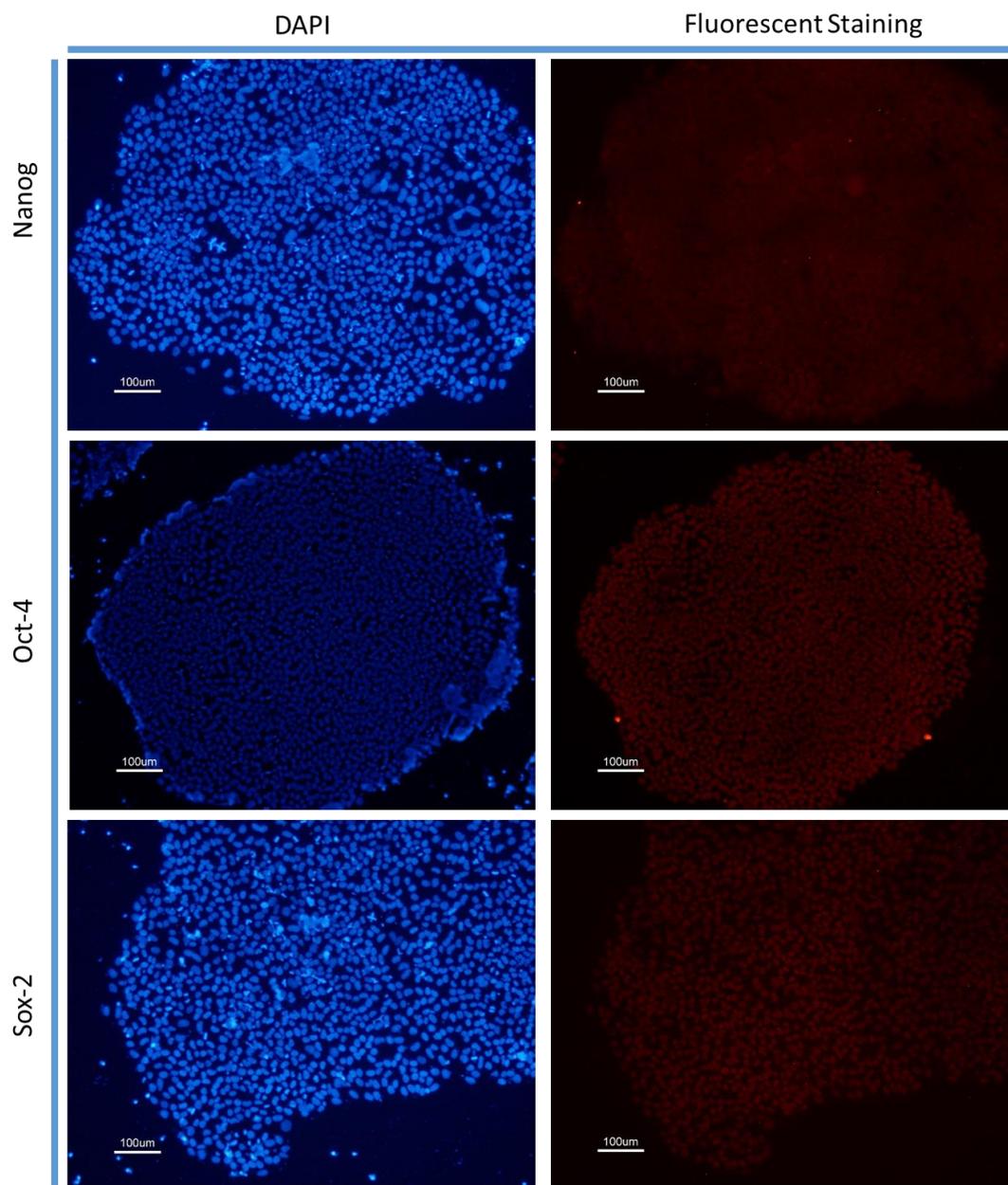


Figure IV.6: Intracellular staining of the expanded Cre-Sort hiPSC line. The cells were stained on the 120th passage after about 2 months of culture at SCBL. DAPI nuclear staining images are also provided as controls for the fluorescent staining photos. Scale: 100 µm.

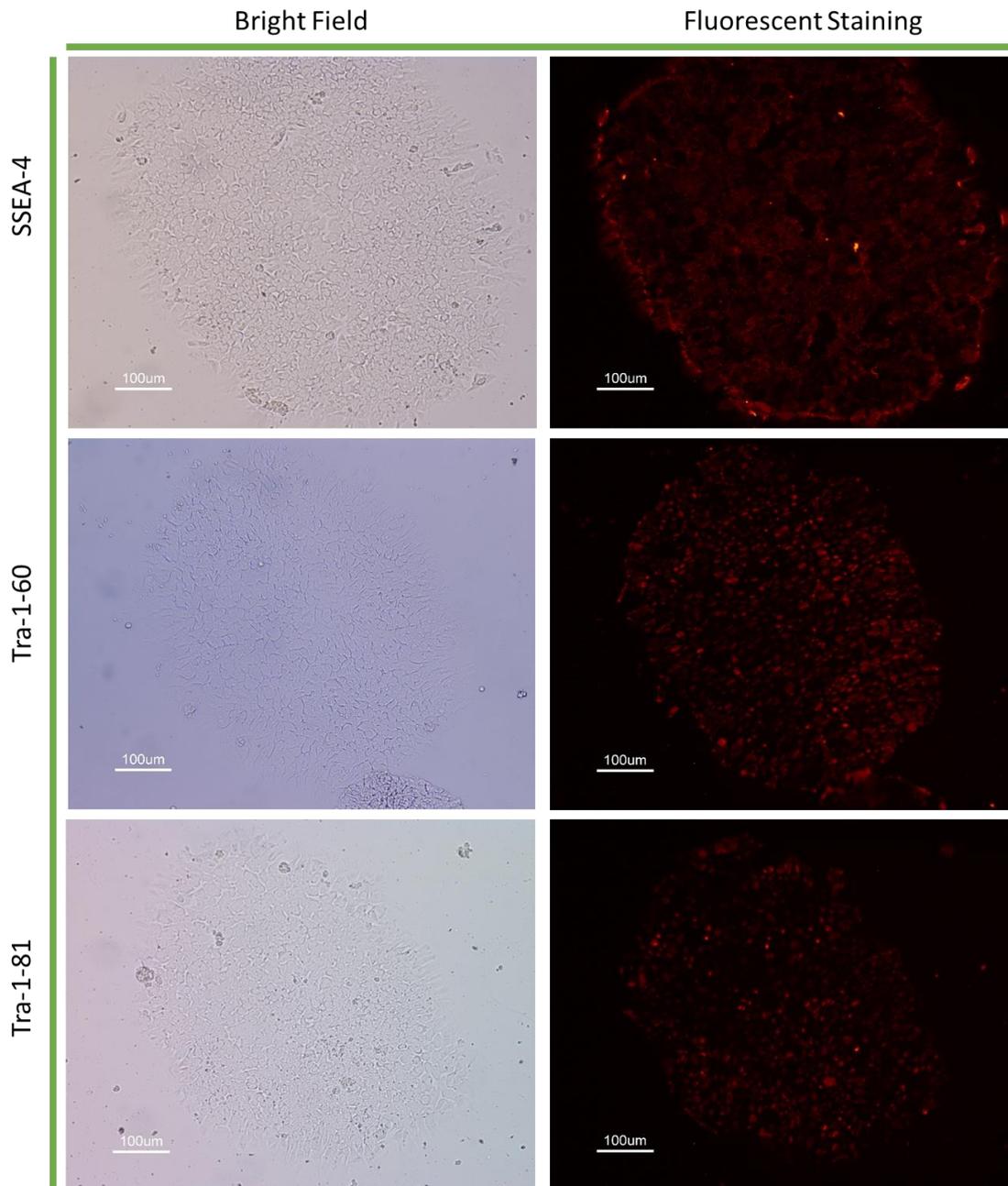


Figure IV.7: Extracellular staining of the expanded Cre-Sort hiPSC line. The cells were stained on the 120th passage after about 2 months of culture at SCBL. Bright Field images are also provided as controls for the fluorescent staining photos. Scale: 100 µm.

From the fluorescent microscopy pictures of Figure IV.6, it was possible to observe that there exists a clear and constant expression of all the considered markers. A reduced amount of the cells show reduced expression of the markers, usually they can be found on the periphery of the considered colonies, as can be seen in the comparison of the DAPI staining picture correspondent to the Oct-4

staining picture. A few of the cells found at the periphery of the colony show clear DAPI staining identifying their nucleus but those same cells are missing from the Oct-4 staining picture. Another point of note is the staining of the cells for SSEA-4. As we can see by comparing the fluorescent staining picture to the corresponding Bright Field picture, all cells show some expression of this marker. This was clearly seen in the rest of the well for these cells therefore supporting our original belief that the results obtained from Flow Cytometry for this marker are not the most reliable in this study and that the real percentage of SSEA-4+ cells is most likely superior to 60%.

IV.2 hiPSCs Differentiation into Neural Progenitor Cells

As said before, in order to claim that a cell line is truly pluripotent, it is not enough to prove that it expresses all the required markers of pluripotency. Although it is a necessary step, it is not conclusive in itself. To lend a bigger strength to it we must also access the cell line's differentiation potential into all three embryonic germ lines.

For this study, the Cre-Sort cell line was also differentiated into Neural Progenitor Cells using a differentiation protocol established at the SCBL and based on the dual-SMAD inhibition protocol [109]. This protocol requires the cells to be cultured for 12 days with the N2B27 culture medium supplemented with both SB and LDN small molecules. After the 12 days of culture, the cells were marked for Pax6 and Nestin expression and photos were taken of fluorescent microscopy to visually verify the cellular differentiation (Figure IV.7).

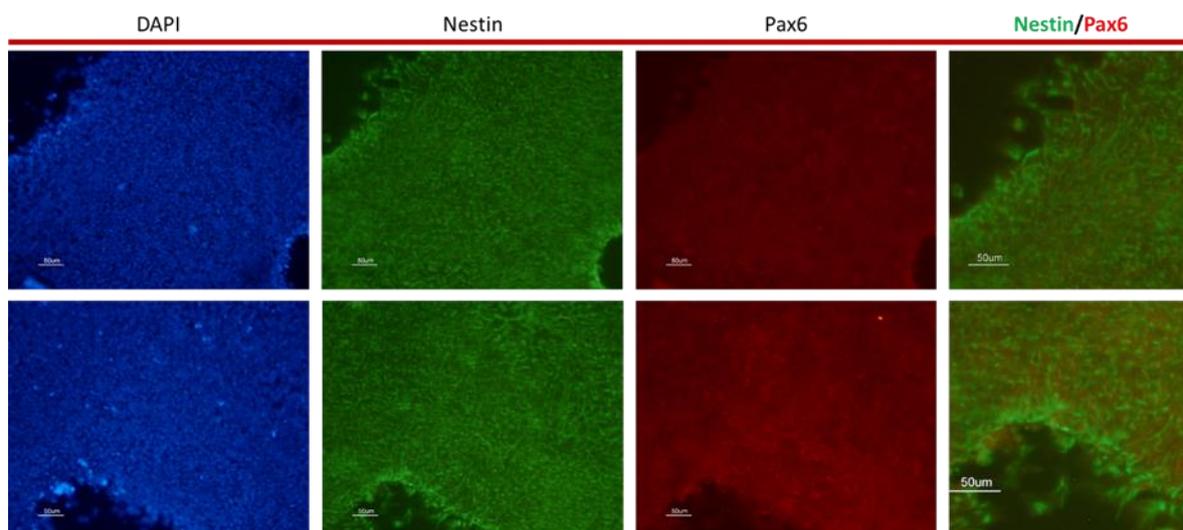


Figure IV.7: Fluorescent microscopy pictures of the Cre-Sort hiPSC line after commitment into the neural lineage. Cells were differentiated using N2B27 medium supplemented with SB and LDN in a Matrigel™ coated well. The pictures were stained for DAPI, Nestin and Pax6. The last pictures to the right are a close-up of the superimposed pictures of Nestin and Pax6 staining

Pax6 is an essential transcription factor in NSCs that regulates 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 their differentiation into neurons and oligodendrocytes [110]. Nestin is an intermediate filament protein, commonly used as a marker for NSCs as it is extensively expressed in multipotent NSCs. It is an essential protein for the proper survival and self-renewal of these cells both *in-vitro* and *in-vivo* [111].

These markers are therefore useful for identifying the differentiation of hPSCs into Neural Progenitor cells since they are expressed during this differentiation and guide cells through their differentiation path, especially Pax6. As we can see from the pictures obtained in fluorescent microscopy (Figure III.7) there is a pretty clear expression of these markers in all of the cells. Nestin can be seen giving rise to several strands in the cells cytoplasm as expected and it was also verified that Pax6 marks, although less prevalently, the nucleus of the differentiated cells. The final pictures show the expression of both proteins and confirms the information obtained from the other images. DAPI staining allows us to see that there are no cells outside the ones expressing both markers.

V. Conclusions

With this study it was possible to develop a feeder-free and serum-free protocol for the expansion of the model Cre-Sort hiPSC line using Matrigel™ coated culture wells and mTeSR™1 culture medium.

The used culture system was capable of expanding the considered cell line. As it was shown by the Growth Kinetics assay, the cells were capable of reaching 6 fold their initial seeding number after 8 days of culture. Although the cells expanded, the speed at which they did so was extremely slow. Usually when working with hiPSCs, most protocols advise to passage the cells every 3-4 days, however the growth kinetics of this line show that after 3-4 days of expansion the cells have not reached the beginning of the exponential phase making it fruitless to passage those cells.

The used culture system was also apparently capable of maintaining the cell line's pluripotent potential. We say apparently because it was not proven fully. The cells expanded using this protocol showed satisfactory levels of expression of several pluripotency markers such as transcription factors Nanog, Sox-2 and Oct-4 and cell surface markers, such as Tra-1-60, Tra-1-81 and SSEA4. All these are strong indicators of pluripotency. The cells were also capable of differentiating into a homogenous population of Neural Progenitor cells by using a 12-day differentiation protocol with N2B27 culture medium supplemented with the small molecules LDN and SB. The differentiated cells presented the expression of both Pax6 and Nestin, markers of Neural progenitor cell differentiation.

In conclusion, the used expansion protocol was capable of expanding the Cre-Sort cell line under serum-free and feeder-free conditions. Also all pointers tested indicate that the expanded cells maintained their pluripotent potential. This way this culture protocol becomes a viable choice to expand this specific cell line obtained after genetic editing and reprogramming protocols. This cell line, like any other future cell line to help people with genetic diseases, was obtained from the considered patient. Using zinc-finger nucleases the genome of the cell was cut in a specific location of relative genetic stability, and the cells were then infected with a lentiviral vector carrying the corrected gene of interest. This way this cell line was now capable of displaying a healthy phenotype. Finally they were subjected to the entire reprogramming procedure. These cells were therefore heavily modified and their growth *in-vitro* was affected, as we have shown in this study. This result is important as a first step towards developing new strategies for cell therapy. The Cre-Sort cell line was a corrected iPSC line from a patient with FA. Since this protocol was shown to be able to expand this special line it made it clear that it is possible to use this line for cell therapy of this specific condition for this specific patient. Much must still be done, but as a proof-of-concept this study certainly contributed as a first step toward the creation of the base technology for personalized medicine.

VI. Future Work

This work performed in this thesis showed that it is possible to expand special genetically corrected hiPSC lines, such as the Cre-Sort cell line, using a standard expansion protocol for iPSCs. Although this is a step forward towards the eventual application of these cells in personalized medicine there is still much to do, including as a continuation of this study.

Future work would consist of optimizing the expansion protocol for this cell line, the current growth rate of the cell line in this protocol is very low and plagued with several problems such as spontaneous differentiation among others. One first step would be to tailor make a protocol that would maximize growth and minimize spontaneous differentiation. This protocol would also need to be developed in a completely xeno-free way, with a known substrate for cell growth, instead of Matrigel™ coated wells, and a completely defined xeno-free media such as mTeSR™2 or E8. Another future work based on this study would be to completely characterize the pluripotent potential of this cell line. For this objective, it would be necessary to check a broader array of cellular markers as well as attempt differentiation into all the three germ layers. It would also mean running teratoma formation assays in immune-compromised mice and a karyotype analysis for genetic aberrations. Finally, considering that the main application of these cells would be for the treatment of bone marrow failure in FA patients, a study of the differentiation of these cells to hematopoietic stem cells would be necessary to conduct and in a long-term goal even a clinical trial and experimentation.

As a final possible future work, the scale-up of the culture procedure for these cells to bioreactor culture systems [112], these could even include expansion as cell aggregates or even using microcarriers. This along with protocols for the selection and purification of the target cells are likely candidates. We must never forget the real objective behind works like these, the translation of the laboratory techniques into clinical use. Any future work that could contribute to this overarching goal is a possible and worthy continuation to this project.

VII. References

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