A COMPARATIVE STUDY OF CELL CULTURE CONDITIONS DURING CONVERSION FROM PRIMED TO NAIVE HUMAN PLURIPOTENT STEM CELLS

Master's Degree in Biomedical Research

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ABSTRACT

Since the successful reprogramming of human somatic cells into induced pluripotent stem cells (hiPSCs), stem cells have opened up a large field of research to generate new therapies due to their ability to differentiate into any cell type.

Embryonic stem cells (ESC) are pluripotent cells which give rise to all somatic cell types in the embryo. Their self-renewal ability and plasticity allows for *in vitro* generation of many distinct cell types, raising new challenges for regenerative medicine and therapies.

The "naive" state of cell pluripotency is the result of cells that come from the preimplantation epiblast *in vivo.* This state was observed in mouse embryonic stem cells and was characterized by a high proliferation and differentiation capacity as well as a global DNA hypomethylation. Human embryonic stem cells (hESCs) are derived from the inner cell mass of preimplantation embryos and correspond to a later stage called "primed" stage of embryonic development. The conversion of this "primed" human embryonic stem cells (hESCs) to a "naive" state is desirable as their characteristics would facilitate many techniques such as gene editing and a more efficient differentiation. The main objective of this conversion is to facilitate the application of cell therapies to be able to use them in clinical treatments to model different diseases, such as human primary immunodeficiencies related to NK cell defects.

In the present study, the main objective is to compare and evaluate different culture conditions for conversion from primed to naive state of an hES cell line called ES-2. The different culture conditions are based on different conversion media (Gafni, Fine and T2iLGö) in both feeder and feeder-free cells conditions.

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1. INTRODUCTION

1.1. STEM CELLS

Stem cells are unspecialized cells of the human body with the capacity to both differentiate and multiply into any cell type. They are able to self-renewal and exist both in adult cells and in embryos. Exists different steps of specialization and the stem cell potency is reduced with each degree. *Totipotent stem cells* have the ability to both differentiate and divide into the cells of whole organisms. These cells are capable to form both embryo and extra-embryonic structures. One example of a totipotent cell is a zygote, which is form when the sperm fertilizes an egg. Once the fertilized egg is formed, 4 days later the blastocyst's inner cell mass becomes pluripotent. *Pluripotent stem cells* can be differentiated into the three germ layers and germ cells but not the placenta. Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of preimplantated embryos and their culturing is very promising to overcome many diseases and regenerative medicine. *Multipotent stem cells* can be differentiated into cells of specific cell lineages. An example is an haematopoietic stem cell which can derivate into any cell type of the blood.

Finally, the most committed step belongs to *unipotent stem cells*. These cells are able to form only one cell type of their own linage, for example cells of the nervous system (Ota, 2008) (Fig. 1).
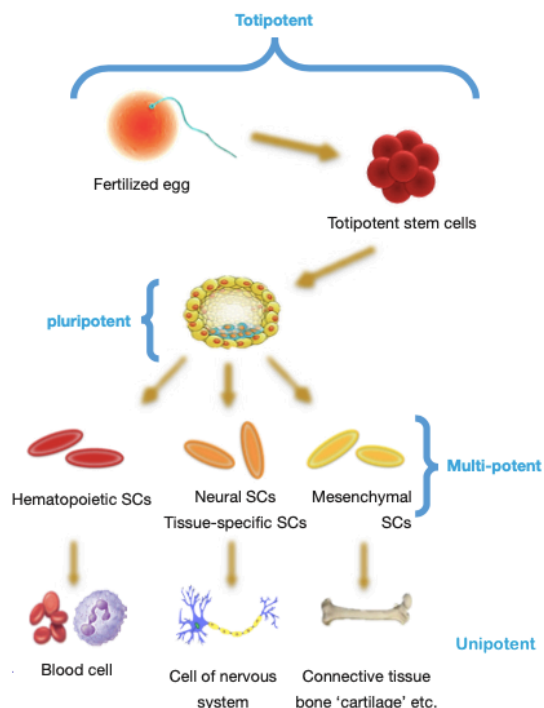
Figure 1. Stem cells types diagram (Obtained from (Rajabzedeh, Fathi, and Farahzadi 2019)).

1.2. PLURIPOTENT STEM CELLS

As mentioned above, pluripotent stem cells are able to form cells of the embryonic lineage but not of the extra-embryonic structures. For further investigations, is necessary the isolation and the generation of pluripotent stem cells, mainly embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

1.2.1. Human induced pluripotent stem cells (iPSCs)

In 2006 a Japanese group formed by Takahashi and Yamanaka discovered induced pluripotent stem cells, another dimension of stem cell field. The combination of four transcription factors (Sox2, Oct4, cMyc and Klf4) introduced by retroviruses into murine fibroblasts, induced reprogramming of the somatic cells to a pluripotent state. These cells were similar to embryonic stem cells (ESC) and were called induced pluripotent stem cells or iPS cells (Fig. 2).

Induced pluripotent stem cells grow as a colony, mainly on top of a monolayer of murine inactivated fibroblasts. They express many pluripotency markers such as TRA-1-80, TRA-1-60, SSEA-4, Sox2, Oct4 and Nanog. The expression of TRA-1-60 and TRA-1-80 are useful to distinguish fully reprogrammed colonies from those that are only partially reprogrammed.
In 2007 Takahashi et al. and Yu et al. generated human induced pluripotent stem cells from human fibroblasts. In this attempt they replaced Klf4 and cMyc for Nanog and LIN28. Results showed once again that they behave as ES cells and analysis of DNA methylation showed highly similar global scale methylome of ES cells and iPS cells.
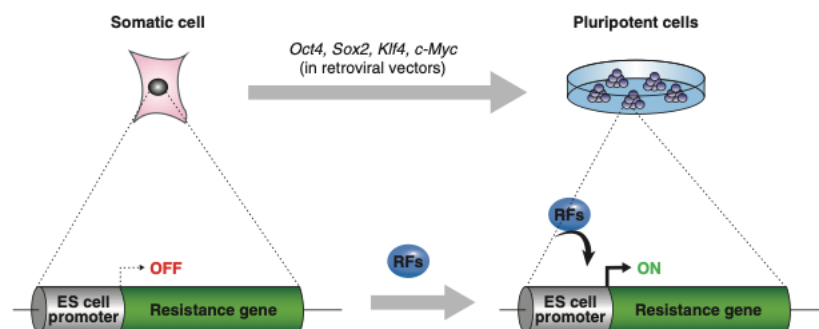

Figure 2. Strategy to derive iPS cells (Obtained from (Hochedlinger and Jaenisch 2015)).

These cells are not only derived from fibroblasts, many researchers have generated iPS from other somatic cells asserting the possibility to reprogram cells of different origins. Even though iPS cells can be differentiated into ectoderm, mesoderm and endoderm, the future goal is to

discover the most efficient, accessible and safest way to reprogram cells for future clinical applications.

One possible clinical application of iPS is in multifactorial diseases. iPS cells can be generated from Parkinson disease fibroblasts and differentiate into neurons, nevertheless, these induced cells do not generate any disease-specific benefit. This may be due to the environmental factors that affect and accelerate the disease phenotype.

Moreover, patient specific-iPS cells can be a powerful system for identifying drug candidates of certain diseases and for regenerative medicine as well (Fig. 3).
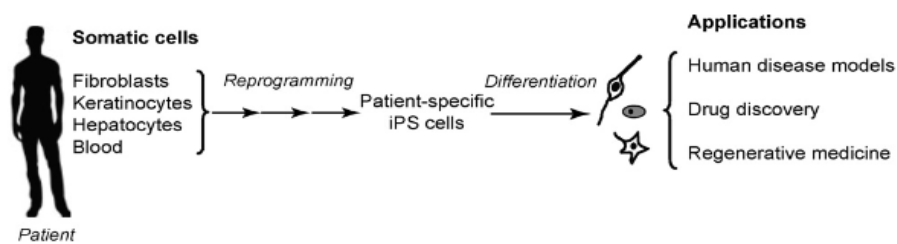
Figure 3. The generation of iPS cells and their applications (Obtained from (Rajabzedeh et al. 2019)).

Although iPS cell therapy is a promising new technology for regenerative medicine, there are still many issues that need to be addressed before they are used in clinical therapies. It has not yet been proven that iPS cells are safe for humans. What has been found is that the main disadvantage is tumour formation; this may be due to reactivation of oncogenes after reprogramming, the origin of the cell, or other factors such as aberrant DNA methylation. However, the great advantage of generating patient-compatible iPS cells is that would overcome the immunological rejection and ethical problems faced by embryonic stem cells (hESCs) (Rajabzedeh et al. 2019).

1.2.2. Human embryonic stem cells (hESCs)

A blastocyst is generated by multiple mitotic cell divisions during early embryogenesis of a diploid zygote after the fertilization of an egg. The blastocyst is composed of two different layers of cells: the inner cell mass (ICM), which is the main responsible of the development of a foetus and the trophoblast, which forms the extra-embryonic tissues such as the placenta, the chorion and the umbilical cord (Vazin and Freed 2010).

In 1975, Sherman et al. studied the development of mouse blastocysts. They examined the differentiation and growth process of the inner cell mass and the trophoblast cells in long-term

cell cultures. These cells were termed ESCs (embryonic stem cells) and since they could derive ESC from mouse, the next challenge was the obtention of a human embryonic pluripotent stem cell line. Nevertheless, at the beginning, protocols used to the obtention of these cells had only been proved successfully in mouse. This led them to believe that rodents had unusual early embryonic development that was different from humans. In the process of blastocyst formation, mouse and human embryos development are morphologically quite similar. In humans, the blastocyst implants into the uterus about 7–9 days postfertilization, however, in rodent embryos, at around embryonic day 4-5. Implantation leads to decidualization, a widely conserved response between mice and humans. This process consists of changes in the stromal cells of the maternal endometrium under the influence of the steroid hormones estrogen and progesterone (Hemberger, Hanna, and Dean, 2020) (Fig. 4).
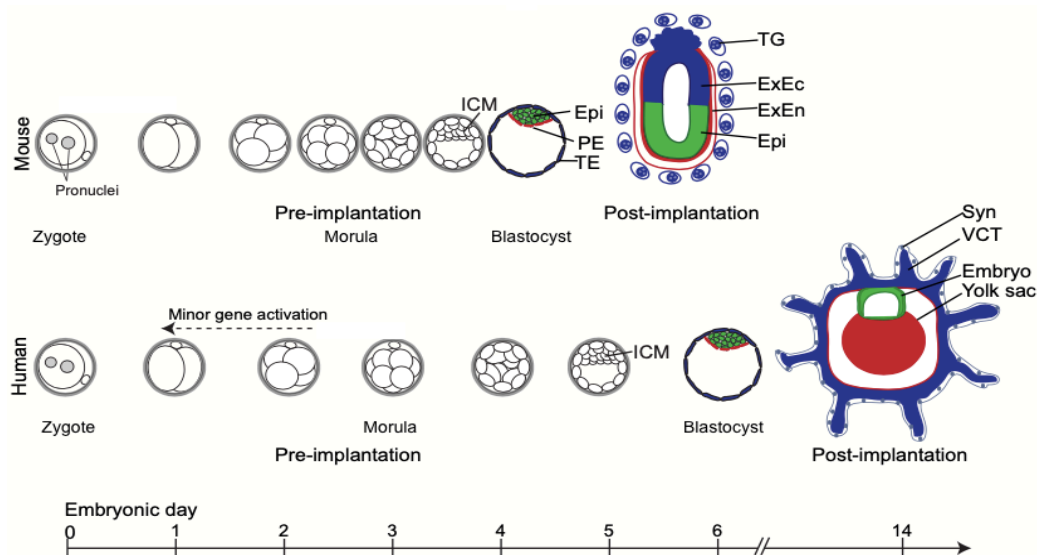
Figure 4. Early embryonic development mouse vs human (obtained from (Niakan et al. 2012)).

Over the years many cells were reported to show transient similar appearance to mouse embryonic cells. However, all the experiments performed to obtain cultures of pluripotent cells directly from a mouse embryo have been unsuccessful. Cells from embryos of an early post-implantation stage seem to be the best candidates to direct obtention of pluripotent cells in culture. As a result, although these embryos are difficult to isolate, and as the cell number in the isolated epiblast is small, in 1981 the first stable mouse embryonic stem cell lines was successfully derivated (Evans and Kaufman, 1981) (Fig. 5).

Finally, in 1998 James Thomson managed to obtain human embryonic stem cells derived from ICM by in vitro fertilization (Fig. 5).

| mESC | hESC |
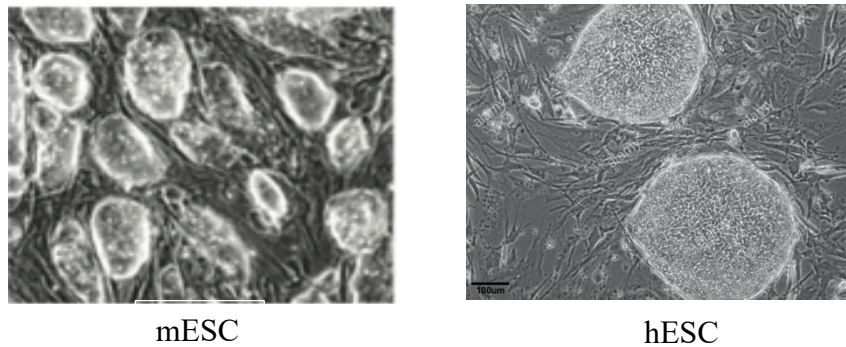
Figure 5. Mouse vs human pluripotent ES cell line derived from blastocyst (Obtained from (Hochedlinger and Jaenisch 2015) and from https://www.alamy.es/).

Once the term "ES cell" was introduced and the properties of mouse ES cells understood, Thomson proposed that primate ES cells should include some essential characteristics: 1) Derivation from the human inner cell mass either from the preimplantation or preimplantation stage 2) Prolonged undifferentiated proliferation 3) Stable developmental potential with the capacity to form of all three embryonic germ layers even after being cultured for a long time 4) hES cells must maintain a normal karyotype even after several multiplications of the original population 5) hES cells must express the molecular markers of pluripotent cells (Table 1).

Marker	Function
OCT4	Maintenance of pluripotency, coordinated control of transcriptional, and post-transcriptional machinery of ICM cells with SOX2.
SOX2	Maintenance of pluripotency, coordinated control for transcriptional, and post- transcriptional machinery of ICM cells with OCT4.
NANOG	Maintenance of pluripotent gene products, formation of binary transcription complex with OCT4.
BMP4	Maintenance of pluripotency, self-renewal, and cellular specification via coordination with OCT4 and SOX2.
TRA1-60/81	hES cell antigens carried by podocalyxin membrane protein.
SSEA-3/4	Stage-specific embryonic sphingolipids present during preimplantation development on hESCs.

Table 1. hES cell markers and each function in hESC. (Obtained from Dupont et al. 2019).

The markers of the table above are not the only ones present in this cell type, there are also p38 inhibitors (GDF3), factors responsible for maintenance of pluripotent genes and products (ESG1), among others. Moreover, Thomson discovered that human embryonic stem cell lines expressed high levels of telomerase activity. Telomerase is a ribonucleoprotein involved in the maintenance of extensive replication by the addition of repeats to chromosome ends. This protein expression is highly correlated with immortality in human cell lines and it is present at high levels in germ line and embryonic tissues. The high level of telomerase activity expressed in human ES cell lines suggests that their replicative potential will exceed that of somatic cells.

Human ES cell lines express pluripotency surface markers: (SSEA)–3, SSEA-4, TRA-l-60, TRA-1- 81 and alkaline phosphatase (Thomson 1998). hESCs capacity for self-renewal and pluripotency is maintained by a network of different transcription factors such as OCT4, SOX2 and NANOG. OCT4 and SOX2 operate in tandem regulating each own expression and they operate as a heterodimer. Furthermore, NANOG is crucial as it monitors and maintains the expression of OCT4 and SOX2 by interacting with their respective genes, *Pou5f1* and *Sox2*. BMP4 also assists in maintaining pluripotency and ES cell self-renewal via inhibition of the extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, responsible for downstream signalling of mitogens and growth factors that induce cellular division and differentiation, for example, LIF, FGF, and BMP (Dupont et al. 2019).

The main difference between a stem cell and a differentiated cell is represented in the cells' DNA. In the differentiated cell, the DNA is arranged loosely with working genes. When the signal enters in the cell, those genes that are no longer needed are shut down while genes required for the specialized function will still be active. In table 1 the functional genes for the maintenance of pluripotency are shown and among them, Oct3/4 and Sox2 are necessary for the generation of induced pluripotent stem cells (iPSCs) (Ota, 2008).

Stem cells have the potential to become one of the most effective and important therapy in medicine. Aside from the valuable information that can offer in understanding human development, human stem cells play an important role in regenerative medicine. Many diseases such as cancer are caused by a wrong differentiation or cell division and for that reason, stem cell therapies may result a new way of treating currently incurable diseases. However, although stem cells appear to be an ideal solution for medicine, there is a huge obstacle to overcome in the future: the ethical concern. Moreover, immunological tolerance between the patient's body

and stem cells is another problem that needs to be solved. At the moment, the most frequent haplotypes of ESCs can be selected in order to avoid immune rejection.

1.3. PLURIPOTENT STATES

The comparative analysis of hESCs (from preimplantation blastocyst) and mouse embryonic stem cells revealed many molecular differences. At first and for many years it was thought to be due to differences between species but in 2007 a post-implantated epiblast was isolated and found to be more similar to hESCs than mESCs. This led them to conclude that hESCs correspond to a later embryonic development stage (Brons et al. 2007).

During preimplantation development the zygote is transiently regulated. Here, we consider how unrestricted cells are first generated and then prepared for lineage commitment. So that two pluripotency states can be defined: naive and primed (Fig. 6) (Nichols and Smith, 2009).
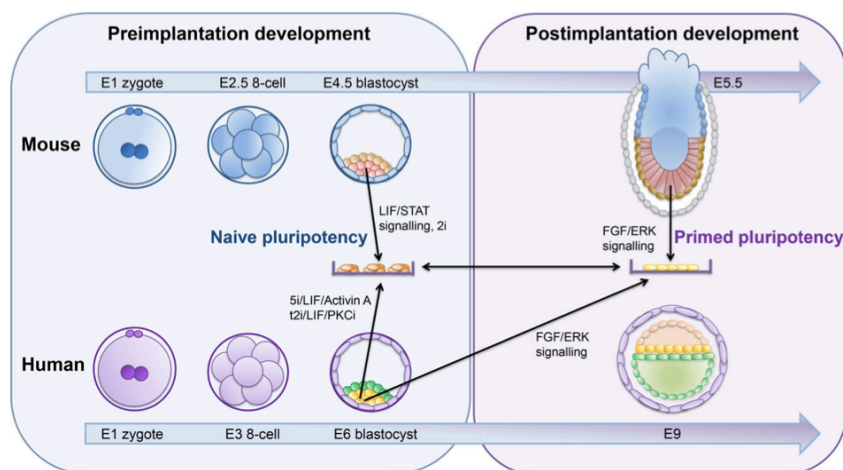
Figure 6. States of pluripotency in mouse (top) and human (bottom): naive and primed (Obtained from (Eguizabal et al. 2019).

Primed and naive pluripotent states represent a continuous network of configurations rather than an immovable state. Within both states, different degrees of naive state or primed state can be found, differing in various characteristics between them.

1.3.1. Primed state in human ESCs

In preimplantation state in mouse, the pluripotent epiblast compartment within the inner cell mass (ICM) of the blastocyst constitutes a naive or ground state of mESCs pluripotency. After implantation, transcription factors associated with naive pluripotency are downregulated and

pluripotent cells become primed for differentiation in response to signals from the surrounding extraembryonic tissues. This primed state is more committed than the naive state. Human embryonic stem cells (hESCs) derived under conventional conditions are thought to represent a primed pluripotent state, and were shown to correspond transcriptionally to the late post-implantation epiblast in a non-human primate model.

This priming scenario in human ESCs shows low levels of expression of naive pluripotency markers (KLF17 and DPPA3), cells in primed state reflect distinct pluripotency associated gene patterns, XIST-coated inactive X chromosome (XaXi), poor cloning efficiency, flat morphology, DNA hypermethylation (H3K27me3), more condensed chromatin and DNA less accessible, activin/FGF-responsive, generation of energy through glycolysis and a reduced tolerance to single cell dissociation and very limited ability to participate in chimera formation (Blair, Wray, and Smith 2011).

Furthermore, although different culture systems do display markers variability, Trusler et al. provided to researchers a resource of human naive and primed pluripotent stem cell markers.

After analysing a panel of surface markers, they proposed to create a smaller group to discriminate between the main specific stages: naive and primed. The study by Shakiba et al. showed that the naïve cells had a reduced level of CD24 surface expression when compared with primed hESCs. CD75, CD7, CD77 and CD130 were positive only in naive-like cells whereas CD24, CD57, CD90 and HLA-A,B,C were positive only in primed hESCs (Trusler et al. 2018).

1.3.2. Naive state in human ESCs

The newly discovered naive or ground state pluripotency is a cellular state that resembles preimplantation epiblast cells. Referring its characteristics, it does not present the problems that conventional primed human pluripotent stem cells can offer. These problems are variable differentiation capability, difficult cell-passing and low gene editing efficiency. In mice, mESCs come from the inner cell mass of the preimplantation blastocyst. When combining and maintaining with leukemia inhibitory factor (LIF) and protein kinases, mESCs are described as being in a naive state. When injected back into an early embryo, these cells contribute to all lineages without tumorigenesis. In recent years the main objective is to develop strategies for capturing hESCs in a naive pluripotent state or to convert primed cells for future treatments.

A key difference between naive and primed states is their differentiation potential. Moreover, respiration differs between the two cell types: primed cells are almost completely glycolytic, whereas naive cells use mitochondrial respiration. Naive cells survive more of single cell passaging than primed cells and they also differ in the doubling time.

In the naive stage the colonies form rounded 3D, whereas in primed stage colonies grow in flat monolayers and are inefficient in forming chimeras; corresponding to the transition of naive epiblast cells toward a more committed state in vivo. This gives us information about the role that diffusion signalling-molecules and cell-cell adhesion play in the different pathways of each state. In general, there are more active enhancers in naive state than in primed state. Although both enhancers are activated, the proximal enhancer corresponds mainly in primed cells and distal enhancer in naive state. DNA methylation has been correlated with the ability to maintain naive pluripotency and concretely, have been shown to be hypomethylated. There is an upregulation of pluripotency markers (KLF2 and KLF4) and X-inactivation by heterochromatin formation is only shown in primed cells. Naive cells have a reactivated X chromosome (XaXa pre-X inactivation). Naive cells show downregulation of committed markers (CD24 for example) and the expression pattern of some naive-associated genes, differ from mouse to human.

In conclusion, the naive pluripotency state has great potential due to the following main features: (1) it is very useful as an *in vitro* model of early human embryonic development, (2) naive culture media are more favourable in certain aspects since the high homogeneity and the low variability during differentiation of multiple lineages. This advantage allows that X chromosome abnormalities (*de novo* mutations for example) can be avoided by resetting female primed ESCs. Researchers are optimizing culture conditions for naive cell derivation and maintenance for future cell therapies (Dodsworth, Flynn, and Cowley 2015).

1.4. CULTURE CONDITIONS

1.4.1. Feeder layer conditions

Feeder layer are composed of adherent growth viable and bioactive cells that enhance self-renewal and proliferation of other cells. These feeder layer cells act as a substratum to the medium on which other cells are grown by releasing key growth factors required for stem cell maintenance. Nevertheless, feeder cells have also other important roles such as detoxifying the

medium or synthetizing cytokines and extracellular matrix proteins (ECM) that are needed to control the cell growth acting as a substrate for a better attachment of the cells. The leukemia inhibitory factor (LIF) is required for maintaining both the developmental and proliferation potential of non- primate stem cells. For that reason, it is a cytokine used as an additive for culture medium of human and mouse ESC. Embryonic stem cells express LIF receptor and the binding of LIF activates JAK-STAT pathways, essential for stem cell maintenance.

To carry out the expansion and proliferation of cultured target cells, feeder layer must provide some active signals and factors avoiding an overgrowing of the culture. This means that target cells that are grown above feeder cells have to be metabolically active expressing specific ligands or cytokines but without an excessive multiplication. To avoid feeder cells division, although in recent year have been developed new methods, g-irradiation (GI) and mitomycin-C (MC) are the most commonly successful treatments (Llames et al. 2015).

In 1998, Thomson cultured hESCs using MEF (murine embryonic fibroblasts) as feeder layers. With an *in vitro* purpose, animal feeder cell does not constitute an important problem. However, the use of animal feeder cells like MEF in clinical applications may compromise the culture due to the possibility of cross-transfer of animal pathogens and immunogens. For that reason, researchers found an alternative culture conditions, growing hESCs in human feeder layers, such as human foreskin fibroblasts mitotically inactivated (HFFi), which are easily obtained (Oliveira et al. 2018).

1.4.2. Feeder-free layer conditions

Although many cell types are completely feeder layer cell dependent on physical contact for survival and expansion, some other cell types can grow in feeder-free conditions. These feeder-free conditions are formed by the coating of the culture plate with an extracellular protein such as laminin, collagen, fibronectin, vitronectin or a mixture of the extracellular matrix components (Matrigel). Indeed, coating proteins can be produced in bacteria or in a cell-free system, and supplemented with a xeno-free media conditioned by feeder cells that are commercially available (Essential E8 medium) (Chen et al. 2011).

2. OBJECTIVES

The breakthrough discovery of induced pluripotent stem cells (iPSCs) allows us to use this technology in the study of human diseases. However, since human embryonic stem cells (hESCs) were first derived in 1998, hESCs also represent a big challenge. With a better characterization of hES pluripotent states, we might be able to better understand the pathogenesis of different diseases and facilitate the discovery of new therapeutic agents and/or cell therapy for future treatments. The observation of the advantages that pre-blastocyst implantation state "naive" has over post-implantation state "primed" have made naive state a better candidate for further re-differentiation. For that reason, many studies focus on establishing optimal culture conditions for the transition from primed to naive state in human embryonic pluripotent stem cells (hESCs).

The aims of this project are:

1. To establish and maintain a hESCs line with different naive conversion media in both feeder and feeder-free conditions.

2. To determine which culture conditions are the most favourable for naive conversion.

3. METHODOLOGY

3.1. CULTURE OF PRIMED hESCs

Although in this project the ES-2 cell line is converted to the naive state with two different media conditions, a back-up line of hESCs is grown in primed state.

3.1.1. Feeder conditions

Primed ES-2 cell line grown on feeder cells were cultured in hES medium with the following composition: KO-DMEM (Life Technologies) supplemented with 20% of Knockout Serum Replacement (KSR; Life Technologies), 1% non-essential amino acids (Life Technologies), 1% glutaMAX (Life Technologies), 1% penicillin/streptomycin (Pen/Strep; Life Technologies), 50 µM β-mercaptoethanol (Life Technologies) and 10 ng/ml fibroblast growth factor 2 (FGF2; Miltenyi).

At the beginning, as soon as the cells were thawed, took almost two weeks to grow and to pass them as clumps. From that moment on, cells are ready for being passed after 7 days in culture. Primed ES-2 cells were mechanically passaged as clumps by scratching the colonies with a STRIPPER$^®$ micropippeter. The feeder layer consists of irradiated HFF-1 cells seeded the day prior to cell passage on gelatine-coated plates and incubated at 37 °C overnight. The medium was changed daily with the exception of the day the ES-2 cells are passed to a new plate, which are cultured for 48 hours with 3 ml of hES medium.

When flow cytometry was performed, the medium was aspirated and 1mL of basal hES media was added. The colonies were then mechanically scraped off and disaggregated into a single cell solution using a micropipette. Subsequently, the cells were centrifuged at 1000 rpm for 4 minutes at 4 ° C.

3.1.2. Feeder-free conditions

Primed ES-2 in feeder-free condition were cultured onto 1% vitronectin dissolved in PBS (Phosphate Buffered Saline) coated plates in Essential 8^{TM} medium (Life Technologies); E8 medium contains insulin, selenium, transferrin, L-ascorbic acid, FGF2, and TGFβ in DMEM/F12 with pH adjusted with NaHCO3. Cells are ready for being passed after 4-5 days in culture. These cells are also mechanically passaged as clumps by scratching the colonies with a STRIPPER$^®$ micropippeter. 1% vitronectin in PBS were added to coat the plates at least

30 minutes before cell passage. Then, picked-up colonies were passed into a new vitronectin coated plate in a total volume of 3 mL with E8 medium. Medium was changed daily with the exception of the day the cells are passaged to a new plate and cultured for 48 hours with 3 ml of E8 medium.

When flow cytometry was performed, the medium was aspirated and cells were treated with 0,5 mL of ethylenediaminetetraacetic acid (EDTA) 0.5 mM, which indirectly suppresses cell-to-cell connections by chelating divalent cations. Cells were incubated at 37 °C for 3-4 minutes. Without aspirating the EDTA, 1 mL of basal E8 medium was added and cells were disaggregated with the micropipette into single cell. Subsequently, the cells were centrifuged at 1000 rpm for 4 minutes at 4 ° C.

3.2. CONVERSION CULTURE

As mentioned before, several conversion media were tested for naive conversion and maintenance of hESC cell line. In this project, a common medium has been used in both conditions (feeder and feeder-free) and each condition also has an additional culture medium (Fig.7).
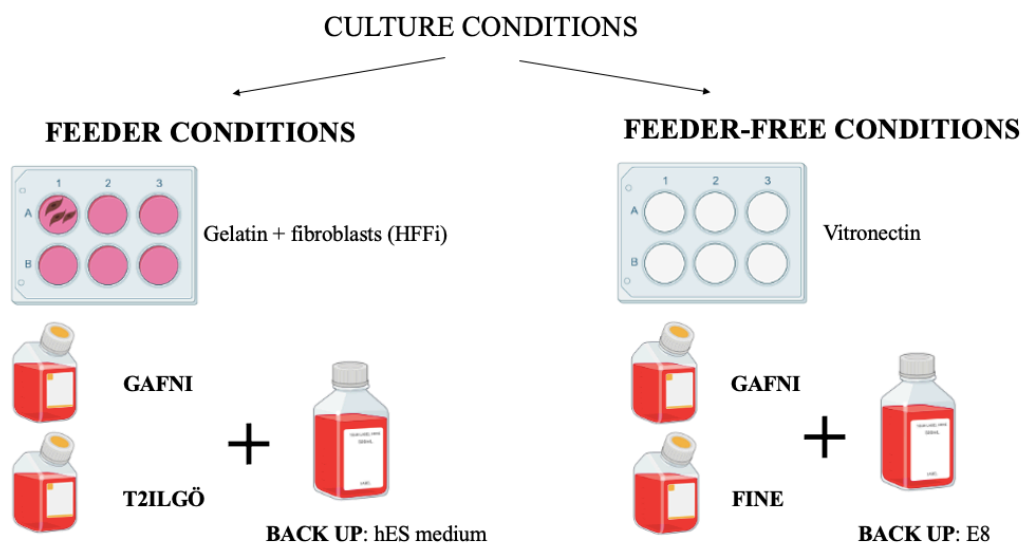

Figure 7. Diagram of the two different growing conditions used in this project. Within each condition there are two different media: in feeder condition Gafni and T2iLGö and in feeder-free condition Gafni and Fine.

3.2.1. GAFNI conversion medium

The naive Human Stem Cell Medium (NHSM), also known as Gafni Medium, has been reported to enable naive conversion in both feeder and feeder-free conditions in hESC cell lines.

Gafni medium consists of KO-DMEM (Life Technologies), 18% KSR (Life Technologies), 12.5 µg/mL recombinant human insulin (Sigma), 1% glutaMAX (Life Technologies), 1% nonessential amino acids (NEAA; Life Technologies), 1% Pen/Strep (Life Technologies) and 0.1 mM β-mercaptoethanol (Life Technologies) supplemented with 20 ng/mL recombinant human LIF (hLIF; Peprotech), 8 ng/mL FGF2 (Miltenyi Biotec), 1 ng/mL TGF-β1 (Peprotech), 1 µM PD0325901 (ERK1/2i, Axon Medchem), 3 µM CHIR99021 (GSK3bi, Axon Medchem), 10 µM SP600125 (JNKi, Axon Medchem), 2 µM BIRB796 (p38i, Axon Medchem), 5 µM Y-27632 (ROCKi, Axon Medchem) and 5 µM Go6983 (PKCi, Axon Medchem) (Gafni et al. 2013).

The medium was aspirated and naive hESCs on feeder layers were passaged every 6-7 days by trypsinization (trypsin breaks protein bonds between the cells). For that, cells were washed with 1x PBS and 500 µL of 0.05% Trypsin were added to each well of hESCs. Cells were incubated at 37°C for 4 minutes. When flow cytometry was performed, 1 mL of basal hES medium was added on the top of the trypsin to inactivate it. Cells were detached with a 1-mL micropipette doing up and down slowly and centrifuged 4 minutes at 1000 rpm at 4ºC to single cells (Fig. 8).
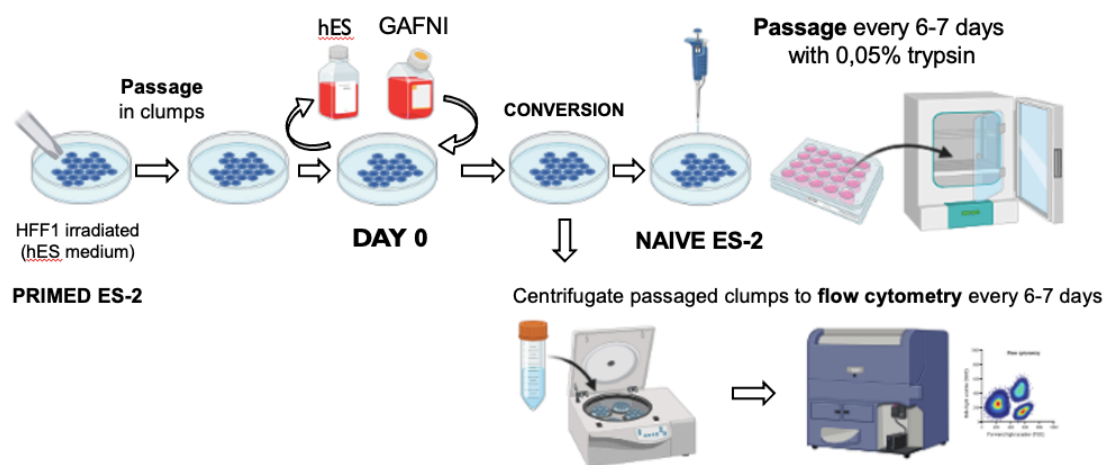
Figure 8. Protocol of naive conversion from primed ES-2 onto feeder condition with GAFNI medium.

Naive hESCs in Gafni condition grown on vitronectin-coated (feeder-free) plates were passaged with EDTA. Cells were washed with 1x PBS and 500 µL of EDTA 0.5 mM were added to each well. Cells were incubated at 37 °C for 3-4 minutes. For cells that continue the naive state conversion, EDTA was aspirated and 3 mL of GAFNI medium were added. Further, cells were disaggregated slowly with a 1-mL pipette doing up and down slowly and seeded on

new vitronectin-coated plates. However, for those plates used for cytometry, EDTA was not aspirated and 1 mL of GAFNI medium was added. Then, 1-mL micropipette was used to break clumps and cell were centrifuged 4 minutes at 1000 rpm at 4ºC. Medium was changed daily in the case of the naive-conversion plates and cells were passaged every 3-4 days (Fig. 9).
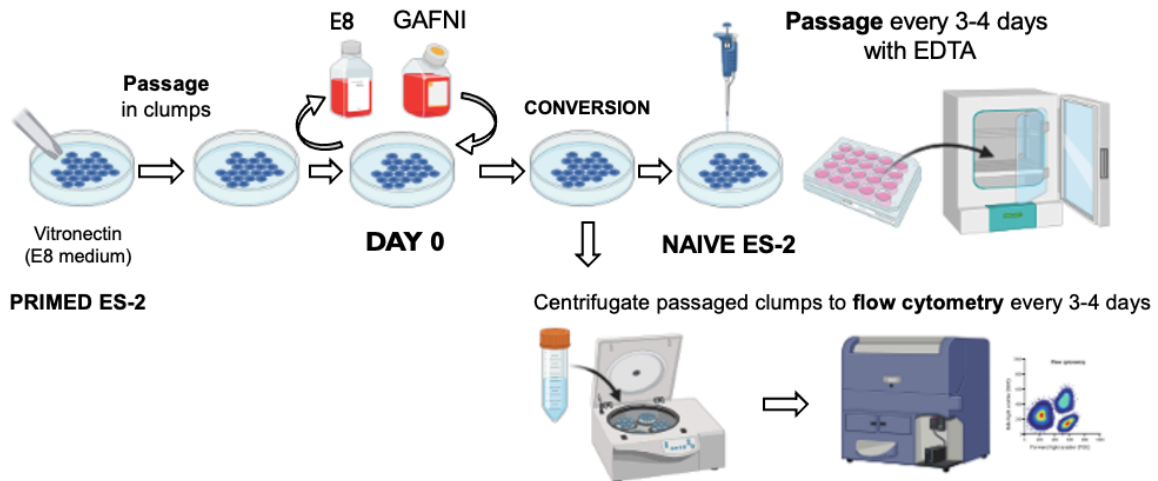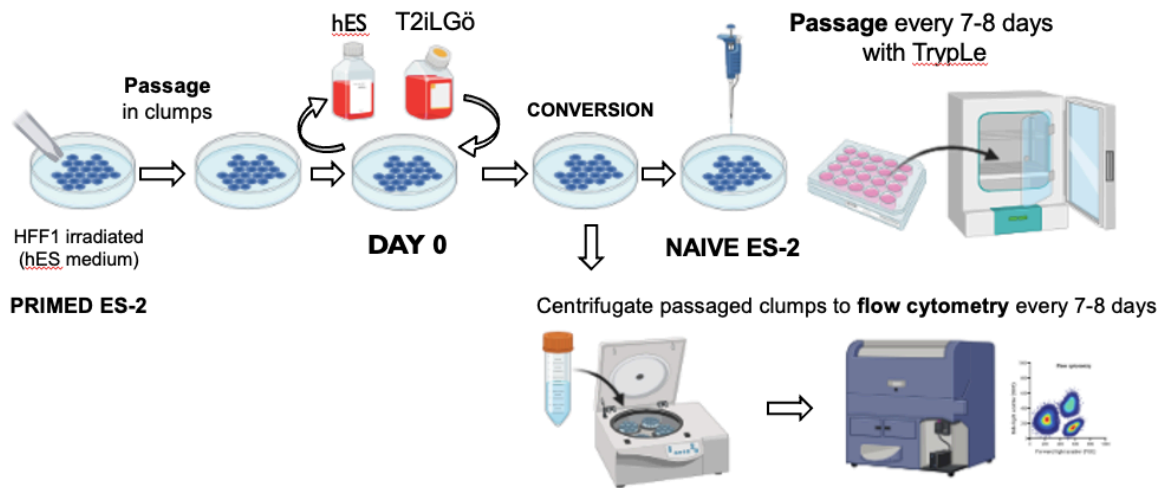

Figure 9. Protocol of naive conversion from primed ES-2 onto feeder-free condition with GAFNI medium.

3.2.2. T2iLGö conversion medium

T2iLGö medium has been established as a naive conversion medium for stem cells grown on feeder condition. This culture media includes DMEM/F12 (Life Technologies), 1% N2 (Life Technologies), 1% B27 (Life Technologies), 1% NEAA (Life Technologies), 1% glutaMAX (Life Technologies), 0.1 mM β-mercaptoethanol (Life Technologies), 50 μg/mL bovine serum albumin (BSA; Sigma) and 0.5% Pen/Strep (Life Technologies) supplemented with 20 ng/mL hLIF (Peprotech), 1 μM PD0325901 (Axon Medchem), 1 μM CHIR99021 (Axon Medchem) and 5 μM Gö6983 (Axon Medchem) (Kilens et al. 2018).

Naive hESCs were passaged using TrypLe solution (which breaks protein bonds between the cells). Cells were washed with 1x PBS and 500 μL of 0,05% TrypLe were added to each well of hESCs. Cells were incubated at 37 °C for 4 minutes. Without aspirating the TrypLe, 1 mL of basal hES medium was added. Cells were disaggregated with a 1-mL micropipette and centrifuged at 1000 rpm for 4 minutes to single cells. After aspirating the supernatant, cells were resuspended in T2iLGö medium and seeded on fresh HFF-1 feeder layer. Medium was changed daily. Cells were passaged every 7-8 days.

When flow cytometry was performed, hESCs were passaged using TrypLe solution and without aspirating the TrypLe, 1 mL of basal hES medium was added. Cells were centrifugated 1000 rpm for 4 minutes at 4ºC (Fig.10).
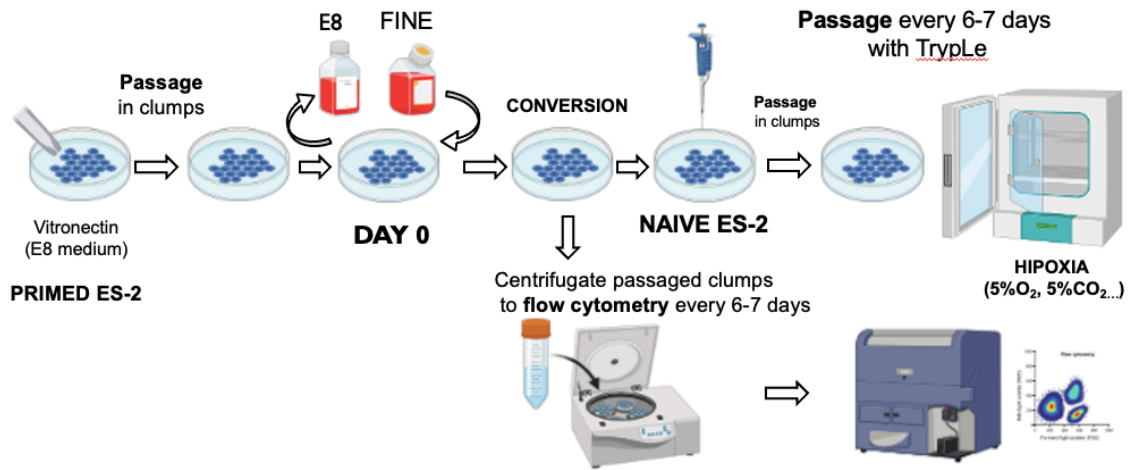

Figure 10. Protocol of naive conversion from primed ES-2 onto feeder condition with T2iLGö medium.

3.2.3. FINE conversion medium

Feeder-independent naive hESCs (FINE) medium is a xeno-free feeder-free medium that includes DMEM/F12 and Neurobasal medium (1:1 ratio, Life Technologies), 1% N2 (Life Technologies), 1% B27 (Life Technologies), 1% glutaMAX (Life Technologies), 1% NEAA (Life Technologies), 0.1 mM β-mercaptoethanol (Life Technologies), and 62.5 ng/mL BSA (Sigma) supplemented with 0.1 µM dasatinib (BDR- ABL/SRCi, STEMCELL), 0.1 µM AZD5438 (CDK1/2/9i, Tocris), 0.1 µM SB590885 (BRAFi, Sigma), 1 µM PD0325901 (Axon Medchem), 10 µM Y-27632 (Axon Medchem), 20 ng/mL hLIF (Peprotech), 20 ng/mL activin A (Peprotech), and 8 ng/mL FGF2 (Miltenyi Biotec) (Szczerbinska et al. 2019).

Naive hESCs were passaged with TrypLe solution. Cells were washed with 1x PBS and 500 µL of TrypLe were added to each well of hESCs. Cells were incubated at 37 °C for 3 minutes. TrypLe was aspirated and washed with 1x PBS. After adding 1 mL of FINE medium, cells were disaggregated with a 1-mL micropipette and centrifuged at 1000 rpm for 4 minutes to single cells. After aspirating the supernatant, cells were resuspended in FINE medium and seeded on fresh HFF-1 feeder layer. Medium was changed daily. Cells were passaged every 6-7 days.

When flow cytometry was performed, hESCs were passaged using TrypLe solution, without aspirating the TrypLe, 1 mL of basal hES medium was added. Cells were centrifugated 1000 rpm for 4 minutes at 4ºC (Fig. 11).

Figure 11. Protocol of naive conversion from primed ES-2 onto feeder-free condition with FINE medium.

3.3. CHARACTERIZATION OF NAIVE COLONIES

In order to determine the conversion from the primed to the naive state of the hESCs, colony morphology was monitored under the microscope together with the expression analysis of different primed and naive markers by flow cytometry.

3.3.1. Morphology

Referring back to the differences in colonies' morphology, primed hESCs form compact and flat colonies whereas naive hESCs form dome-shaped colonies. Pictures of the colonies were taken every 3-6 days during conversion with the Leica DMi8 inverted microscope (Leica Microsystems) from day 0 up to day 30 and day 33 in feeder and feeder-free conditions respectively.

3.3.2. Expression of primed and naive markers

Flow cytometry is a very common tool used for researchers in different fields such as immunology, molecular biology, bacteriology, virology, cancer biology and infectious disease monitoring. Is a technology that provides rapid multi-parametric analysis of live cells in a fluid mixture utilizing lasers as light sources. Fluorescent light labelled cells are detected by

photodiodes or photomultiplier tubes and these signals are analysed by a computer and saved in a standardized format (.fcs) data files. Human embryonic stem cell populations can be analysed based on their fluorescent characteristics (Figure 12).

Many fluorescent reagents are used in flow cytometry. These include fluorescently conjugated antibodies, fluorescent expression proteins, DNA binding dyes, viability dyes and ion indicator dyes.
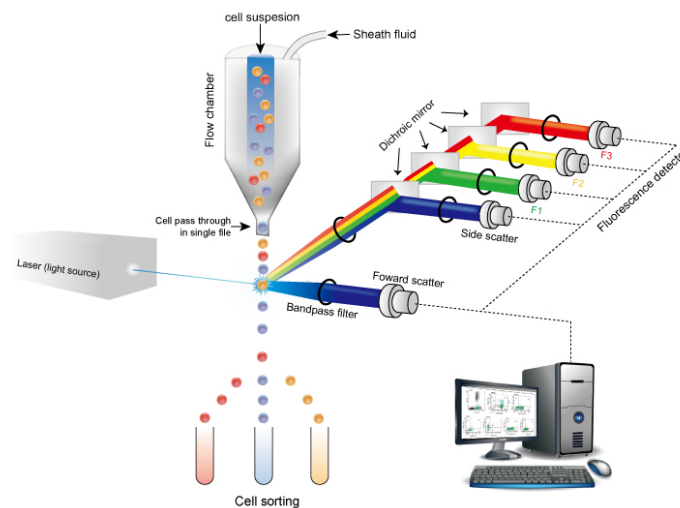
Figure 12. Scheme of flow cytometer procedure.

Cells were recollected and centrifuged. Then, after discarding the supernatant, cells were resuspended in blocking PBS (PBS+10% fetal bovine serum (FBS)) so that there were 2 cytometry tubes with 0.5 mL of staining PBS per sample, plus an unstained tube. Cells were centrifuged at 1500 rpm, 4°C for 4 minutes, and after discarding the supernatant, they were washed again under the same conditions. Next, they were resuspended in 1 mL of PBS and 1 μL of the viability marker (*Life/Death Fixable kit*, Invitrogen) was added. The unstained tube was resuspended in 400 μL of PBS and kept on ice. The samples were incubated on ice at darkness for 30 minutes. Afterwards, they were washed twice (1500 rpm, 4°C for 4 minutes) with PBS; the first time with the 1 mL in which they were kept and the second time with 500 μL. After decanting the supernatant, the selected surface marker antibody was added directly over the residual volume. Samples were incubated on ice at darkness for 30 minutes. Cells were washed twice (1500 rpm, 4°C for 4 minutes) with 500 μL of PBS. Following the last wash, cells were resuspended in 400 μL of PBS. Samples were vortexed briefly before placing them in the cytometer.

The experiments were conducted in a BD FACSCantoTM II Flow Cytometer (BD Biosciences). BD FACSDivaTM Software (BD Biosciences) was used for flow cytometer setting up and data acquisition, while data was analysed with the FlowJoTM v.10.5.3 Software (BD Biosciences).

For describing of the pluripotency status of the samples the following surface biomarkers were selected (Table 2):

- PRIMED: **CD24** (post-implantation state marker) and **CD57** (mature NK and T cell marker).
- NAIVE: **CD75** and **CD130** (LIF co-receptor).
- STEMNESS: **CD90** (related to cell-cell adhesion and communication).
- PLURIPOTENCY: **SSEA-4** (pluripotency marker and is considered a defining factor for hESCs).

Markers								
	488 nm laser				**635 nm laser**		**405 nm laser**	
	FITC	**PE**	**PerCP-Cy5-5A**	**PE-Cy7**	**APC**	**APC-Cy7-A**	**Pacific Blue**	**BV510**
Tube 1	CD75	CD24		CD90	CD57	Viability	CD130	
Tube 2		SSEA-4				Viability		

Table 2. Designed panel of primed and naive markers to study by flow cytometry. Surface antibodies and viability marker.

4. RESULTS

4.1. NAIVE CONVERSION IN FEEDER CONDITIONS

4.1.1. GAFNI CONVERSION MEDIA

4.1.1.1. Morphology

The morphology is one of the main features to observe in the conversion from primed colonies to naive colonies. The day 0 represents primed hES-2 in feeder condition forming flat colonies. Cells grown on Gafni medium were converted in 29 days although around day 6 the transition to naive state was significantly appreciated (Fig. 13).

After passing primed colonies around two times, naive colonies with Gafni medium started to grow very fast, in multitude and with a very round and small form.

DAY 0   DAY 6   DAY 9

DAY 14   DAY 21   DAY 29
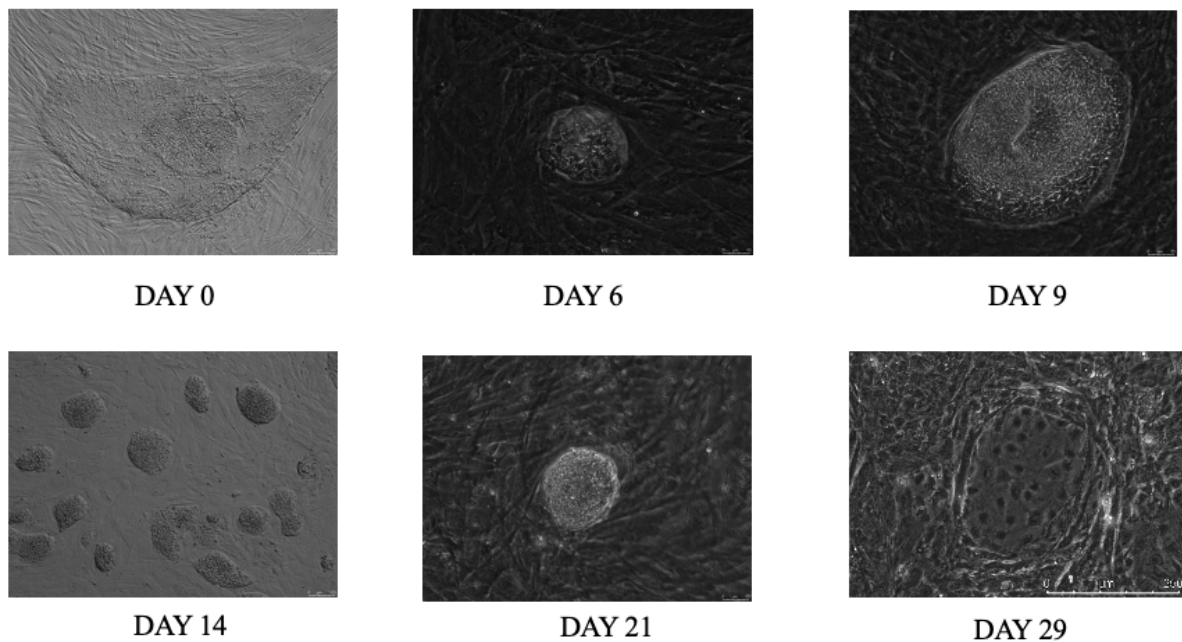
Figure 13. ES-2 colonies grown in feeder condition over time with Gafni medium. Images taken with light microscope throughout the naive conversion during 30 days. Microscope magnification= 10x.

4.1.1.2. Primed and naive status, cell viability, stemness and pluripotency

In addition to morphology, in order to study the correct conversion of the colonies to the naive state, different markers of primed, naive, cell viability, stemness and pluripotency are analysed by flow cytometry.

In cells grown on Gafni medium and feeder conditions, there was not a great decrease in primed markers in CD24+/CD57+ population over the 31 days. Curiously, the CD24+/CD57+ population went up around day 10 and 17 but decreased again around day 24. CD24+/CD57- plot is not very informative during the days since there is a low increase at day 3 and at day 24 but afterwards at day 31 there is a very notorious decrease (1,17%). CD24-/CD57+ population were significantly increased compared to the control at day 0. This increase may be related to the "pathway" that the colonies take to reach the two negative primed markers since after this increase of CD57 marker, cells become double negative and CD57 marker also decrease (Fig. 14).
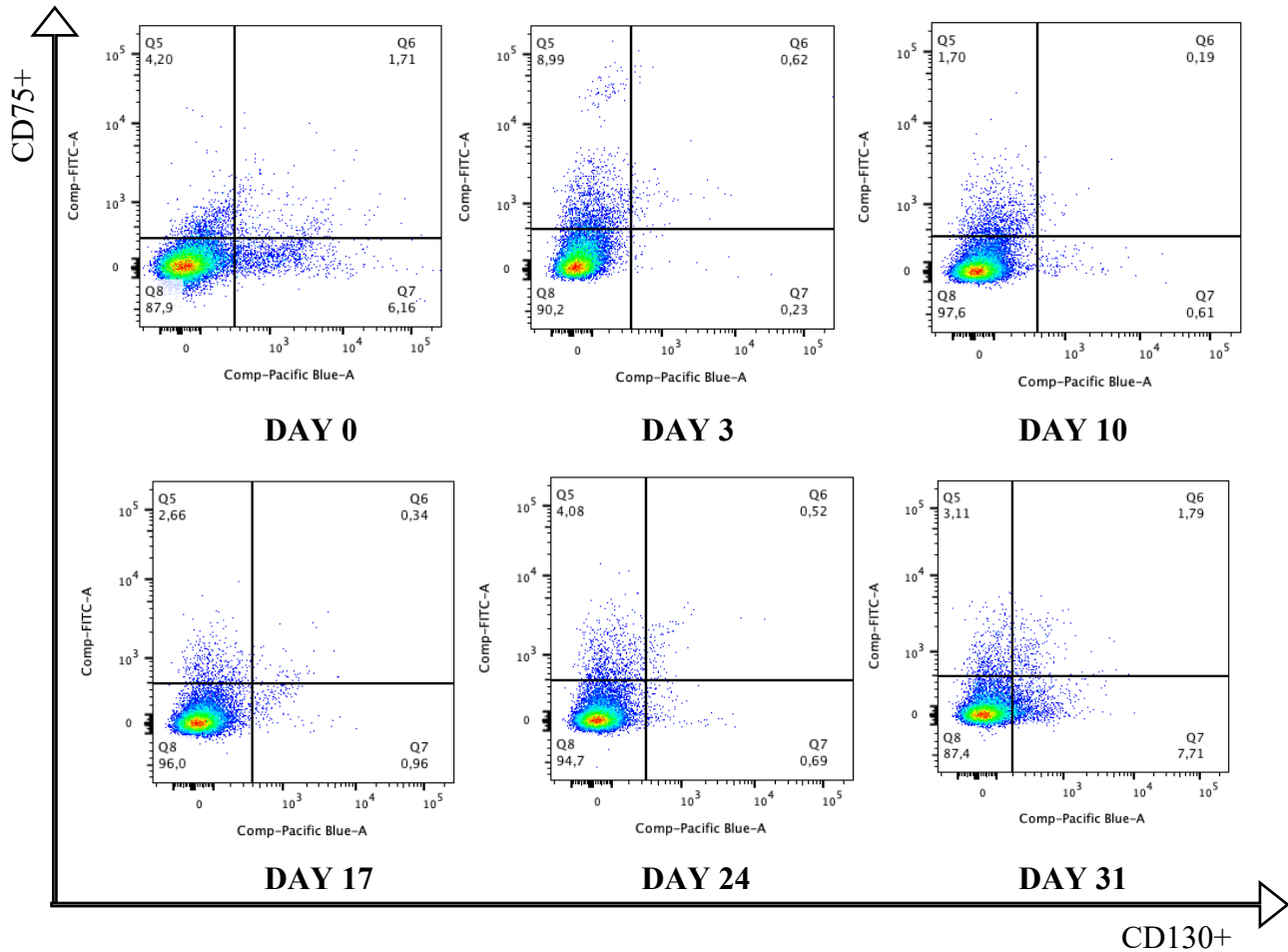
- **Primed markers**
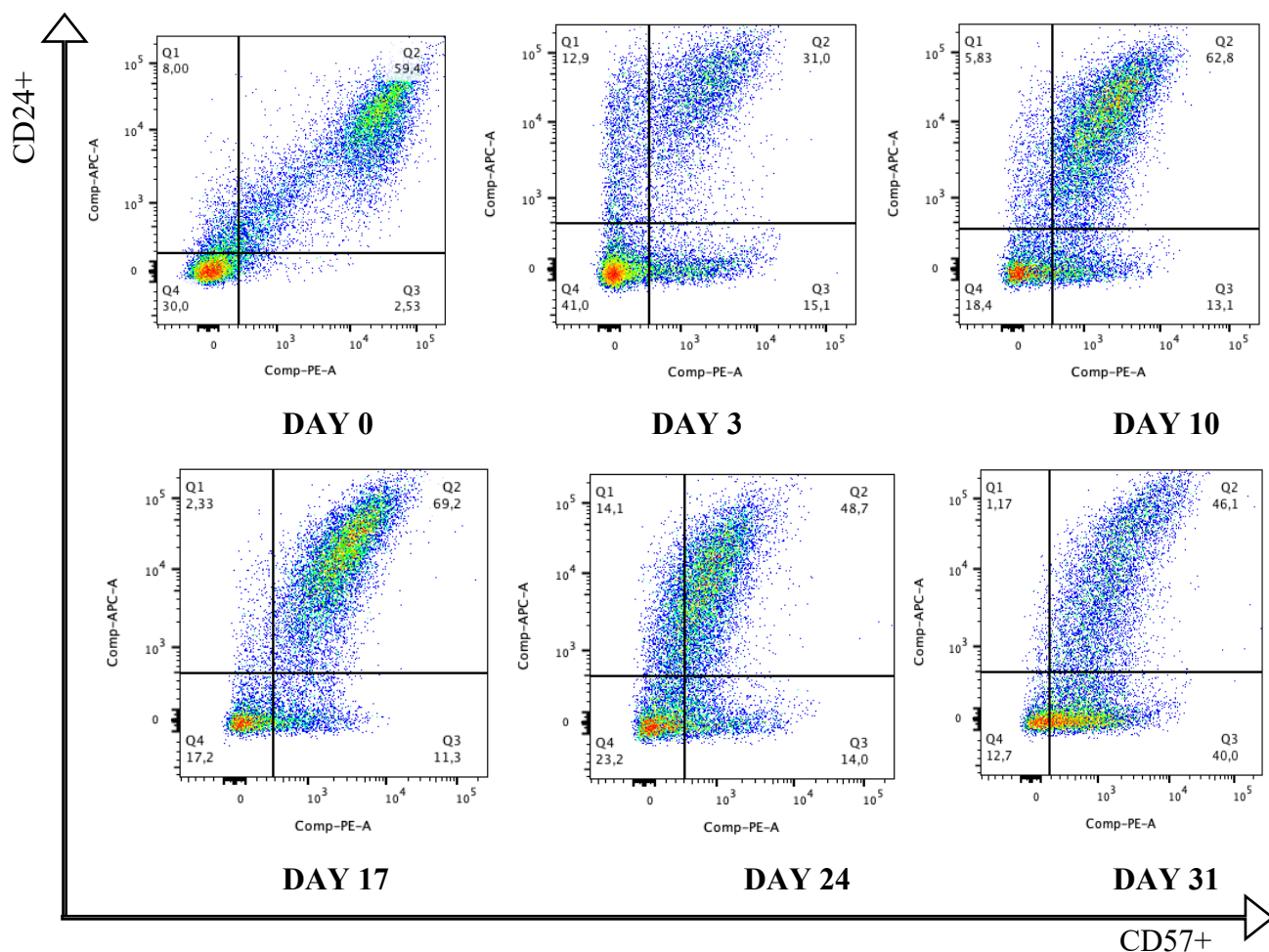
Figure 14. Expression of primed markers in feeder conditions with Gafni medium over time during naive conversion. Samples were stained with primed markers CD24 and CD57 and flow cytometry was performed at day 0, 3, 10, 17, 24 and 31 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

In contrast, an increase in naive markers was expected during the conversion, but there is a few or no increase. There may be a slight increase in the CD75+/CD130- population around day 3 but compared to the control, at day 31 CD75+/CD130+ population remained practically the same (Fig. 15).

- **Naive markers**

Figure 15. Expression of naive markers in feeder conditions with Gafni medium over time during naive conversion. Samples were stained with naive markers CD75 and CD130 and flow cytometry was performed at day 0, 3, 10, 17, 24 and 31 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

Cells grown on Gafni medium showed significantly lower levels of expression of the stemness marker CD90 compared to day 3 (Fig. 16).

- **CD90+**

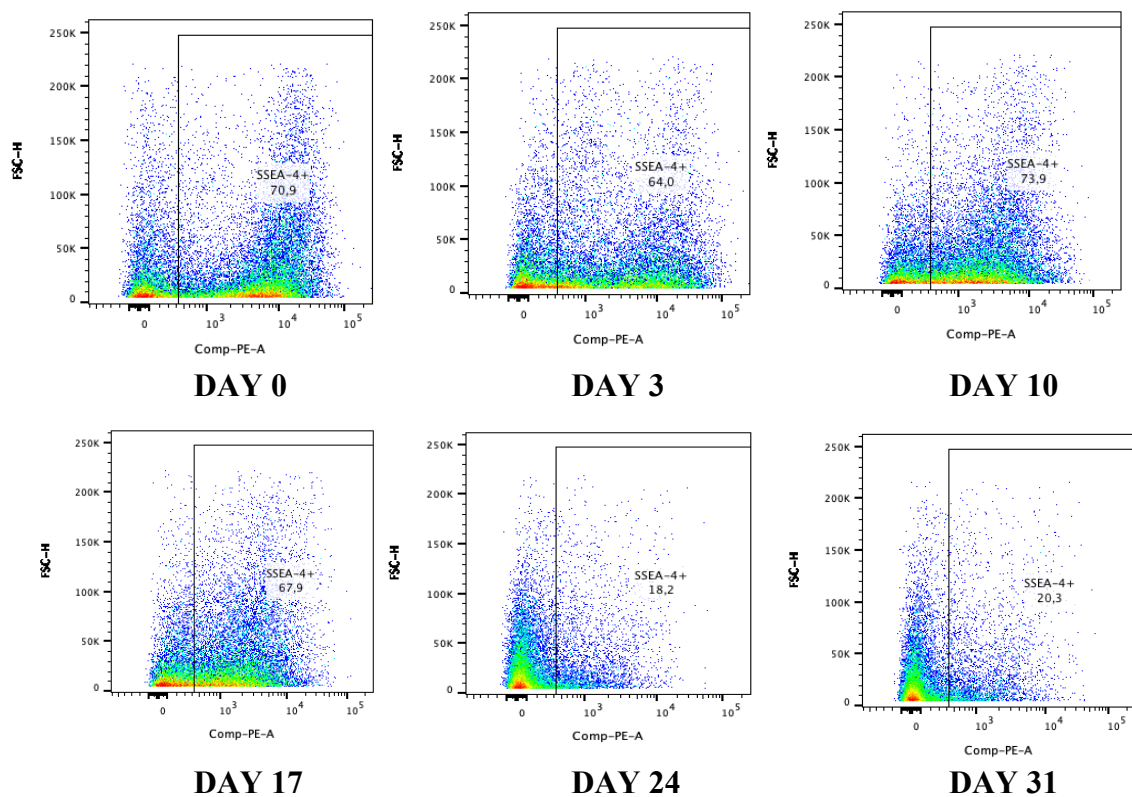**DAY 3**   **DAY 10**   **DAY 24**
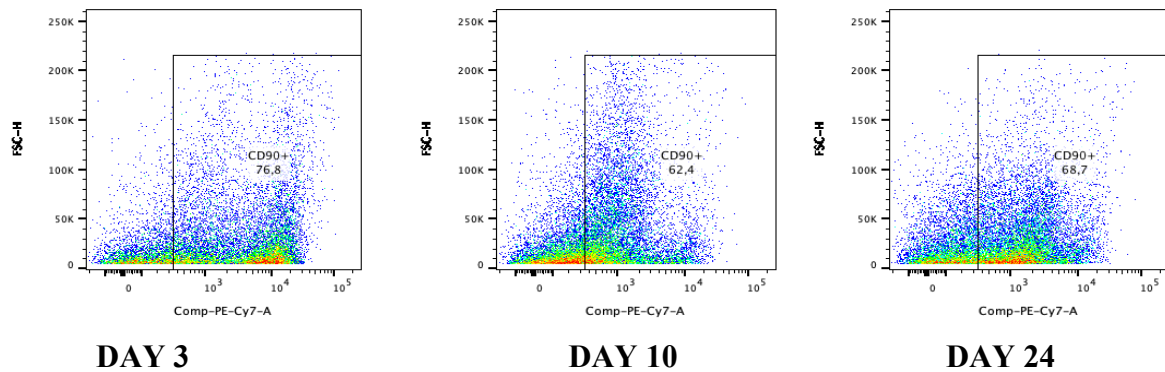
Figure 16. Expression of the stemness marker CD90 in feeder condition over time naive expression. Flow cytometry was performed at day 3, 10 and 24 during conversion on Gafni medium.

Regarding the expression of the pluripotency marker SSEA-4, cells grown on Gafni medium showed an increase of the marker around day 10, however, afterwards, consistently SSEA-4 expression decreased over time (Fig. 17).

- **SSEA-4+**

DAY 0   **DAY 3**   **DAY 10**

DAY 17   **DAY 24**   **DAY 31**
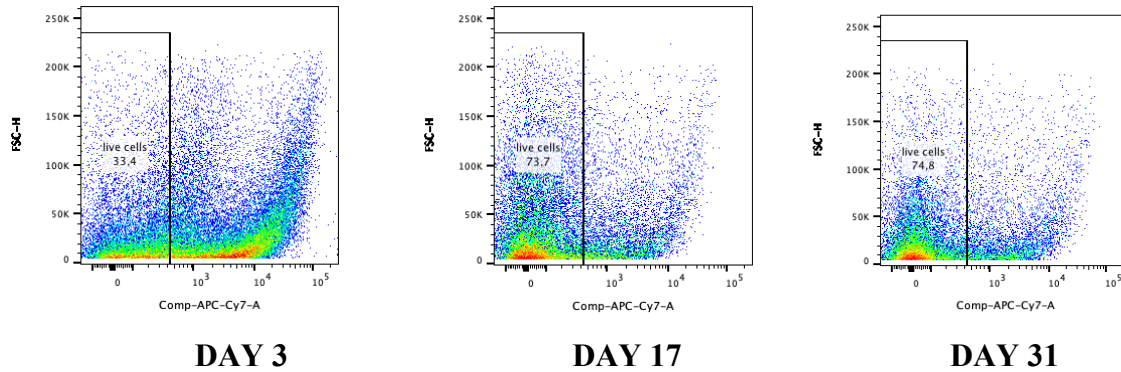
Figure 17. Expression of the pluripotency marker SSEA-4 in feeder condition over time naive conversion. Flow cytometry was performed at day 0, 3, 10, 17, 24 and 31 during conversion on Gafni medium.

In the early days during conversion, when primed medium is replaced, cell viability decreased significantly, however, at day 31 cell viability increased in cells grown on Gafni medium (Fig. 18).

- **Cell viability**

| DAY 3 | DAY 17 | DAY 31 |

Figure 18. Cell viability of ES-2 grown in feeder condition over time during naive conversion. Flow cytometry was performed at day 3, 17 and 31 during conversion on Gafni medium.

4.1.2. T2ILGÖ CONVERSION MEDIA

4.1.2.1. Morphology

Similar to what was observed above in feeder condition with Gafni medium, ES-2 colonies grown on T2ilGö transformed from flat colonies to dome-shaped colonies. Thanks to the contrast of the feeder layer formed fibroblasts, the nuclei are in dark black and is clearly visible colonies fully transited to a naive state (Fig. 19).
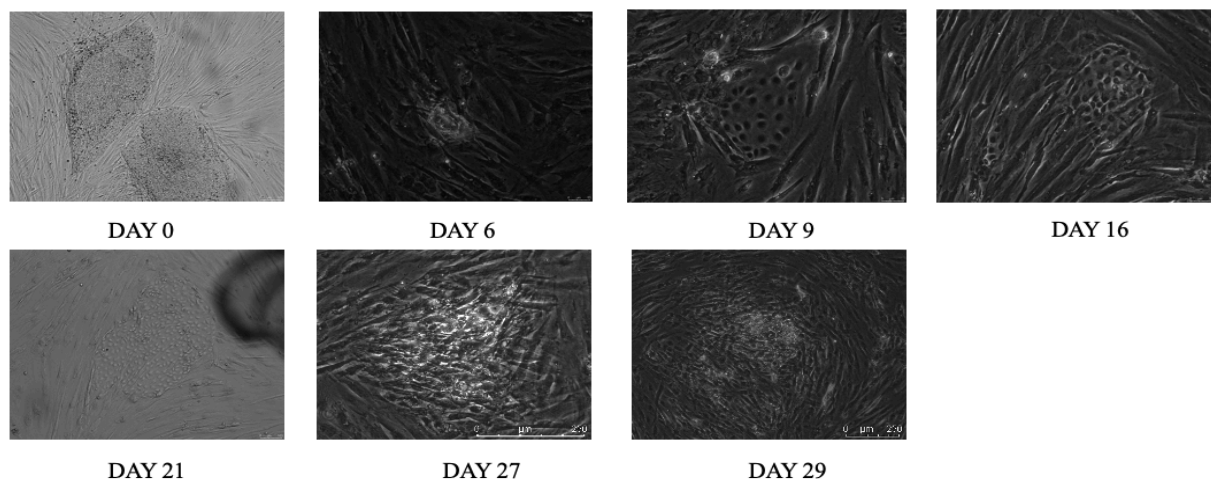

Figure 19. ES-2 colonies grown in feeder condition over time with T2ilGö conversion medium. Images taken with the light microscope throughout the naive conversion during 30 days. Microscope magnification= 10x and 4x.

4.1.2.2.   Primed and naive status, cell viability, stemness and pluripotency

As with the previous condition, stemness, viability, pluripotency and conversion markers were analysed during the transition to the naive state.

Compared to the control at day 0, for colonies grown on T2ilGö medium the CD24-/CD57+ population consistently increase during naive conversion until day 31. Moreover, interestingly the CD24+/CD57+ population decreased favourably during conversion over time (Fig. 20).

- **Primed markers**
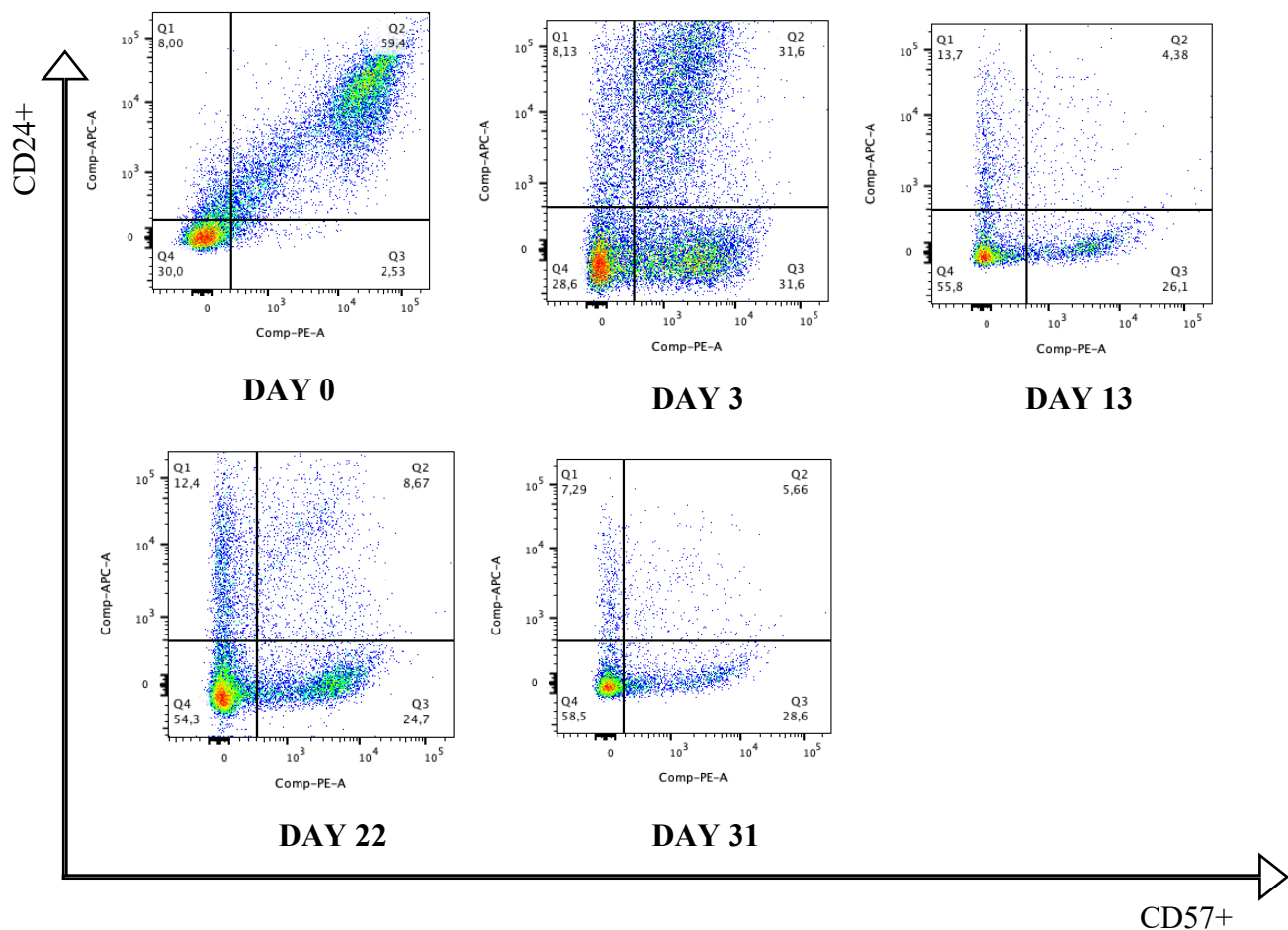
Figure 20. Expression of primed markers in feeder conditions with T2ilGö medium over time during naive conversion. Samples were stained with primed markers CD24 and CD57 and flow cytometry was performed at day 0, 3, 13, 22 and 31 during conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

In contrast, as expected from the expression of primed pluripotency markers, naive markers have not increased significantly. There was a slight increase in the CD75+/CD130- population and CD75+/CD130+ population over time (Fig. 21).
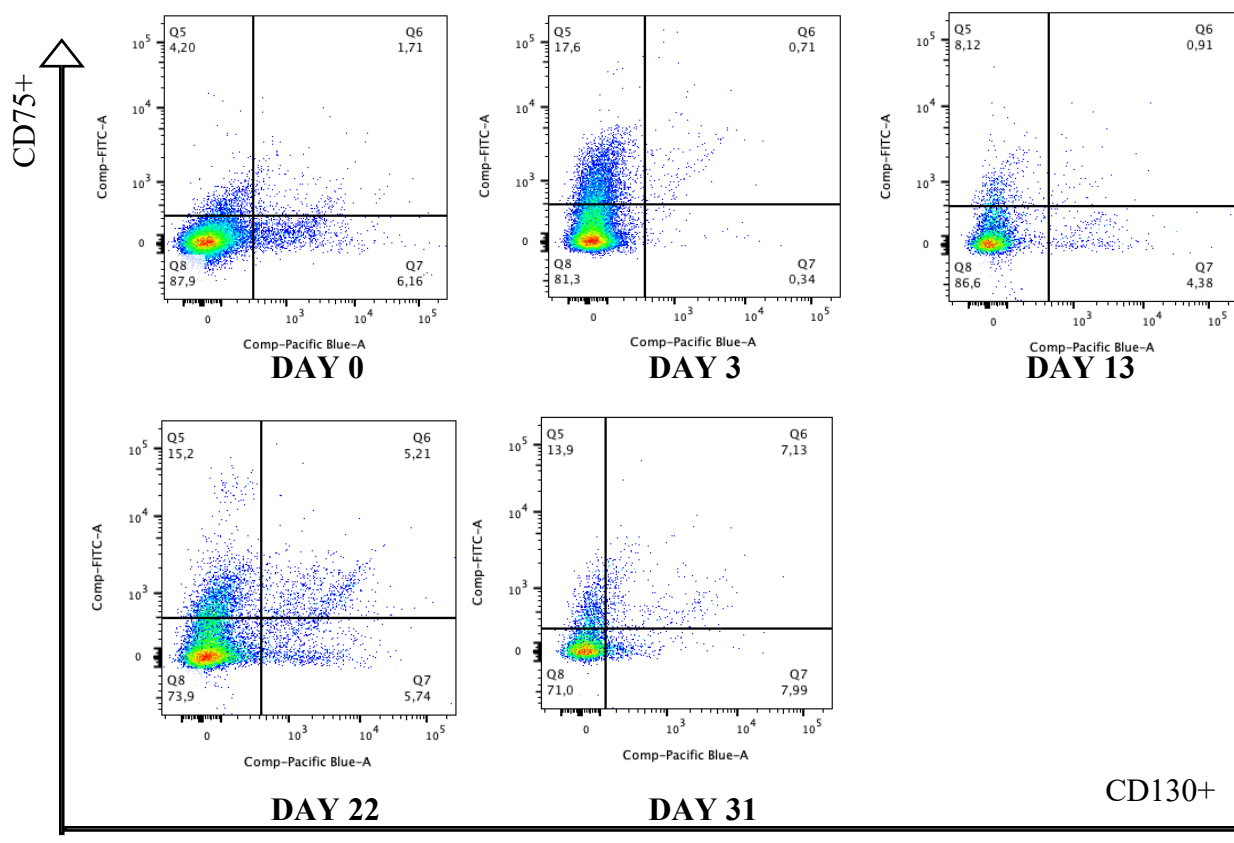
- **Naive markers**

Figure 21. Expression of naive markers in feeder conditions with T2ilGö medium over time during naive conversion. Samples were stained with naive markers CD75 and CD130 and flow cytometry was performed at day 0, 3, 13, 22 and 31 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

Stemness marker CD90 decreased notoriously during naive conversion (Fig. 22).

- **CD90+**
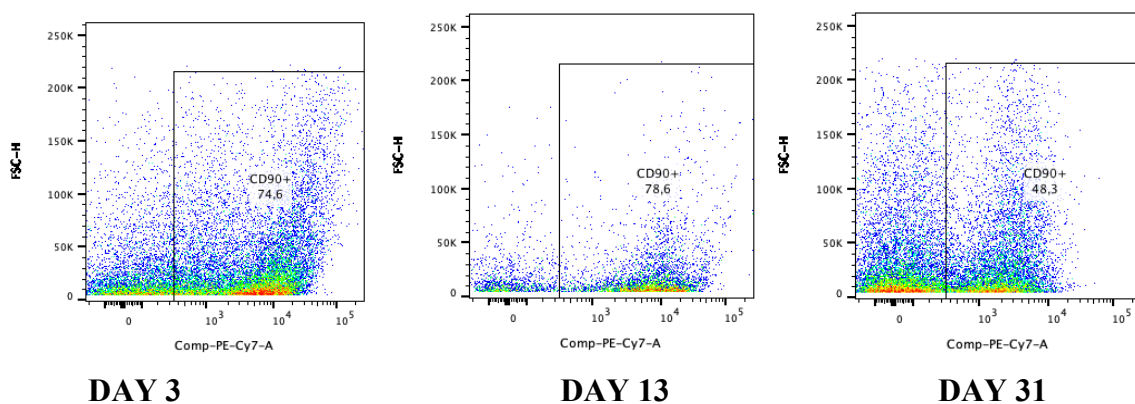
Figure 22. Expression of the stemness marker CD90 in feeder condition over time naive expression. Flow cytometry was performed at day 3, 10 and 24 during conversion on Gafni medium.

In the early days during conversion, cells grow on T2ilGö medium showed higher levels of expression of the pluripotency marker SSEA-4 compared to the control of day 0. However, SSEA-4+ cell population decreased significantly afterwards up to 31 days (Fig. 23).

- **SSEA-4+**

DAY 0   **DAY 3**   **DAY 13**

DAY 24   **DAY 31**
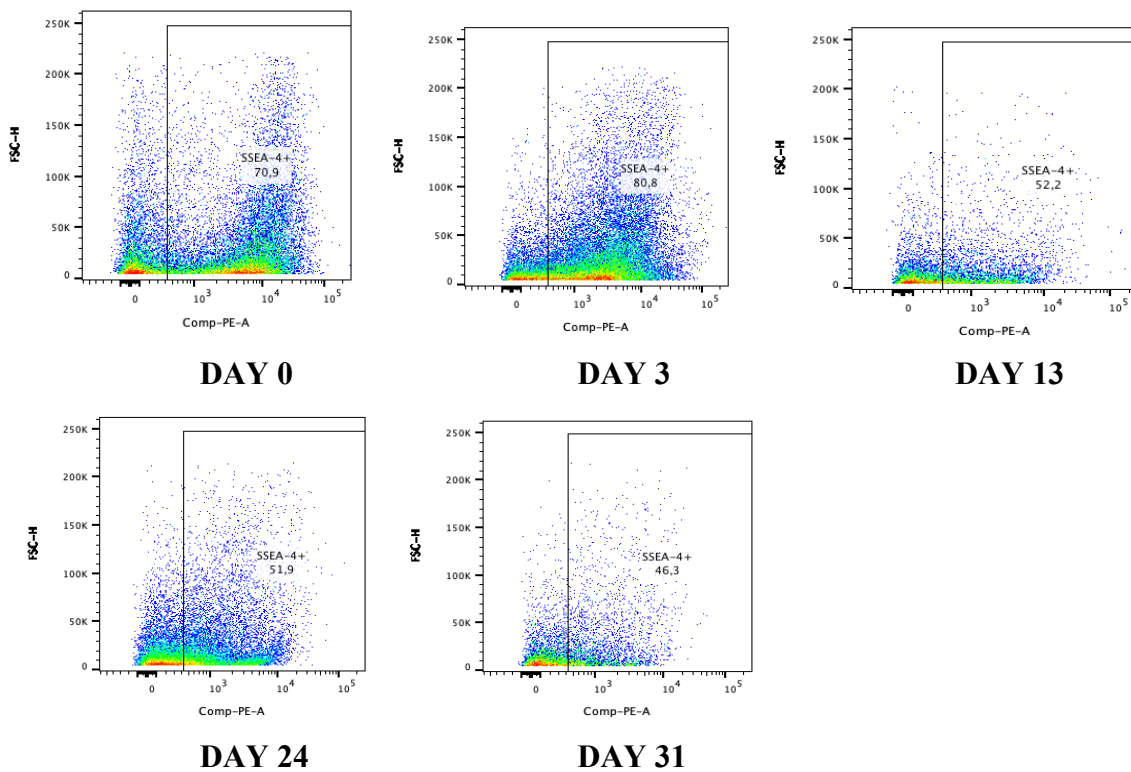
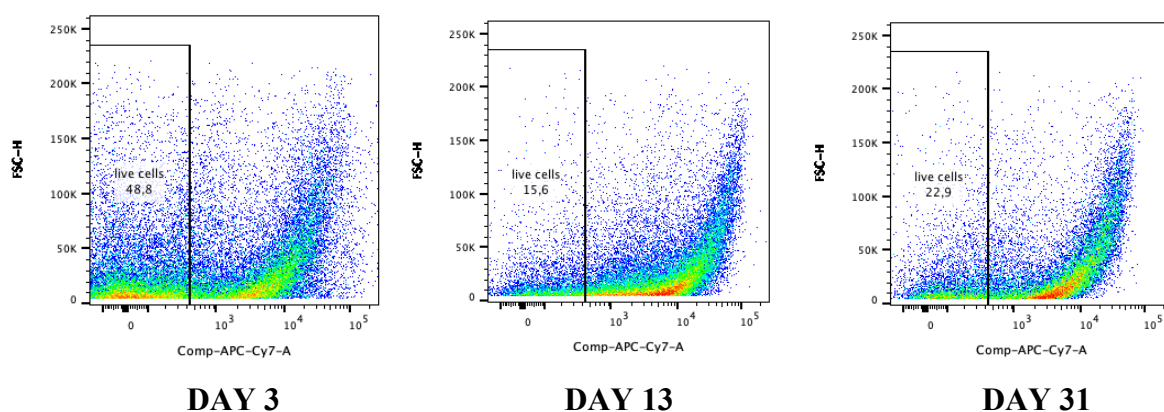**Figure 23. Expression of the pluripotency marker SSEA-4 in feeder condition over time naive conversion.** Flow cytometry was performed at day 0, 3, 13, 24 and 31 during conversion on T2ilGö medium.

Lastly, as mentioned above in cell morphology, unfortunately cell viability decreased during the naive conversion (Figure 24).

- **Cell viability**

**DAY 3**   **DAY 13**   **DAY 31**

Figure 24. Cell viability of ES-2 grown in feeder condition over time during naive conversion. Flow cytometry was performed at day 3, 13 and 31 during conversion on T2ilGö medium.

4.2. NAIVE CONVERSION IN FEEDER-FREE CONDITIONS

4.2.1. GAFNI CONVERSION MEDIA

4.2.1.1.   Morphology

In feeder conditions, HFF-1 fibroblasts act as a helper for the correct growth and proliferation of the colonies. In this case, in feeder-free conditions, it is only a coating of vitronectin that helps them to grow. The fact that they are not onto a monolayer of fibroblasts the morphology change under the microscope is noticeable. Colonies in the primed state are squarer and bigger, when are transformed to naive state, however, they are much rounder and smaller. As can be seen in the figure 25, cells grown on Gafni medium convert in about 5 days.

DAY 0   DAY 5   DAY 14

DAY 22   DAY 30
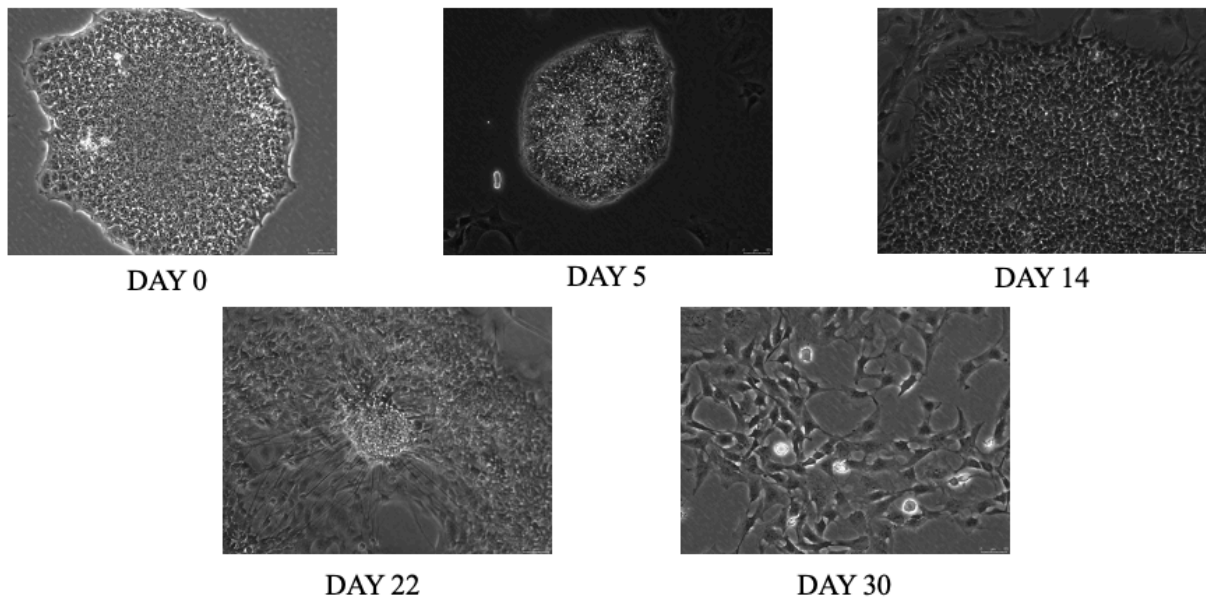
Figure 25. ES-2 colonies grown in feeder condition over time with Gafni medium. Images taken with the light microscope throughout the naive conversion during 30 days. Microscope magnification= 10x.

4.2.1.2.   Primed and naive status, cell viability, stemness and pluripotency

As referred to the cytometry results in feeder conditions, colonies grown on Gafni medium in feeder-free conditions show their possible trajectory to become CD24-/CD57-. There was a decrease in CD24+/CD57+ population while there was a considerably increase in CD24+/CD57- population on Gafni culture condition over time (Fig. 26).

- **Primed markers**
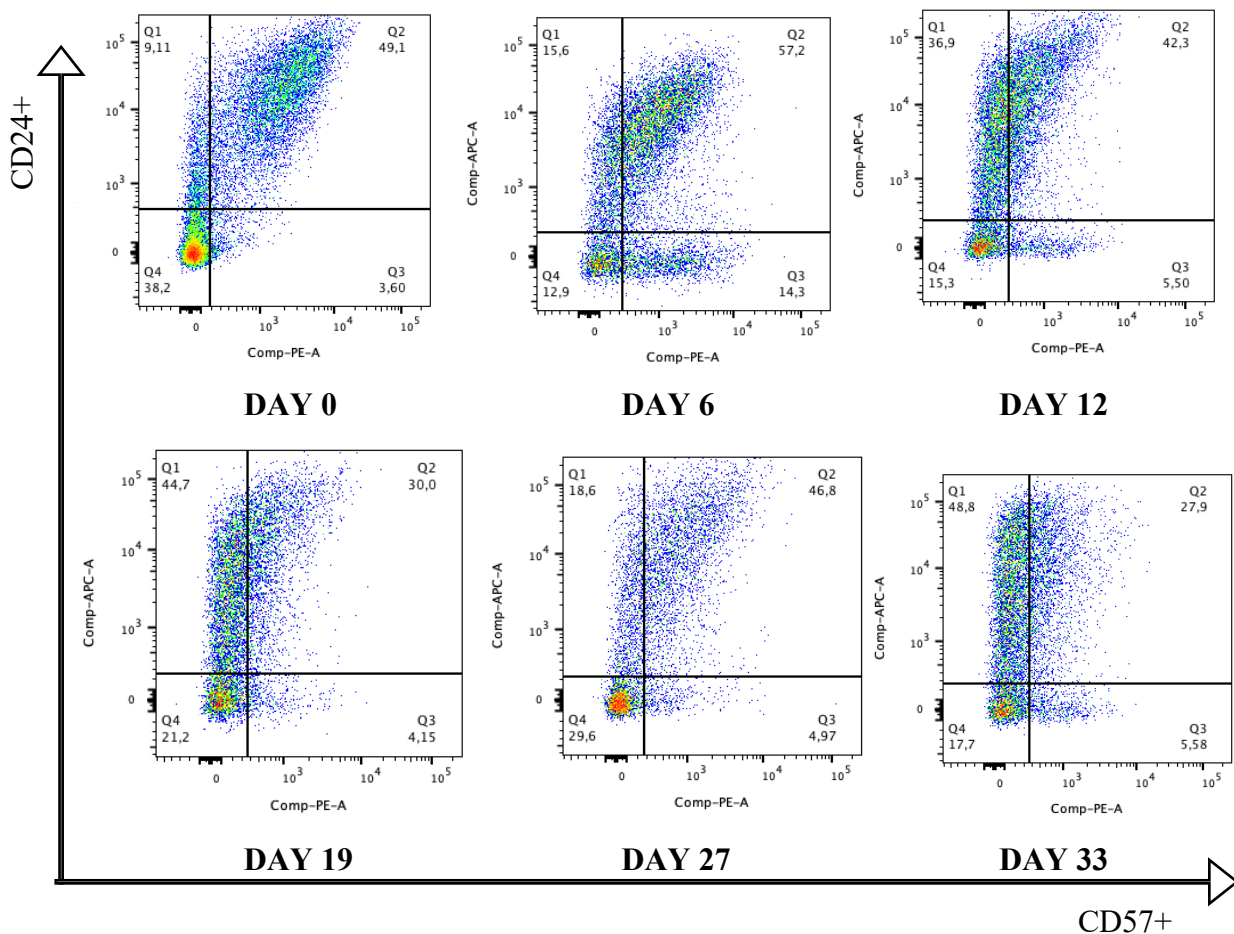
Figure 26. Expression of primed markers in feeder-free conditions with Gafni medium over time during naive conversion. Samples were stained with primed markers CD24 and CD57 and flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

On the contrary, double positive cells expressing naive-associated markers was not increased barely. At day 27 CD75-/CD130+ population increased but surprisingly, afterwards, at day 33 decreased practically totally (Fig.27).

- **Naive markers**
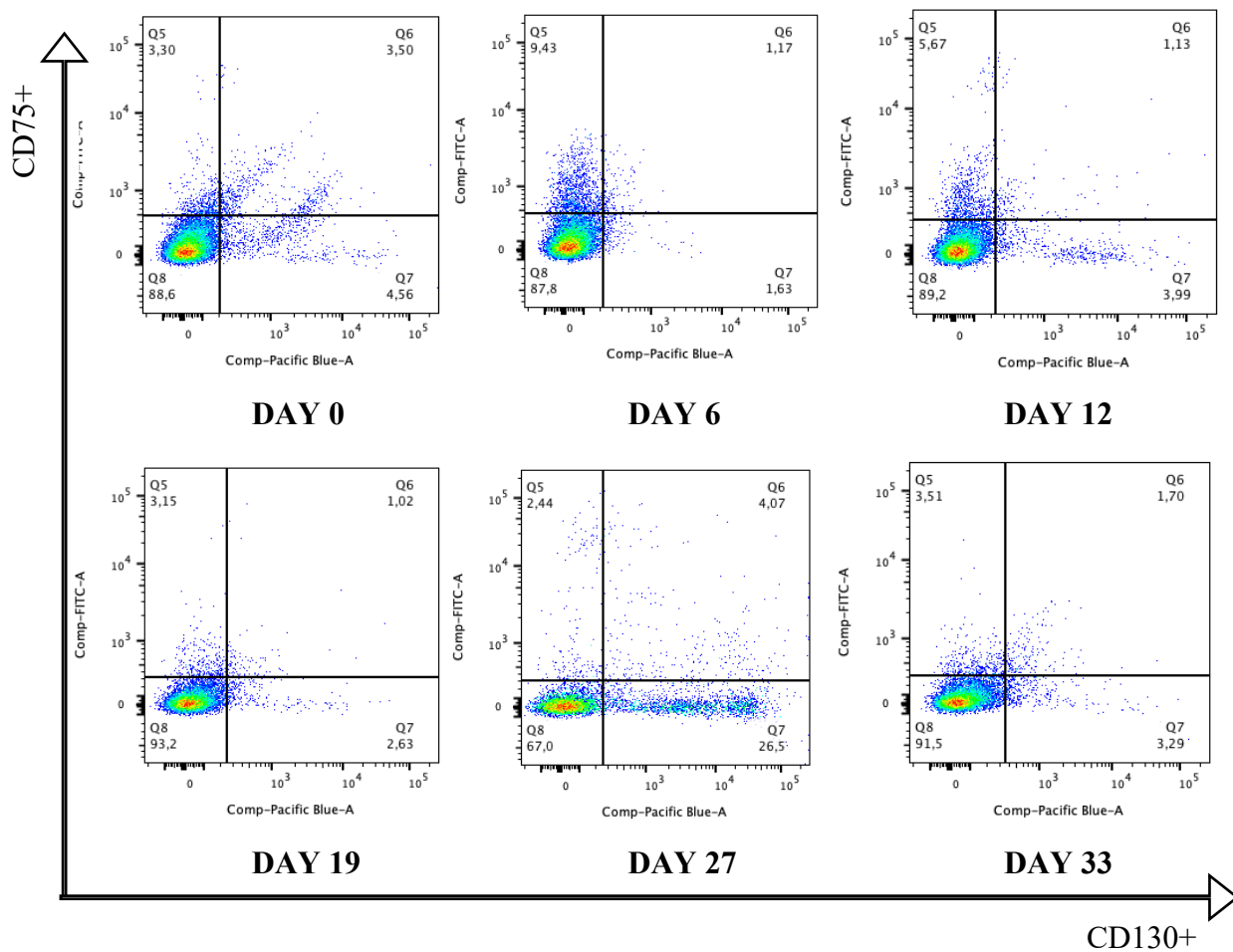
Figure 27. Expression of naive markers in feeder-free conditions with Gafni medium over time during naive conversion. Samples were stained with naive markers CD75 and CD130 and flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

Remarkably, for cells grown on Gafni medium in feeder-free conditions, the stemness marker CD90 increased during the naive conversion (Fig. 28).

- **CD90+**

DAY 0   **DAY 19**   **DAY 27**
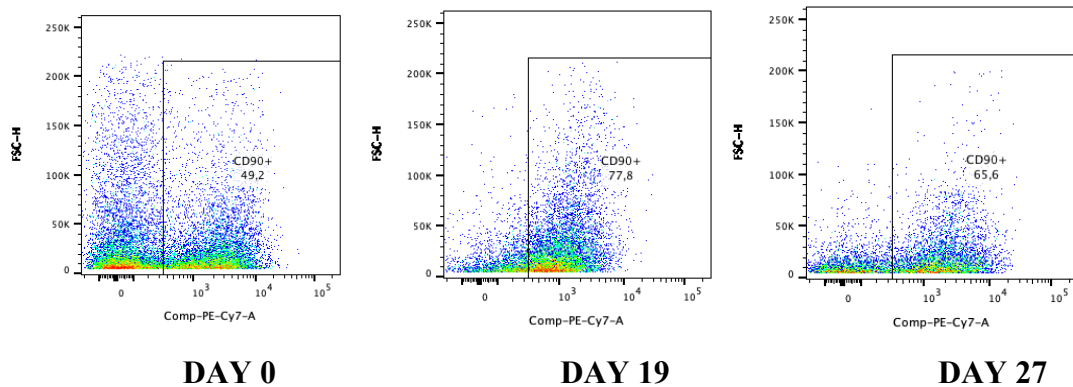
Figure 28. Expression of the stemness marker CD90 in feeder-free condition over time naive expression. Flow cytometry was performed at day 0, 19 and 27 during conversion on Gafni medium.

Compared to the control of day 0, primed hES-2 colonies notoriously decreased expression of pluripotency marker SSEA-4 during naive conversion until 33 days (Fig.29).

- **SSEA-4+**

DAY 0   **DAY 6**   **DAY 12**

DAY 19   **DAY 27**   **DAY 33**
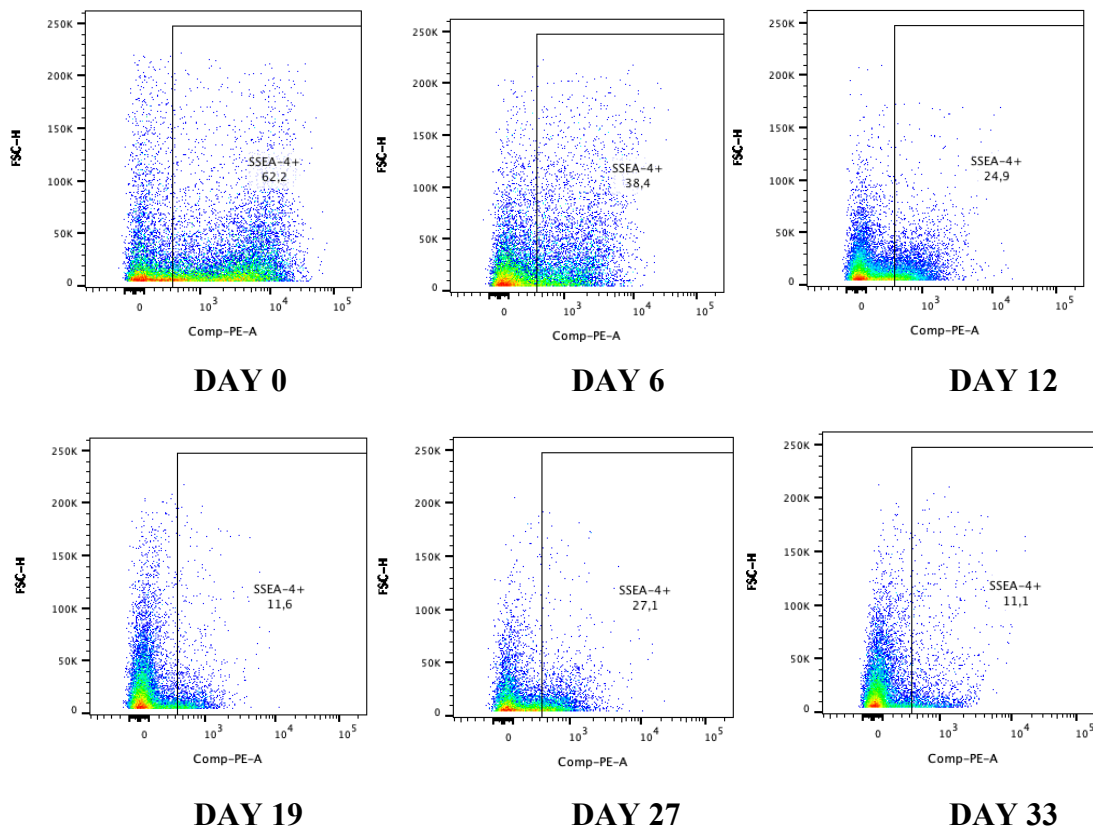
Figure 29. Expression of the pluripotency marker SSEA-4 in feeder-free condition over time naive conversion. Flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during conversion on Gafni medium.

Despite the cells undergo several passages, cell viability remains fairly constant from day 0 (Fig. 30).

- **Cell viability**

DAY 0   **DAY 19**   **DAY 33**
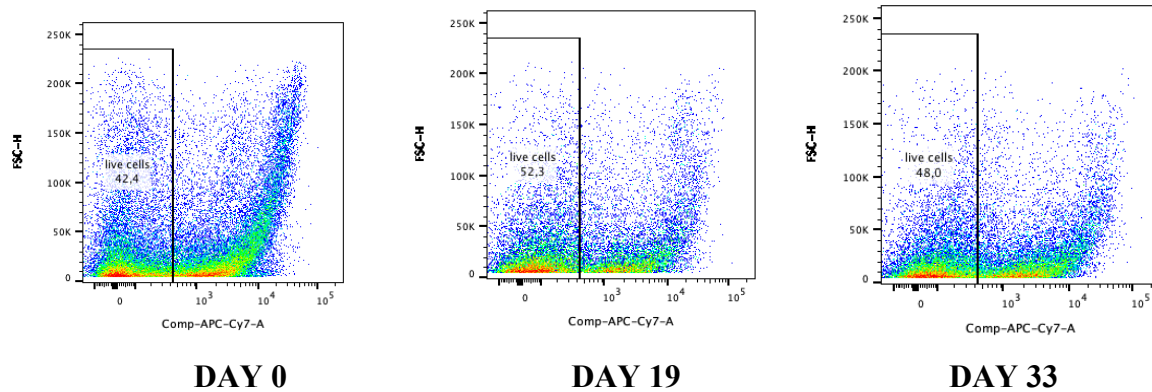
Figure 30. Cell viability of ES-2 grown in feeder-free condition over time during naive conversion. Flow cytometry was performed at day 0, 19 and 33 during conversion on Gafni conversion medium.

4.2.2. FINE CONVERSION MEDIA

4.2.2.1. Morphology

ES-2 colonies with FINE medium in feeder-free conditions are fully converted in about 5 days. The morphology of the colonies changes from flat colonies to dome-shaped colonies and in addition, the colonies form a stellate structure during conversion to a naive state up to 30 days (Fig.31).

DAY 0   DAY 5   DAY 14

DAY 22   DAY 30
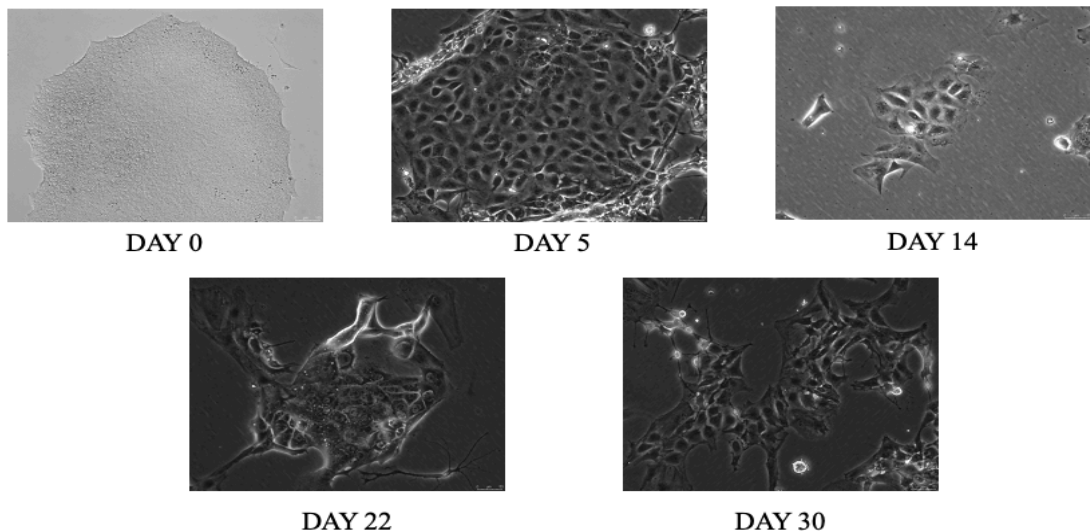
Figure 31. ES-2 colonies grown in feeder-free condition over time with Fine medium. Images taken with the microscope throughout the naive conversion during 30 days. Microscope magnification= 10x and 4x.

4.2.2.2.   Primed and naive status, cell viability, stemness and pluripotency

To evaluate the correct transition to naive state of the ES-2 cells, it is necessary to determine cell viability, stemness and conversion markers among others.

Flow cytometry results showed that, comparing to the control, the decrease in primed markers of ES-2 cells on Fine medium in feeder-free conditions is very notorious. Although CD24+/CD130+ population went up at day 6, started decreasing greatly by day 12 suggesting a considerable conversion to CD24-/CD130- (Fig. 32).

- **Primed markers**
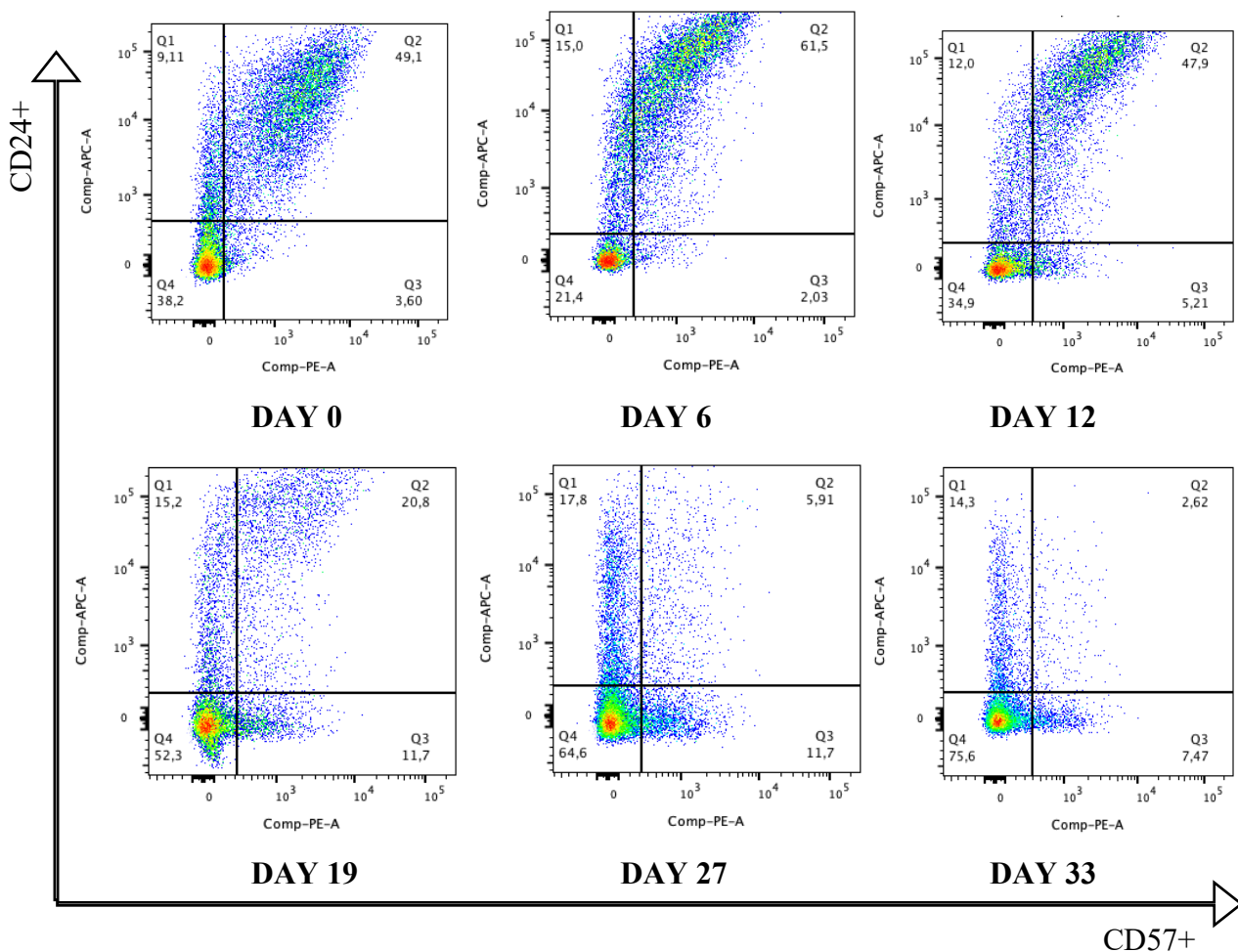
Figure 32. Expression of primed markers in feeder-free conditions with Fine medium over time during naive conversion. Samples were stained with primed markers CD24 and CD57 and flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

As for the naive markers CD75 and CD130, there is a slight increase especially in CD75-/CD130+ population around day 19, however, the changes on doble positive populations are not very significant (Fig. 33).

- **Naive markers**
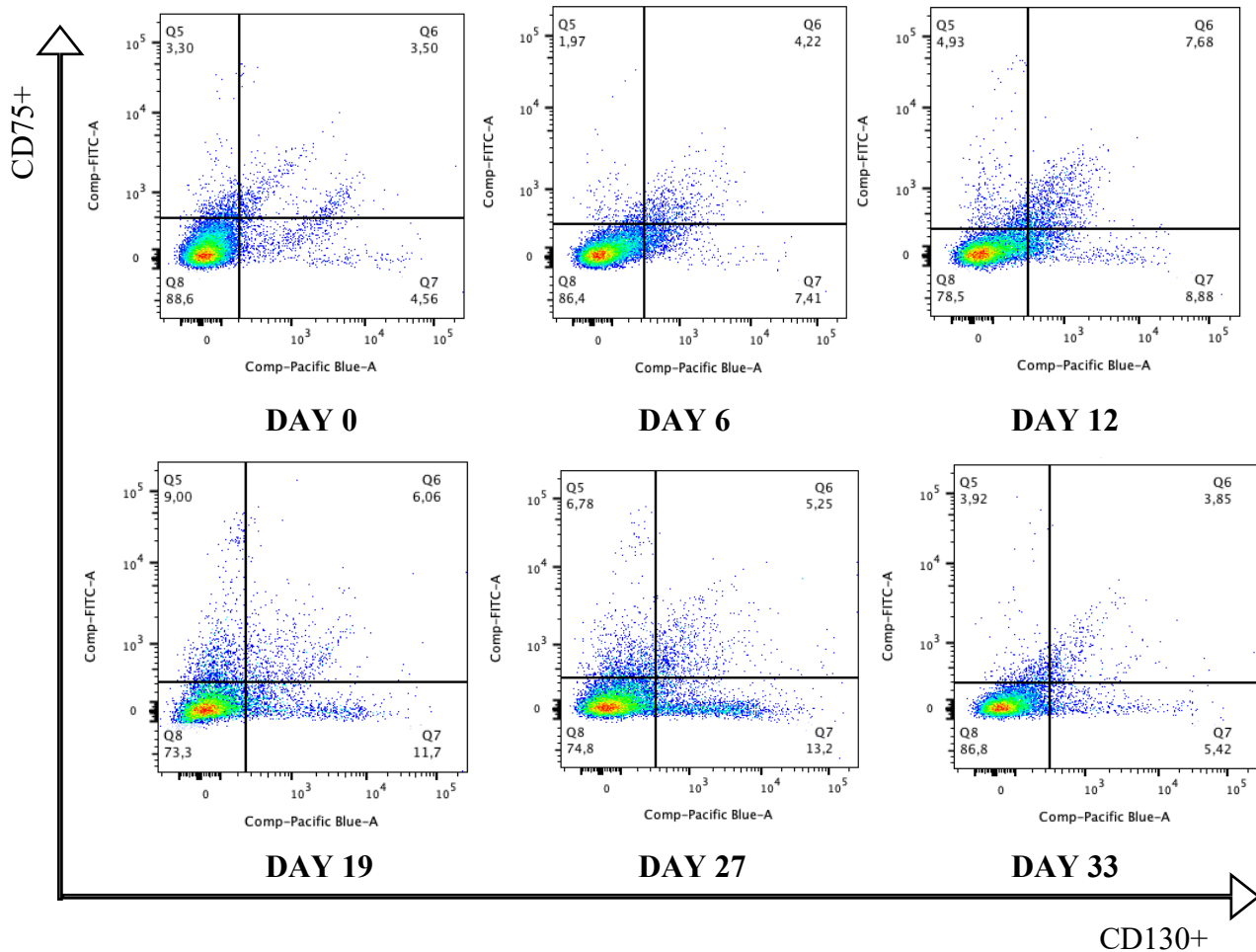
Figure 33. Expression of naive markers in feeder-free conditions with Fine medium over time during naive conversion. Samples were stained with naive markers CD75 and CD130 and flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during naive conversion. The numbers in the plots indicate the percentage of total cells analysed by flow cytometry.

Compared to the control of day 0, stemness marker CD90 decreased very significantly over time (Fig. 34).

- **CD90+**

DAY 0   **DAY 12**   **DAY 33**
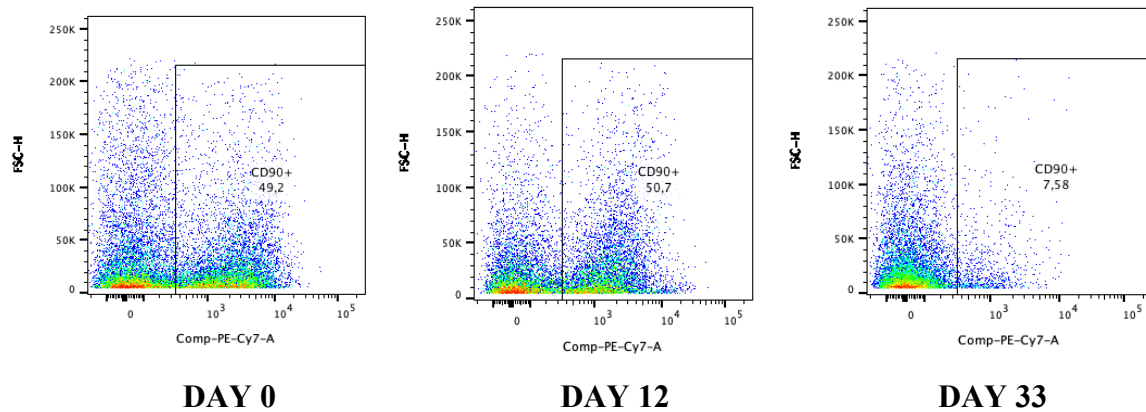
Figure 34. Expression of the stemness marker CD90 in feeder-free condition over time naive expression. Flow cytometry was performed at day 0, 12 and 33 during conversion on Fine medium.

Moreover, although there is a little increased around day 19, pluripotency marker SSEA-4 decreased over time compared to the control of day 0 (Fig. 35).

- **SSEA-4+**

DAY 0   **DAY 6**   **DAY 12**

DAY 19   **DAY 27**   **DAY 33**
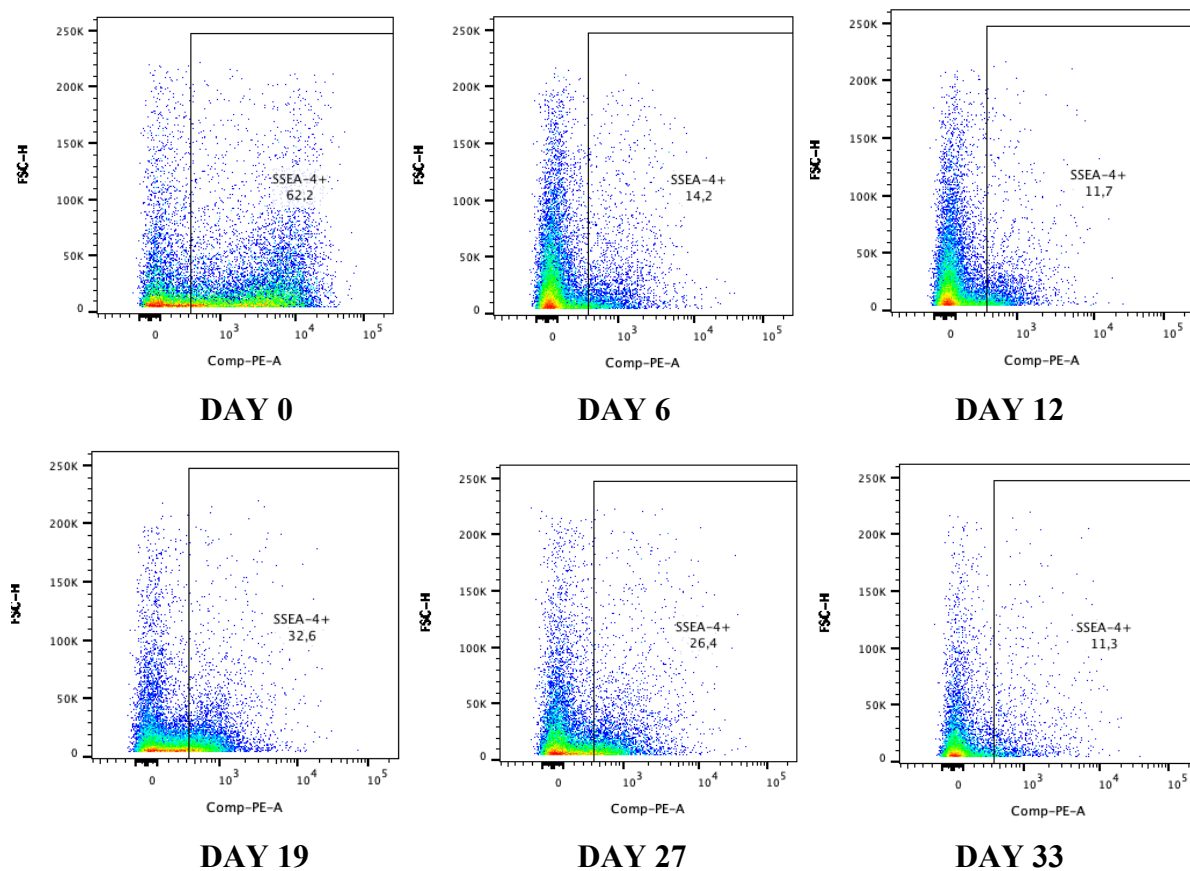
Figure 35. Expression of the pluripotency marker SSEA-4 in feeder-free condition over time naive conversion. Flow cytometry was performed at day 0, 6, 12, 19, 27 and 33 during conversion on Fine medium.

Lastly, for cells grown on Fine medium, cell viability decreased a few during naive conversion from day 0 to day 33 (Fig. 36).

- **Cell viability**

| DAY 0 | DAY 12 | DAY 33 |
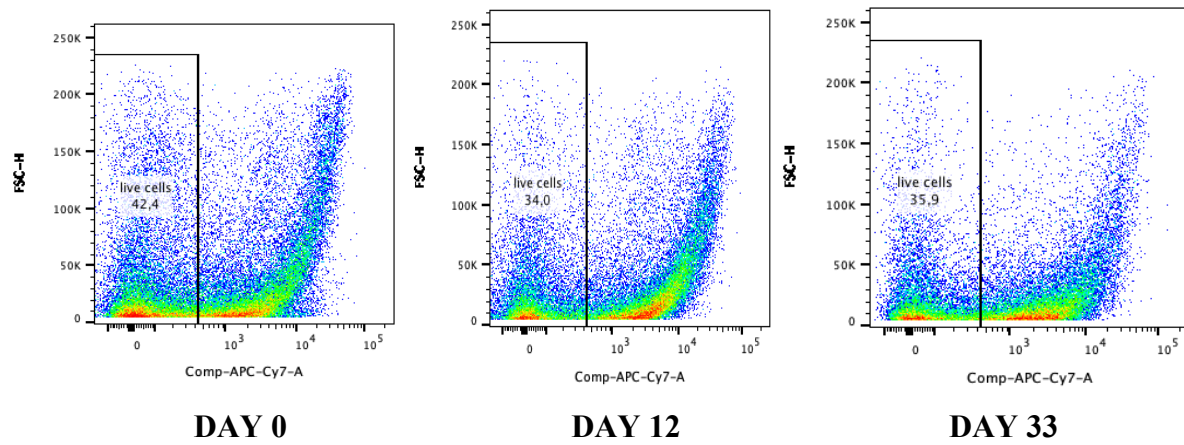
Figure 36. Cell viability of ES-2 grown in feeder-free condition over time during naive conversion. Flow cytometry was performed at day 0, 12 and 33 during conversion on Fine medium.

4.3. SUMMARY OF RESULTS

Finally, a summary of results of the conversion of primed ES-2 cells to naive state in Gafni (feeder and feeder-free), T2ilGö (feeder) and FINE (feeder-free) media are represented in table 3 and 4.

	Viability	SSEA4	CD90	CD24-/CD57-	CD24+/CD57+	CD75-/CD130-	CD75+/CD130+
Control	43,0%	70,9%	31,1%	30,0%	59,4%	87,9%	1,71%
Gafni D3	33,4%	64,0%	76,8%	41,0%	31,0%	90,2%	0,62%
Gafni D17	73,7%	67,9%	72,1%	17,2%	69,2%	96,0%	0,34%
Gafni D31	74,8%	20,3%	68,7%	12,7%	46,1%	87,4%	1,79%
T2iLGo D3	48,8%	80,8%	74,6%	28,6%	31,6%	81,3%	0,71%
T2iLGo D13	15,6%	52,2%	78,6%	55,8%	4,38%	86,6%	0,91%
T2iLGo D31	22,9%	46,3%	48,3%	58,5%	5,66%	71,0%	7,13%

Table 3. Summary of results of the analysis of the conversion to naive state in feeder conditions. In this project different markers of primed, naïve, cell viability, stemness and pluripotency are analysed by flow cytometry.

	Viability	SSEA-4	CD90	CD24-/CD57-	CD24+/CD57+	CD75-/CD130-	CD75+/CD130+
Control	72,9%	62,2%	49,2%	38,2%	49,1%	88,6%	3,50%
Gafni D6	42,4%	38,4%	70,1%	12,9%	57,2%	87,8%	1,17%
Gafni D19	52,3%	11,6%	77,8%	21,2%	30,0%	93,2%	1,02%
Gafni D33	48,0%	11,1%	92,5%	17,7%	27,9%	91,5%	1,70%
FINE D6	42,4%	14,2%	54,3%	21,4%	61,5%	86,4%	4,22%
FINE D19	34,0%	32,6%	23,3%	52,3%	20,8%	73,3%	6,06%
FINE D33	35,9%	11,3%	7,58%	75,6%	2,62%	86,8%	3,85%

Table 4. Summary of results of the analysis of the conversion to naive state in feeder-free conditions. In this project different markers of primed, naïve, cell viability, stemness and pluripotency are analysed by flow cytometry.

5. DISCUSSION

Both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are pluripotent, which means they are capable to develop into all cells of the adult body (somatic and germline cells). The study of pathways underlying mouse embryonic development have been studied in depth and used as a model to understand the differentiation of human pluripotent stem cells. However, application of mouse development to the human system has not always been successful because of fundamental differences in development between species (Trusler et al. 2018).

It has been 8 years since the terms "primed" and "naive" were first proposed as pluripotency states of stem cells. These two different pluripotent states exhibit slightly different properties, such as different culture requirements to maintain their self-renewal capacity and differences in the molecular networks underlying the pluripotent state (Rossant and Tam 2017). ESCs derived from the inner cell mass of preimplantation embryos correspond to the later stage called "primed". The "naive" state is the result of cells less committed that come from the preimplantation epiblast in vivo and was characterized by a high capacity for proliferation, differentiation and global hypomethylation. The conversion of hESC to a "naive" state is desirable as their characteristics would facilitate many techniques such as gene editing and a more efficient differentiation. For this reason, in this study we evaluated the conversion and maintenance of a primed hES cell line (ES-2) into its naive counterpart pluripotent state using different naive conversion media (Gafni, T2iLGö and FINE) in feeder and feeder-free cell systems. To that end, morphology, cell viability and expression of specific markers (stemness, primed and naive markers) were examined.

Regarding the morphology, ES-2 cell colonies were successfully converted from compact flat colonies (primed) to round dome-shaped ones (naive) as described Abu-Dawud and colleagues (Abu-Dawud et al. 2018). This change in colony morphology is due to the effect of the different conversion media added in the two different conditions: feeder and feeder-free systems. After analysing the naive conversion process during 30 days, we could observe that, in general, the cells grown in feeder-free conditions acquired a naive morphology faster than cells grown in feeder conditions. Specifically, conversion was shown around 5 days on feeder-free conditions (FINE and Gafni medium), whereas 6-7 days on feeder conditions. Llames et al. in 2015 proposed that this slower conversion into the naive morphology may be due to the high metabolism of feeder cells (fibroblasts) that influence the culture environment.

Interestingly, when using Gafni medium (in both feeder and feeder-free conditions) more round small colonies arise faster compare to T2iLGö and FINE media.

Concerning cell viability, cells grown with Gafni medium (feeder and feeder-free conditions) maintain a quite higher viability rate during naive conversion. However, cell viability does not remain constant during the first 30 days of conversion. At the beginning of the conversion, the viability is relatively low in both Gafni feeder and Gafni feeder free conditions (33% and 42% respectively), however, as days go by, these values increase (up to 74% and 48%) especially in the feeder condition. The opposite tendency occurs with T2iLGö and FINE media: during the first days after naive media addition, viability values are 48% and 42% respectively, but during the transition to the naive state and after 30 days, viability decreases to suboptimal values (22% and 35%).

To fully determine and evaluate the conversion process from primed to naive status, it is necessary to analyze several markers to identify naive human ES cells.

SSEA-4 is one of the multiple cell surface glycolipid antigens expressed in the early embryo that are exquisitely and finely regulated during development. These antigens provide valuable markers of ES cells (Wright and Andrews 2009). The expression of SSEA-4 cell surface marker indicates colonies' metabolic program. Primed ES-2 cells metabolically rely on glycolysis which is shown by an elevated SSEA-4 expression level, while naive cells in oxidative phosphorylation have a more reduced expression of this marker. For this reason, its expression decreases significantly during naive conversion among all culture conditions (different culture media and feeder vs feeder-free system), although there was a peak of SSEA-4 expression around day 9 for the four conditions. A very interesting fact is shown by the results is that cells grown in feeder-free conditions show exactly the same levels of SSEA-4 marker after conversion (11%) while in feeder-free conditions its expression levels increase up to 20% and 46% for the Gafni and T2iLGö medium respectively.

Apart from the pluripotency marker SSEA-4, the stemness marker CD90 is also a determining factor for naive conversion. Cell expressing the surface protein CD90 (CD90+ cells) have been discovered in several tumors exhibiting similar properties of cancer stem cells such as proliferation, differentiation and metastasis in immunodeficient organisms. CD90 is encoded by Thy-1 and is a small GPI-anchored protein, particularly abundant on the surface of mouse

thymocytes and peripheral T cells. The exact biological role of CD90 has remained controversial, however, it has been proposed to mediate cell-cell interactions in various cell types (Haeryfar and Hoskin 2004). Cells grown on FINE medium showed the lowest levels of these stemness marker (7%) after 30 days during conversion, while Gafni condition, either feeder and feeder-free system, showed significantly higher levels of CD90 (68% and 65%). Furthermore, cells grown in T2iLGö medium showed lower levels of expression (48%) compared to the control (74%). The fact that fibroblasts express CD90 might explain the high expression levels of this marker in cells grown in feeder conditions.

Lastly, primed pluripotency markers (CD24/CD57) and naive pluripotency markers (CD75/CD130) were also analysed according to the findings of Trusler and colleagues (Trusler et al. 2018). In all conditions (Gafni with and without feeder, T2ilGö and FINE) we found decreased expression of primed markers CD24+/CD57+ over time. Cells grown in the T2ilGö medium (feeder system) yield colonies with a very low expression of primed pluripotency markers, however, FINE medium in feeder-free system, yield colonies with the lowest primed pluripotency markers expression. In addition, T2ilGö and FINE media are the best conditions due to the lowest value of double positive primed markers after 30 days (5% and 2% respectively), indicating that the conversion to the naive pluripotent state has been successful. Interestingly, cells growing in feeder conditions show a more marked increase of the CD57 marker (which also is a marker for mature NK and T cells; (Sivori et al. 2020)), suggesting that this is the mechanism why which cells become double negative, increasing one of the markers until they arrive to the CD24-/CD57- and loose completely expression of CD57 (double negative cells). However, in feeder-free conditions, CD24 is the marker that increases the most until it becomes naive colonies. CD24 is a small cell surface sialoglycoprotein highly expressed in ovarian and breast among other tissues. CD24 it is involved in cell adhesion, cell-matrix interactions and metastasis, modulating growth and differentiation of the cells. This indicates that CD24 could be a significant marker in conversion to naive state since has been also linked to the post-implantation stage of embryo development (Jaggupilli and Elkord 2012).

The discovery of cell surface markers that are expressed by naive human pluripotent stem cells (hPSC) makes possible the identification and isolation of these cells. Regarding the results obtained in the expression of naive pluripotency markers, cells expressed low levels of CD75 and CD130 markers. These markers values decrease in all conditions except in cells grown on Gafni feeder free which increases slightly (control cells 88% and after 30 days 91%). This data

is not very representative as the decrease was relatively low. Moreover, a second informative cell surface marker is CD130, which is characteristic of naive cells, but not of primed cells. CD130 expression is induced in primed to naive conversion, and differentiates a broad population of cells of which only a subgroup of them are also CD75 positive. In fact, CD130 is the most informative cell surface marker as it is expressed in the inner cell mass (ICM) of human blastocysts and the its expression is enhanced when the embryo is in a preimplantation state. CD130 acts in the transduction of signals in response to the leukaemia inhibitory factor (LIF) or the interleukin 6 (IL6). The interactions between the cytokine receptor and its ligands results in the activation of JAK proteins and the corresponding downstream pathways (STAT3, PI3K and RAS) (Goodwin, Laslett, and Rugg-Gunn 2020). These observations suggest a clear role of the cytokine signalling pathway (P38i/JNKi among others) characteristic of naive human pluripotent cells (Bayerl et al. 2021).

In summary our results show that SSEA-4 and CD90 markers appear to be indicative of conversion to naive state. In addition, cells cultured with T2ilGö medium (feeder condition) and FINE medium (feeder-free condition) express very low levels of primed markers (CD24/CD57) while cells cultured in Gafni medium in both conditions (feeder and feeder-free) do not express high levels of these markers in the primed to naive conversion. As for the naive markers, results conclude that they are not indicative of conversion as their levels do not increase significantly in any of the conditions. This project leads to the conclusion that the most optimal medium for conversion may be FINE medium (feeder-free conditions). However, a more detailed analysis of these pathways would be favourable to understand the signalling requirements and downstream effectors of hPSC.

6. CONCLUSIONS

- Having regard to the results obtained in this project, leads to conclude that human embryonic stem cells grown in a feeder layer system show a more successful conversion to naive pluripotent state than cells grown in feeder-free systems.

- Gafni medium (either in a feeder or a feeder-free system) confers greater cell viability compared to the other conversion media (FINE and T2iLGö).

- Among the conditions used along with feeder cells, cells grown in a T2ilGö medium yield colonies with the highest naive pluripotency markers (CD130 and CD75) expression, lowest primed pluripotency (CD24 and CD57) and higher stemness marker (CD90) compared to the Gafni condition in feeder system. However, pluripotency associated marker (SSEA-4) is lower in Gafni under feeder conditions than in T2ilGö medium.

- Among the conditions used along feeder-free conditions, cells grown in FINE medium yield colonies with the highest naive pluripotency markers (CD130 and CD75) expression, lowest primed pluripotency (CD24 and CD57) and primed pluripotency associated (SSEA-4 and CD90) compared to the Gafni condition.

- Through primed to naïve conversion, ES-2 cells grown in feeder and feeder-free conditions, show an unexpected increase of CD57 and CD24 markers respectively. These expression patterns change upon stabilization of the naive pluripotent state in which the expression of both, CD57 and CD24, decreases.

- Further experiments, with the same culture conditions used in this work, will help to decipher how the conversion towards the naive pluripotent state is similar between hES and hiPS.

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