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Ot Massafret Surinyach

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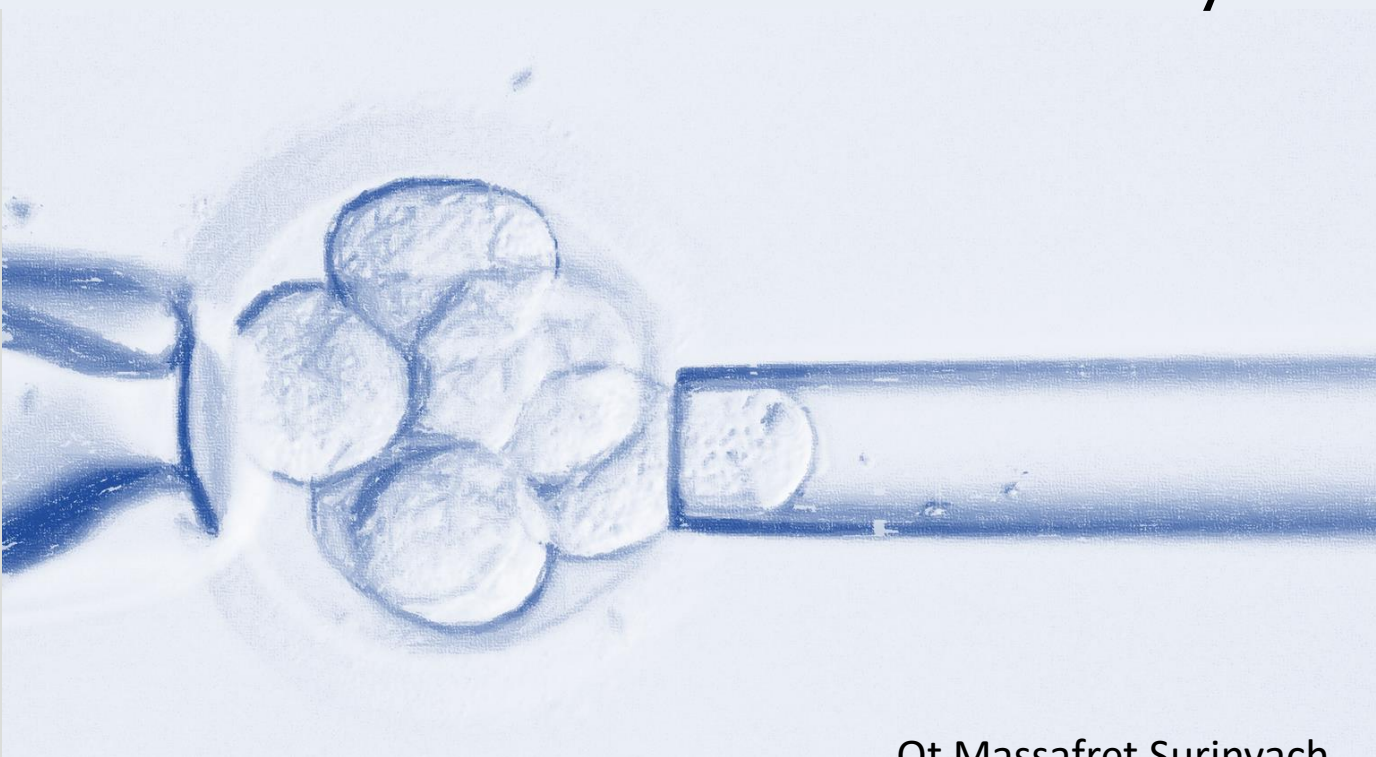

Ot Massafret Surinyach

Doctoral Thesis · 2022

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Unravelling the pluripotency state of human embryonic stem cells derived from single blastomeres of 8-cell embryos

Ot Massafret Surinyach

Memòria presentada per optar al Grau de Doctor per la Universitat Autònoma de Barcelona

Tesi doctoral inscrita en el Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biociències, Universitat Autònoma de Barcelona

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Bellaterra · 2022

El Dr. **Josep Santaló Pedro**, catedràtic del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona,

i la Dra. **Elena Ibáñez de Sans**, professora agregada del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona,

CERTIFIQUEN

Que **Ot Massafret Surinyach** ha realitzat sota la seva direcció el treball d'investigació que s'exposa en la memòria titulada "**Unravelling the pluripotency state of human embryonic stem cells derived from single blastomeres of 8-cell embryos**" per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

Que aquest treball s'ha dut a terme a la Unitat de Biologia Cel·lular del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona.

I perquè així consti, signen el present certificat.

Dr. Josep Santaló Pedro Dra. Elena Ibáñez de Sans Ot Massafret Surinyach

Bellaterra, 14 de març de 2022

Durant la realització d'aquesta tesi, Ot Massafret Surinyach ha estat beneficiari d'una beca FI-2017 concedida per l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) de la Generalitat de Catalunya.

A la meva família
i a la Mireia

“Anàvem lents perquè anàvem lluny”

Summary

Abstract

Embryonic stem cells (ESCs) can have at least two states of pluripotency: naïve and primed, with an expression profile resembling that of the preimplantation and postimplantation epiblast, respectively. Naïve ESCs have some advantages over primed ESCs that make them more suitable for potential clinical uses, such as a greater differentiation potential and a greater proliferation capacity and clonogenicity. They are also a better *in vitro* model than primed hESCs for the study of preimplantation developmental events. However, the existence of intermediate states of pluripotency has been recently reported, and therefore pluripotency should indeed be considered as a continuum where different types of ESCs are allocated according to their characteristics. Human ESCs (hESCs) traditionally derived from blastocyst stage embryos show a primed pluripotency state, but hESCs can also be derived from single blastomeres of 8-cell embryos, although their pluripotency state is unclear. Since they are obtained from embryos at an early stage of development, they could be more prone to present naïve pluripotency characteristics. Therefore, the objective of this thesis was to determine the pluripotency state of hESCs derived from single blastomeres.

In a first study, the initial objective was to derive new hESC lines from single blastomeres (bm-hESCs) and from whole blastocysts (bc-hESCs) with the highest possible efficiency. The results indicated that the use of inhibitors of GSK3β and ROCK had a positive effect on hESC derivation efficiencies. We also observed that single blastomeres, like blastocysts, organized to form a post-inner cell mass intermediate (PICMI) during the first days of the derivation process.

Next, different naïve pluripotency indicators were analysed in bm-hESCs, bc-hESCs (primed control), and a naïve-converted hESC line (naïve control). Our bm-hESCs showed a significantly higher clonogenicity than bc-hESCs, although lower than naïve hESCs, and also showed a significantly higher expression of some naïve pluripotency marker genes in early culture passages. No differences

were observed between bm-hESCs and bc-hESCs for the rest of the indicators analysed, whereas naïve hESCs were significantly different. These results indicated that bm-hESCs would be in a primed state of pluripotency, although closer to the naïve end of the pluripotency continuum than bc-hESCs.

Finally, in order to continue the previous work, the last objective was to analyse the transcriptome and the differentiation potential of bm-hESCs. First, we observed that the transcriptional profile of bm-hESCs was similar to that of bc-hESCs but significantly different from that of naïve hESCs. However, bc-hESCs overexpressed genes related to nervous system development, embryonic pattern specification, and embryonic epithelial morphogenesis with respect to bm-hESCs, whereas bm-hESCs overexpressed mitochondrial genes involved in cellular respiration. These results were consistent with those obtained in the first work with regards to the pluripotency state of hESCs. Referring to the differentiation potential, no differences were observed between bm-hESCs, bc-hESCs and naïve hESCs when treated as groups, but a large heterogeneity was observed between different hESC lines.

All in all, the results obtained in this thesis indicate that bm-hESCs present a pluripotency state slightly closer to the naïve end of the pluripotency continuum than bc-hESCs, which makes single blastomeres an interesting alternative as a source for hESC derivation.

Resum

Les cèl·lules mare embrionàries (ESCs) poden presentar almenys dos estats de pluripotència: *naïve* i *primed,* amb un perfil d'expressió gènica semblant al de l'epiblast preimplantacional i postimplantacional, respectivament. Les ESCs *naïve* presenten alguns avantatges respecte les *primed* que les fan més adients per a possibles usos clínics, com per exemple un major potencial de diferenciació i una major capacitat de proliferació i d'expansió clonal. També constitueixen un millor model *in vitro* per l'estudi dels esdeveniments del desenvolupament preimplantacional. Malgrat això, recentment s'ha demostrat l'existència d'estats de pluripotència intermedis i, per tant, la pluripotència s'ha de considerar com un espectre continu on els diferents tipus d'ESCs es posicionen en funció de les seves característiques. Les ESCs humanes (hESCs) tradicionalment derivades a partir d'embrions en estadi de blastocist presenten pluripotència *primed*. Les hESCs també poden ser derivades a partir de blastòmers aïllats d'embrions a 8 cèl·lules, però no es coneix amb certesa quin és el seu estat de pluripotència. Tenint en compte que provenen d'embrions en un estadi de desenvolupament primerenc, podrien ser més propenses a presentar característiques de pluripotència *naïve*. Per això, l'objectiu d'aquesta tesi ha estat determinar l'estat de pluripotència de les hESCs derivades a partir de blastòmers aïllats.

En un primer treball, l'objectiu inicial fou derivar noves línies de hESC a partir de blastòmers aïllats (bm-hESCs) i de blastocists sencers (bc-hESCs) amb la major eficiència possible. Els resultats obtinguts van indicar que l'ús dels inhibidors de GSK3β i de ROCK va tenir un efecte positiu en les eficiències de derivació de hESCs. També es va observar que els blastòmers aïllats, com els blastocists, s'organitzaven formant un *post-inner cell mass intermediate* (PICMI) durant els primers dies del procés de derivació.

A continuació es van analitzar diferents indicadors de pluripotència *naïve* en les bm-hESCs, les bc-hESCs (control *primed*) i una línia de hESCs convertida a *naïve* (control *naïve*) Les bm-hESCs van presentar una clonogenicitat

significativament superior a la de les bc-hESCs, encara que inferior a la de les hESCs *naïve*, i també van presentar una expressió significativament superior d'alguns gens marcadors de pluripotència *naïve* a passatges primerencs de cultiu. En la resta d'indicadors, no es van observar diferències entre les bm-hESCs i les bc-hESCs, mentre que les hESCs *naïve* van ser significativament diferents. Aquests resultats van indicar que les bm-hESCs es trobarien en un estat de pluripotència *primed*, tot i que més proper a l'extrem *naïve* de l'espectre de la pluripotència que les bc-hESCs.

Finalment, per tal de continuar amb el treball anterior, l'últim objectiu va consistir en analitzar el transcriptoma i el potencial de diferenciació de les bm-hESCs. Primer, es va observar que el transcriptoma de les bm-hESCs era semblant al de les bc-hESCs però significativament diferent al de les hESCs *naïve*. No obstant, les bc-hESCs van sobreexpressar gens relacionats amb el desenvolupament del sistema nerviós, l'especificació de patrons embrionaris i la morfogènesi de l'epiteli embrionari respecte les bm-hESCs, mentre que les bm-hESCs van sobreexpressar gens mitocondrials involucrats en la respiració cel·lular. Aquests resultats van anar en la línia dels obtinguts en el primer treball en referència a l'estat de pluripotència de les hESCs. En quant al potencial de diferenciació, no es van observar diferències entre les bm-hESCs, les bc-hESCs i les hESCs *naïve* com a grups, però si que es va observar una gran heterogeneïtat entre diferents línies.

En conjunt, els resultats obtinguts en aquesta tesi indiquen que les bm-hESCs presenten un estat de pluripotència lleugerament més proper a l'extrem *naïve* de l'espectre de la pluripotència que les bc-hESCs, cosa que converteix els blastòmers aïllats en una alternativa interessant com a punt de partida per a la derivació de hESCs.

Abbreviations and acronyms

2i	2 inhibitors cocktail (CHIR99021 and PD0325901)
AMPK	AMP-activated Protein Kinase
bc-hESCs	blastocyst-derived human Embryonic Stem Cells
bFGF	basic Fibroblast Growth Factor
bm-hESCs	blastomere-derived human Embryonic Stem Cells
BMP	Bone Morphogenic Protein
EBs	Embryoid Bodies
ECCs	Embryonal Carcinoma Cells
ECM	Extracellular Matrix
EGCs	Embryonic Germ Cells
EGF	Epidermal Growth Factor
EpiLCs	Epiblast-Like Cells
EpiSCs	Epiblastic Stem Cells
EPSCs	Expanded Potential Stem Cells
ERK	Extracellular-Regulated Kinase
ESCs	Embryonic Stem Cells
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FSCs	Formative Stem Cells
FTW	FGF, TGF-β and Wnt signalling pathways
GSK3β	Glycogen Synthase Kinase 3β
HDACi	Histone Deacetylase inhibitors
hESCs	human Embryonic Stem Cells
HFFs	Human Foreskin Fibroblasts
hiPSCs	Human induced Pluripotent Stem Cells
HSCs	Hematopoietic Stem Cells
ICM	Inner Cell Mass

IGF2 Insulin Growth Factor 2

iPSCs induced Pluripotent Stem Cells

JNK c-Jun N-terminal Kinase

JAK Janus Kinase

LIF Leukaemia Inhibitory Factor

MAPK Mitogen-Activated Protein Kinase

MEFs Mouse Embryonic Fibroblasts

MEK MAPK/ERK Kinase

mEpiSCs mouse Epiblastic Stem Cells

mESCs mouse Embryonic Stem Cells

NCM Naïve Conversion Medium

NHSM Naïve Human Stem cell Medium

NT-hESCs Nuclear Transfer-human Embryonic Stem Cells

PCA Principal Component Analysis

PGCs Primordial Germ Cells

PGT Preimplantational Genetic Testing

PI3K Phosphatidylinositol 3 Kinase

PICMI Post-Inner Cell Mass Intermediate

PKC Protein Kinase C

PLCβ Phospholipase C Beta

ROCK Rho-associated protein kinase

RSCs Rosette-Like Stem Cells

SCs Stem Cells

STAT3 Signal Transducer and Activator of Transcription 3

TE Trophectoderm

TFE3 Transcription Factor E3

TGFβ Transforming Growth Factor Beta

XCI X Chromosome Inactivation

Introduction

1. What are Stem Cells?

Stem cells (SCs) are undifferentiated cells that have the potential to differentiate into other cell types under certain conditions. They are also able to self-renew, which means they can divide indefinitely without losing their undifferentiated state (Figure 1).

Thanks to these properties, SCs are ideal for the research on the mechanisms of cellular differentiation and are considered a promising tool for the development of therapies in the field of regenerative medicine.

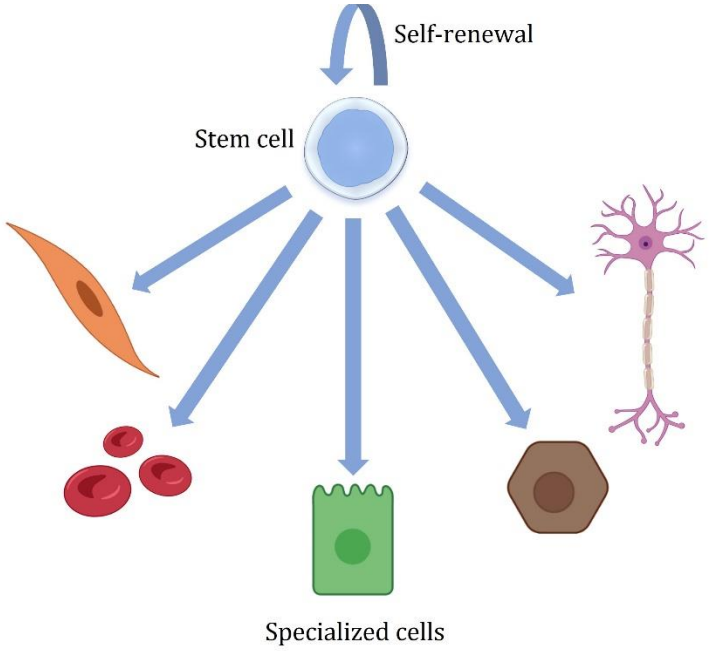

Figure 1. Representation of the defining properties of stem cells. Stem cells can differentiate into more specialised cell types (A) and can also self-renew and produce more undifferentiated stem cells (B).

2. The concept of Stem Cells potency

The potency of SCs is defined as the capacity to differentiate into other cell types. Not all the SCs have the same potency: while some have a broad potential, being

able to differentiate into all the cell types of the organism and generate a new individual, others have a narrower potential and can become just a few specific cell types or even only one.

2.1. Totipotent Stem Cells

During the first developmental stages of the embryo, its blastomeres have the potential to differentiate into all the cell types of the adult organism and even into extraembryonic tissues. These cells are totipotent, and they have the ability to generate a whole new individual by themselves (Weissman, 2000; Lanza and Atala, 2013). Totipotency is observed just after the fertilisation of the oocyte and during the few first divisions of the embryo, albeit it progressively decreases and lasts until the beginning of the first differentiation event (Morris et al., 2012) (Figure 2).

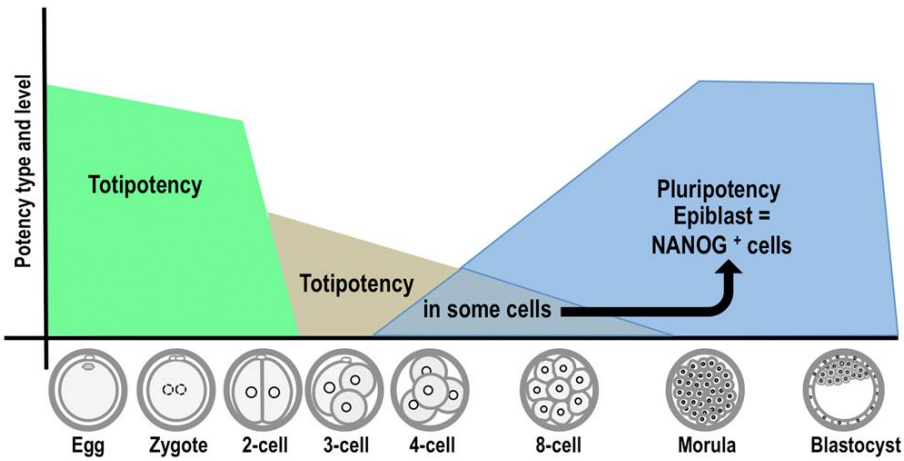

Figure 2. Evolution of the potency of embryonic cells through preimplantational development. Reprinted by permission from Bioscientifica Limited: Society for Reproduction and Fertility. Reproduction. Totipotency continuity from zygote to early blastomeres: a model under revision. Boiani et al. Copyright (2019).

The first totipotent-like cells described *in vitro* were termed 2c-like cells, a rare and transient subpopulation of cells within embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) cultures resembling the totipotent blastomeres of the mouse embryo at the 2-cell stage (Macfarlan et al., 2012).

These 2c-like cells were shown to express a large number of genes that are restricted to the 2-cell stage embryo, and did not express the pluripotency markers *Oct4*, *Sox2* and *Nanog*. Moreover, 2c-like cells contributed to both embryonic and extraembryonic tissues after injection into a recipient blastocyst (Macfarlan et al., 2012; Choi et al., 2017). Another type of cells that can contribute to embryonic tissues and also to extraembryonic lineages in chimaera assays have been derived from mouse (Yang et al., 2017) and pig (Gao et al., 2019) embryos. These so-called expanded potential stem cells (EPSCs) have also been obtained in humans by conversion from already established human ESCs (hESCs) (Gao et al., 2019).

More recently, two studies describing the generation of SCs resembling the murine 2-cell embryo were published. The first one reported the obtention of totipotent blastomere-like cells from mouse ESCs (mESCs) by spliceosomal repression (Shen et al., 2021). The authors of the study claimed that these cells were more similar to blastomeres from the 2- and 4-cell embryos at the transcriptional level than both EPSCs and 2c-like cells. The second study described the obtention of the so-called totipotent-like SCs, which were even more similar to the blastomeres from 2-cell embryos. Moreover, they could be generated by chemically resetting mESCs and also directly derived from 2-to-8-cell embryos (Yang et al., 2022). All in all, these SC types constitute the closest *in vitro* approach to the totipotent cells found in the early embryo.

2.2. Pluripotent Stem Cells

Pluripotent SCs are those SCs that can differentiate into the three embryonic germ layers (ectoderm, mesoderm and endoderm), but cannot form extraembryonic tissues such as the placenta (Wobus and Boheler, 2005). *In vivo*, pluripotent SCs can be found in the epiblast of the embryo at the blastocyst stage, after the segregation into the CDX2-positive trophectoderm (TE) and the OCT4-positive inner cell mass (ICM). The cells of the ICM will as well segregate into two different tissues: the GATA6-positive primitive endoderm, or hypoblast, which will differentiate into extraembryonic structures like the yolk

sac, the allantois and the amnion, and the NANOG-positive epiblast, which will remain pluripotent and will eventually give rise to all the cell types of the adult organism (Gilbert, 2000; Arnold and Robertson, 2009).

In vitro, several types of pluripotent SCs have been established, which will be described below.

2.2.1. Embryonic Stem Cells

ESCs are derived from the inner cell mass of the embryo at the blastocyst stage or from single cells of cleavage stage embryos. They share two main properties: pluripotency and self-renewal. They can differentiate into cells from the three embryonic germ layers (ectoderm, mesoderm and endoderm) and they can divide and generate daughter cells indefinitely without losing their undifferentiated state.

The properties and characteristics of ESCs will be further discussed later in this thesis.

2.2.2. Embryonic Germ Cells

Embryonic germ cells (EGCs) are derived from primordial germ cells (PGCs), a population of cells formed during the first hours of gastrulation. PGCs *in vivo* do not respond to differentiation-promoting factors like other cells of the embryo. Instead, they remain undifferentiated and migrate to genital ridges where they form mature germ cells. These cells will eventually differentiate into functional gametes (Saffman and Lasko, 1999; Lanza and Atala, 2013). In mice, the main factors that promote the formation of PGCs *in vivo* are bone morphogenic proteins 4 (BMP4) and 8 (BMP8), which are produced by the extraembryonic ectoderm.

The derivation of both mouse and human EGCs from PGCs is achieved by culture of the isolated PGCs in the presence of stem cell factor, also known as kit ligand, fibroblast growth factor 2 (FGF2), also known as basic FGF (bFGF), and leukaemia inhibitory factor (LIF). However, there are some differences between mouse and human EGCs. Mouse EGCs do not require stem cell factor nor bFGF

for long-term maintenance; just a 12-hour exposure is enough for mouse EGC derivation in the presence of LIF, serum and feeder cells. By contrast, human EGC are dependent on bFGF to maintain their pluripotent state (Donovan and de Miguel, 2003).

2.2.3. Embryonal Carcinoma Cells

Embryonal carcinoma cells (ECCs) are the pluripotent cells found in teratocarcinomas. Teratocarcinomas are malignant tumours derived from germ cells that form usually in the testicles or ovaries. They are composed of differentiated somatic and embryonic cells, and a group of pluripotent SCs that constitute the ECCs (Andrews et al., 2005).

The identification of pluripotent cells within mouse teratocarcinomas and their subsequent isolation and culture *in vitro* in the late 1960s and early 1970s (Finch and Ephrussi, 1967; Kahan and Ephrussi, 1970; Evans, 1972; Bernstine et al., 1973) set the basis for the posterior derivation of ESC lines more than a decade later. These ECCs are thought to be the malignant counterpart of the ICM, since they have similarities in the expression of surface antigens and can contribute to chimeric mice after injection into a blastocyst (Artzt et al., 1973; Papaioannou et al., 1975). Generally, ECCs provide a good model to study carcinogenesis, cell differentiation and self-renewal *in vitro*, and are cheaper and easier to culture than ESCs (Abu Dawud et al., 2012). However, these cells are extremely heterogeneous, and their differentiation potential can sometimes be restricted (Przyborski, 2001). Moreover, human ECCs are highly aneuploid, even more than mouse ECCs, which limits their potential for clinical uses (Draper et al., 2003).

2.2.4. Epiblastic Stem Cells

Epiblastic stem cells (EpiSCs) are derived from the postimplantational epiblast of embryos at day 5.5-6.5 in mice. They share some features with mESCs: they can differentiate into cell types from the three germ layers and express pluripotency markers *Oct4*, *Sox2* and *Nanog* (Brons et al., 2007).

Mouse EpiSCs (mEpiSCs) also drive the formation of teratomas when injected in adult mice but have little to no potential of contributing to the ICM when injected into recipient blastocysts, rarely generating chimeric animals (Tesar et al., 2007). Moreover, unlike mESCs, mEpiSCs are dependent on FGF/mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) and Activin/Nodal signalling pathways in order to maintain their pluripotency (Vallier et al., 2009; Kojima et al., 2014). These properties make mEpiSCs more similar to hESCs than to mESCs.

2.2.5. Induced Pluripotent Stem Cells

iPSCs are pluripotent cells obtained by reprogramming of adult somatic cells. The first iPSC line was generated in 2006 (Takahashi and Yamanaka, 2006) by ectopic overexpression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* (the so-called Yamanaka's cocktail) on mouse embryonic fibroblasts (MEFs). Although impactful, the first methodologies used to reprogram somatic cells into iPSCs had several issues including the very low efficiency, which prevented large-scale production, and the presence of the transcription factor *c-Myc*, which acts as a proto-oncogene and was seen to cause tumorigenesis in up to 50% of the chimeric mice obtained after iPSC transplantation (Okita et al., 2007; Wernig et al., 2008).

The main interest of iPSCs is the possibility of developing autologous regenerative therapies without immune rejection while overcoming most of the ethical issues associated with the obtention of ESC, which will be discussed later in this thesis. Theoretically, the use of a patient's own cells should not drive any rejection after transplantation of these cells. However, the immunogenicity of iPSC-derived cells has been an important concern for the development of therapies involving iPSCs.

In 2011, Zhao and co-workers showed that iPSCs derived from MEFs from the mouse inbred B6 strain could activate an immune response when injected subcutaneously in mice of the same strain. The immune response was strong enough to prevent the formation of teratomas, which are typically observed after injection of pluripotent SCs. The genes Hormad1 and Zg16, which were

expressed by iPSCs but not by ESC, were found to be the cause of the immunorejection (Zhao et al., 2011). Contrarily, in 2013 two independent studies did not find immunorejection after iPSC injection in mice (Araki et al., 2013; Guha et al., 2013). Araki and colleagues used *in vivo* terminally differentiated iPSCs and ESCs into skin and bone marrow cells, whereas Guha and colleagues differentiated their iPSCs and ESCs *in vitro* into precursor cells from the three germ layers. They did not find increased immunorejection of iPSC-derived cells when compared to ESC-derived cells after injection into syngeneic mice nor did they observe an overexpression of *Hormad1* and *Zg16* in iPSCs-derived cells. It was suggested that the results from Zhao et al., 2011 could be caused by a poor experimental design, since they only used one ESC line as a control and used undifferentiated iPSCs, which would never be used for clinical therapies. Additionally, the pluripotency of the iPSCs was not tested, and it is known that incompletely reprogrammed iPSCs can drive immune responses (Okita et al., 2011; Araki et al., 2013; Guha et al., 2013). Finally, another conclusion from these studies was that non-integrative episomal vectors were less immunogenic than retroviral integrative vectors (Zhao et al., 2011; Kaneko and Yamanaka, 2013).

Four years after their first study, other results from Zhao and colleagues demonstrated that the immune response could vary depending on the transplanted human iPSC (hiPSC)-derived cell type. They stated that, whereas autologous hiPSC-derived smooth muscle cells caused a strong immune response, retinal-pigmented epithelium cells were tolerated in humanised mice (Zhao et al., 2015). Moreover, the cell type from which iPSCc are generated can also affect the immunogenicity of the iPSCs-derived cells (Vanneaux, 2020). Therefore, proper reprogramming and differentiation protocols are crucial to obtain safe clinical-grade hiPSCs.

Nevertheless, several clinical trials with autologous and allogenic hiPSCs are currently in progress, with the first results mainly pointing out that transplanted cells appear to be safe for the patients (Bragança et al., 2019; Vanneaux, 2020).

2.3. Multipotent Stem Cells

Multipotent SCs are restricted to differentiate into cell types of one specific lineage. Most of the adult SCs are multipotent. These adult SCs can be found in a large variety of tissues of a developed individual, such as the brain, bone marrow, blood, blood vessels, skeletal muscles, skin, and liver. These cells remain quiescent in these tissues until a disease or injury occurs. Then, they differentiate into the damaged cell types while also self-renewing their own population (Hima Bindu and Srilatha, 2011). The most well-known type of multipotent stem cells are adult hematopoietic stem cells (HSCs), which are the most transplanted stem cell type.

2.4. Unipotent Stem Cells

Unipotent cells, also called precursor cells, can only become their own specific cell type, but still have the ability so self-renew. Examples of unipotent cells are those found in most epithelial tissues, which can self-renew indefinitely to regenerate damaged cells during the whole life of the individual (Blanpain et al., 2007; Hima Bindu and Srilatha, 2011).

3. Applications of Stem Cells

Thanks to their unique properties, SCs are considered a promising tool for both research and clinical applications.

ESCs are the *in vitro* equivalent of the epiblast of the embryo, which can differentiate into the three germ layers. Since the epiblast is a very transient structure, ESCs, which can self-renew indefinitely in culture, are good models for studying early development and cellular differentiation (Zhu and Huangfu, 2013; Doğan, 2018). Understanding the mechanisms, molecules and signalling pathways involved in cell differentiation can give rise to more efficient protocols to generate progenitor cells for clinical therapies.

Recently, structures called blastoids, which display all the cell lineages present in the human blastocyst, have been generated from naïve hESCs or hiPSCs (Liu et al., 2021; Yu et al., 2021a) and from EPSCs (Fan et al., 2021; Sozen et al., 2021) by sequential differentiation in 3D cultures. Blastoids constitute a good model to study the events that occur in early human embryogenesis, including early cell fate decision or differentiation events and implantation. Additionally, they can be a source of pluripotent SCs and trophoblastic SCs. Although the initial reported efficiencies of blastoid formation were low (around 10%), a recent study described the generation of blastoids with 70% efficiency by triple inhibition of the Hippo pathway (Kagawa et al., 2021). Nevertheless, it remains uncertain if blastoids can keep a stable epigenetic status, including the maintenance of imprinted regions, to become a viable model for lineage development (Niemann and Seamark, 2021).

Pluripotent SCs are also useful for disease modelling. It is possible to obtain pluripotent SCs carrying mutations that cause genetic diseases. The two main approaches to obtain these cells are: 1) deriving ESCs from embryos that have been diagnosed by preimplantational genetic testing (PGT) and carry chromosomal abnormalities or mutations that cause a known genetic disease (Maury et al., 2012; Zhu and Huangfu, 2013) or 2) reprogramming mutated somatic cells from patients with a known disease (Wu and Hochedlinger, 2011; Zhu and Huangfu, 2013). Differentiated cells from pluripotent SCs carrying disease-associated mutations can be a valuable tool for drug screening assays (Kropp et al., 2017) and for disease modelling (Martín, 2016) (Figure 3). Additionally, hESCs or hiPSCs can be genome-edited with systems like CRISPR/Cas9 (Soldner and Jaenisch, 2018). These genome-edited pluripotent SCs could be used to generate blastoids, which can model the effect of different mutations on the early embryonic development (Yu et al., 2021a).

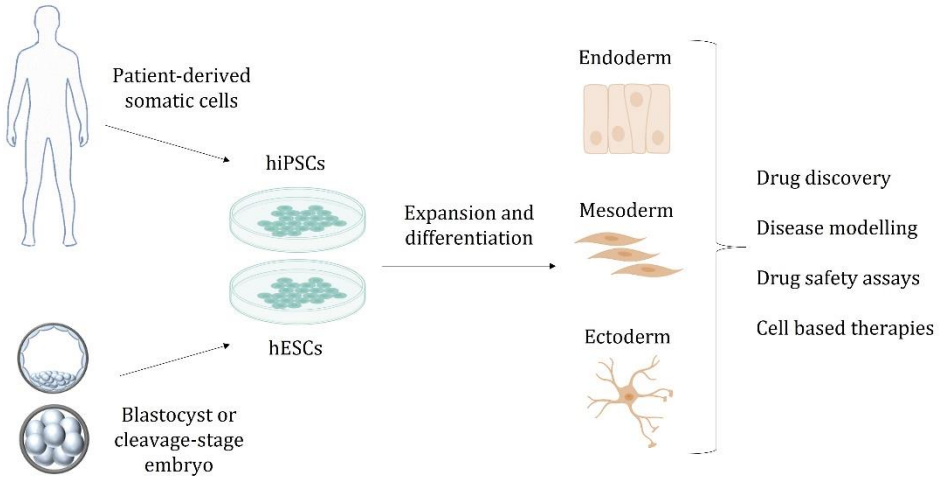

Figure 3. Overview of the different applications of human pluripotent stem cells. Both hiPSCs and hESCs can be differentiated into cells from the three germ layers. These differentiated cells can be used for disease modelling and drug discovery and safety assays, and also in regenerative cell-based therapies. Adapted from Kropp et al., 2017. Creative commons CC-BY-NC-ND 4.0.

However, the most interesting and promising application of pluripotent SCs is the possibility of engrafting and replacing somatic cells of adult tissues that have been lost by degeneration or injury (Doğan, 2018; Nguyen et al., 2018a). The first hESC-based clinical trial was performed in 2010 and involved the use of hESC-derived oligodendrocyte precursors to treat spinal cord injury (Lebkowski, 2011). Since then, many other trials have been started, mainly phase I/II trials focused on the use of retinal-pigmented epithelium cells derived from hESCs, ESCs generated by nuclear transfer (NT-ESCs) or iPSCs, hESC-derived neural precursor cells for treating Parkinson's disease, hESC-derived cardiomyocytes to support heart regeneration and hESC-derived pancreatic beta cells to treat type 1 diabetes (Nguyen et al., 2018).

Despite the achievement of some promising results, there are still a few concerns with the use of human pluripotent SCs for clinical therapies. First, incomplete differentiation of the cells or the presence of undifferentiated cells in the transplanted population can give rise to teratomas (Nguyen et al., 2018). To circumvent this situation, several purification protocols based on flow-

cytometry (Tang et al., 2013) and on cytotoxic antibodies targeting surface markers of the undifferentiated cells (Choo et al., 2008) have been proposed. Another potential issue is the genomic instability that ESCs cultured in vitro can acquire as an adaptation to culture conditions (Jacobs et al., 2016). Moreover, iPSCs are exposed to genomic changes that can occur during the reprogramming process and to genetic variations present in the source cells (Nguyen et al., 2018). Finally, allogenic transplantation of hESCs can drive an immunologic rejection response in the host individual. iPSCs are a better alternative in this case because they can be the basis for autologous therapies, thereby minimizing the risk of rejection (Liu et al., 2017b). Nevertheless, as previously mentioned, they have a higher chance of carrying genomic abnormalities (Yoshihara et al., 2017).

An alternative approach for potential autogenic transplantations is the use of hESCs derived from embryos generated by somatic cell nuclear transfer. These embryos are obtained when a metaphase II oocyte is enucleated, and the nucleus of a donor cell (usually fibroblasts) is injected into the cytoplasm of the oocyte. Then, the oocyte is activated, and it can develop into a functional blastocyst from which hESCs can be derived (Tachibana et al., 2013). Like hiPSCs, NT-hESCs carrying specific mutations can be generated. However, the need for large numbers of good-quality human oocytes, and the fact that therapeutic cloning is restricted in many countries limit the potential of NT-hESCs (Gouveia et al., 2020).

Several therapies that use adult SCs such as bone-marrow derived HSCs, mesenchymal SCs, and neural SCs have also been developed. Allogenic and autologous HSC transplants are currently an option for the treatment of most inherited blood cell diseases, like immune deficiencies, and acquired diseases, like blood cancers. Mesenchymal SCs are also under research for the treatment of autoimmune diseases and other immune affections like Crohn's disease, and neural progenitor cells are being studied for the treatment of Parkinson's disease (Gurusamy et al., 2018).

Finally, pluripotent ESCs can also be used for tissue engineering. The generation of 3D organoids that mimic different organs from the human body can provide better approaches than 2D cell cultures for disease modelling, drug screening or even for the replacement of damaged organs (Kretzschmar and Clevers, 2016; Kim et al., 2020). Organoids can be derived from pluripotent SCs including ESCs and iPSCs, and from multipotent adult SCs. They can be composed of epithelial cells, or of both epithelial and mesenchymal cells (Kretzschmar and Clevers, 2016). As of today, organoid cultures of several organs of the body have been generated. It has been possible to obtain organoids of salivary glands, lungs, mammary glands, liver, kidneys, pancreas, stomach, intestine, fallopian tubes, endometrium, bladder and prostate from adult SCs. Moreover, it has also been possible to obtain organoids of brain, retina, thyroid gland and blood vessels from pluripotent SCs (Kim et al., 2020).

4. Embryonic Stem Cells

4.1. General characteristics

In 1981, it was shown that the pluripotent cells of the ICM could proliferate indefinitely *in vitro* under the form of ESCs (Evans and Kaufman, 1981; Martin, 1981). The first ESC lines were obtained from the ICM of mouse blastocysts and were cultured on a feeder layer with the addition of LIF to help them maintain their pluripotent state. Since then, the methodologies and requirements for the derivation and culture of new ESC lines have been a subject of study in order to improve derivation efficiencies and standardise the properties of the ESCs produced.

Although mESCs are the most well-known and standardised SCs, ESCs have also been obtained in a relatively efficient way from rats (Buehr et al., 2008; Li et al., 2008), non-human primates (Thomson et al., 1995, 1996) and humans (Thomson et al., 1998). Nevertheless, ESC derivation from other mammals, like farm animals, has been extremely inefficient (Ogorevc et al., 2016).

In the mouse, ESCs are defined by two main properties: 1) the ability to form teratomas when injected subcutaneously in immunosuppressed mice and 2) the capacity of contributing to the germ line when injected into the ICM of recipient blastocysts to form chimeric mice. Nevertheless, for ethical reasons it is not possible to generate chimeric individuals in humans, therefore this is a non-suitable criterion for defining hESCs.

Nonetheless, both mouse and human ESC are pluripotent and share the ability to differentiate into the three germ layers (ectoderm, mesoderm and endoderm). The maintenance of the pluripotent state in ESCs is controlled by a large network of transcription factors that are governed by OCT4 (also known as POU5F1), the homeobox protein NANOG and the high mobility group box transcription factor SOX2. These three transcription factors together regulate the expression of several target genes that regulate pluripotency in ESCs (Johnson et al., 2008).

The procedures for ESC derivation and their properties will be further explained in section 5, focusing on hESCs.

4.2. The state of pluripotency

4.2.1. Naïve and primed pluripotency

When hESCs were first derived in 1998 (Thomson et al., 1998), it was obvious that several differences between them and mESC lines existed, and these disparities were thought to be species-specific. However, in 2007 mEpiSCs were first derived from the mouse postimplantation epiblast, and they had a surprising resemblance to hESCs (Brons et al., 2007; Tesar et al., 2007). This suggested that the differences found between mouse and human ESC could be due to distinct developmental origins rather than just species-specific variations. Whereas mESCs display a gene expression pattern consistent with the preimplantation epiblast, that of mEpiSCs mostly resembles the postimplantation epiblast.

It was suggested that the observed differences between mESCs and mEpiSCs or hESCs could be explained by defining the existence of two distinct states of pluripotency: the naïve state, represented by mESCs, and the primed state, represented by mEpiSCs and hESCs (Nichols and Smith, 2009). Note that most of the characteristics defining the naïve and primed states have been described in the mouse, in which both types of SCs were first obtained. Moreover, although it is now possible to generate naïve hESCs, the populations obtained to date are very heterogeneous, as their characteristics differ depending on the protocols used for their generation (see section 5.3.2).

Naïve mESCs and primed mEpiSCs and conventional hESCs have several differential traits that allow the definition and identification of both states of pluripotency. Both naïve and primed cells are pluripotent because they are able to self-renew and differentiate into the three embryonic layers, but since primed cells represent a later stage of development, their differentiation potential is more restricted. This is evidenced by the fact that naïve mESCs can efficiently contribute to the germline of chimeric mice after injection into a recipient blastocyst, whereas primed mEpiSCs generally do not have this capacity (Brons et al., 2007; Tesar et al., 2007). For ethical reasons, this procedure cannot be applied to hESCs, but most of the attempts to generate chimeras from primate ESCs, which also display a primed pluripotency, have failed so far (Boroviak and Nichols, 2017). Only Chen and colleagues were able to generate chimeras in cynomolgus monkeys, after converting their ESCs to a naïve state (Chen et al., 2015b).

Naïve and primed cells also differ in their morphology: naïve mESCs grow as compact, dome-shaped small colonies, whereas mEpiSCs and hESCs form large, flat colonies (Figure 4).

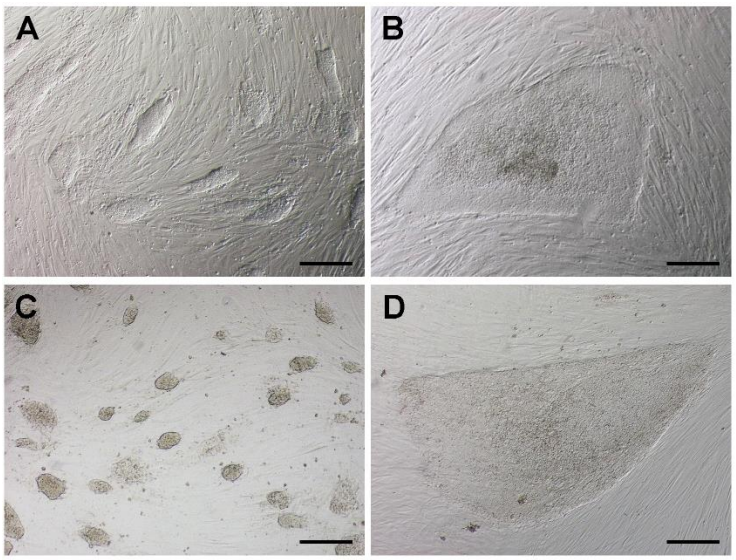

Figure 4. Different morphologies of naïve and primed ESC colonies. A) mESCs forming small, dome-shaped colonies. B) mEpiSCs forming a large, flat colony. C) naïve hESCs cultured in 2i+FGF medium forming small dome-shaped colonies. D) conventional hESCs forming a large, flat colony. Scale bars: 100 μm.

Referring to their proliferative capacity, naïve cells display faster doubling times than primed cells. Moreover, primed cells, unlike naïve, have low survival rates after single-cell passaging, and therefore they need to be passaged as cell clumps (Thomson et al., 1998; Tesar et al., 2007).

The signalling pathways involved in the maintenance of naïve and primed pluripotency also differ. LIF/Signal transducer and activator of transcription 3 (STAT3) signalling is known to be essential for the maintenance of mESC pluripotency (Smith et al., 1988; Williams et al., 1988). However, hESCs and mEpiSCs do not respond to LIF signalling and they rely on FGF and transforming growth factor β (TGFβ)/Activin pathways (Thomson et al., 1998; Humphrey, 2004; Brons et al., 2007; Tesar et al., 2007). Inhibition of the MAPK/ERK pathway and activation of Wnt signalling have a beneficial effect for the maintenance of naïve pluripotency in mice. The addition of MAPK/ERK kinase (MEK) inhibitor PD0325901 and glycogen synthase kinase 3β (GSK3β) inhibitor CHIR99021 (together referred as 2i cocktail) to the culture media with or even

without LIF can maintain mESCs in the so-called naïve ground state of pluripotency (Ying et al., 2008), mimicking the transcriptional profile of the E4.5 preimplantation epiblast and yielding a less heterogeneous population of mESCs than when cultured in serum/LIF medium (Boroviak et al., 2015). On the other hand, although hESCs differentiate after exposure to 2i alone (Hanna et al., 2010), most of the protocols described for generating naïve hESCs incorporate 2i among other components, suggesting that MAPK/ERK and Wnt signalling also play a role in the maintenance of human naïve pluripotency.

There are also several epigenetic features that distinguish naïve and primed cells (Figure 5). In general, genomic DNA of naïve cells is hypomethylated, whereas primed ESCs have a hypermethylated genome. These differences are evident in mESCs, since 2i/LIF ground state mESCs have widespread DNA hypomethylation, even losing imprinting marks (Yagi et al., 2017), whereas the non-ground state but yet naïve mESCs cultured in serum/LIF have much higher levels of methylation, although lower than that of mEpiSCs (Hackett et al., 2013). This characteristic is especially surprising since this pattern does not match that of their *in vivo* counterparts. In fact, both pre- and postimplantation mouse epiblast cells present a globally hypomethylated DNA (Veillard et al., 2014; Takahashi et al., 2018). Moreover, an increase of H3K27me3 repressive marks in the promoter regions of developmental genes is observed in hESCs and mEpiSCs compared to mESCs (Gafni et al., 2013; Hackett et al., 2013).

The activation status of one of the X chromosomes in female cells is also a distinguishing trait between naïve and primed cells. Naïve mESCs display two active X chromosomes, whereas female mEpiSCs have already undergone X chromosome inactivation (XCI) process (Guo et al., 2009). Most of the conventional hESC lines also display one inactive X chromosome (Figure 5), although the XCI status in female hESCs can vary (Hoffman et al., 2005), and epigenetic marks of XCI like H3K27me3 accumulation or *XIST* cloud can be lost during extended *in vitro* culture in a process known as erosion (Mekhoubad et al., 2012). This erosion seems to be regulated by *XACT*, which normally coats the active X chromosome but also coats the inactive X chromosome just before it

loses its XCI marks. As for gene expression, it is reported that in eroded X chromosomes, genes in H3K27me3 domains are reactivated, while those mapped within H3K9me3 domains remain inactive (Vallot et al., 2015). The eroded X chromosome in primed hESCs does not undergo XCI upon cell differentiation, which may be problematic regarding the clinical use of female hESCs due to the lack of proper dosage compensation (Vallot et al., 2015; Sahakyan et al., 2017; Takahashi et al., 2018).

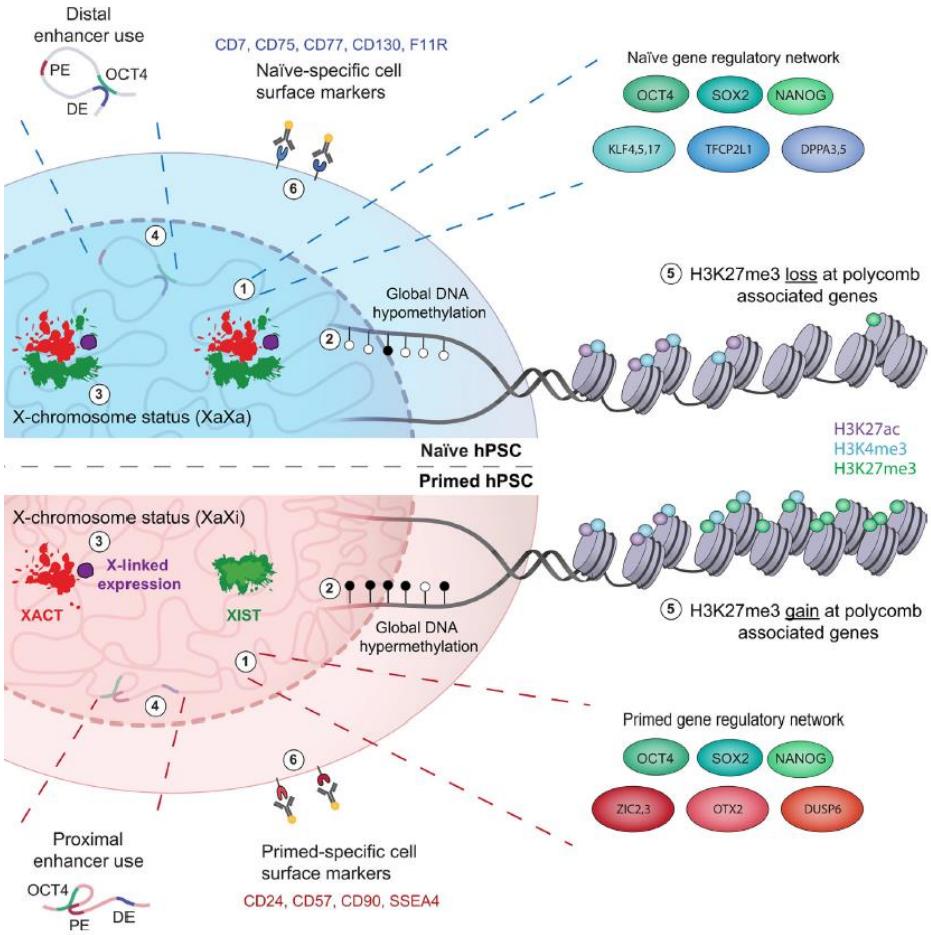

Figure 5. Overview of differential characteristics of naïve and primed hESCs. From Collier et al., 2018. Creative commons license CCBY 4.0.

Additionally, the regulation of *OCT4* gene expression varies between naïve and primed pluripotent cells. The *OCT4* gene has three cis-regulatory elements that

regulate its expression: the proximal promoter, the proximal enhancer and the distal enhancer, which regulate its expression (Yeom et al., 1996). In naïve cells, *OCT4* expression is controlled by the distal enhancer, whereas in primed cells the proximal enhancer is preferentially activated over the distal one (Tesar et al., 2007; Gafni et al., 2013) (Figure 5). This is consistent with their in vivo counterparts, since the distal enhancer regulates *OCT4* in the ICM and the proximal enhancer does it in the postimplantation epiblast of the mouse embryo (Yeom et al., 1996).

Naïve and primed ESCs also have differences in their metabolism. Whereas naïve cells use both mitochondrial respiration (oxidative phosphorylation) and glycolysis to generate energy, primed cells are almost exclusively glycolytic (Zhou et al., 2012; Takashima et al., 2014). Mouse ESC have a much higher basal oxygen consumption rate than mEpiSCs and hESCs, whereas the latter two have higher extracellular acidification rate, which is correlated with glycolytic activity. Moreover, the mitochondrial membrane potential is lower in primed cells, and they down-regulate the expression of most cytochrome c oxidase genes, which is also observed in cells from the mouse epiblast *in vivo* (Zhou et al., 2012).

Another marker used to distinguish naïve and primed ESCs is the intracellular localization of transcription factor E3 (TFE3). TFE3 is predominantly, although not exclusively, localized inside the nucleus in naïve ESCs. Contrarily, it is restricted to the cytoplasm in differentiating mESCs and primed ESCs (Betschinger et al., 2013; Gafni et al., 2013).

Regarding gene expression, both naïve mESCs and primed mEpiSCs and hESCs express the core pluripotency markers *OCT4*, *NANOG* and *SOX2*. However, some genes like *Essrb*, *Klf2*, *Klf4*, *Klf5*, *Zfp42*/*Rex1*, *Dppa3*/*Stella*, *Tfcp2l1*, *Fgf4*, *Tbx3* or *Cdh1* are upregulated in mESCs compared with mEpiSCs, whereas mEpiSCs express higher levels of lineage commitment factors like *Otx2*, *Zic2*, *Brachyury* (*T*), *Dnmt3b* or *Fgf5* (Kumari, 2016; Weinberger et al., 2016). Despite both being considered primed cells, conventional hESCs show some differences with

mEpiSCs in their gene expression pattern. For example, they retain some expression of *KLF4*, *REX1* and *PRDM14*, which are considered naïve markers in the mouse, and do not express the postimplantation marker *FGF5* (Ávila-González et al., 2016). Moreover, transcriptional analysis of human and primate embryos revealed some differences in the gene expression pattern of the epiblast compared to that of mouse embryos. For example, human and marmoset epiblasts lack expression of *ESRRB* or *KLF2*, and express *KLF17*, which is absent in the mouse epiblast (Blakeley et al., 2015). Extensive analysis of human postimplantation embryos is difficult due to ethical limitations, but a study in marmoset postimplantation embryos revealed that genes expressed in the preimplantation epiblast and not in postimplantation stages included *TFCP2L1*, *KLF5*, *KLF17*, *NODAL* and *SOX15*. *KLF4* and *DNMT3L* were also drastically downregulated upon implantation (Nakamura et al., 2016). Recently, a 3D culture system for human embryos for up to 14 days was developed. Consistent with results in cynomolgus monkey embryos, the human epiblast was found to downregulate *DNMT3L*, *TFCP2L1*, *KLF4* and *KLF17* after implantation, while upregulating the primed marker *CD24* (Xiang et al., 2020). This suggests that the true naïve pluripotent state in primates might be unique and different from that of the mouse.

The fact that conventional hESCs display a primed state of pluripotency despite being derived from the ICM of the blastocyst like naïve mESCs is somehow surprising. Several authors have tried to shed light on this issue by focusing on the existing differences in the embryonic development between mice and humans. Mouse embryos can be kept in a dormant state in a process called diapause. Diapause, or delayed implantation, is a developmental arrest of the embryo at the late blastocyst stage that happens in order to ensure that the birth of the offspring occurs at a time within the most favourable environmental conditions (Daniel, 1970). Thus, cells of the mouse epiblast have mechanisms to be held at a pluripotent state; otherwise, they would rapidly initiate differentiation. It was proposed that this feature could provide a time window in which this naïve pluripotent state would be more easily captured *in vitro*. On

the other hand, in human embryos, as well as in many other mammalian embryos, such mechanisms do not exist. Since in these cases cells from the early embryo rapidly undergo differentiation, this could make it harder to establish the naïve state *in vitro* in species other than the mouse (Nichols and Smith, 2011; Rossant, 2015).

The formation of the postimplantation epiblast also differs between mice and humans. After implantation, the mouse embryo forms a structure called the egg cylinder, in which the epiblast cells organize themselves into a cup-shaped structure surrounded by the hypoblast. This process needs directed apoptosis of the internal epiblast cells. In contrast, primate and human epiblasts form a flattened simple structure called embryonic or epiblast disc (Figure 6).

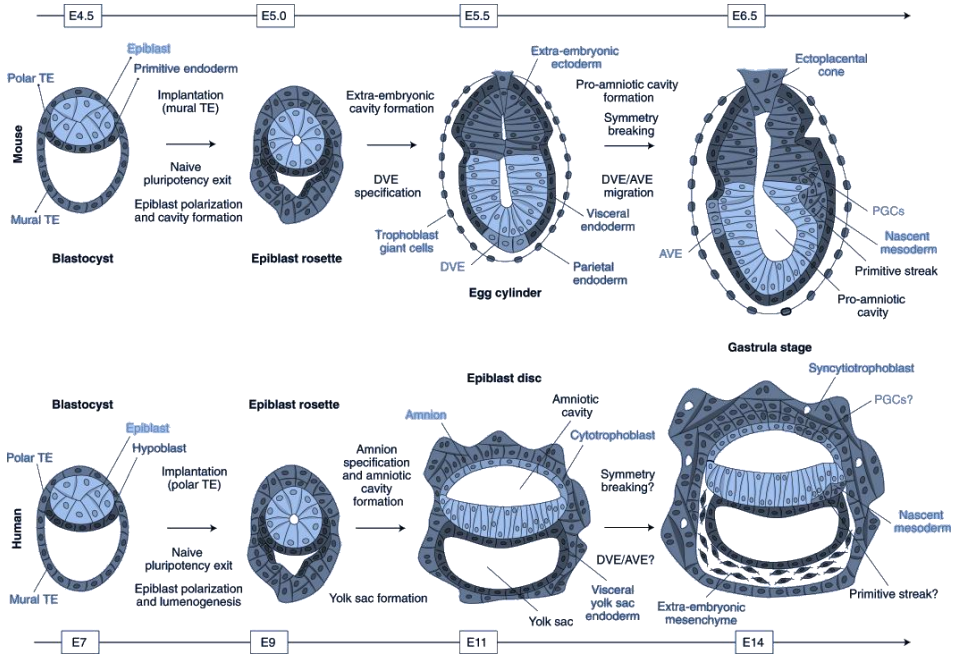

Figure 6. Early embryonic development in mouse and human embryos. Adapted by permission from Springer Nature. Nature Cell Biology. Deconstructing and reconstructing the mouse and human early embryo. Shahbazi and Zernicka-Goetz. Copyright (2018).

These differences could make it easier for non-rodent ESCs to progress *in vitro* to a more primed state (Nichols and Smith, 2009). Additionally, in the human embryo, the amnion arises from the postimplantation epiblast, and the amniotic epithelium interacts directly with the epiblast cells. It has been proposed that this interaction can influence the differentiation of the epiblast towards the neuroectoderm (Ávila-González et al., 2021). This could be reflected in hESCs, the *in vitro* counterpart of the postimplantation epiblast.

4.2.2. Intermediate pluripotency states

The idea of the existence of two well-defined pluripotent states has been recently challenged with the characterization of some types of cells that display a pluripotent state that differs from the naïve or primed states. The first hint of the existence of an intermediate pluripotent state was found back in 2011, when epiblast-like cells (EpiLCs) were described as transient cells resembling the pre-gastrulating mouse epiblast but distinct to mEpiSCs. These cells showed a high competence for PGC specification (Hayashi et al., 2011). Six years later, this intermediate pluripotent state was described as formative pluripotency. It has been hypothesised that this formative pluripotency represents a capacitation state during which cells exit the naïve state and acquire competence for lineage differentiation before entering the primed state (Smith, 2017).

The epiblast of the mouse embryo forms a rosette structure at E5.0, when the embryo is implanted (Bedzhov and Zernicka-Goetz, 2014). It has been proposed that epiblast cells at the rosette stage (E5.0-E5.5) display an intermediate pluripotent state between the preimplantation naïve state and the postimplantation primed state. Cells from the rosette stage can be captured *in vitro* as rosette-like SCs (RSCs) with a combination of LIF, Wnt inhibitor IWP-2 and MEK inhibitor PD325901. RSCs present high expression of the naïve marker *Klf4* and the primed marker *Otx2*, and do not express *Oct6*, consistent with the epiblast at E5.0 (Neagu et al., 2020).

More recently, two studies described the establishment of formative pluripotent SCs in mouse and human. Kinoshita and colleagues reported that activin A at

low concentrations, Wnt inhibitor XAV939 and a pan-retinoic acid receptor inverse agonist could be used to derive formative SCs (FSCs) from the mouse E5.5 epiblast. Additionally, culturing naïve hESCs in these conditions could produce human FSCs resembling the human epiblast at E10-12. (Kinoshita et al., 2021). On the other hand, Yu and colleagues used FGF2, Activin A and either WNT3A or a GSK3β inhibitor to activate the FGF, TGF-β and Wnt signalling pathways (FTW) and generate FTW-mESCs from mouse embryos and FTW-hiPSCs. Like mouse FSCs, the expression profile of FTW-mESCs correlates with the E5.5-6.0 mouse epiblast. However, the transcriptome of human FTW-PSCs resembles the E8.0 epiblast (Yu et al., 2021b).

5. Human Embryonic Stem Cells

The first hESC lines were obtained in 1998 from isolated ICMs from 5 human blastocysts (Thomson et al., 1998), 17 years after the derivation of the first mESC lines. As explained before, despite both being derived from preimplantation embryos, hESCs and mESCs show important differences, especially in their morphology, gene expression and culture requirements. Conventional hESCs are known to share more similarities with mEpiSCs than with mESCs (Tesar et al., 2007), and thereby they are considered to represent a primed state of pluripotency. Nonetheless, it is currently possible to also generate naïve hESCs. In this section, the procedures for the derivation and maintenance of conventional and naïve hESC lines will be detailed.

5.1. Sources of hESCs

Human ESCs can be derived from whole embryos, usually at the blastocyst stage, or from isolated blastomeres of cleavage-stage embryos, typically at the 4 or 8-cell stages.

5.1.1. Derivation of hESCs from blastocysts

Derivation of hESCs from blastocysts can be performed by isolating the ICM (Figure 7A) or by whole embryo culture after the removal of the zona pellucida (Figure 7B) (Kim et al., 2005).

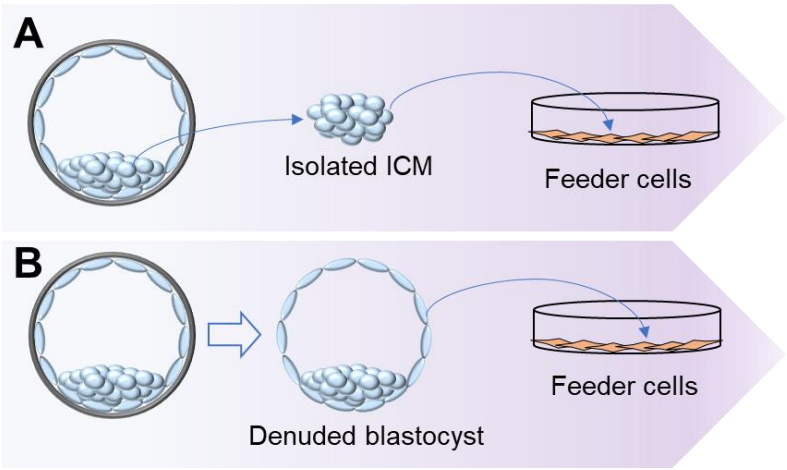

Figure 7. Representation of the two main methodologies for the derivation of hESCs from blastocysts. A) hESC derivation with ICM isolation. B) hESC derivation through whole embryo culture,

The ICM can be isolated from blastocysts by several methods. The first method used was immunosurgery, and it has been routinely applied for several years. Nevertheless, a major inconvenient of this method is that it requires the presence of animal serum in the media used for the immunosurgery, thus making the hESC lines obtained with this procedure unsuitable for clinical purposes (Khan et al., 2018). Mechanical isolation of the ICM using fine metallic needles has also been reported (Ström et al., 2007). It is a completely xeno-free method, but it is a very laborious procedure (Khan et al., 2018). Finally, the most promising method for obtaining xeno-free hESC lines from the ICM is by laser-assisted isolation. This method was first described in mice (Tanaka et al., 2006) and then in humans (Turetsky et al., 2008). In this last study, 3 hESC lines were derived from 8 isolated ICMs of blastocysts that had previously been diagnosed with a PGT on a biopsied blastomere.

Derivation of hESCs from whole embryo culture is especially indicated for low-quality blastocysts in which the ICM is difficult to distinguish (Kim et al., 2005). The main advantage of the whole embryo derivation is its simplicity, since the only manipulation that must be done to the embryo is removing its zona pellucida by incubating with pronase or Tyrode's acidic solution (Heins et al., 2004). However, it is not clear if the derivation efficiency from whole blastocysts is equal to that from isolated ICMs.

In 2012, O'Leary and colleagues described the existence of a transient structure during the first few days of the hESC derivation process from whole blastocysts. This structure was called the post-inner cell mass intermediate (PICMI). PICMI formation was reported to be essential and sufficient for the establishment of a hESC line, since virtually all the karyotypically normal PICMIs were able to give rise to a hESC line and none of the outgrowths without PICMI were able to generate hESCs (O'Leary et al., 2012).

The PICMI arises 2-3 days after blastocyst plating and it increases in size until 7 days after plating. Morphologically, it is a round-shaped structure composed of small epiblast-like cells surrounded by a thin layer of GATA6-positive hypoblast-like cells. For proper hESC derivation, the PICMI must be mechanically isolated from the surrounding trophectodermal cells and plated onto a new feeder layer. Then, the PICMI flattens and hESC-like cells begin to grow (O'Leary et al., 2012, 2013; van der Jeught et al., 2015). Using this approach, the efficiency of PICMI formation from blastocysts with good quality ICMs was 21,3% (47 PICMIs out of 221 blastocysts) whereas the generation of hESC lines from further passaged PICMIs was 80,6% (25 hESC lines out of 31 PICMIs) (O'Leary et al., 2012).

The PICMI has a unique molecular and transcriptional signature that differs from that of the human ICM and from hESCs themselves. It has been shown to express genes from both early and late epiblast, and early germ cell markers (O'Leary et al., 2012). A more exhaustive transcriptional analysis confirmed the upregulation of germ cell markers and showed that the PICMI expresses

markers of both primed and naïve pluripotency. It was demonstrated that naïve-related properties like the expression of extracellular matrix (ECM)-related components and LIF, BMP, Wnt and phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathways in the ICM of the embryo are progressively downregulated during the derivation process, whereas FGF and Activin/Nodal signalling are upregulated in the hESC primed state, with the PICMI being the crucial turning point. Since the ICM progresses *in vitro* to form the PICMI, these findings suggest that the formation of the PICMI during the derivation process could be what causes hESCs to adopt their primed pluripotent state resembling mEpiSCs, which are derived directly from the mouse postimplantation epiblast (Figure 8). The PICMI could be a starting point for the obtention of hESCs in distinct pluripotent states and for PGC differentiation (Warrier et al., 2018).

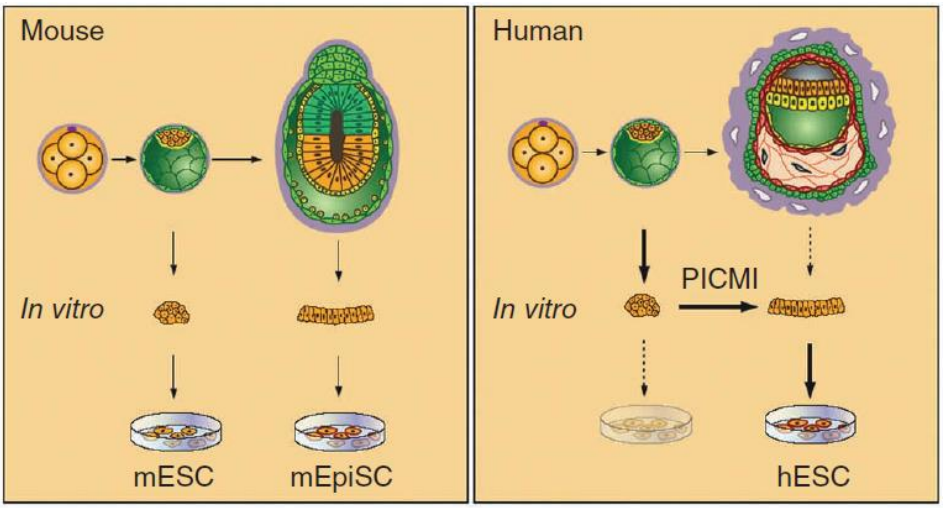

Figure 8. Schematic representation of the derivation of pluripotent cells from mouse and human embryos. Reprinted by permission from Springer Nature: Nature Biotechnology. Tracking the progression of the human inner cell mass during embryonic stem cell derivation. O'Leary et al. Copyright (2012).

5.1.2. Derivation of hESCs from single blastomeres

There are some ethical issues regarding the obtention of ESCs from human embryos, as the traditional methodology for hESC derivation from embryos at

the blastocyst stage inevitably involves the destruction of the embryo used. To avoid these ethical concerns, protocols for the derivation of ESCs from single blastomeres isolated from cleavage-state embryos were developed first in mice (Delhaise et al., 1996) and then in humans (Klimanskaya et al., 2006).

The derivation of ESCs from single blastomeres does not necessarily involve the destruction of the embryo, since the biopsy of one or two blastomeres from a day 3 human embryo, typically around the 8-cell stage (Figure 9), barely affects its viability (Magli et al., 1999). Indeed, embryo biopsy is widely used for PGT in fertility clinics, and this approach could be used to obtain autologous hESC lines if the biopsied embryo is afterwards transferred and a child is born. Nevertheless, as hESC derivation efficiencies from single blastomeres are very low at this moment, there is still a long way to go. On the other hand, the use of all the blastomeres of an embryo, although causing its destruction, may increase the chance of obtaining at least one ESC line from that embryo by as many times as blastomeres the embryo has. This approach can also lead to a sensible reduction of the total number of embryos needed (González et al., 2011; Vila-Cejudo et al., 2019).

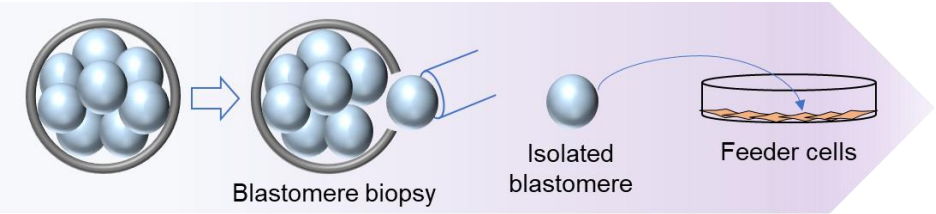

Figure 9. Representation of the hESC derivation process from single blastomeres.

Klimanskaya and colleagues obtained the first hESC lines from single blastomeres in 2006 (Klimanskaya et al., 2006). Since then, the methodologies and culture conditions for the derivation of hESC lines from single blastomeres have been optimised to maximise its success rate.

The first approach involved co-culturing the isolated blastomeres in the same drop with the parental embryos and later co-culturing the blastomere-derived

aggregates with GFP-positive established hESC colonies on inactivated MEFs, which allowed the derivation of 2 hESC lines out of 91 blastomeres (efficiency rate 2.2%) (Klimanskaya et al., 2006). Two years later, the same group showed that hESC co-culture was not necessary to derive hESC lines from single blastomeres if they were cultured for 24 h on laminin to recreate the ICM niche and prevent TE differentiation. They claimed to have achieved a 50% efficiency, although it was just one hESC line out of two blastomere-derived aggregates. Moreover, one of the aggregates came from two biopsied blastomeres (Chung et al., 2008).

Ilic and colleagues also showed in 2009 that co-culture of the biopsied blastomeres with the parental embryo was not necessary to obtain hESC lines. They derived hESC lines from biopsied blastomeres cultured on low passage inactivated human foreskin fibroblasts (HFFs) with minimal exposure to xenomaterials (Ilic et al., 2009). In the same year, Geens and colleagues reported the derivation of 2 hESC lines out of 16 blastomeres (12.5%) from four 4-cell stage embryos (Geens et al., 2009).

By then, all the studies had been performed using high quality embryos and sequential culture of the blastomeres, letting them form aggregates before seeding them onto feeder cells. In 2013, Yang and colleagues demonstrated that hESC lines could be derived from blastomeres from low quality embryos by direct plating onto feeder cells in standard hESC medium. However, their success rate was low, around 1% (Yang et al., 2013).

In the same year, Taei and colleagues stated that culturing the embryos in media supplemented with GSK3β inhibitor CHIR99021 and Rho-associated protein kinase (ROCK) inhibitor Y-27632 from the 4-cell stage increased the hESC derivation efficiency from single blastomeres to 13% (8 hESC lines out of 61 blastomeres). They also used low and fair quality embryos and direct plating of the blastomeres for these experiments, although they used MEFs as feeder cells and pointed out that they could not obtain a single hESC line using HFFs (Taei et al., 2013).

Despite being more ethically acceptable, hESC derivation from single blastomeres still has some disadvantages over derivation from blastocysts, the main one being its lower efficiency. In humans, derivation from blastocysts can yield success rates of up to 52 % (Ilic et al., 2007; Chen et al., 2009a), much higher than in hESC derivation from single blastomeres.

5.2. Maintenance of hESC pluripotency

Culture conditions for hESCs have evolved greatly since they were first derived in 1998. Initial protocols included the co-culture of hESCs with MEFs as feeder cells or over an animal-derived matrix in medium containing foetal calf serum (FCS). In these systems, the exposure of hESCs to animal products was high, thus making these derived lines unsuitable for clinical applications due to the risk of immunorejection or infection transmitted by animal pathogens. Since then, several culture protocols that minimize the exposition of hESCs to xeno-products, while still maintaining their pluripotency, have been developed in order to generate clinical-grade hESC lines.

5.2.1. Feeder layers and feeder-free systems

Feeder cells support long-term maintenance of hESCs in an undifferentiated state by two means. First, they provide a surface for the attachment of the hESC colonies by expressing cellular adhesion molecules and producing an ECM. Second, they secrete growth factors that maintain the pluripotent state of hESCs, such as TGF-β, bFGF or Activin A (Greber et al., 2007; Eiselleova et al., 2008).

As previously mentioned, MEFs were the first feeder cells used for the culture of hESCs, and they are still widely used nowadays. However, the presence of animal-derived fibroblasts in hESC culture may not be desirable since they can be the source of potential pathogenic products and diminish the suitability of the hESC lines for clinical applications (Hovatta et al., 2003; Cobo et al., 2008). To overcome this, several human cell types have been successfully used as feeder cells for hESC culture, including fallopian tube cells (Bongso et al., 1994), fetal muscle cells, fetal and adult skin cells (Richards et al., 2002, 2003), foreskin

fibroblasts (Amit et al., 2003; Hovatta et al., 2003), bone marrow cells (Cheng et al., 2003), placental cells (Genbacev et al., 2005), endometrial cells (Lee et al., 2005), and umbilical cord cells (Cho et al., 2010). Fibroblast-like cells derived autologously from hESCs have also been used (Chen et al., 2009b). It is reported that the ability of human feeder layers to support hESC growth varies depending on the cell line used, but some of them are as supportive as MEFs (Richards et al., 2003).

An advantage of human feeders over MEFs is that they can support hESC growth at late feeders' passages (up to 16), whereas MEFs are most optimally used at passages 4-6 because, after that timespan, they enter into senescence (Richards et al., 2003; Meng et al., 2008). Especially, HFFs, the most used human feeder cell type, are more durable than MEFs after inactivation. Indeed, inactivated HFFs can last for more than 2 weeks, whereas inactivated MEFs begin to deteriorate after one week. Moreover, the stability of HFFs, which can be propagated in culture for more than 20 passages, reduces the batch-to-batch variability associated with MEF feeder layers, which need to be replaced and prepared more frequently (Ma et al., 2012).

The secretion of growth factors from different types of human feeder layers was compared with that of MEFs. Eiselleova and colleagues found differences in the production of bFGF and Activin A. Human feeders produced significant amounts of bFGF, whereas bFGF production from MEFs was almost undetectable. On the other hand, MEFs produced significantly higher amounts of Activin A than human feeders. They correlated these differences in Activin A secretion with the overexpression of the pluripotency marker SSEA-3 by hESC cultured over MEFs (Eiselleova et al., 2008). Consistent with these findings, Yang and colleagues also stated that HFFs, but not MEFs, produce significant levels of bFGF, therefore hESCs could be cultured on HFFs without exogenous bFGF supplementation (Yang et al., 2016).

Culture systems that do not rely on feeder cells were developed to overcome the variability associated with feeder cells. These feeder-free culture systems

allowed researchers to better understand the requirements of ESCs for maintaining their pluripotent state.

The first feeder-free hESC culture was described by Xu and colleagues in 2001. They used Matrigel as a substrate in combination with MEF-conditioned medium (Xu et al., 2001). Matrigel is a gelatinous mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells that contains several ECM components like laminin, nidogen, collagen IV, proteoglycans, and growth factors like TGF-β and epidermal growth factor (EGF), among others (Kleinman et al., 1982; Kleinman, 2001). The main drawbacks of the use of Matrigel are the animal origin of their components, which compromises the clinical safety of the cultured hESCs, and the batch-to-batch variability, which may affect the reproducibility of the experiments. Nonetheless, it has become the standard for feeder-free hESC cultures in research labs all over the world.

To overcome the issues of Matrigel, defined systems that use components of the human ECM and recombinant proteins were described. Xu and colleagues already highlighted the ability of laminin to maintain hESC in an undifferentiated state in MEF-conditioned medium (Xu et al., 2001), and it was later reported that the addition of Activin A to hESC cultures over laminin could replace MEF-conditioned medium (Amit et al., 2004). Human ESC culture was also possible using human fibronectin as substrate in combination with growth factors like bFGF and TGFβ (Beattie et al., 2005). Since then, several xeno-free culture systems using laminin as a substrate have been described, mainly in combination with the commercial xeno-free and chemically defined medium TeSR1. Other ECM components that have been used as a substrate are E-cadherin and vitronectin (reviewed in Desai et al., 2015).

Further optimization of the feeder-free cultures was achieved with synthetic substrates, which include peptide or protein-based systems, polymers, and polymers in conjunction with biomolecules. Some of the successful approaches were the utilization of surfaces containing the heparin-binding peptide GKKQRFRHRNRKG, isolated from vitronectin (Klim et al., 2010), and the

synthetic polymers PMEDSAH and APMAAm, which are reported to support undifferentiated growth of hESC for at least 20 passages (Villa-Diaz et al., 2010; Irwin et al., 2011).

5.2.2. Signalling modulation

The development of the first feeder-free defined media systems allowed the identification of critical factors for the maintenance of pluripotency of hESCs (Ohtsuka and Dalton, 2008). Moreover, researchers had to overcome the fact that hESCs seemed to have culture requirements that severely differed from those of mESCs. One of the first differences between hESCs and mESCs to be detected was that the former do not need LIF to maintain their pluripotency, whereas the latter require LIF for their maintenance in culture through STAT3 activation (Dahéron et al., 2004; Humphrey et al, 2004). As previously described (section 4.2.1), mouse embryos are subject to diapause, or delayed implantation mechanism. LIF signalling is thought to enable the diapause in mouse embryos, thereby maintaining the epiblast cells in an undifferentiated state (Nichols et al., 2001). Since human embryos do not have the capacity to enter diapause, human ICM cells might not respond to LIF signalling in the same way as mouse ICM cells (Dahéron et al., 2004; Humphrey, 2004). Nevertheless, LIF is commonly used in most of the media formulations described to support human naïve hESCs.

In humans, almost all the protocols developed for the culture of ESCs include exogenous supplementation with bFGF. It can activate at least four signalling pathways, including Janus kinase/STAT (JAK/STAT), phosphoinositide phospholipase Cγ (PLCγ), PI3K and MAPK/ERK pathways (Dailey et al., 2005; Lanner and Rossant, 2010).

bFGF has an effect on the feeder cells that support hESC growth. It acts on the feeder cells by inducing the expression of several components of the TGFβ/Activin signalling pathway, which is also known to support pluripotency in hESCs. Moreover, bFGF can also induce the expression of TGFβ and insulin growth factor 2 (IGF2) in hESC-derived fibroblast cells to generate a pluripotency-maintaining niche (Greber et al., 2007). Another study reported

that endogenous expression of bFGF from hESCs is essential to maintain them in an undifferentiated state, whereas exogenous supplemented bFGF supports hESC cloning efficiency by enhancing survival and adhesion, although it has a redundant effect on the maintenance of the pluripotency (Eiselleova et al., 2009).

Another important difference between mouse and human ESCs is the role of MAPK/ERK signalling. Inhibition of MAPK/ERK signalling by PD0325901, in conjunction with GSK3β inhibitor CHIR99021, stabilizes mESCs and keeps them in the so-called ground state of pluripotency (Ying et al., 2008). However, the role of MAPK/ERK signalling is less clear in hESCs. The involvement of ERK in maintaining hESC pluripotency might only happen at low concentrations. Dalton and colleagues suggested that a crosstalk mechanism with the PI3K/AKT pathway regulates the concentration of ERK. At low FGF concentrations, ERK is kept under a threshold level required to promote differentiation. At high concentrations, FGF can activate the PI3K/AKT pathway, in which AKT suppresses the activity of ERK, also maintaining its levels under the threshold level and thereby maintaining the pluripotency of hESCs (Dalton, 2013).

The role of Wnt signalling in hESCs has also been controversial. As mentioned above, inhibition of the member of the β-catenin degradation complex GSK3β, which results in the activation of the canonical Wnt pathway, promotes a ground state of pluripotency in mESCs (Ying et al., 2008). However, research in hESCs has produced contradictory results. Some authors believe that activation of Wnt signalling maintains hESCs in a pluripotent state (Sato et al., 2004; Ullmann et al., 2008), whereas others suggest that Wnt signalling promotes hESC differentiation (Dravid et al., 2005; Sumi et al., 2008; Bone et al., 2011; Davidson et al., 2012). Singh and colleagues suggested that the main reason for this disparity of results is that authors may have overseen the fact that multiple pools of GSK3β exist within the cell, and that it can act in a Wnt-independent manner (Singh et al., 2012). It is believed that at least two pools of GSK3β exist: one being regulated by Wnt ligands and the other by AKT in the PI3K pathway (Ding et al., 2000; Wu and Pan, 2010). Apart from its role in the Wnt canonical

pathway, it is believed that GSK3β also phosphorylates and inhibits c-MYC (Gregory et al., 2003) (Figure 10). The addition of low concentrations of GSK3β inhibitors to the culture media can stimulate the proliferation of hESCs, but at higher inhibitor concentrations, hESCs differentiate (Tsutsui et al., 2011). Moreover, Singh and colleagues suggested that there might be different threshold inhibitory concentrations for GSK3β in each pathway. They proposed that the GSK3β from the pool regulated by AKT has a lower inhibition threshold, so low GSK3β inhibitors concentrations could enhance self-renewal of hESCs by stabilizing c-MYC, whereas higher inhibitor concentrations could promote differentiation by stabilizing β-catenin through the canonical Wnt pathway (Singh et al., 2012; Dalton, 2013).

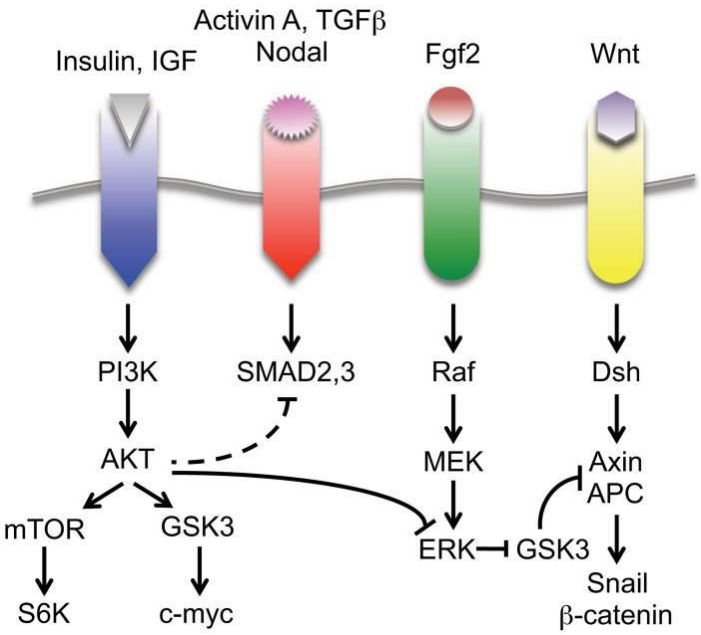

Figure 10. Summary of the different signalling pathways involved in the maintenance of pluripotency in hESCs and their crosstalk mechanisms. Reprinted by permission from Elsevier. Current Opinion in Cell Biology. Signaling networks in human embryonic stem cells. Dalton S. Copyright (2013).

Regarding the TGFβ superfamily ligands, the first reports agreed that Activin A/Nodal signalling through SMAD2/3 was essential for the maintenance of the pluripotency in hESCs (Beattie et al., 2005; James et al., 2005; Vallier et al., 2005;

Xiao et al., 2006). By contrast, it was shown that BMP signalling through SMAD1/5/8 had the opposite effect: it promoted differentiation to TE, and BMP signalling had to be inhibited to maintain hESC pluripotency (Xu et al., 2002, 2005). This again contrasts with mESCs, in which BMP signalling is known to block differentiation into neuroectodermal lineages and sustain self-renewal (Ying et al., 2003; Morikawa et al., 2016).

Finally, despite some signalling pathways like MAPK/ERK, LIF/STAT3, TGFβ/Activin and Wnt having opposite or different effects in hESCs and mESCs, there is a general consensus that both types of ESCs require an active PI3K/AKT signalling pathway in order to maintain their pluripotency and self-renewal. It can be activated by several ligands like IGF, EGF or even FGF at high concentrations (Yu and Cui, 2016).

PI3K/AKT signalling is thought to maintain the pluripotent state in hESCs by several mechanisms, by cross-talking with other signalling pathways (Figure 10). First, it regulates the levels of SMAD2/3, a downstream effector of the TGFβ/Activin/Nodal signalling pathway. When PI3K/AKT signalling is active, it keeps SMAD2/3 in low activity levels compatible with self-renewal whereas, when inhibited, SMAD2/3 is highly activated and promotes differentiation of hESCs into mesendodermal lineages (Singh et al., 2012; Dalton, 2013; Yu et al., 2015). Second, active PI3K/AKT signalling suppresses ERK1,2 activity. Since it has been reported that ERK inhibits GSK3β, this leads to a stabilization of GSK3β activity. This prevents Wnt pathway effectors like β-catenin from being activated, thereby maintaining the pluripotent state (Singh et al., 2012; Dalton, 2013). Third, PI3K/AKT pathway activation could inhibit GSK3β from a pool unrelated to the Wnt canonical signalling. This may stabilize the transcription factor C-MYC, which regulates the expression of several pluripotency-associated genes (Figure 10). This is known to happen in mESCs but it is less clear in hESCs (Ohtsuka and Dalton, 2008; Singh et al., 2012; Dalton, 2013).

5.3. Naïve pluripotency in hESCs

5.3.1. Advantages and disadvantages of naïve hESCs

The generation of naïve hESCs has been achieved by several groups in the recent years, as detailed in the next section. Naïve hESCs have several advantages over their primed counterparts. Their increased single-cell clonogenicity and faster doubling times could facilitate the rapid obtention of a large number of cells for drug screening applications (Kumari, 2016). Naïve cells are also reported to have higher efficiency of homologous recombination than primed cells, which is useful for gene targeting modifications (Gafni et al., 2013).

Naïve hESCs are appropriate to study some characteristics of early preimplantational embryogenesis, such as the role of different signalling pathways or the epigenetic mechanisms involved in the first differentiation events (Collier and Rugg-Gunn, 2018). For instance, female lines serve as a good model for X chromosome dampening, a mechanism of dosage compensation observed in the cells of the TE, epiblast and primitive endoderm in the preimplantational human embryo but not in the mouse embryo. This phenomenon consists of a partial reduction of transcriptional activity in both X chromosomes, probably mediated by the long non-coding RNA *XIST* (Petropoulos et al., 2016; Sahakyan et al., 2017).

Recently, it was reported that hESCs can differentiate into extraembryonic lineages (TE and primitive endoderm), and pluripotent cells that can differentiate into TE-like cells have already been established (Gao et al., 2019). This capacity to generate TE cells is reported to be higher in naïve than in primed hESCs (Ávila-González et al., 2021). Therefore, naïve hESCs can be a valuable tool for the study of all the cell lineages of the early human embryo. Plus, it is known that conventional hESC or hiPSC lines are quite heterogeneous and present a differentiation bias, thereby reducing their potential to efficiently become cells from the three germ layers. Nevertheless, although naïve hESCs should have a broader differentiation potential than primed hESCs, their main limitation is that they are less responsive to differentiation stimuli, and an initial

priming or capacitation step is necessary in order to perform directed differentiation from naïve hESCs (Weinberger et al., 2016; Rostovskaya et al., 2019).

Another remarkable feature is that the eroded state of the X chromosome in female primed hESCs can be reverted if hESCs are converted to the naïve state prior to differentiation. It was reported that once conventional hESCs presenting X chromosome erosion were converted to naïve in 5iLAF conditions (Theunissen et al., 2014), they were able to undergo XCI again upon differentiation (Sahakyan et al., 2017). Since the presence of an eroded X chromosome poses a limitation for the use of female hESCs for clinical purposes due to altered dosage compensation, naïve female hESCs might be more appropriate in this scenario.

On the other hand, among the drawbacks of naïve hESCs are their increased susceptibility to develop chromosomal abnormalities during culture and the loss of methylation in imprinted regions (Pastor et al., 2016). This instability could be problematic for the clinical use of these cells. Note that these characteristics are also observed in naïve ground state mESCs cultured in 2i/LIF media. Since most of the protocols for obtaining naïve hESCs also include these components, the abnormalities may be caused by the culture conditions. Another hypothesis is that genomic instability may be intrinsic to the naïve state, since it is a transient state *in vivo* and it is not naturally prepared or programmed to be maintained for a long time (Weinberger et al., 2016).

5.3.2. Generation of naïve hESC lines

During the last decade, several approaches to generate naïve hESC lines have been designed (Table 1; Figure 11). The first naïve-like cells were obtained by conversion from primed hESCs in 2010, combining the ectopic overexpression of *OCT4*, *KLF2* and *KLF4* with culture in 2i/LIF conditions (2iL) (Hanna et al., 2010). This work also demonstrated that hESCs cannot be maintained in 2i/LIF alone, since the withdrawal of the transgene expression caused differentiation. This differentiation was palliated with the addition of forskolin. Three years

later, the same group developed a media formulation that consisted of a combination of inhibitors for MEK, GSK3β, protein kinase C (PKC), ROCK, c-Jun N-terminal kinase (JNK) and p38, along with LIF. This media was named naïve human stem cell media (NHSM) and it supported the conversion of conventional hESCs and hiPSCs to a naïve state, as well as direct naïve hESC derivation from human blastocysts without the use of transgenes (Gafni et al., 2013).

Since then, several other transgene-dependent and transgene-free protocols were described during the next 3-4 years. The methodologies relying on ectopic gene expression include the one reported by Takashima and colleagues, which used short-term overexpression of *NANOG* and *KLF2* and a culture media composed of titrated 2i with LIF and PKC inhibition by Gö6983, termed t2iLGö (Takashima et al., 2014), the protocol described by Chen and colleagues, which relied on lentiviral-mediated overexpression of *STAT3* and culture in 2iL conditions (Chen et al., 2015a), and the protocol described by Valamehr and colleagues, which used a non-integrative episomal reprogramming of fibroblasts with *OCT4*, *SOX2* and *SV40LT* followed by culture in the presence of LIF, bFGF, MEKi, GSK3βi and ROCKi to generate hiPSCs with properties resembling naïve ground state mESCs (Valamehr et al., 2014).

The transgene-free protocols described are based on combinations of several inhibitors and small molecules. In 2013, Chan and colleagues reported that MEKi, GSK3βi and AMP-activated protein kinase inhibitor (AMPKi) (3i cocktail)/LIF conditions (3iL) could support the maintenance of hESCs and hiPSCs in a pluripotent state that shared an expression signature with the naïve epiblast (Chan et al., 2013). Ware and colleagues showed that exposure of hESCs to histone deacetylase inhibitors (HDACi) SAHA and sodium butyrate followed by culture in 2i/bFGF (2iF) or 2iL allowed the establishment of naïve hESCs. This work also reported the derivation of a naïve hESC line from a blastocyst, albeit at an extremely low efficiency (Ware et al., 2014). The same year, Theunissen and colleagues described a combination of inhibitors that also supported the conversion of established hESCs into a naïve state and the direct derivation from blastocysts. This formulation included MEKi, GSK3βi, SRCi,

BRAFi and ROCKi (5i cocktail) along with LIF and Activin A with or without bFGF (5iLAF and 5iLA, respectively) (Theunissen et al., 2014). Duggal and colleagues described a protocol for a rapid conversion of established hESCs into a naïve state using a naïve conversion medium (NCM) that included 2i, bFGF, forskolin and ascorbic acid (Duggal et al., 2015). The next year, two additional studies reporting the generation of naïve hESCs from conventional cultures were published. Both included modifications of the 2i/LIF conditions: one added forskolin and lysophosphatidic acid, a Hippo pathway inhibitor (Qin et al., 2016), and the other added XAV939, a Wnt pathway inhibitor (2iLXAV) (Zimmerlin et al., 2016).

Derivation of naïve hESCs from isolated cells of the ICM of a human blastocyst has also been reported. Guo and colleagues used a modification of the t2iLGö media described by themselves (Takashima et al., 2014), adding the ROCK inhibitor Y-27632 and ascorbic acid for increased survival. This formulation was named t2iLGöY (Guo et al., 2016). One year later, the same group reported a protocol for the conversion of established hESCs into a naïve state maintained in t2iLGö medium after exposure of hESCs to the HDACi valproic acid. They claimed that these chemically reset lines were transcriptionally similar to the ones obtained by overexpression of *NANOG* and *KLF4* by Takashima et al. (2014) and to the ones previously derived from isolated cells of the ICM (Guo et al., 2017).

More recently, Hu and colleagues described a method for naïve conversion of primed hESCs based on promoting the nuclear translocation of TFE3. They performed a transient inhibition of mTOR by torin1 or rapamycin for 3 hours followed by culture in a medium containing 2i, LIF and human insulin (2iLI). Their naïve-like cells showed an expression profile similar to the naïve Elf1 line derived in 2iF conditions (Ware et al., 2014), as shown by the principal component analysis (PCA) of the RNA sequencing data (Hu et al., 2020).

Table 1. Summary of the protocols described for the generation of naïve hESC.

Reference	Media formulation	Ectopic gene expression	Derivation from embryos
Hanna et al., 2010	MEKi, GSK3i, LIF (2iL)	Yes (OCT4, KLF2 and KLF4)	No
Chan et al., 2013	MEKi, GSK3i, AMPKi, LIF (3iL)	No	No
Gafni et al., 2013	MEKi, GSK3i, PKCi, JNKi, ROCKi, p38i, bFGF, LIF, Activin A or TGF-β1 (NHSM)	No	Yes
Takashima et al., 2014	MEKi, GSK3i, PKCi, LIF (t2iLGö)	Yes (*KLF4* and *SOX2*)	No
Theunissen et al., 2014	MEKi, GSK3i, SRCi, BRAFi, ROCKi, LIF, Activin A with or without bFGF (5iLAF/5iLA)	Yes (*KLF2* and *NANOG*) / No	Yes
Valamehr et al., 2014	MEKi, GSK3i, LIF, bFGF, ROCKi	Yes (*OCT4*, *SOX2* and *SV40LT*)	No
Ware et al., 2014	MEKi, GSK3i, bFGF (2iF) after exposure to HDACi for 1-3 passages (HDACi+2iF)	No	Yes
Chen et al., 2015a	MEKi, GSK3i, LIF (2iL)	Yes (*STAT3*)	No
Duggal et al., 2015	MEKi, GSK3i, bFGF, forskolin, ascorbic acid (NCM)	No	No
Qin et al., 2016	MEKi, GSK3i, LIF, forskolin, lysophosphatidic acid	No	No
Zimmerlin et al., 2016	MEKi, GSK3i, LIF, tankyrase inhibitor (2iLXAV)	No	No
Guo et al., 2016	MEKi, GSK3i, PKCi, LIF, ROCKi, ascorbic acid (t2iLGöY)	No	Yes
Guo et al., 2017	MEKi, GSK3i, PKCi, LIF (t2iLGö) after exposure to HDACi	No	No
Hu et al., 2020	MEKi, GSK3i, LIF, insulin after exposure to mTORi	No	No

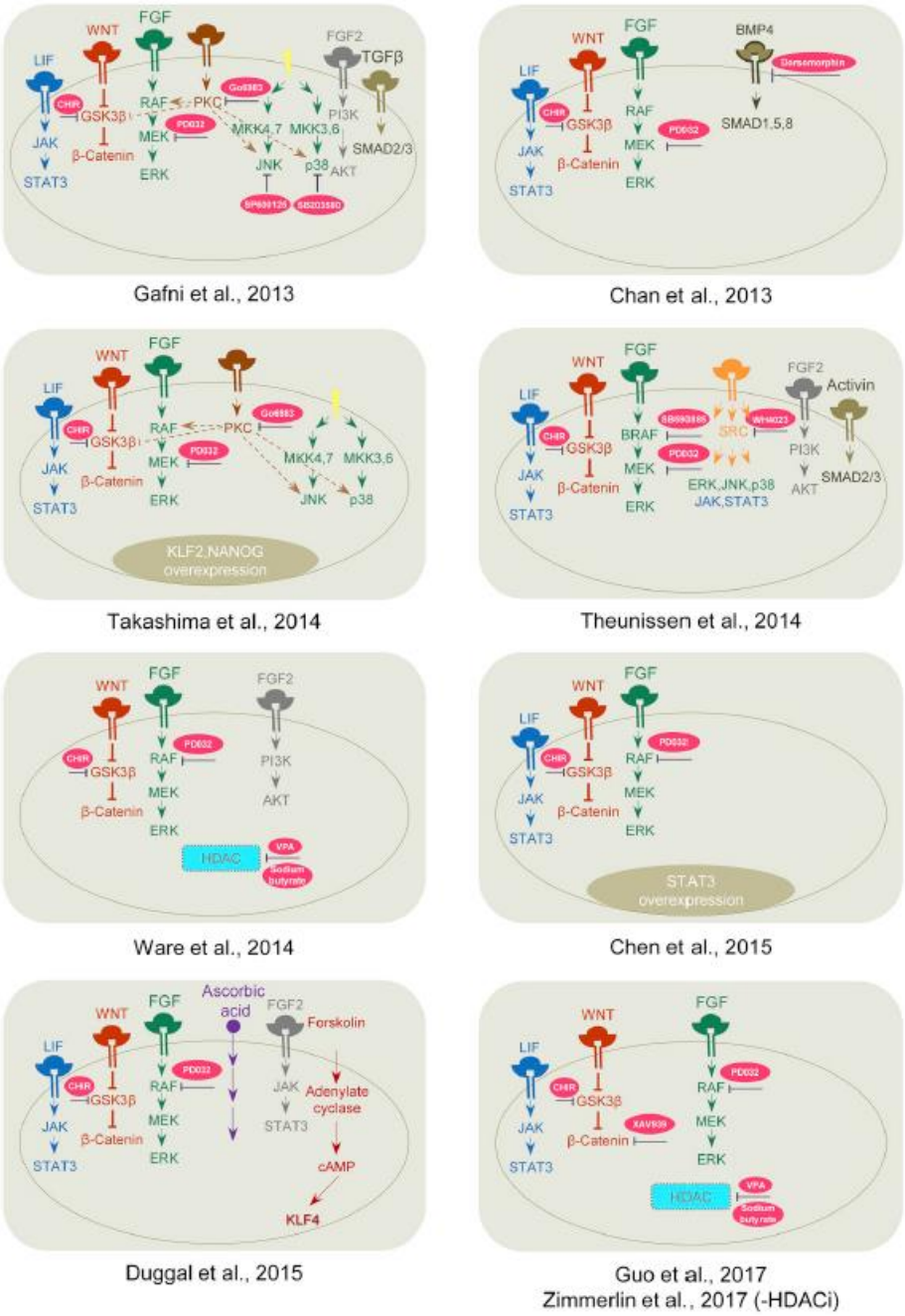

Figure 11. Representation of some of the protocols described to obtain naïve hESCs. Reprinted by permission from Elsevier. Experimental Cell Research. Signal regulators of human naïve pluripotency. Taei et al. Copyright (2020).

5.3.3. Looking for the real human naïve pluripotency state

The existence of a wide variety of naïve-like hESC populations obtained by different methodologies and by targeting different signalling pathways has led to the question of which is the "real" human naïve pluripotency state. To find it out, several groups carried out studies comparing the transcriptome and the molecular and epigenetic characteristics of most of the different populations of naïve hESCs between each other and also with naïve mESCs and the human and primate embryo.

The first analysis was performed by Huang and colleagues in 2014, just after the first protocols for obtaining naïve hESCs were published. Their analysis included naïve hESCs obtained in 2iL (Hanna et al., 2010), NHSM (Gafni et al., 2013), 3iL (Chan et al., 2013), HDACi+2iF (Ware et al., 2014), t2iLGö (Takashima et al., 2014) and 5iLA/5iLAF (Theunissen et al., 2014) conditions. They found that the naïve hESCs obtained in t2iLGö and in 5iLA/5iLAF were the most closely related in terms of gene expression. Moreover, these two sets of naïve hESCs were the most similar to mESCs cultured in 2i/LIF and their transcriptional profile corresponded to that of the human preimplantational blastocyst (Huang et al., 2014).

Consistent with that, Pastor and colleagues found that the naïve hESCs obtained in t2iLGö (Takashima et al., 2014) and in 5iLAF (Theunissen et al., 2014) were the ones that mostly upregulated the preimplantation epiblast-specific genes. In another work, Liu and colleagues reprogrammed human fibroblasts into naïve hiPSCs using the NHSM (Gafni et al., 2013), t2iLGö (Takashima et al., 2014) and 5iLAF (Theunissen et al., 2014) formulations. They confirmed that t2iLGö and 5iLAF cells clustered together and close to the human ICM with respect to their transcriptional profiles, but NHSM cells had an intermediate expression pattern between primed hESCs and the ICM (Liu et al., 2017c). Another group also determined that 5iLA cells and t2iLGö cells showed a gene expression profile more similar to that of the human epiblast (Stirparo et al., 2018).

Another study compared the transcriptional profile of different naïve hESC populations with the cynomolgus monkey embryo, which resembles the human embryo during early pre- and postimplantational development. Consistent with the previous findings, the naïve hESCs obtained in t2iLGö (Takashima et al., 2014) and in 5iLA/5iLAF (Theunissen et al., 2014), in addition to the ICM-derived naïve cells in t2iLGöY (Guo et al., 2016), showed a transcriptional profile more closely related to the preimplantation epiblast, whereas naïve cells generated in NHSM (Gafni et al., 2013) and 3iL (Chan et al., 2013) correlated to the postimplantation embryo, like conventional hESCs (Nakamura et al., 2016).

The developers of the NCM (Duggal et al., 2015) performed a comparison of different naïve hESCs that included their NCM cells, those obtained in NHSM (Gafni et al., 2013) and the ones generated in HDACi+2iF (Ware et al., 2014), termed RT. They reported that NCM and NHSM cells showed a stronger upregulation of naïve markers than RT cells. In the transcriptional analysis, the naïve cells clustered all together and separated from primed hESCs. However, in this study they were not compared with mESCs or preimplantation embryos. Regarding the differentiation potential of the naïve hESCs, all of them differentiated robustly into the three germ layers from embryoid bodies (EBs), except for NHSM cells, which showed inefficient differentiation into mesoderm. However, none of the naïve cells were able to differentiate into functional cell types when directed terminal differentiation protocols were applied (Warrier et al., 2017). The same year it was reported that NHSM cells showed efficient trilineage differentiation, whereas naïve 5iLAF and t2iLGö cells could only differentiate efficiently into mesoderm (Liu et al., 2017c).

Based on their gene expression profiles, Taei and colleagues suggested a classification for the different naïve hESCs in which 5iLAF and t2iLGö cells were named “bona-fide naïve hESCs” whereas the rest were classified as “intermediate naïve hESCs” (Taei et al., 2020). Since it was reported that 5iLAF and t2iLGö cells (“bona-fide naïve hESCs”) had a rather low differentiation potential *in vitro* and needed a capacitation step before being able to efficiently differentiate into cells from the three germ layers (Lee et al., 2017; Liu et al.,

2017c; Rostovskaya et al., 2019), a relationship between the degree of naïvety and the ability to differentiate has been suggested. Intermediate naïve cells are known to be more responsive to differentiation stimuli. For instance, naïve hESCs obtained in 2iLXAV medium, as well as RT, NCM and NHSM cells, all classified as intermediate naïve, can differentiate efficiently into cells from the three germ layers without a capacitation step (Zimmerlin et al., 2016; Warrier et al., 2017).

Regarding the methylation status, it has been reported that cells cultured in 5iLAF and t2iLGö show a global genome hypomethylation and a loss of methylation in imprinted regions, whereas cells obtained in NHSM show little change in methylation levels and remain intact in imprinted regions. Additionally, widespread chromosomal abnormalities in naïve cells cultured in 5iLAF after 14 passages have been reported (Pastor et al., 2016; Liu et al., 2017c).

On the other hand, contrarily to previous studies, Yousefi and colleagues analysed naïve-like hESC generated with 9 different protocols (Hanna et al., 2010; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014; Duggal et al., 2015; Guo et al., 2016; Qin et al., 2016) and found that all the naïve cells were generally homogeneous at the transcriptional level, since none of the naïve cell populations clustered separately on the PCA (Yousefi et al., 2019). However, no comparisons with the human embryo were carried out.

All in all, it seems that the pluripotent state *in vitro* highly depends on the small molecules, growth factors and inhibitors used in the culture media. But how about the embryonic origin? As of today, all the groups that reported direct derivation of naïve hESCs used embryos at the blastocyst stage cultured with different combinations of inhibitors and media supplements (Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014; Guo et al., 2016). It is unclear whether the derivation of hESCs from single blastomeres of pre-compaction embryos can yield hESCs with a different pluripotent state than that of hESCs derived from

blastocysts, and how the different embryonic origin can affect the establishment of the pluripotent state *in vitro*. In this context, a work that studied the XCI status of different hESC lines found some indications that female hESCs derived from single blastomeres could present both X-chromosomes active, a typical feature of naïve ESCs, at very early culture passages (Geens et al., 2016). Since these hESC lines come from an early developmental stage, it might be easier to recapitulate the naïve state *in vitro* in hESCs derived from single blastomeres of 8-cell embryos than in blastocyst-derived hESCs. This thesis will try to find an answer to this question.

Hypothesis and Objectives

Hypothesis

Human ESCs derived from single blastomeres of 8-cell embryos could present naïve pluripotency characteristics due to the early developmental stage of the source embryo, but these naïve pluripotency characteristics would be lost during extensive culture.

Objectives

The main objective of this thesis is to determine the effect of the developmental stage of the source embryo on the pluripotency state of hESCs.

To achieve this objective, three secondary objectives were proposed:

1. To derive hESCs from single blastomeres of 8-cell embryos and from whole blastocysts at the highest efficiency possible.

2. To determine the state of pluripotency of blastomere-derived hESCs and evaluate the effect of prolonged culture on their pluripotency state.

3. To analyse the transcriptome and the differentiation potential of blastomere-derived hESCs and compare them with those of blastocyst-derived hESCs.

Results

Study 1

The pluripotency state of human Embryonic Stem Cells derived from single blastomeres of eight-cell embryos

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Introduction

Embryonic stem cells (ESCs) can exist in at least two different states of pluripotency: naïve and primed (Nichols and Smith, 2009). Mouse ESCs (mESCs), which are derived from preimplantation embryos (Evans and Kaufman, 1981; Martin, 1981), are said to present a naïve pluripotency, whereas epiblastic stem cells (mEpiSCs), derived from the post-implantation epiblast (Brons et al., 2007; Tesar et al., 2007), are thought to be in a primed state of pluripotency. Among the features that distinguish naïve from primed cells are the presence of two active X chromosomes in female cells, an hypomethylated genome, a bivalent glycolytic/oxidative metabolism, the expression of several pre-implantation epiblast specific genes and the capacity to efficiently contribute to the germline of chimeric mice after injection into a recipient blastocyst (Nichols and Smith, 2009; Kumari, 2016; Weinberger et al., 2016). *In vitro*, primed stem cells are dependent on basic fibroblast growth factor (bFGF) and Activin A and, contrary to naïve cells, they do not respond to LIF signalling. Furthermore, naïve cells form dome-shaped colonies, in contrast to the large, flattened colonies of primed cells, have a faster doubling time and an increased single-cell clonogenicity (Tesar et al., 2007; Kumari, 2016). Human ESCs (hESCs), despite being derived from pre-implantation embryos, share more similarities with mEpiSCs than with mESCs (Brons et al., 2007; Tesar et al., 2007; Nichols and Smith, 2009). Therefore, they are classified as primed stem cells.

Naïve stem cells have some practical advantages over primed stem cells, and they are thought to be a more suitable starting point for potential clinical applications. For instance, their higher single-cell clonogenicity and faster doubling time can lead to a rapid obtention of a large number of cells for drug screening (Kumari, 2016). In addition, naïve stem cells are more suitable for gene-targeting studies due to their higher efficiency of homologous recombination (Zwaka and Thomson, 2003; Gafni et al., 2013). Finally, primed ESCs usually show a differentiation bias towards one or more cell lineages (Osafune et al., 2008), whereas naïve ESCs display a broader differentiation

potential, since they represent an earlier stage of development (Nichols and Smith, 2009; Weinberger et al., 2016).

During the last decade, several groups have developed protocols to obtain hESCs in a naïve pluripotency state, similar but still not equal to that of mESCs (Hanna et al., 2010; Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014; Chen et al., 2015; Duggal et al., 2015; Guo et al., 2016, 2017; Qin et al., 2016; Hu et al., 2020). Most of these protocols are based on the 2i conditions, a combination of GSK3β and MEK inhibitors that maintains mESCs in the so-called ground state of pluripotency (Ying et al., 2008). Some of these groups have even achieved direct derivation of a few lines of naïve hESC lines from preimplantation embryos (Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014; Guo et al., 2016), although efficiency rates, when specified, were very low (e.g. 1 line out of 128 embryos in Ware et al., 2014).

Nonetheless, the mechanism by which the naïve pluripotent cells of the inner cell mass (ICM) transition to the primed pluripotency of the hESCs established *in vitro* is not fully understood. O'Leary and colleagues described a transient epiblast-like structure which can be observed during hESC derivation from blastocysts a few days after plating. They called this structure the post-inner cell mass intermediate (PICMI) and stated that its formation was a necessary step for the generation of a new hESC line (O'Leary et al., 2012). The PICMI was shown to express markers of both early and late epiblast, and it was proposed to be a turning point between the naïve pluripotency observed in the pre-implantation epiblast of the embryo and the primed pluripotency observed in hESCs (van der Jeught et al., 2015; Warrier et al., 2018).

While it is well known that the pluripotency state of hESCs can vary depending on the small molecules and growth factors added to the culture media, a potential effect of the developmental stage of the preimplantation embryo from which the hESCs are derived is unclear. hESCs were first derived from blastocysts (Thomson et al., 1998), but derivation from isolated blastomeres

from cleavage stage embryos has also been achieved by several groups (Klimanskaya et al., 2006; Chung et al., 2008; Geens et al., 2009; Ilic et al., 2009; Taei et al., 2013; Yang et al., 2013). In 2016, Geens and colleagues studied an XX hESC line derived from an isolated 8-cell blastomere and found that, at early culture passages, the cells showed a random X-chromosome inactivation (XCI), while at later passages this XCI was skewed. This observation suggested that either the original blastomere had both X-chromosomes active or that a re-activation event happened very early in the derivation process (Geens et al., 2016). Since the presence of two active X-chromosomes is a characteristic feature of naïve stem cells, this observation raises the possibility that hESCs derived from cleavage stage blastomeres present a more naïve pluripotency state, at least in early passages, although the naïve characteristics would be gradually lost during prolonged culture.

To test this hypothesis, in the present work we derived new hESC lines from single blastomeres from 8-cell stage human embryos at D3 as well as from whole blastocysts using the same conditions, and we analysed their behaviour in culture, gene expression, mitochondrial activity, and DNA methylation status in order to assess the influence of the developmental stage of the donor embryo on the pluripotency state of hESCs at early culture passages.

Materials and methods

Ethics statement

For this work, surplus embryos from couples undergoing assisted reproduction treatments were donated under informed consent after obtaining the mandatory authorization from the *Departament de Salut de la Generalitat de Catalunya* (Project #02/2017). Embryos were collected from different reproduction centres in Barcelona (Spain).

Embryo thawing and culture

A total of 264 cryopreserved embryos from 52 couples were used for this study: 10 embryos were cryopreserved at the 2PN stage, 129 at D2, 61 at D3, and 64 at D5 or D6.

Vitrified embryos were thawed using the Irvine Vit Kit-Thaw (Irvine Scientific, USA) following manufacturer's instructions. Ultra-rapid frozen embryos were thawed using the Global® Blastocyst Fast Freeze® Thawing Kit (LifeGlobal, Denmark) following manufacturer's instructions. Slow frozen embryos were thawed as follows: the straw was removed from liquid N_2 and held for 40 s at room temperature (RT) and 40 s in a water bath at 30ºC. After that, the content of the straw was emptied directly on a petri dish and incubated for 15 min at RT. Then the embryos were transferred to a drop of KSOM-H media (made in-house) and incubated for 15 min at 37ºC.

Embryos were then cultured in drops of Global Total medium (LifeGlobal, Denmark) covered by mineral oil at 37ºC, 5% CO_2 until they reached the required developmental stage. GSK3β inhibitor CHIR99021 (Axon Medchem, Netherlands) and ROCK inhibitor Y-27632 (Stemcell Technologies, Canada) were added to the culture media when indicated.

Feeder cells culture and inactivation

Human Foreskin Fibroblasts (HFF-1, ATCC®SCRC-1041™) were used as feeder cells. HFF were cultured for expansion in DMEM (Gibco, USA) containing 10% Foetal Bovine Serum (FBS; Gibco, USA). They were inactivated by incubating with 10 μg/mL mitomycin C (Fisher Scientific, USA) for 3 h. Inactivated HFFs (iHFFs) were then seeded on either 4 well plates for the derivation of hESCs from whole blastocysts or in 50 μL drops on 60 mm petri dishes for the derivation of hESCs from single blastomeres.

Derivation and culture of hESC

In this study, hESC derivation was performed from whole blastocysts and from single blastomeres isolated from 6 to 10-cell embryos (mostly 8-cell embryos) using a protocol based on Taei et al., 2013.

Blastocysts at D5 or D6 of development were incubated for approximately 1 min in Tyrode's Acidic Solution (Sigma, USA) to remove their zona pellucida. Denuded blastocysts were then seeded in 4-well plates onto a monolayer of iHFFs.

For the isolation of single blastomeres, 6- to10-cell embryos were placed into drops of PBS containing 1% of Bovine Serum Albumin (BSA; Sigma, USA) and they were biopsied using a micromanipulator (Olympus, Japan/Eppendorf, Germany). First, a small hole in the zona pellucida was drilled by pipetting Tyrode's Acidic Solution. Then, blastomeres were aspirated individually with a 40-50 nm pipette containing PBS + 1% BSA solution and placed in the same drop. Blastomeres were individually seeded onto a monolayer of iHFF in 50 µL media drops and monitored every day from day 3 onwards to check for cell division.

Medium for hESC derivation consisted of KO-DMEM (Gibco, USA) containing 20% Knockout Serum Replacement (KSR; ThermoFisher, USA), 2 mM L-glutamine (BioWest, USA), 1x MEM-non essential amino acids (Gibco, USA), 50 mM 2-mercaptoethanol (Gibco, USA), 1x ITS-X (Gibco, USA), 10000 U/mL Penicillin - 10 mg/mL Streptomycin (Gibco, USA) and 4 ng/mL human Fibroblast Growth Factor-basic (bFGF; Gibco, USA). When indicated, 1 mM ROCK inhibitor Y-27632 (Y; Stemcell Technologies, Canada) and 3 mM GSK3β inhibitor CHIR99021 (CH; Axon, Netherlands) were added to the medium. In experiments of hESC derivation from single blastomeres in naïve conditions, 3 mM CH, 0.5 mM MEK inhibitor PD0325901 (Axon, Netherlands) and 10 ng/mL bFGF were added to the hESC medium (2iF medium). All culture procedures were performed in a humidified incubator at 37ºC and 5% CO_2 in air.

The presence or absence of a PICMI was evaluated daily from day 3 onwards for blastocyst outgrowths and from day 5 onwards for blastomere outgrowths.

Blastocyst outgrowths were passaged mechanically at day 6-7, while blastomere outgrowths were passaged at day 10-12. In both cases, if a PICMI was observed, it was mechanically separated from differentiated cells and passaged individually. If a PICMI was not observed, the whole outgrowth was passaged. Medium was changed every other day and hESC colonies were passaged either mechanically or enzymatically as small clumps with Trypsin-EDTA (BioWest, USA) every 6-7 days.

Conversion of a pre-existing primed hESC line

As a positive control for our experiments, we generated a naïve hESC line from an established blastocyst-derived hESC line (ES[10] line, 46XX obtained from Barcelona Stem Cell Bank) using the protocol described by Ware and colleagues (Ware et al., 2014). Briefly, hESCs were first cultured for 2 passages in hESC medium supplemented with 0.1 mM sodium butyrate (Sigma, USA) and 50 nM SAHA (Santa Cruz, USA), and then passaged as single cells and maintained in 2iF medium. This naïve-converted hESC line could be maintained in a naïve state in culture for more than 25 passages in 2iF medium.

Characterization of hESC by immunostaining

Putative newly generated hESC lines were characterized by immunofluorescence of the pluripotency markers OCT4 and SOX2. Differentiation markers AFP (endoderm), SMA (mesoderm) and TUJ1 (ectoderm) were also analysed after inducing spontaneous differentiation by culturing colonies in DMEM supplemented with 10% FBS without feeder cells for 7-10 days.

hESC colonies grown on coverslips were fixed with 4% paraformaldehyde (PFA) for 20 min at RT and then washed 3 times with 1X PBS for 5 min. After that, cells were permeabilized and blocked in a PBS solution containing 0.2% sodium

azide (Sigma, USA), 0.5% Triton X-100 (Sigma, USA), and 3% goat serum (BioWest, USA) for 30 min at 37ºC. Then, cells were incubated with the primary antibody overnight in a wet chamber at 4ºC. The next day, cells were washed three times with 1X PBS for 5 min each before adding the corresponding secondary antibody and incubating for 2h at RT in the dark. All the antibodies were diluted in a PBS-based solution containing 0.2% sodium azide, 0.1% Triton X-100, and 3% goat serum.

After that, 10 µg/ml Hoechst 33258 (Molecular Probes - Invitrogen, USA) diluted in Vectashield (Vector Laboratories, USA) was added as a nuclear counterstain and coverslips were mounted on slides. The preparations were kept at -20ºC until they were analysed on an epifluorescence microscope (Olympus BX61, Japan). Images were obtained using the Cytovision software (Applied Imaging, Inc., USA).

Primary antibodies used in this study were mouse monoclonal anti-OCT4 (Santa Cruz, USA sc-5279, dilution 1:50), rabbit polyclonal anti-SOX2 (Merck, USA AB5603, dilution 1:200), mouse monoclonal anti-AFP (R&D Systems, USA MAB1368, dilution 1:200), mouse monoclonal anti-SMA (Sigma, USA A5228, dilution 1:200), and mouse monoclonal anti-TUJ1 (Covance, USA MMS-435P, dilution 1:500).

Secondary antibodies used were chicken anti-mouse IgG Alexa Fluor 488 (Molecular Probes, USA A-21200, dilution 1:500) and goat anti-rabbit IgG Alexa Fluor 594 (Molecular Probes, USA A-11037, dilution 1:500).

Alkaline Phosphatase assay

The Alkaline Phosphatase (ALP) assay was performed using a two-component buffered ALP substrate containing a BCIP (5-bromo-4-chloro-3-indolyl phosphate) analogue and nitro blue tetrazolium (NBT) (Sigma, USA). hESC colonies were fixed with 4% PFA for 1 min. Then, they were washed twice with 1X PBS and washed twice again with a 1:1 mixture of both components. Finally, the fixed colonies were incubated in the same mixture for a maximum of 10 min.

Images of the blue stained pluripotent colonies were obtained with an Olympus IX71 inverted microscope.

Fluorescence In-Situ Hybridisation

Fluorescence In-Situ Hybridisation (FISH) was used to determine the sex of the newly derived hESC. Briefly, 20 ng/µL Karyomax Colcemid (Gibco, USA) was added to hESC cultures at 70-80% of confluence and cells were incubated for 7 h at 37ºC 5% CO_2. Next, hypotonic solution (0,075M KCl) was added drop by drop in agitation and incubated at 37ºC for 12 min. Finally, fixative solution (3 vol methanol: 1 vol acetic acid) prepared in-house was added also drop by drop in agitation, and metaphase extensions were prepared onto slides.

The slides were washed twice in 2xSCC for 3 min and dehydrated with a battery of 70%, 90% and 100% ethanol for 2 min each. Then, hybridisation was performed with X, Y and 18 probes (Cytocell, United Kingdom) in a Hybrite slide stainer (Vysis, USA) as follows: 5 min at 75ºC followed by an overnight incubation at 37ºC.

The next day, the coverslips were removed carefully, and the slides were washed with 0.4xSSC/0.3%NP-40 at 73ºC for 1 min followed by 2xSSC/0.1%NP-40 at RT for 1 min. Finally, DAPI II (Abbott Molecular, USA) was added as a counterstain.

The presence of X and Y chromosomes in the metaphases was assessed with an epifluorescence microscope (Olympus BX61, Japan).

Single-cell clonogenicity assay

For the clonogenicity assay, hESC colonies were trypsinised into individual cells, and 5,000 cells were seeded in 3 wells of a 4-well plate onto feeder cells. Three days after plating for 2iF cultures or 6-7 days for standard medium cultures, the number of colonies per well was assessed and the single cell clonogenicity was expressed as the number of colonies per well over the number of plated cells.

Doubling time assay

Cells were seeded in two wells of a 4-well plate and collected during the exponential growth phase at 4-5 days of culture (initial time point) and 42-60 h later (final time point). The initial and final number of cells were determined with a Neubauer chamber and doubling time was calculated using the following formula:

$$DT = \frac{time \times log\,(2)}{log\,(\frac{final\ number\ of\ cells}{initial\ number\ of\ cells})}$$

TFE3 intracellular localization analysis

Cells were fixed and immunostained using the aforementioned protocol. In this case, a rabbit polyclonal anti-TFE3 primary antibody (Sigma, USA HPA023881, dilution 1:200) and a goat anti-rabbit IgG Alexa Fluor 594 secondary antibody (Molecular Probes, USA A-11037, dilution 1:500) were used.

The ImageJ software was used for the calculation of nuclear *vs* cytoplasmatic ratios of TFE3 staining (TFE3 ratio). Nuclei and surrounding cytoplasmic regions were selected on the fluorescence images and the mean grey value was measured for the two compartments of each cell. The TFE3 ratio was measured for 100 cells from several different colonies in each hESC line.

Mitochondrial activity analysis

As a measure for mitochondrial activity, the mitochondrial membrane potential was quantified using the tetramethylrhodamine ethyl ester (TMRE) mitochondrial membrane potential assay kit (Abcam, United Kingdom). Briefly, 100 nM TMRE were added directly to hESC cultures and incubated at 37 ºC, 5% CO_2 for 20 min. After washing twice with 1X PBS, images were obtained with a Texas red filter on an inverted epifluorescence microscope (Olympus, Japan). The mean fluorescence intensity was quantified as mean gray value on a minimum of 10 colonies from each hESC line using the ImageJ software.

RNA extraction and real-time quantitative PCR

For the gene expression analysis, 40-50 hESC colonies were mechanically separated from the feeder cells, manually picked and trypsinised into individual cells.

Total RNA from samples was isolated using the Maxwell® RSC simplyRNA Tissue Kit (Promega, USA) following the manufacturer's instructions. RNA concentration and purity were assessed with a Nanodrop (ThermoFisher, USA). Then, 1 µg of total RNA was retrotranscribed (RT) to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, USA). The RT reaction was performed as follows: 5 min at 25ºC followed by 30 min at 42ºC, 5 min at 85ºC and cooled down to 4ºC.

The qPCR reactions were performed in triplicate with a SYBR green Supermix (Bio-Rad) on a CFX96 Thermocycler (Bio-Rad, USA). Five ng of cDNA were used in a total reaction volume of 20 µL per well. Primers were either selected from the literature or designed in-house (Table I). All primers were tested and those with efficiency values ranging from 90 to 115% were validated. Analysed genes included naïve pluripotency markers *REX1*, *PRDM14*, *DNMT3L*, *KLF2*, *KLF4*, *KLF17*, *TFCP2L1* and *STELLA*; and primed pluripotency markers *DNMT3B*, *OTX2*, *ZIC1*, *ZIC2* and *CD24*. Housekeeping genes *GAPDH*, *RPL13A* and *RPLP0* were used for normalization of gene expression levels, after their selection among 10 candidate genes using the geNorm algorithm. A non-template control (NTC) was added for each gene.

The reaction program consisted of a denaturation step of 3 min at 95ºC followed by 40 cycles of 5 s at 95ºC (denaturing) and 30 s at 60ºC (annealing and extension). The melt curve of the reaction products was obtained with a last step consisting of an increment of 0.5ºC every 5 s from 65ºC to 95ºC.

Table I. Primers used in qPCR analysis.

Gene	Forward (5'→3')	Reverse (5'→3')
CD24	CTCCTACCCACGCAGATTTATTC	AGAGTGAGACCACGAAGAGAC
DNMT3B	GGCAAGTTCTCCGAGGTCTCTG	TGGTACATGGCTTTTCGATAGGA
DNMT3L	GGCCCTTCTTCTGGATGTTCGT	ATGGTGACTGGCTCCATCTCCA
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
KLF17	CTCCTGCTGCTGGTCCTTAG	ACAGTTGCCACGTCCAGTG
KLF2	TACACCAAGAGTTCGCATCTG	CCGTGTGCTTTCGGTAGTGG
KLF4	CGAACCCACACAGGTGAGAA	GAGCGGGCGAATTTCCAT
OTX2	GAGGTGGCACTGAAAATCAAC	TCTTCTTTTTGGCAGGTCTCA
PRDM14	TGAGCCTTCAGGTCACAGAG	ATTTCCTATCGCCCTTGTCC
REX1	CCTGCAGGCGGAAATAGAAC	GCACACATAGCCATCACATAAGG
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTGTCAA
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC
STELLA	GGCGGAGTTCGTACGCATGAAAGA	GACACGCAGAAACTGCAGGGACA
TFCP2L1	GCTCTTCAACGCCATCAAA	CAGGGGCACTCGATTCTG
ZIC1	AAACTGGTTAACCACATCCGC	CTCAAACTCGCACTTGAAGG
ZIC2	CACCTCCGATAAGCCCTATCT	GGCGTGGACGACTCATAGC

DNA extraction and 5mC/5hmC quantification

For DNA extraction, hESC colonies from 4-5 wells of 4-well plates were mechanically separated from the feeder cells, manually picked and trypsinised into individual cells. Genomic DNA was extracted using the Gentra Puregene Cell Kit (Qiagen, Germany) following the manufacturer's instructions. Then, its concentration and purity were assessed with a Nanodrop (ThermoFisher).

For the quantification of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), the Fluorometric Methylated DNA Quantification Kit (Abcam, United Kingdom) and the Fluorometric Hydroxymethylated DNA Quantification Kit (Abcam, United Kingdom) were used, respectively, following the manufacturer's instructions. Relative Fluorescence Units (RFUs) were measured in a Spark®

multimode microplate reader (Tecan, Switzerland) at 530nm excitation/590nm emission. To determine the exact amount of 5mC or 5hmC in the hESC samples, a calibration curve was obtained with control samples containing known concentrations of 5mC or 5hmC. Samples were measured in duplicate.

Experimental design

Three experimental groups were defined for the hESC derivation from both blastomeres and blastocysts. In the first group, named CHY, embryos were cultured in the presence of GSK3β inhibitor CHIR99021 (CH) and ROCK inhibitor Y-27632 (Y) from the 4-cell stage until plating onto iHFFs. Then, the same inhibitors were added to the hESC culture media. Y was removed 6 days after plating and CH was removed 12 days after plating. bFGF was added either from day 0 or 6 days after plating. To determine the effect of the embryo culture with CH and Y on the hESC derivation efficiency, we designed a second group, named NT, which was equivalent to the CHY but removing the inhibitors from the embryo culture medium before the derivation process. Finally, as a negative control, we performed hESC derivation without any of the two inhibitors during the whole process (NT-NT group) (Fig. 1).

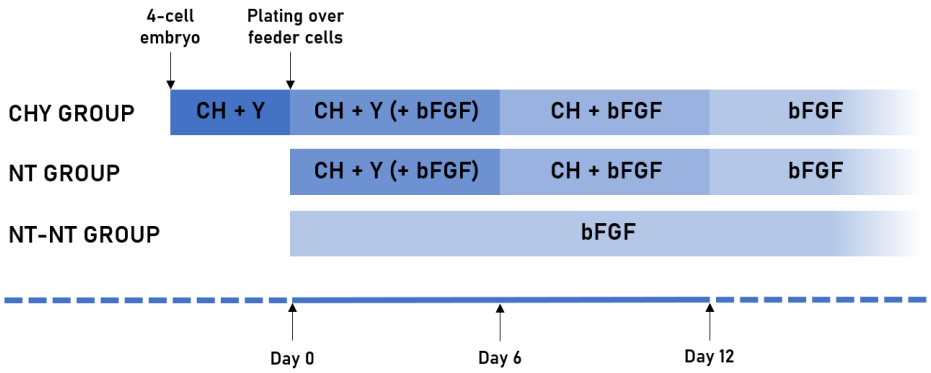

Figure 1. Representation of the culture conditions for each of the three groups of hESC derivation from blastomeres and blastocysts. CH: CHIR99021, Y: Y-27632, bFGF: basic fibroblast growth factor.

For the analysis of naïve pluripotency indicators, all blastomere-derived hESC lines (bm-hESCs) and 6 blastocyst-derived hESC lines (bc-hESCs) were used, along with the naïve-converted hESC line. All the tests were performed at low culture passages (3-5) for bm-hESCs and bc-hESCs to minimize the effect of culture conditions on their pluripotency state, and at passage 12-15 for naïve-converted hESCs. Moreover, to assess the influence of the time the ESC lines remained in culture on the expression of pluripotency marker genes, qPCR analysis for naïve and primed pluripotency markers were repeated at passage 15 in bc-hESCs and bm-hESCs and compared to naïve converted hESCs.

Statistical Analysis

hESC derivation efficiencies were expressed as the number of hESC lines obtained out of the number of blastocysts or blastomeres seeded. Efficiency values for each group were compared by performing a Fisher exact test.

The TFE3 ratios for each group were compared by performing a Kruskal-Wallis test followed by a Dunn's post-hoc test.

The clonogenicity and doubling time assays' values, the mean TMRE fluorescence intensity per colony values and the mean percentages of genomic 5mC and 5hmC in each group were compared by performing a one-way ANOVA test followed by a Tukey HSD post-hoc test.

In the qPCR experiments, relative expression levels were calculated using the $\Delta\Delta C_q$ method. Either a *t* test or a one-way ANOVA test followed by a Tukey HSD post-hoc test was applied on the relative expression values of each gene.

The statistical tests were performed using the GraphPad Prism 7 software, except for the qPCR results, in which the CFX Maestro software (Bio-Rad, USA) was used. In all cases, differences with p values lower than 0.05 were considered statistically significant.

Results

Generation of outgrowths and PICMIs from single blastomeres and whole blastocysts

Once blastomeres were seeded, cell division (Fig. 2a) and outgrowth (Fig. 2b) formation rates were assessed during the next days. No statistical differences were observed among groups in the cell division rates, but outgrowth formation rates were significantly higher in the CHY and NT groups (23.7% and 13.1%, respectively) than in the NT-NT group (1.5%; Table II).

PICMIs were observed in some outgrowths during the derivation process from single blastomeres. Usually, the PICMI could be first detected as a round-shaped structure with small pluripotent-like cells at day 6-7 and kept growing until the first passaging at day 10-12. Different morphologies of PICMIs could be observed, either ball-like (Fig. 3a) or more flattened (Fig. 3b). The PICMI was mechanically separated from the rest of the outgrowth and was plated in another well over fresh iHFFs. After the first passaging, pluripotent cells began to emerge from the PICMI (Fig. 3c), and eventually formed a hESC colony (Fig. 3d). We observed the formation of a total of 5 PICMIs from the plated single blastomeres, most of them (4/5) in the CHY group. In our hands, 4 out of these 5 PICMIs (80%) gave rise to a hESC line, and no hESC lines could be derived from single blastomeres without previous formation of a PICMI (Table II).

Referring to PICMI formation from plated blastocysts, a total of 15 PICMIs were observed among the different groups. From these, a total of 11 hESC lines were obtained (73.3% of PICMIs), mostly from the CHY and NT groups. Again, no bc-ESC lines could be obtained without previous PICMI formation (Table III).

These results indicate that the PICMI formation is a necessary previous step for the establishment of a new hESC line both from a single blastomere and from a whole blastocyst.

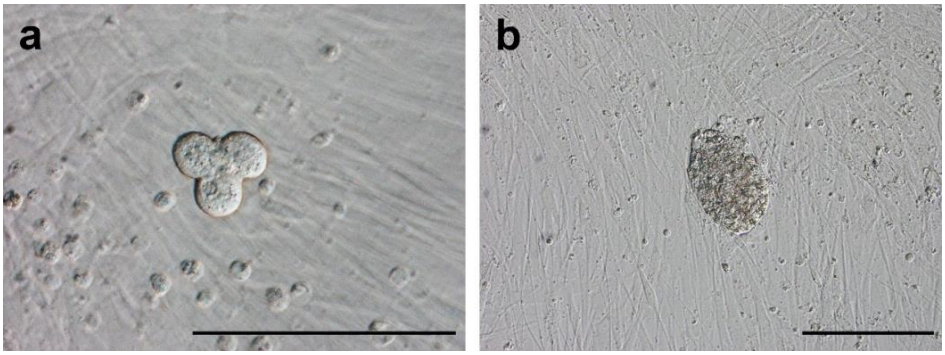

Figure 2. Initial steps of the hESC derivation process from a single blastomere a) Single blastomere seeded onto ihFF, which divided into 3 cells after 48 h in culture. b) Formation of the initial outgrowth at day 5. Scale bars: 200 µm.

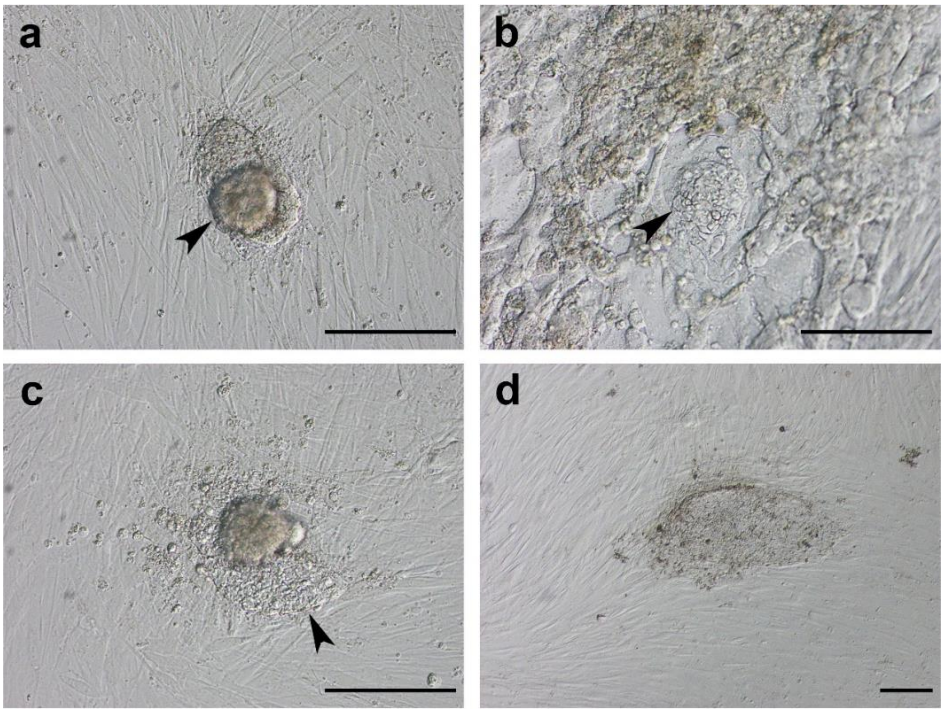

Figure 3. PICMI formation during hESC derivation from single blastomeres. a) Ball-like PICMI. b) Flat PICMI. c) hESC-like cells (arrow) growing from the PICMI after mechanical passaging. d) Initial hESC colony at passage 2. Scale bars: 200 µm.

Table II. Outgrowths and PICMI formation and hESC derivation efficiencies from single blastomeres. Different letters indicate significant differences with $p<0.05$.

		Plated blastomeres	Cell division	Outgrowths	PICMIs	hESC lines
CHY group	bFGF from day 6	115	81 (70.4%)	32 (27.8%)	1 (0.9%)	0
	bFGF from day 0	83	52 (62.7%)	15 (18.1%)	3 (3.6%)	3 (3.6%)
	TOTAL	**198**	**133 (67.2%)**	**47 (23.7%) [a]**	**4 (2,0%)**	**3 (1.5%)**
NT group	bFGF from day 6	120	85 (70.8%)	19 (15.8%)	1 (0.8%)	1 (0.8%)
	bFGF from day 0	56	29 (51.8%)	4 (7.1%)	0	0
	TOTAL	**176**	**114 (64.8%)**	**23 (13.1%) [b]**	**1 (0.6%)**	**1 (0.6%)**
NT-NT group	bFGF from day 0	**134**	**78 (58.2%)**	**2 (1.5%) [c]**	**0**	**0**

Table III. Outgrowths and PICMI formation and hESC derivation efficiencies from whole blastocysts.

	Plated blastocysts	Outgrowths	PICMIs	hESC lines
CHY group	22	21 (95.5%)	5 (22.7%)	**5 (22.7%)**
NT group	26	26 (100%)	4 (15.4%)	**4 (15.4%)**
NT-NT group	25	23 (92%)	6 (24%)	**2 (8%)**

Effects of GSK3βi, ROCKi and bFGF on hESC derivation efficiencies from single blastomeres and whole blastocysts

A total of four hESC lines were generated from isolated blastomeres (n=508), three from the CHY group (1.5%) and one in the NT group (0.6%). No hESC lines could be derived in the control group (NT-NT) despite the numbers of plated blastomeres in each group were equivalent.

The need for exogenous bFGF during the derivation process was assessed by adding bFGF to the hESC culture media either from day 0 (simultaneously with blastomere plating) or from day 6. In the NT group, no hESC lines could be derived when bFGF was added from day 0, whereas 1 hESC line was obtained

with bFGF added from day 6, at an efficiency of 0.8%. However, in the CHY group, the highest hESC derivation efficiency among all groups was achieved at 3 hESC lines out of 83 blastomeres (3.6%) when adding bFGF from day 0 (Table II). This indicates that FGF should be added to the media at the time of plating to maximize the efficiency of hESC derivation from single blastomeres.

Considering the results obtained in the derivation from single blastomeres, bFGF was added to the media from day 0 in all groups of hESC derivation from whole blastocysts. In this case, no significant differences were observed in terms of derivation efficiency between groups, although the CHY group was again the one that yielded the highest efficiency. In total, 5 hESC lines were derived from blastocysts in the CHY group (22.7%), 4 hESC lines could be established from the NT group (15.4%) and 2 hESC lines were obtained in the NT-NT group (8%) (Table III).

Characterization of hESC lines

All putative hESC lines expressed pluripotency markers OCT4 and SOX2 (Fig. 4a-b). All lines were also positive for differentiation markers AFP (endoderm), α-SMA (mesoderm) and TUJ1 (ectoderm) after being cultured in differentiation-prone conditions, thereby confirming their pluripotent state (Fig. 4c-e). Moreover, all lines were positive for alkaline phosphatase (Fig. 4f-g).

A FISH was performed to determine the sex of each hESC line. All 4 of the bm-hESC lines were male, while 3 out of 8 bc-hESC lines analysed were male and 5 were female.

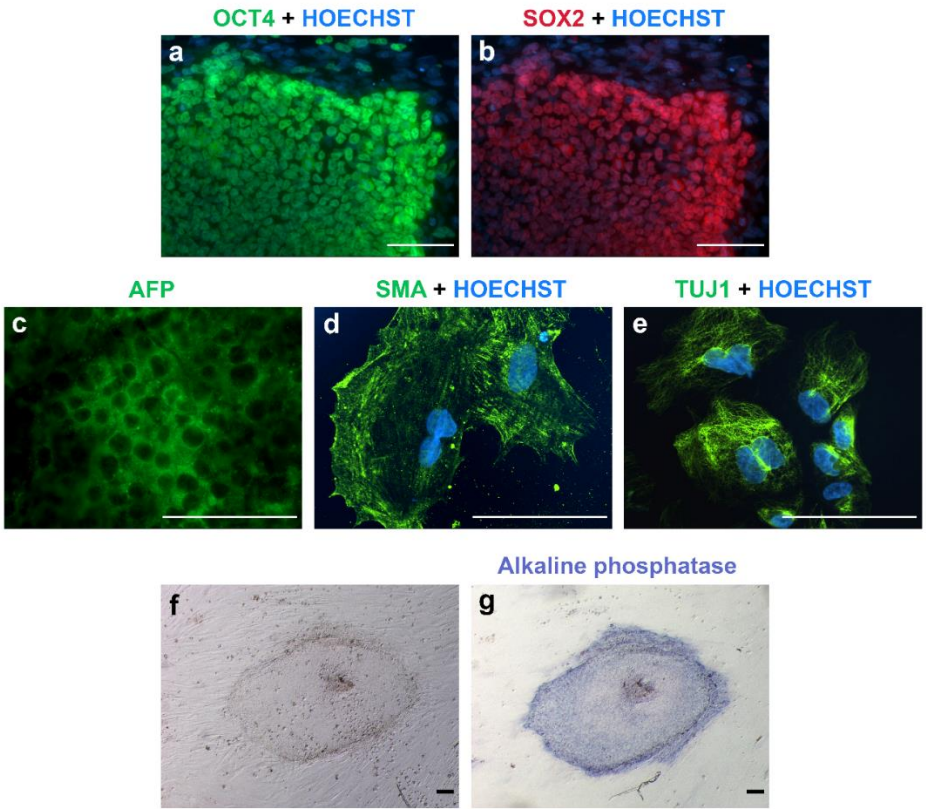

Figure 4. Characterization of putative hESC lines. a-b) Immunofluorescence for pluripotency markers OCT4 and SOX2. c-e) Immunofluorescence for differentiation markers AFP (endoderm), SMA (mesoderm) and TUJ1 (ectoderm). f-g) Image of a hESC colony before (f) and after (g) the alkaline phosphatase assay. Scale bars: 200 μm.

hESC morphology and behaviour in culture

Bm-hESCs and bc-hESCs both formed large, flattened colonies from the second passage onwards (Fig.5a-b). Most of the colonies remained undifferentiated with well-defined edges, although when cells were passaged mechanically as small clumps, a few colonies began to show differentiation at the centre after 5-6 days. By contrast, naïve-converted hESCs in 2iF media formed small dome-shaped colonies and did not show signs of differentiation for more than 25 passages (Fig. 5c).

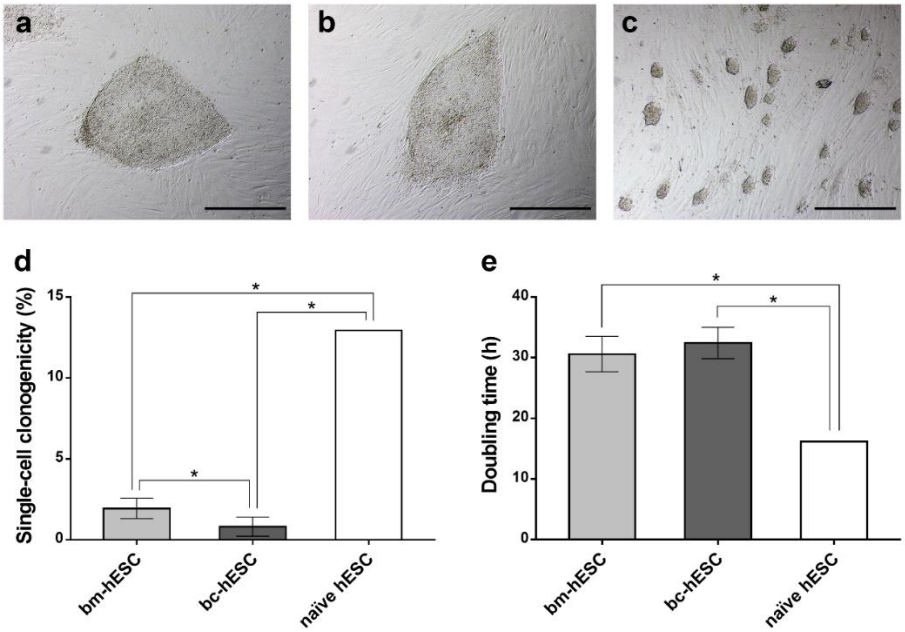

Figure 5. hESC colony morphology and behaviour in culture. a,b,c) Images of hESC colonies from a blastomere-derived hESC line, a blastocyst-derived hESC line and a naïve-converted hESC line, respectively. Scale bar: 500 µm d) Single-cell clonogenicity expressed as number of colonies out of number of plated cells. e) Mean cell population doubling time. Mean±SD, *p<0.05.

Bm-hESCs showed a significantly higher clonogenicity after trypsinization into single cells than bc-hESCs (1.93%±0.63 vs 0.81%±0.59), although still significantly lower than that of naïve-converted hESCs (12.93%) (Fig. 5d). Referring to proliferation capacity, no differences were observed in the doubling times between bm-hESCs and bc-hESCs (30.6 h±2.93 vs 32.42 h±2.58), whereas naïve-converted hESCs proliferated significantly faster (16.19 h) (Fig. 5e).

Analysis of naïve pluripotency indicators

Immunofluorescence of TFE3 showed a preferential cytoplasmic staining in all the bm-hESCs and bc-hESCs lines analysed, while naïve-converted cells exhibited a nuclear enrichment of TFE3 (Fig 6a). This was confirmed by fluorescence quantification. No significant differences were found in the nuclear

vs cytoplasmic ratios of TFE3 between bm-hESCs and bc-hESCs (0.587±0.161 vs 0.588±0.215, respectively), whereas this ratio was found to be significantly higher in the naïve-converted hESC line (1.799±0.422) (Fig. 6b).

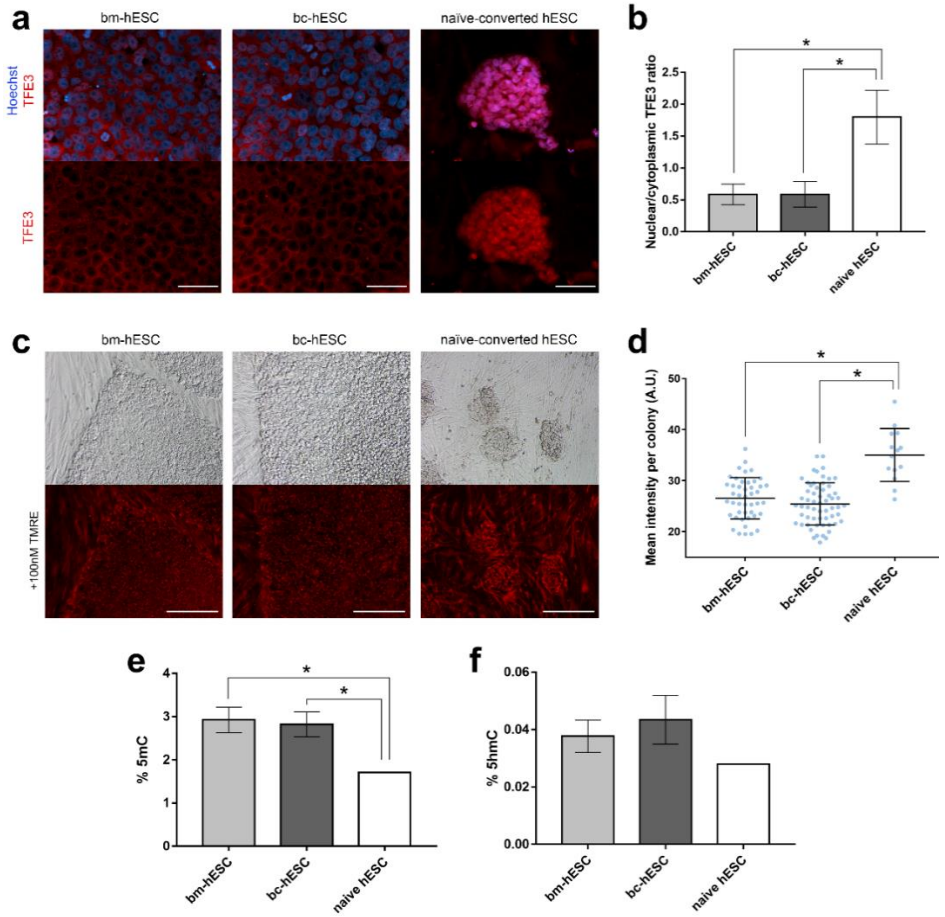

Figure 6. Analysis of naïve pluripotency indicators. a) Immunofluorescence of TFE3 (red) counterstained with Hoechst33258 (blue) for a bm-hESC line, a bc-hESC line and a naïve-converted hESC line. Scale bars: 100 μm b) Fluorescence quantification of the TFE3 immunofluorescence. Expressed as mean±SD. c) TMRE staining (red) for a bm-hESC line, a bc-hESC line and a naïve-converted hESC line. Scale bars: 200 μm. d) Fluorescence quantification of the TMRE staining. Each point represents a single hESC colony. Expressed as mean±SD. e) Quantification of global 5mC. f) Quantification of global 5hmC. * $p<0.05$.

Mitochondrial activity was analysed by staining with TMRE. Bm-hESCs and bc-hESCs showed no statistical differences in the intensity of staining with TMRE

(26.52±4.05 vs 25.67±3.97, respectively), whereas it was significantly more intense in the naïve-converted hESC line (35.03±5.2) (Fig. 6c-d). This indicates a higher mitochondrial membrane polarization in naïve-converted hESCs when compared to the other groups.

Finally, global genomic DNA methylation and hydroxymethylation was assessed by an ELISA-based fluorescent quantification of 5mC and 5hmC. No significant differences were found in the genomic 5mC levels between bm-hESCs and bc-hESCs (2.93%±0.3 vs 2.83%±0.29, respectively), whereas the genome of naïve-converted hESCs showed significantly lower levels of methylated cytosines (1.71%) (Fig. 6e). As for the percentage of 5hmC, again no differences were found between bm-hESCs and bc-hESCs (0.038%±0.006 vs 0.043%±0.008, respectively). The value was also lower in naïve hESCs (0.028%) but, in this case, the difference was not statistically significant (Fig. 6f).

An analysis of the X chromosome inactivation status on bm-hESC lines was initially planned, but unfortunately it could not be performed because all bm-hESC lines obtained were male.

Expression of naïve and primed pluripotency genes

To determine whether bm-hESCs have a more naïve expression profile than bc-hESCs at early culture passages and the influence of the culture on pluripotency state, qPCRs were performed at passages 5 and 15 in both bm-hESC and bc-hESC lines. Naïve-converted hESCs at passage 15 were used as a positive control for both passages. Eight naïve pluripotency markers and 5 primed pluripotency markers were analysed. Results showed that, at passage 5, the expression of the naïve marker DNMT3L was significantly higher in bm-hESCs than in bc-hESCs, although lower than in the naïve-converted positive control line. Expression of naïve markers PRDM14, REX1 and STELLA in bm-hESCs was also higher than in bc-hESCs but equivalent to the naïve-converted positive control (Fig. 7a). Most of these differences disappeared at passage 15, when only expression of DNMT3L remained significantly higher in bm-hESCs, which became then

equivalent to the naïve-converted positive control line (Fig. 7c). None of the hESC lines expressed detectable levels of KLF17 (data not shown). As for primed markers, no significant differences were observed between bm-hESCs, bc-hESCs and naïve-converted positive control groups neither at passage 5 nor at passage 15 (Fig.7b,d).

Additionally, when comparing the expression levels of naïve and primed pluripotency genes in bm-hESCs at passage 15 with respect to passage 5, we observed that the expression of REX1 and STELLA significantly decreased at passage 15 (data not shown).

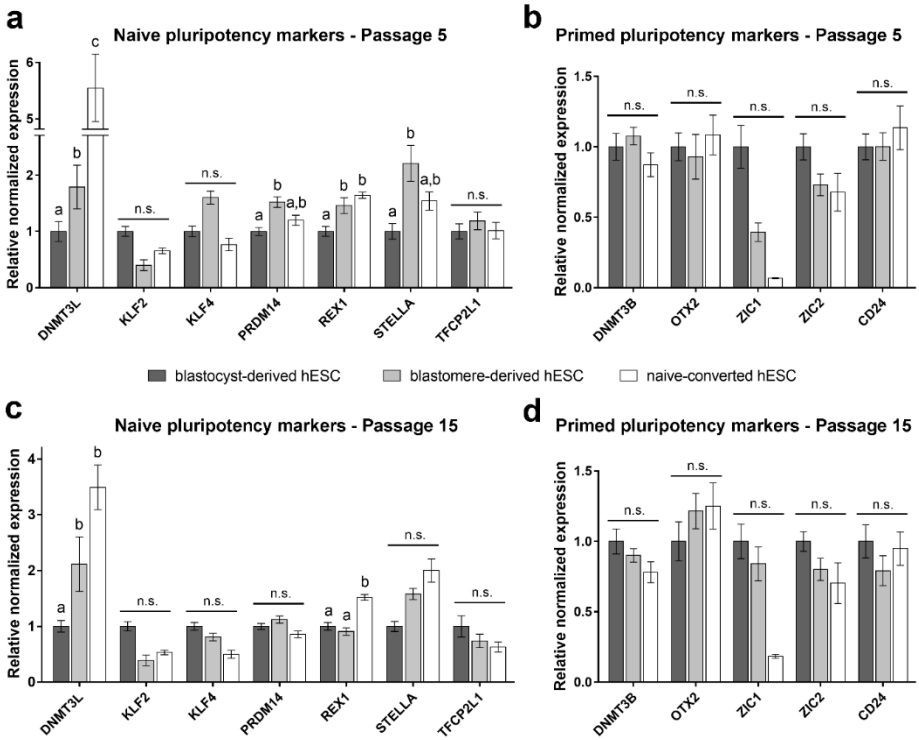

Figure 7. qPCR results for the expression of naïve and primed pluripotency associated genes in bc-hESC, bm-hESC and naïve-converted hESC. Expressed as mean±SD a,b) Expression of naïve and primed pluripotency genes at passage 5. c,d) Expression of naïve and primed pluripotency genes at passage 15. Different letters indicate significant differences with $p<0.05$.

hESC derivation from single blastomeres in naive conditions

We attempted to derive hESC lines from single blastomeres directly in naïve conditions using 2iF media. To maximize efficiency, 4-cell embryos were cultured in the presence of CH + Y until the 8-cell stage, like in the CHY group, and after embryo biopsy single blastomeres were seeded onto feeder cells in 2iF medium. Out of 161 blastomeres plated, 16 formed an outgrowth (10%) and 3 of them gave rise to a PICMI (1.9%). All 3 PICMIs could be mechanically separated from the non-pluripotent cells of the outgrowth, but they rapidly differentiated after the first passaging and no hESC lines could be obtained.

Discussion

Our results confirmed that the use of GSK3β inhibitor CHIR99021 and ROCK inhibitor Y-27632 has a beneficial effect on the derivation of hESCs from single blastomeres. This is consistent with the results obtained by Taei and colleagues (Taei et al., 2013), although they reported a higher derivation efficiency (up to 13% of the blastomeres seeded). In their work, bFGF was not present in the media for the first 6 days of the derivation process and mouse embryonic fibroblasts (MEFs) were used as feeder cells. They tested different types of feeder cells, including HFFs, and were not able to derive any hESC line when using HFF as feeder cells. Here, our results demonstrate that hESC lines can be derived from single blastomeres by direct plating over HFFs using GSK3β and ROCK inhibitors.

Human feeder cells are preferred for hESC derivation over MEFs because they avoid the presence of xeno-products in the culture media, which can be potentially pathogenic or harmful (Hovatta et al., 2003; Cobo et al., 2008). Additionally, HFFs are more stable and durable than MEFs (Richards et al., 2003; Meng et al., 2008; Ma et al., 2012), and survive longer after inactivation. Some studies reported differences between HFFs and MEFs in their production and secretion of growth factors to the culture media (Eiselleova et al., 2008; Yang et al., 2016). These differences may explain the lower efficiencies obtained in this

study and the differences in the requirements for bFGF supplementation at the beginning of the derivation process.

Several markers and indicators were used in the present study to characterize the pluripotency state of bm-hESCs in relation to that of known primed (bc-hESC lines) and naïve (naïve-converted bc-hESC line) controls. We first analysed the localization of TFE3, a transcription factor which is known to exit the nucleus at the onset of ESC differentiation (Betschinger et al., 2013). Our bm-hESC lines displayed a cytoplasmic localization of TFE3, similar to that of bc-hESC lines, whereas the naïve-converted hESC line showed a nuclear enrichment of TFE3. This is consistent with other reports in mouse (Betschinger et al., 2013) and human pluripotent cells (Gafni et al., 2013). bm-hESCs also presented a significantly lower mitochondrial activity than naïve-converted hESCs, as measured by staining with TMRE. It has been reported that primed stem cells have a preferentially glycolytic metabolism, and thereby show a low mitochondrial activity, whereas naïve stem cells are bivalent, leaning on both glycolysis and oxidative phosphorylation for energy production (Zhou et al., 2012). Hence, our results are consistent with previous observations in mEpiSCs and mESCs (Zhou et al., 2012), and naïve hESCs as well (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015). Moreover, bm-hESCs and bc-hESCs showed higher 5mC and 5hmC levels than naïve-converted hESCs. It is known that naïve mESCs show a hypomethylated genome with respect to their primed counterparts (Hackett et al., 2013; Leitch et al., 2013). 5hmC is the product of the oxidation of 5mC catalysed by TET enzymes. In several studies performed in mESCs, oxidation of 5mC into 5hmC was identified as one of the mechanisms involved in the demethylation observed during the serum to 2i transition (Hackett et al., 2013; Leitch et al., 2013; von Meyenn et al., 2016). The reduction of 5mC and 5hmC levels in our naïve hESCs cultured in 2i + FGF medium is consistent with previous findings in naïve hESCs too (Takashima et al., 2014; Pastor et al., 2016). The results from the analysis of these three indicators all point towards bm-hESCs presenting a state of pluripotency close to the primed pluripotency state.

Nonetheless, despite having similar population doubling times, bm-hESCs displayed a higher single-cell clonogenic capacity than bc-hESCs, though lower than that of naïve-converted hESCs. It has been widely reported that primed mEpiSCs and hESCs display lower population doubling times when compared to naïve hESCs and have low survivability when dissociated into single cells, therefore they need to be passaged as small cell clumps (Schatten et al., 2005; Brons et al., 2007). By contrast, naïve hESCs, as well as mESCs, have an increased single-cell clonogenicity with respect to their primed counterparts (Warrier et al., 2017). The increased single-cell clonogenicity of bm-hESCs implies that a high number of cells could be obtained more rapidly and easily than when using bc-hESCs.

Moreover, the qPCR data showed that the expression of DNMT3L, PRDM14, REX1 and STELLA was significantly higher in bm-hESCs than in bc-hESCs at early passages. DNMT3L is a catalytically inactive DNA methyltransferase which plays a dual role in ESC, either promoting DNA methylation in housekeeping genes or repressing it in promoters of bivalent genes (Neri et al., 2013). Unlike the other members of its family, DNMT3A and DNMT3B, DNMT3L is strongly upregulated in most of the naïve hESC populations and contributes to confer them the capacity to differentiate into different cell types by keeping bivalent epigenetic signals that may quickly activate or inhibit different sets of genes (Jenkins and Carrell, 2012). PRDM14 is reported to promote naïve pluripotency in mESCs by repressing FGF signalling and de novo DNA methylation (Grabole et al., 2013; Yamaji et al., 2013), while REX1 is also associated with the maintenance of naïve pluripotency in mESCs (Kalkan et al., 2017a). Both are downregulated in mEpiSCs (Ghimire et al., 2018). Although PRDM14 and REX1 are expressed in conventional hESCs and are thought to be involved in the core pluripotency circuit (Son et al., 2013; Seki, 2018), most of the human naïve cell populations do upregulate both PRDM14 and REX1 (Warrier et al., 2017; Taei et al., 2020). This suggests that they could also have a role in the maintenance of human naïve pluripotency. STELLA, also known as DPPA3, is also a marker of naïve pluripotency in mESCs. It regulates DNA methylation by interacting with

de novo methyltransferases DNMT3A and DNMT3B, although its exact mechanism is unclear (Zhao et al., 2019). It is expressed at high levels in mESCs and strongly downregulated in mEpiSCs (Tesar et al., 2007; Bao et al., 2009). In humans, STELLA is expressed in the epiblast, but it is downregulated during the transition to primed hESCs (Yan et al., 2013). Interestingly, STELLA is expressed at significantly higher levels in the PICMI than in hESCs (Warrier et al., 2018). The higher levels of STELLA expression in our bm-hESCs at early passages indicate a slower downregulation of STELLA in bm-hESCs when compared to bc-hESCs during the derivation process.

Our results also showed that most of the differences in the expression of naïve markers between bm-hESCs and bc-hESCs present at early passages disappeared at passage 15, with only DNMT3L remaining upregulated in bm-hESCss, at levels equivalent to those of the naïve-converted hESCs. Expression of REX1 and STELLA decreased in bm-hESCs over the culture passages, which suggests that prolonged culture could have an effect on the pluripotent state of bm-hESCs, making them more similar to primed hESCs. Taken together, these results indicate that bm-hESCs display a slightly more naïve expression profile than bc-hESCs at low culture passages but become more similar at later passages. Further research would be needed to determine if the observed differences in the expression of naïve markers can have an impact on the differentiation potential of bm-hESCs.

Note that our naïve hESCs generated by the protocol described by Ware and colleagues (Ware et al., 2014) did not show a marked upregulation of several naïve-associated transcription factors like some other naïve hESC populations, as already reported (Theunissen et al., 2014; Warrier et al., 2017; Taei et al., 2020). In fact, they have been recently classified as intermediate naïve (Taei et al., 2020).

Finally, results from the hESC derivation attempts in naïve conditions showed that 2iF medium supported the growth of pluripotent cells from single blastomeres until the formation of the PICMI with a similar efficiency to that

obtained under standard conditions when using GSK3βi and ROCKi. However, these cells were unable to progress further as undifferentiated cells. This suggests that the pluripotent cells of the PICMI may need different culture conditions to self-renew and to form a hESC colony. It also reinforces the hypothesis of the PICMI being the turning point between the naïve pluripotency of the preimplantation embryo and the primed pluripotency of the established hESCs *in vitro* (van der Jeught et al., 2015; Warrier et al., 2018). To the best of our knowledge, direct derivation of naïve hESCs from single blastomeres had not been previously attempted, and derivation from blastocysts in naïve conditions is reported to be very inefficient, at 1 out of 128 embryos (Ware et al., 2014). Other groups that achieved naïve hESC derivation from blastocysts have not reported their efficiencies (Gafni et al., 2013; Theunissen et al., 2014). Obtaining naïve hESCs via conversion of already established conventional hESC lines is still the most efficient approach. However, since several protocols which use different routes to obtain naïve hESCs have been described, it is unclear if any of the available naïve populations really represents the gold standard for human naïve pluripotency.

To sum up, in this work we derived new hESC lines from single blastomeres and from whole blastocysts and analysed their behaviour in culture, metabolism, genetics and epigenetics to evaluate their pluripotency state. Our study detected moderate changes in the expression of naïve pluripotency-associated genes and in the single-cell clonogenicity of the bm-hESC lines at early passages. This indicates that bm-hESCs and bc-hESCs are not identical in terms of their pluripotent state. The pluripotent state of ESCs should be seen as a wide continuous spectrum instead of the conventional two-state model of naïve vs primed pluripotency. In this context, bm-hESCs would fall into this spectrum in a position closer to the naïve state than bc-hESCs. Moreover, the fact that differences in the expression levels of naïve markers were only observed at early passages suggests that they are probably caused by the distinct embryonic origin of bm-hESC (8-cell stage, early preimplantation embryo development) and bc-hESC lines (blastocysts, late preimplantation embryo development)

rather than being induced by culture conditions. This situation might reflect an intrinsic higher plasticity in differentiation capacity of cells from early stages of embryo development when compared to later stages, in which some differentiation decisions have already been taken.

Acknowledgements

We thank Hospital Universitari Dexeus, Hospital Quirónsalud, Clínica Sagrada Família and Clínica Eugin for the kind donation of surplus embryos. We also thank Sandra Alonso, Jonatan Lucas and María Jesús Álvarez for their supportive work. Ot Massafret was beneficiary of a FI-2017 fellowship by Generalitat de Catalunya.

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Study 2

The transcriptional profile and differentiation potential of single blastomere-derived human embryonic stem cells

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Introduction

Embryonic stem cells (ESCs) were first derived from the inner cell mass (ICM) of murine embryos at the blastocyst stage in 1981 (Evans and Kaufman, 1981; Martin, 1981) and, 17 years later, the first human ESC (hESC) lines were obtained (Thomson et al., 1998). Since then, the methods for establishing new ESC lines have been optimised to maximise their efficiency and circumvent some ethical issues.

Standard ESC derivation from blastocysts implies the destruction of the source embryo. As an alternative, derivation of ESCs from single blastomeres from embryos at precompaction stages has also been described in mice (Delhaise et al., 1996) and humans (Klimanskaya et al., 2006). Since the removal of one or two blastomeres from an 8-cell embryo barely affects its viability, as demonstrated in preimplantation genetic testing procedures, ESC derivation from single blastomeres can avoid embryo destruction. On the other hand, by using all the blastomeres of the embryo individually to start the derivation process, there should be more chances to obtain at least one ESC line from a specific embryo. This approach could reduce the number of embryos needed to establish hESC lines (González et al., 2011; Vila-Cejudo et al., 2019).

In the pre-implantation human embryo, blastomeres are thought to be totipotent at least until the 4-cell stage, since the embryonic genome activation process lasts until between the 4- and 8-cell stages in humans (Braude et al., 1988). After that, a polarization event occurs that eventually causes the first specification of the blastomeres into trophectoderm (TE) and inner cell mass (ICM). Later, during the late blastocyst stage, a second cell fate specification takes place, in which a subpopulation of cells from the ICM gives rise to the pluripotent epiblast and another one forms the primitive endoderm (Niakan et al., 2012). Accordingly, differences exist between the transcriptomes of 8-cell stage embryos and cells from the ICM (Yan et al., 2013; Blakeley et al., 2015; Boroviak et al., 2018; Stirparo et al., 2018). Additionally, blastomeres from the

8-cell embryo display a hypomethylated DNA whereas cells from the ICM have already gone through a re-methylation process (Fulka et al., 2004).

Mouse ESCs derived from pre-implantation embryos, either at cleavage-stages or blastocysts, have a transcriptional signature which resembles the pre-implantation epiblast and are classified as naïve ESCs. Contrarily, hESCs derived from the ICM of blastocysts resemble the post-implantation epiblast and are considered to display a primed state of pluripotency, showing a more restricted differentiation potential (Nichols and Smith, 2009). Regarding hESCs derived from single blastomeres, the analysis of their transcriptome has provided contradictory results. Some authors reported that blastomere-derived hESCs (bm-hESCs) and blastocyst-derived hESCs (bc-hESCs) share similar transcription profiles (Giritharan et al., 2011; Galan et al., 2013), whereas another study did find significant differences in their transcriptomes (Zdravkovic et al., 2015). The latter work also reported that bm-hESCs had an increased capacity to differentiate into trophectodermal lineages, which could be indicative of a more naïve pluripotent state. In this sense, recent results from our research group indicate that bm-hESCs and bc-hESCs are not identical regarding their pluripotency state. The former appear to be slightly closer to the naïve end of the pluripotency continuum than the latter at early passages, although extensive culture erases these differences (Massafret et al., 2022). All in all, it is still unclear whether the different developmental stage of the source embryo can lead to different gene expression patterns in hESCs or, by contrast, hESCs acquire similar characteristics during the derivation and culture processes, regardless of their embryonic origin.

In this work we used bm-hESC and bc-hESC lines obtained and cultured in the same conditions, as well as a naïve-converted hESC line, to determine the effect of the developmental stage of the source embryo on their transcriptional profile and differentiation potential, and whether a correlation between these parameters and differences in their pluripotency state can be established.

Materials and methods

Feeder cells culture and inactivation

Human Foreskin Fibroblasts (HFF-1, ATCC®SCRC-1041™) were used as feeder cells. HFF cultures were expanded in DMEM (Gibco) containing 10% Fetal Bovine Serum (FBS; Gibco). Inactivation was performed by incubating with 10 µg/mL mitomycin C (Fisher Scientific) for 3 h. Inactivated HFFs (iHFFs) were then seeded on 4-well plates for the culture of hESCs.

hESCs culture

In this study we used four bm-hESC lines (bm-6.1, bm-23.3, bm-26.5 and bm-31.5) and four bc-hESC lines (bc-4, bc-17, bc-21 and bc-26) previously derived in our laboratory, as described in Massafret et al. (2022). The four bm-hESC lines were obtained from single blastomeres biopsied from 8-cell stage embryos. Additionally, a naïve-converted bc-hESC line was also used as a naïve pluripotency control. This naïve line was obtained by converting the ES[10] line (46XX, obtained from the Barcelona Stem Cell Bank) into the naïve pluripotency state following the protocol described by Ware et al., (2014), as detailed in Massafret et al. (2022). The naïve-converted hESC line was named hES10 2iF.

Both bm-hESC and bc-hESC lines were cultured on iHFFs in KO-DMEM (Gibco) containing 20% Knockout Serum Replacement (KSR; ThermoFisher), 2 mM L-glutamine (BioWest), 1x MEM-non essential amino acids (Gibco), 50 mM 2-mercaptoethanol (Gibco), 1x ITS-X (Gibco), 10000 U/mL Penicillin - 10 mg/mL Streptomycin (Gibco) and 4 ng/mL basic human Fibroblast Growth Factor (bFGF) (Gibco). Naïve-converted hESCs were cultured in the same medium but containing 10 ng/mL bFGF and supplemented with 0.5 mM PD0325901 (Axon) and 3 mM CHIR99021 (Axon) (2iF medium). All media were changed every other day. Both bm-hESCs and bc-hESCs were mechanically passaged every 6-7 days, whereas naïve-converted hESCs were enzymatically passaged with Trypsin-EDTA (BioWest) every 3-5 days. All bm-hESCs and bc-hESCs were used at passages 6-9, whereas naïve-converted hESCs were used at passage 18.

RNA extraction

For gene expression analysis, 40-50 hESC colonies were mechanically separated from the feeder cells, manually picked and trypsinised into individual cells, and EBs were harvested at day 6 of suspension culture. Very small or irregularly shaped EBs were discarded.

Total RNA from the different samples (hESCs and EBs) was isolated using the Maxwell® RSC simplyRNA Tissue Kit (Promega) following the manufacturer's instructions. RNA concentration and purity were assessed with a Nanodrop (ThermoFisher).

Library preparation and RNA-sequencing

Two µg of total RNA obtained from each hESC line were sent to the Centre Nacional d'Anàlisi Genòmica (CNAG) in Barcelona (Spain) for RNA-sequencing (RNA-seq).

Total RNA was quantified by Qubit® RNA BR Assay kit (Thermo Fisher Scientific) and the RNA integrity was estimated by using RNA 6000 Nano Bioanalyzer 2100 Assay (Agilent).

The RNA-seq libraries were prepared with KAPA Stranded mRNA-Seq Illumina Platforms Kit (Roche) following the manufacturer´s recommendations. Briefly, 500 ng of total RNA was used for the poly-A fraction enrichment with oligo-dT magnetic beads, following the mRNA fragmentation by divalent metal cations at high temperature. The strand specificity was achieved during the second strand synthesis performed in the presence of dUTP instead of dTTP. The blunt-ended double stranded cDNA was 3´adenylated and Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay.

The libraries were sequenced on NovaSeq 6000 (Illumina) in paired-end mode with a read length of 2x51bp, following the manufacturer's protocol for dual indexing. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 3.4.4) and followed by generation of FASTQ sequence files.

RNA-seq processing and analysis

RNA-seq reads were mapped against human reference genome (GRCh38) using STAR software version 2.7.8a (Dobin et al., 2013) with ENCODE parameters. Annotated genes were quantified with RSEM v1.3.0 (Li and Dewey, 2011) using default parameters and the human GENCODE annotation version 38.

Sample to sample distance heatmap was generated with regularized log transformed (rlog) counts, taking all genes into account. Principal Component Analysis (PCA) plot was also generated with rlog counts but only considering the top 500 most variable genes. The heatmap with the top 50 DE genes was performed with the pheatmap R package, using the scaled rlog transformed counts.

Differential expression analysis was performed with DESeq2 v1.26.0 R package (Love et al., 2014) using a Wald test to compare bm-hESC and bc-hESC groups, making sex-specific contrasts. Genes were considered differentially expressed (DE) with an adjusted p-value < 0.05 and absolute fold change |FC| > 1.5. Significant genes were selected to perform a Gene Ontology (GO) enrichment analysis using the PANTHER software. A PANTHER overrepresentation test was performed on the GO Ontology database (DOI: 10.5281/zenodo.5725227) released on November 16th, 2021. The statistical test applied was a Fisher's exact test with a False Discovery Rate correction.

Embryoid body formation

For the generation of embryoid bodies (EBs) from hESC lines, bm-hESC and bc-hESC colonies were mechanically disaggregated in medium-sized cell clumps

and cultured in Nunclon Sphera 12-well plates (ThermoFisher) in hESC medium without bFGF. Naïve-converted hESCs colonies were digested into individual cells with Trypsin-EDTA (BioWest) and seeded in the same 12-well plates in hESC media without bFGF, PD0325901 and CHIR99021. Media were changed every other day and EBs were cultured in suspension for 6 days.

qPCR

One µg of total RNA obtained from both hESC lines and their corresponding EBs was retrotranscribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The RT reaction was performed as follows: 5 min at 25ºC followed by 30 min at 42ºC, 5 min at 85ºC and cooled down to 4ºC.

The qPCR reactions were performed in triplicate with a SYBR green Supermix (Bio-Rad) on a CFX384 Thermocycler (Bio-Rad). Five ng of cDNA were used in a total reaction volume of 10 µL per well. Validated PrimePCR SYBR green assays (Bio-Rad) for *OCT4* and *SOX2* pluripotency markers, *SOX1*, *PAX6* and *NESTIN* ectodermal markers, *T*, *HAND1* and *EOMES* mesodermal markers, and *AFP*, *FOXA2* and *SOX17* endodermal markers were used. For the normalization of gene expression levels, *GAPDH* and *RPLP0* were used as housekeeping genes. A non-template control (NTC) was added for each gene.

The reaction program consisted of a denaturation step of 3 min at 95ºC followed by 40 cycles of 5 s at 95ºC (denaturing) and 30 s at 60ºC (annealing and extension). The melt curve of the reaction products was obtained with a last step consisting of an increment of 0.5ºC every 5 s from 65ºC to 95ºC.

Relative expression levels were calculated using the $\Delta\Delta C_q$ method with the CFX Maestro software (Bio-Rad). Relative expression values in EBs samples were normalized to the expression of the same hESC line before differentiation to calculate the fold change for each gene. Fold change values for each gene were compared between groups with a one-way ANOVA followed by a post-hoc Tukey test. Additionally, gene expression values for each gene in EB samples were compared with their respective undifferentiated hESC lines using a t-test

analysis. Both statistical analyses were performed using the GraphPad Prism 7 software. Differences with $p<0.05$ were considered statistically significant.

Results

Transcriptome analysis

RNA-seq was performed on 4 bm-hESC lines, 4 bc-hESC lines and the naïve-converted hESC line. PCA showed that all the bm-hESC and bc-hESC lines clustered together, except for the bc-hESC line bc-4, and separated from the naïve-converted hESC line (Figure 1A). The same sample distribution could also be observed on the dendrogram obtained from hierarchical clustering, which showed a first bifurcation between naïve-converted hESCs and both bc-hESCs and bm-hESCs. Then 3 subclusters formed, in which the distribution of the bc-hESC and bm-hESC lines did not match their different embryonic origin (Figure 1B). Note that the bm-hESC lines bm-26.5 and bm-31.5 were obtained from embryos from the same cohort and they were, accordingly, the most closely related samples in the dendrogram (Figure 1B).

An effect of the sex of the hESCs lines was observed in the PC1vsPC3 representation of the PCA (data not shown), which unfortunately could not be corrected because the bm-hESCs group included only XY lines, whereas the bc-hESCs group had two XY (bc-17 and bc-21) and two XX (bc-4 and bc-26) hESC lines.

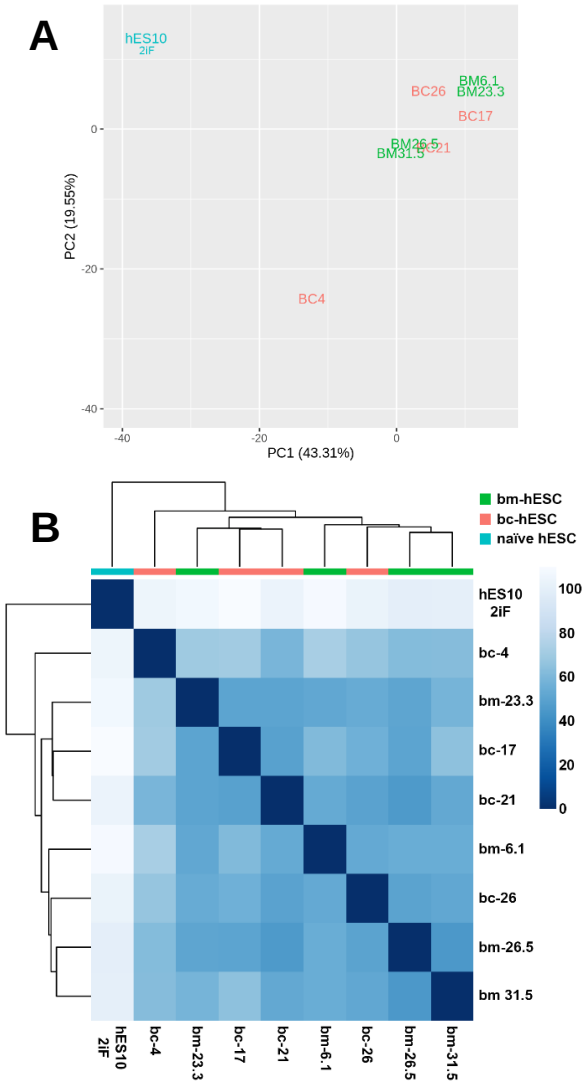


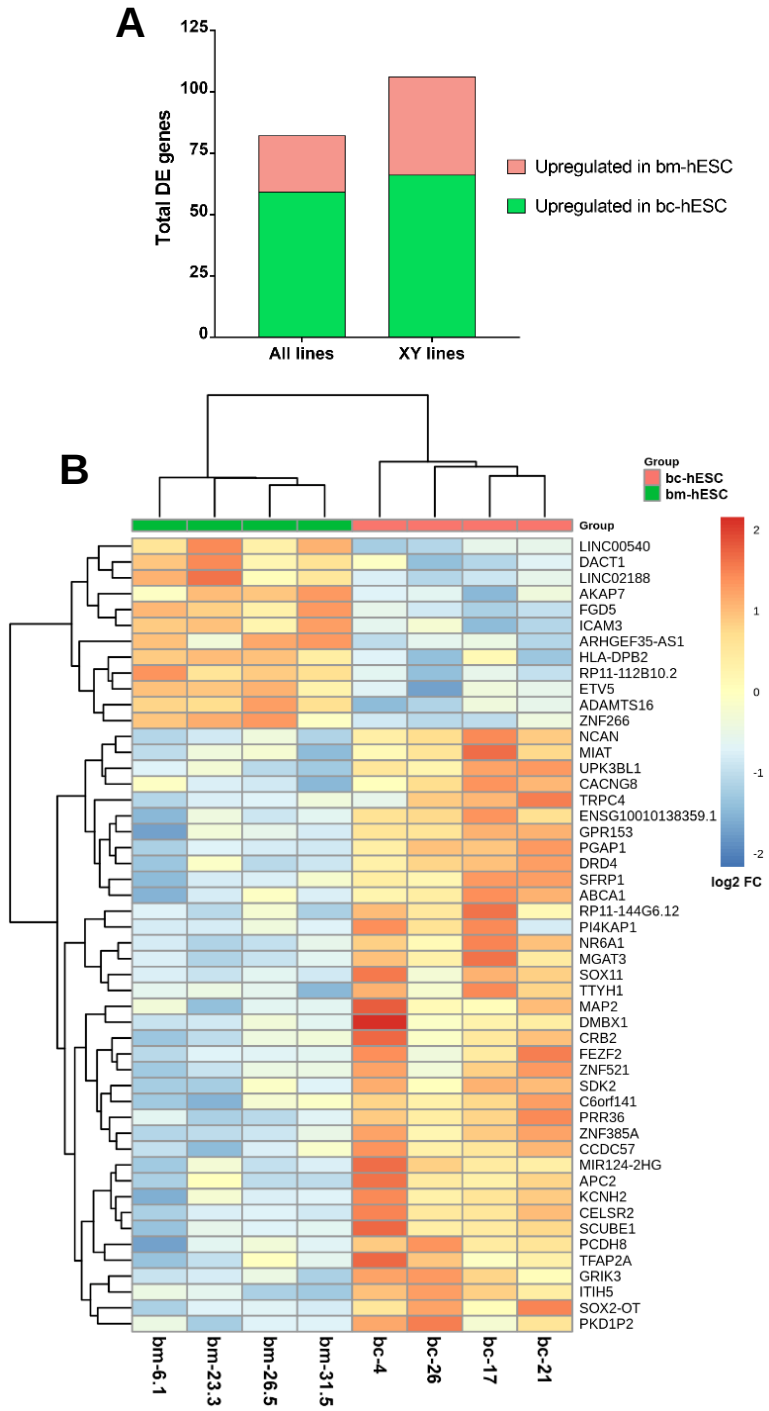
Figure 1. Clustering of the bm-hESC lines (bm-6.1, bm-23.3, bm-26.5 and bm-31.5), bc-hESC lines (bc-4, bc-17, bc-21 and bc-26) and the naïve hESC line hES10 2iF relative to their transcriptional profile. A) Representation of the Principal Component Analysis (PCA). B) Heatmap and hierarchical clustering.

Differential expression analysis

The DE analysis on the bm-hESC and bc-hESC lines showed a total of 82 DE genes with fold change ≥1.5 and $p<0.05$. Out of these, 59 genes were upregulated in bc-hESCs, whereas 23 were upregulated in bm-hESCs (Figure 2A, first column), from which only 15 constituted protein-coding genes that could be

associated with a GO biological process term. The heatmap represents the top 50 DE genes between bm-hESCs and bc-hESCs (Figure 2B). The GO analysis revealed that the DE genes were associated with several biological processes related to the development of the nervous system, embryonic pattern specification, cell signalling and morphogenesis of embryonic epithelium with $p<0.05$ (Table 1). Notably, we found that 22 out of the 82 DE genes were related to the GO term "nervous system development", 21 of them being upregulated in bc-hESCs with respect to bm-hESCs. This set of genes included the transcription factors *TFAP2A*, *SOX11*, *ZNF521*, *FEZF2* and *DMBX1*. All 10 DE genes related to "pattern specification process" were also upregulated in bc-hESCs, including 7 genes involved in the anterior-posterior pattern specification, 6 in morphogenesis of embryonic epithelium and 5 in somite development. Finally, 13 DE genes were associated with "cell-cell signalling", of which 7 were involved in the Wnt signalling pathway. Among them, the Wnt antagonist *DACT1* was overexpressed in bm-hESCs, whereas the member of the β-catenin cytoplasmic degradation complex *APC2* and the inhibitory protein *SFRP1* were overexpressed in bc-hESCs.

Due to the observed effect of sex on the results, we also carried out the same DE analysis excluding the two female lines from the bc-hESCs group. In this scenario, the number of DE genes with fold change >1.5 and $p<0.05$ increased to 106, 66 of which were upregulated in bc-hESCs and 40 in bm-hESCs (Figure 2A, second column). The top-50 DE genes for this comparison were also represented in a heatmap (Figure 2C). The GO analysis on the DE genes indicated that, apart from the aforementioned GO terms, there was an overrepresentation of several GO biological process terms related to mitochondrial respiration and ATP generation, such as "cation transmembrane transport", "cellular respiration", "aerobic respiration", "oxidative phosphorylation" or "ATP synthesis coupled electron transport" with $p<0.05$ (Table 2). Indeed, the bm-hESC lines upregulated 8 mitochondrial genes (*MT-ATP6*, *MT-ATP8*, *MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-ND3*, *MT-ND4* and *MT-ND4L*), all of them coding for proteins involved in the oxidative phosphorylation process.


A
125
100
75
50
25
0
Total DE genes
All lines
XY lines
Upregulated in bm-hESC
Upregulated in bc-hESC
B
Group
bc-hESC
bm-hESC
Group
LINC00540
DACT1
LINC02188
AKAP7
FGD5
ICAM3
ARHGEF35-AS1
HLA-DPB2
RP11-112B10.2
ETV5
ADAMTS16
ZNF266
NCAN
MIAT
UPK3BL1
CACNG8
TRPC4
ENSG10010138359.1
GPR153
PGAP1
DRD4
SFRP1
ABCA1
RP11-144G6.12
PI4KAP1
NR6A1
MGAT3
SOX11
TTYH1
MAP2
DMBX1
CRB2
FEZF2
ZNF521
SDK2
C6orf141
PRR36
ZNF385A
CCDC57
MIR124-2HG
APC2
KCNH2
CELSR2
SCUBE1
PCDH8
TFAP2A
GRIK3
ITIH5
SOX2-OT
PKD1P2
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bc-17
bc-21

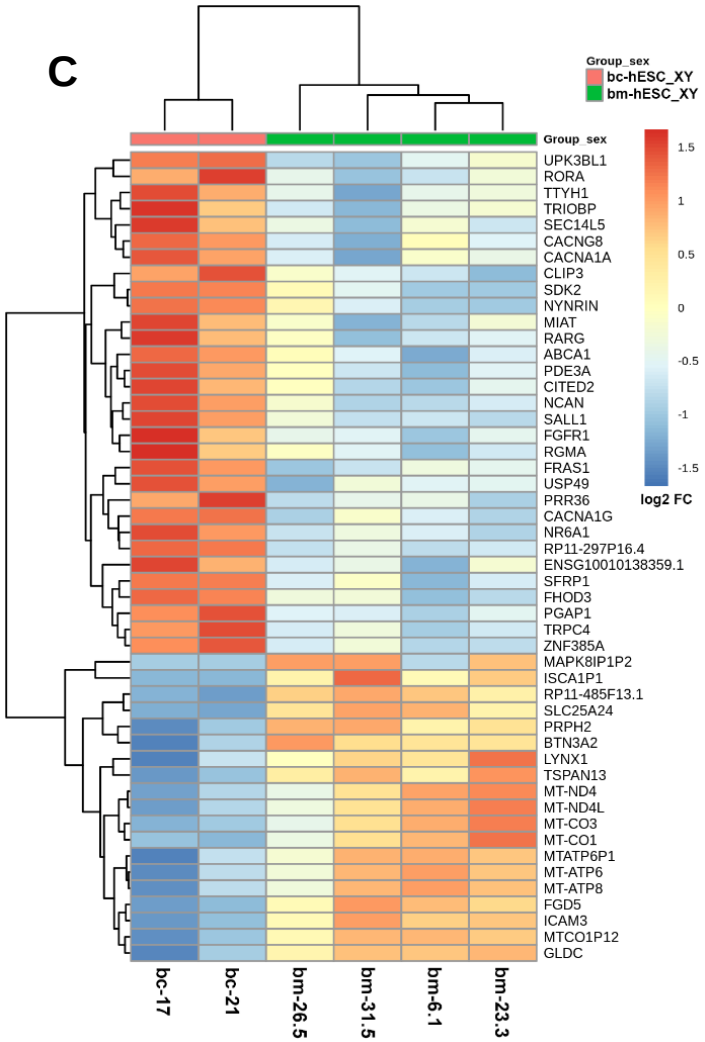

Figure 2. A) Total number of genes genes upregulated in bm-hESCs and in bc-hESCs in both comparisons. B) Top 50 DE genes between all bm-hESC lines (bm-6.1, bm-23.3, bm-26.5 and bm-23.3) and bc-hESC lines (bc-4, bc-17, bc-21 and bc-26) comparison. C) Top 50 DE genes in only XY bm-hESC and bc-hESC lines (all except bc-4 and bc-26) comparison.

Table 1. Top 15 GO biological processes associated with the DE genes in all lines comparison.

GO Biological process	Term size	Sample size	Adjusted p-value (FDR)	Upregulated in bc-hESCs	Upregulated in bm-hESCs
Nervous system development	2195	22	2.32E-03	21	1
Pattern specification process	449	10	2.73E-03	10	0
Regionalization	337	9	2.95E-03	9	0
Multicellular organismal process	6635	39	3.06E-03	34	5
Cell-cell adhesion	536	11	4.02E-03	9	2
Anterior/posterior pattern specification	217	7	1.11E-02	7	0
Somite development	81	5	1.23E-02	5	0
Morphogenesis of embryonic epithelium	149	6	1.25E-02	6	0
Cell surface receptor signaling pathway involved in cell-cell signaling	345	8	1.57E-02	7	1
Cell-cell signaling	1044	13	1.59E-02	11	2
Regulation of biological quality	3704	26	1.77E-02	19	7
Multicellular organism development	4564	29	1.97E-02	26	3
Wnt signaling pathway	276	7	2.18E-02	6	1
Biological adhesion	963	12	2.29E-02	10	2
Cell adhesion	957	12	2.30E-02	10	2

Table 2. Top 15 GO biological processes associated with the DE genes in only XY lines comparison.

GO Biological process	Term size	Sample size	Adjusted p-value (FDR)	Upregulated in bc-hESCs	Upregulated in bm-hESCs
Nervous system development	2195	32	2.88E-05	27	5
Cellular respiration	181	10	1.42E-04	1	9
Aerobic respiration	152	9	2.90E-04	0	9
Oxidative phosphorylation	111	8	2.96E-04	0	8
Energy derivation by oxidation of organic compounds	246	10	8.89E-04	1	9
System development	4222	41	1.42E-03	32	9
Inorganic cation transmembrane transport	582	14	1.45E-03	7	7
Neural tube development	158	8	1.70E-03	7	1
Neural tube formation	107	7	1.86E-03	7	0
Tube morphogenesis	664	14	2.49E-03	11	3
Aerobic electron transport chain	78	6	2.54E-03	0	6
Embryonic epithelial tube formation	127	7	2.59E-03	7	0
ATP synthesis coupled electron transport	83	6	2.68E-03	0	6
Generation of precursor metabolites and energy	392	11	2.70E-03	1	10
Developmental process	5613	48	2.73E-03	34	14

Differentiation capacity of hESCs by EBs formation

To evaluate whether the differences observed at the transcriptome level between bm-hESC and bc-hESC lines can affect their differentiation potential, we measured the changes in the expression of several lineage markers after spontaneous differentiation of the bm-hESCs, bc-hESCs and the naïve-converted

hESC line to EBs for 6 days (Fig. 3A). The qPCR results indicated that, when treated as a whole, no statistically significant differences existed between bm-hESCs, bc-hESCs and naïve-converted hESCs in the expression levels of pluripotent, ectodermal, mesodermal and endodermal markers of the EBs relative to the undifferentiated hESCs (Fig. 3B). However, a large heterogenicity was observed between the different hESC lines, even within the same experimental group. This heterogeneity was already observed when comparing the different hESC lines before differentiation (Fig. 4). Specifically, lines bm-31.5, bc-4 and, to a lesser extent, bc-26 showed a significantly higher expression of most of the lineage markers. Consistently, these hESC lines showed the lowest *OCT4* expression levels, but their expression of *SOX2* was significantly higher than in the other hESC lines (Fig. 4A,B).

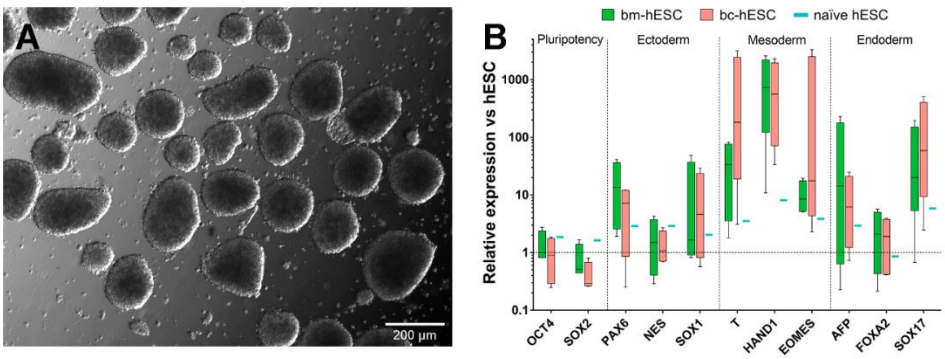

Figure 3. A) Embryoid bodies (EBs) after 6 days of suspension culture. B) Expression of pluripotency and lineage markers in bm-hESC, bc-hESC and naïve hESC-derived EBs normalized to respective undifferentiated hESCs.

Statistically significant differences in the expression of pluripotency and lineage markers were also detected among the EBs generated from the different hESC lines relative to the corresponding hESCs. In the bm-hESCs group, lines bm-6.1 and bm-23.3 differentiated efficiently towards the three germ layers, with the former upregulating all the lineage markers except *FOXA2* and the latter upregulating all of them (Fig. 5A). Line bm-26.5 showed efficient differentiation towards mesoderm and endoderm but only one of the ectodermal markers (*PAX6*) was upregulated after EB formation. Last, bm-31.5 EBs showed

differentiation only towards mesoderm, though the upregulation of its markers was mild when compared to other lines. Regarding the pluripotency markers, line bm-6.1 downregulated both OCT4 and SOX2 after differentiation, lines bm-26.5 and bm-31.5 showed downregulation only of SOX2 and no downregulation of any of the two markers was detected in line bm-23.3. In the bc-hESCs group, line bc-4 differentiated well into mesoderm, although the upregulation of the mesodermal genes was moderate, and only slightly upregulated one out of the three markers for ectoderm (*PAX6*) and two out of three for endoderm (*AFP* and *SOX17*). Line bc-17 showed a marked differentiation propensity towards mesoderm, with a fold change of more than 2000 in the three mesodermal markers. However, it only upregulated one of the ectodermal (*NES*) and two endodermal markers (*FOXA2* and *SOX17*). Line bc-21 differentiated efficiently towards all three germ layers, upregulating all the lineage markers. Finally, line bc-26 strongly upregulated the ectodermal markers *PAX6* and *SOX1*, all the mesodermal markers and the endodermal markers *AFP* and *SOX17*. Pluripotency marker *OCT4* was downregulated in lines bc-4 and bc-21, whereas all lines except bc-21 downregulated *SOX2* after differentiation (Fig. 5B). Lastly, the naïve-converted hESC line hES10, cultured in 2iF media, upregulated all the lineage markers except *FOXA2* after differentiation, but the increase in gene expression was lower than that observed in some bm-hESC and bc-hESC lines. Additionally, naïve hESCs failed to downregulate both pluripotency markers *OCT4* and *SOX2* after differentiation (Figs. 3B and 5C).

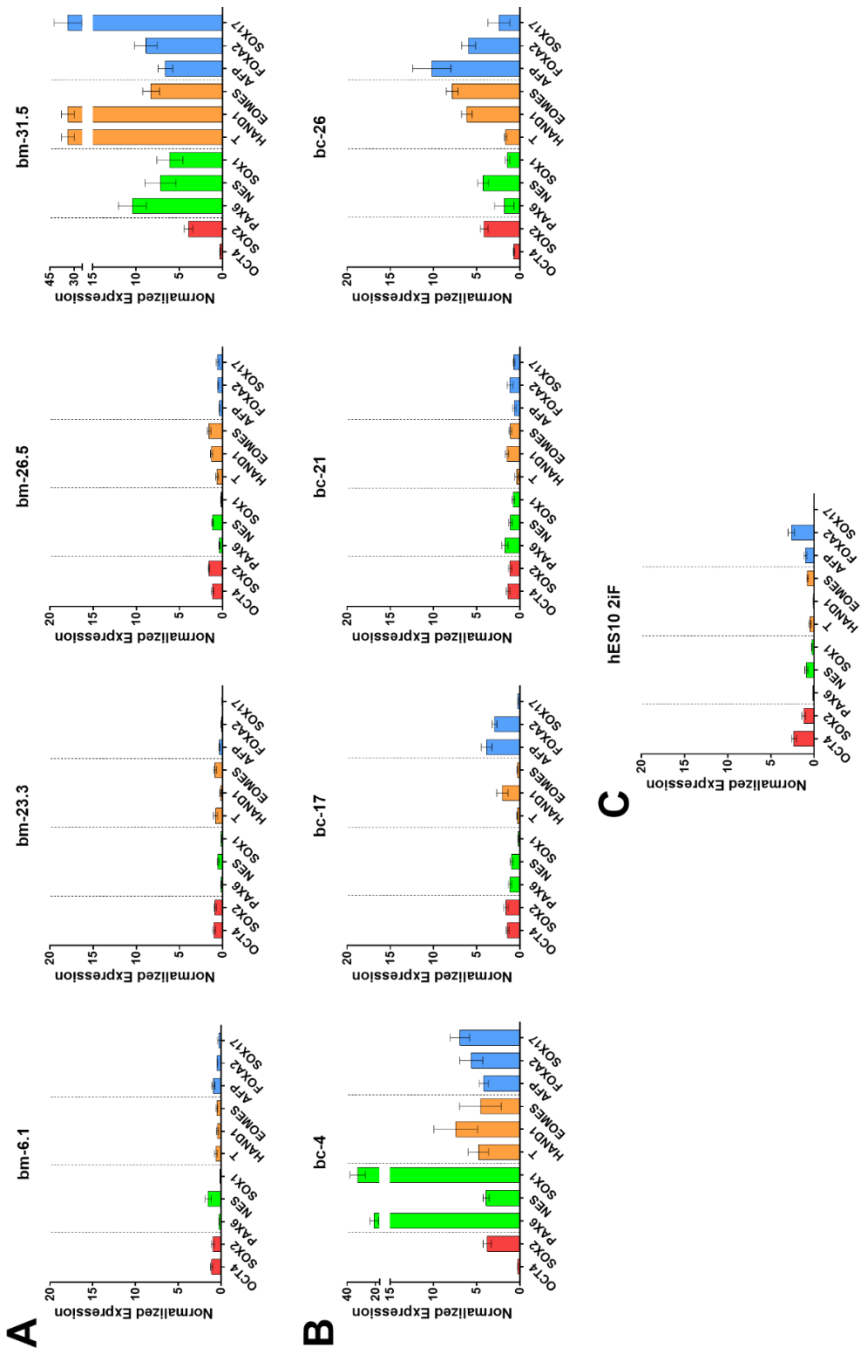

Figure 4. Expression of pluripotency (red), ectoderm (green), mesoderm (orange) and endoderm (blue) markers in bm-ESC lines (A), bc-hESC lines (B) and the naïve-converted hESC line (C) before differentiation.

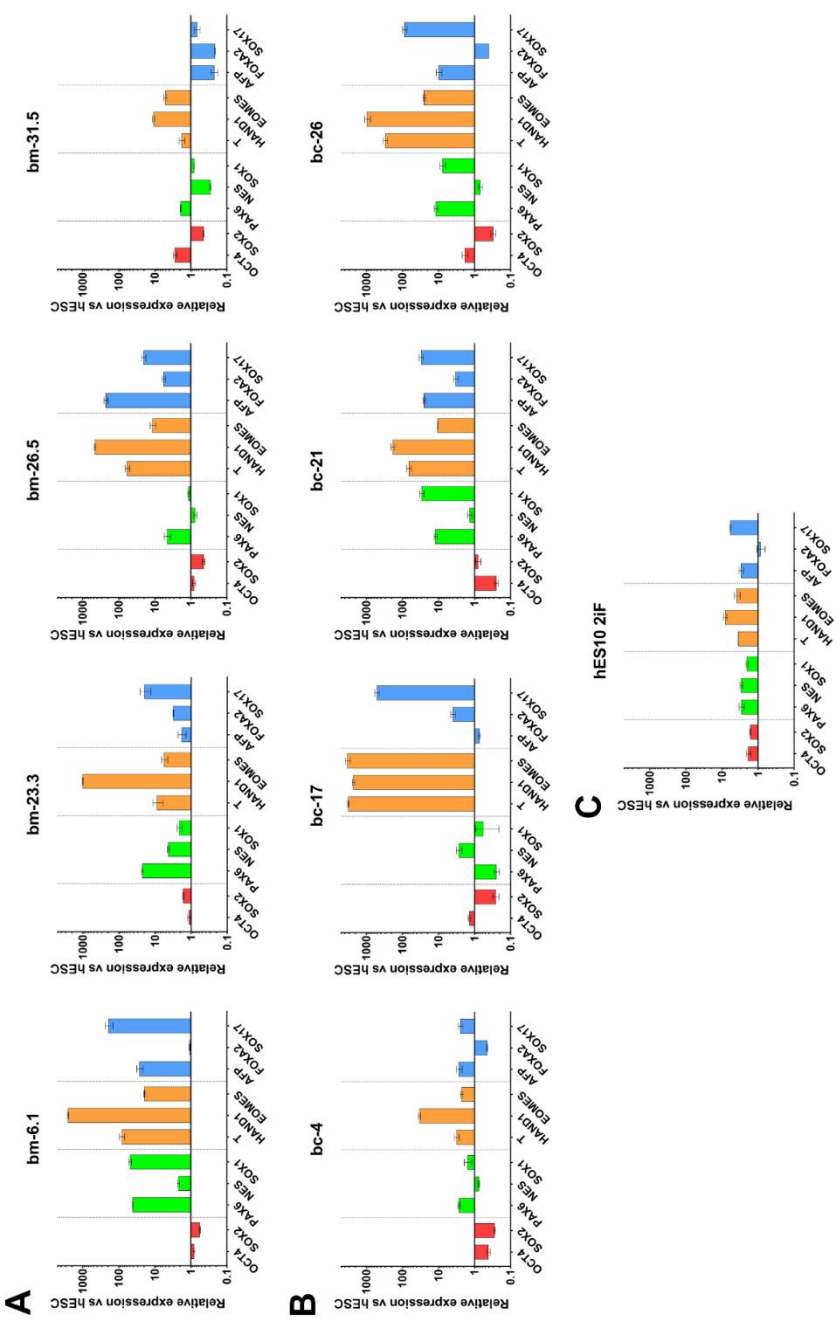

Figure 5. Expression of pluripotency (red), ectoderm (green), mesoderm (orange) and endoderm (blue) markers in EBs normalized to respective undifferentiated hESCs in bm-hESC lines (A), bc-hESC lines (B) and the naïve-converted hESC line (C).

Discussion

Our clustering results indicated that bm-hESCs and bc-hESCs do not show major differences in their transcriptional profiles, and that both differ from naïve hESCs. This suggests that, in spite of their earlier embryonic origin (8-cell stage *vs.* blastocysts stage), bm-hESCs display a pluripotent state similar to that of bc-hESCs, considered to be at a primed pluripotency state. This is consistent with our previous study, in which we observed significant differences between bm-hESCs and naïve hESCs in most of the naïve pluripotency indicators, including cell doubling time and clonogenicity, mitochondrial activity, TFE3 localization and DNA methylation and hydroxymethylation status (Massafret et al., 2022).

The gene expression signature of blastomere and blastocyst-derived hESCs has been examined in only a few other studies. In 2011, Giritharan and colleagues analysed three hESCs lines derived from single blastomeres of 8-cell embryos and two lines derived from blastocysts and did not observe significant differences between bm-hESC and bc-hESC lines in terms of their transcriptional profiles (Giritharan et al., 2011). Galan and colleagues achieved the same results with a similar sample (2 bm-hESC lines and 3 bc-hESC lines) and added that the transcriptome of bm-hESCs and bc-hESCs was different from that of the ICM and of blastomeres from the 8-cell stage embryo (Galan et al., 2013). Contrarily, another study reported that the transcriptome of bm-hESCs differed from that of bc-hESCs, with the former overexpressing several trophectodermal or placental transcripts (Zdravkovic et al., 2015). However, in none of these studies the bm-hESC and bc-hESC lines were established under the same culture conditions. Indeed, culture conditions but also the passage at which hESCs are analysed (not always specified in the publications) could have a profound effect on the results obtained and could explain, at least in part, the differing results among these studies.

Our results from the clustering data are consistent with the first two studies (Giritharan et al., 2011; Galan et al., 2013). Nonetheless, we detected that bc-hESCs overexpressed a set of genes mainly involved in nervous system

development and in embryonic pattern specification with respect to bm-hESCs. After the development of culture conditions that allow the 3D growth of human embryos for 14 days (Xiang et al., 2020), Kinoshita and colleagues observed that conventional hESCs clustered with late human postimplantation epiblast cells (12-14 days old) and were distributed towards the primitive streak anlage in the PCA (Kinoshita et al., 2021), consistent with previous comparisons using the cynomolgus monkey embryo (Nakamura et al., 2016). Interestingly, both the cynomolgus monkey and the human postimplantation epiblast cell populations were found to overexpress genes related to neuron differentiation/nervous system development (Nakamura et al., 2016; Xiang et al., 2020). Moreover, the anterior-posterior axis formation begins at the onset of gastrulation, along with the formation of the primitive streak (Yamaguchi, 2001). Accordingly, genes related to pattern specification processes were found to be overexpressed in gastrulating cynomolgus monkey embryos (Nakamura et al., 2016). The overexpression of genes involved in nervous system development, anterior-posterior pattern specification, morphogenesis of embryonic epithelium and somite development by bc-hESCs in our study may indicate that they better recapitulate the very late postimplantation epiblast of the human embryo than bm-hESCs.

On the other hand, male bm-hESCs overexpressed some mitochondrial genes involved in oxidative phosphorylation and cellular and aerobic respiration. It has been reported that cells from the mouse pre-implantation epiblast use oxidative phosphorylation for energy production. Then, a metabolic switch occurs during implantation, with postimplantation epiblast cells relying almost exclusively in glycolysis. This pattern is also observed *in vitro*, since mESCs and naïve hESCs use oxidative phosphorylation whereas primed epiblastic stem cells (EpiSCs) and hESCs metabolism is almost exclusively glycolytic (Zhou et al., 2012; Takashima et al., 2014). These results, as well as those described above corresponding to the GO terms overexpressed in bc-hESCs, could indicate that bm-hESCs may closer resemble an earlier stage of the human embryo than bc-hESCs (in agreement with their embryonic source) and would be slightly closer

to the naïve end of the pluripotency continuum than bc-hESCs. This is also consistent with previous findings by our group that showed that some naïve pluripotency markers were increased in bm-hESC lines in comparison with bc-hESC ones (Massafret et al., 2022). However, in our previous work we did not observe an increase in the mitochondrial activity of bm-hESCs over that of bc-hESCs when using the tetramethylrhodamine ethyl ester (TMRE) assay kit (Abcam) to assess the mitochondrial membrane potential. It is possible that this assay is not sensitive enough to detect subtle changes in cell metabolism or that the observed upregulation of genes involved in cellular respiration may not cause an actual metabolic change in the cells. Alternatively, the hypothetical metabolic changes could be hidden by changes in the number of mitochondria. Further research is needed to discern among these different possibilities.

Wnt pathway inhibition has been associated with the maintenance of primed pluripotency (Xu et al., 2016; Liu et al., 2017), and we found 7 DE genes associated with this pathway. In particular, we found that bm-hESCs overexpress *DACT1*, a Wnt antagonist (Zhang et al., 2006), whereas bc-hESCs overexpress *APC2*, a member of the β-catenin cytoplasmic degradation complex described in brain cells (Nakagawa et al., 1998), and *SFRP1*, which specifically binds Wnt proteins or Frizzled receptors forming an inhibitory complex (Bafico et al., 1999). These differences suggest that bm-hESCs and bc-hESCs might follow different strategies for Wnt inhibition. Although more investigations are needed, it is tempting to speculate that different strategies for Wnt pathway inhibition could result in different inhibition degrees of Wnt signalling pathway in bm-hESCs and bc-hESCs lines, which in turn could explain the small differences observed in their pluripotency state.

Regarding the differentiation experiments, bm-hESCs and bc-hESCs displayed a similar differentiation potential towards the three germ layers. Naïve-hESCs upregulated all the markers for ectoderm and mesoderm, and the endoderm markers *AFP* and *SOX17* after 6 days of differentiation. However, this upregulation was less pronounced than in most bm-hESC and bc-hESC lines. Moreover, they failed to downregulate both pluripotency markers. This is

consistent with reports indicating that naïve hESCs may need a capacitation or re-priming step for efficient differentiation (Lee et al., 2017; Rostovskaya et al., 2019). Additionally, it has been reported that the presence of 2i makes cells less responsive to differentiation stimuli (Ghimire et al., 2018), and that a 2i withdrawal step before differentiation induction increases the differentiation propensity of mESC cultured in 2i+LIF (Kalkan et al., 2017). Our results seem to corroborate that naïve converted hESCs may require either this pre-treatment or more time to efficiently differentiate into the three germ layers. The less efficient differentiation observed in our naïve hESC line may be caused by a high Wnt activity, due to its prolonged culture under GSK3β inhibition (2i) conditions.

Despite bc-hESCs upregulated genes related to nervous system development, an increased differentiation propensity towards ectoderm was not observed since neither *PAX6*, *NES* nor *SOX1* were overexpressed in bc-hESC-derived EBs when compared to bm-hESC-derived EBs. However, we cannot exclude the fact that bc-hESCs could be more poised for terminal differentiation into neuronal precursors, hindering or not their capacity to generate other mature cell types. Additionally, we did observe several differences in the expression of several lineage markers and in the differentiation potential among some hESC lines, but they were not correlated with a distinct developmental stage of their source embryo. In fact, heterogeneity of hESC lines regarding their differentiation capacities has been previously reported (Osafune et al., 2008; Bock et al., 2011; Sun et al., 2018). This suggests that the observed disparities in the differentiation potential of some hESC lines is an intrinsic characteristic of hESCs rather than an effect of their different embryonic origin. Nevertheless, the origin of this diversity is probably not related to the genetic background of the source embryos since lines derived from embryos of the same cohort (bm-26.5 and bm-31.5), although sharing closely related transcriptome profiles, do not coincide in their differentiation capacity, at least towards endoderm. Moreover, we cannot completely rule out the possibility that this intrinsic heterogeneity

may conceal a milder predisposition in the differentiation potential depending on the different stage of the source embryos.

In summary, our results indicate that the transcriptome profiles of bc-hESCs and bm-hESCs are different from that of naïve converted hESCs, since the latter cluster apart from the other two. Additionally, they demonstrate that the transcriptomes of bc-hESCs and bm-hESCs are not identical, since bc-hESCs mainly upregulate genes related to the nervous system development, embryonic pattern specification processes, the morphogenesis of the embryonic epithelium and Wnt pathway modulation, whereas bm-hESCs mainly upregulate mitochondrial genes involved in oxidative phosphorylation and aerobic respiration. Together with previous observations (Massafret et al., 2022), these results suggest that bm-ESCs could be at a primed pluripotency state, although slightly closer to the naïve end of the pluripotency continuum than bc-hESC lines. Nevertheless, differences in their transcriptional profile do not result in significant differences in their spontaneous differentiation potential towards the three germ layers.

Acknowledgements

We thank Sandra Alonso, Jonatan Lucas and María Jesús Álvarez for their supportive work. Ot Massafret was beneficiary of a FI-2017 fellowship by Generalitat de Catalunya.

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Discussion

The state of pluripotency of ESCs *in vitro* is influenced by several factors, including the developmental stage of the source embryo and the composition of the derivation and culture media. The effect of the first factor can be easily observed in mice, since mESCs derived from pre-implantation embryos display a naïve state of pluripotency, whereas mEpiSCs derived from the post-implantation epiblast are known to be in a primed state of pluripotency (Nichols and Smith, 2009). Contrarily, conventional hESCs derived from the pre-implantation human blastocyst have a transcriptional signature resembling the late post-implantation epiblast (Nakamura et al., 2016; Kinoshita et al., 2021) and are, accordingly, classified as primed pluripotent cells. However, it is important to notice that, although pluripotency has been traditionally considered a dual concept (naïve or primed), these two states probably only represent the extremes of a continuum through which cell lines can be allocated according to their characteristics.

As explained in section 5.3.1, naïve ESCs are a better model than primed ESCs for the study of events occurring during preimplantation embryonic development and have several advantages over primed ESCs, including a higher survivability after single cell passaging, a faster growth rate and a broader differentiation potential, which would make them more suitable for clinical therapeutic uses (Kumari, 2016; Weinberger et al., 2016). Although mESCs are considered the naïve gold standard, their suitability as a model to study early human embryo development and the potentiality of hESCs is limited, due to the aforementioned species-specific notable differences. Therefore, a reliable source of naïve hESCs is needed. In this sense, understanding the pluripotency state of hESCs obtained from different embryonic sources and how they can recapitulate human embryo development and capture its different stages in an *in vitro* environment is of great interest.

Human ESCs derived from single blastomeres of cleavage-stage embryos are particularly appealing. They can be generated without embryo destruction (González et al., 2011; Vila-Cejudo et al., 2019), alleviating the ethical issues involved in hESC derivation, and the earlier developmental stage of the source

embryo (when compared with hESCs derived from blastocysts) could result in hESCs presenting an earlier or more immature pluripotency state. This highlights the need to determine whether bm-hESCs are equivalent to bc-hESCs regarding their pluripotency state or, otherwise, have some particularities that must be considered for potential clinical uses.

In this thesis, we aimed to investigate whether the distinct developmental stage of the source embryo for bm-hESC and bc-hESC derivation (8-cell or blastocyst stage), can lead to the generation of hESC lines with different characteristics or states of pluripotency. For that, we established new hESC lines from both origins under similar conditions and studied several properties of bm-hESCs and bc-hESCs, focusing on a wide variety of naïve pluripotency indicators while also analysing their transcriptional profile and their differentiation potential towards the three germ layers.

1. The hESC derivation process from single blastomeres

In the first work, our initial objective was to efficiently derive new hESC lines both from single blastomeres and from whole blastocysts using the same conditions. To optimize the derivation process, we used a protocol based on that established by Taei and colleagues, who described the efficient derivation of hESCs from single blastomeres of 8-cell embryos by directly plating them onto feeder cells after culturing the source embryos from the 4-cell stage onwards in the presence of GSK3βi and ROCKi (Taei et al., 2013). Our results confirmed the positive effect of these two inhibitors on bm-hESC derivation, since 4 lines were established in the presence of GSK3βi and ROCKi, whereas none could be obtained in standard medium without these inhibitors. Moreover, the addition of GSK3βi and ROCKi also allowed us to obtain high hESC derivation efficiencies from whole blastocysts. Additionally, in contrast to the results reported by Taei and colleagues, we were able to derive bm-hESCs using inactivated HFFs as feeder cells. The use of HFFs instead of MEFs implies a reduction in the exposure

of the hESCs to xeno-products, a step forward towards the derivation of completely xeno-free hESC lines.

During the first days of the derivation process, we monitored the evolution of the plated blastomeres and blastocysts and observed the formation of a PICMI from both single blastomeres and whole blastocysts. So far, the formation of such structure had only been described during hESC derivation from whole blastocysts (O'Leary et al., 2012). In fact, another study describing hESC derivation from single blastomeres reported that colonies emerged rapidly upon plating without the formation of a PICMI (Zdravkovic et al., 2015). In sharp contrast, we describe for the first time that isolated blastomeres go through similar morphological changes during the derivation process than whole blastocysts when the same culture conditions are used, although with a delay of approximately 3 days. This delay may correspond to their different developmental stage at the time of plating (D3 vs D6). This fact may indicate that embryonic cells must reach a given developmental stage to be able to become an established hESC line. Additionally, we did not obtain any hESC line from neither single blastomeres nor whole blastocysts without the previous formation of a PICMI, consistent with the idea that PICMI formation is an essential step for the establishment of a hESC line (O'Leary et al., 2012).

2. The pluripotency state of bm-hESCs

Our second objective was to analyse several naïve pluripotency indicators to determine the pluripotency state of bm-hESCs. We used newly derived bc-hESCs as a primed control and we generated a naïve hESC line by the conversion of an already established hESC line using the protocol described by Ware and colleagues (Ware et al., 2014). This line was used as a naïve control in our experiments.

Our results showed that bm-hESCs and bc-hESCs are not identical regarding their pluripotent state, as we observed a significantly higher single-cell

clonogenicity, although lower than that of naïve hESCs, and a higher expression of several naïve pluripotency-associated markers at early culture passages in bm-hESCs when compared with bc-hESCs. Nevertheless, like bc-hESCs, bm-hESCs showed a high population doubling time, a preferentially cytoplasmic localization of the TFE3 marker, a low mitochondrial membrane potential and high DNA methylation and hydroxymethylation levels, all indicators of a primed state of pluripotency (Zhou et al., 2012; Betschinger et al., 2013; Takashima et al., 2014; Kumari, 2016; Weinberger et al., 2016). These findings suggested that bm-hESCs could be at a primed pluripotency state, although slightly closer to the naïve end of the pluripotency continuum than bc-hESCs. Interestingly, we observed a downregulation of some naïve markers upon prolonged culture, indicating that long-term exposure to standard culture conditions can affect the pluripotency state of bm-hESCs by making them more similar to bc-hESCs.

To test whether naïve pluripotency characteristics could be captured *in vitro* from single blastomeres, we attempted the direct derivation of naïve hESCs from single blastomeres, which, to our knowledge, had never been performed before. Derivation of hESCs lines under naïve conditions has only been reported from blastocysts, yielding extremely low efficiencies (Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014; Guo et al., 2016). We hypothesized that, due to their earlier developmental stage and to the fact that blastomeres from 8-cell embryos have not fully undergone any lineage specification event (Zhu et al., 2021), it could be easier to capture a naïve phenotype *in vitro* from single blastomeres than from whole blastocysts cells. For this, we used the 2iF conditions (Ware et al., 2014) that were reported to allow the derivation of a naïve hESC line from a whole blastocyst (albeit at a very low efficiency, as already mentioned) and had worked well in our hands for the conversion of an already established hESC line.

We observed the formation of PICMIs under naïve conditions, but cells differentiated after the first passaging. The fact that 2iF naïve conditions supported the formation of PICMIs but did not support subsequent undifferentiated hESC growth is consistent with the hypothesis that the PICMI

represents a transitory state between naïve and primed pluripotent states (Warrier et al., 2018). This also indicates that pre- and post-PICMI cells may have different *in vitro* culture requirements and that PICMI formation should probably be avoided to achieve efficient direct naïve hESC derivation from single blastomeres.

3. Transcriptional differences between bm-hESCs, bc-hESCs and naïve converted hESC lines

To complete the results from our first study, in our second work we performed RNA-sequencing to compare the transcriptional profiles of bm-hESCs, bc-hESCs and naïve-converted hESCs. The clustering analysis of the different hESC lines showed that bm-hESCs and bc-hESCs were similar at the transcriptional level, whereas naïve hESCs were significantly different. Our results were consistent with two different studies that reported that the transcriptomes of bm-hESCs and bc-hESCs showed no major differences (Giritharan et al., 2011; Galan et al., 2013), whereas another study described that bm-hESC lines differed from bc-hESC lines at the transcriptional level upregulating genes involved in trophoblast/ectoplacental cone pathways (Zdravkovic et al., 2015). In this last study, bm-hESC lines showed increased potential to form TE spontaneously and were able to generate a trophectodermal SC line. Thus, they correlated the earlier embryonic origin of the bm-hESC lines with an increased plasticity and a relatively more naïve pluripotent state. Nevertheless, in none of the studies mentioned above the bm-hESC and bc-hESC lines were derived in the same conditions, a fact that can introduce bias in the results and may explain the discrepancies between the authors.

Although we found that the transcriptional profiles of bm-hESCs and bc-hESCs were similar, the GO enrichment analysis of the DE genes revealed an upregulation of genes associated with several biological processes. Indeed, genes related to nervous system development, embryonic pattern specification

(including the anterior/posterior pattern specification and somitogenesis) and morphogenesis of embryonic epithelium were overexpressed in bc-hESCs when compared with bm-hESCs. Moreover, when considering only the male hESC lines to avoid the sex bias introduced by the fact that only male lines were obtained from isolated blastomeres, we observed an upregulation of genes involved in cation transmembrane transport, cell respiration and oxidative phosphorylation in bm-hESCs.

A transcriptional study performed in human embryos cultured in vitro until D14 revealed upregulation of genes associated with nervous system development in the post-implantational epiblast population (Xiang et al., 2020). Moreover, the anterior-posterior axis specification occurs with the formation of the primitive streak at the onset of gastrulation (Yamaguchi, 2001), and gastrulating cells from cynomolgus monkey embryos were shown to overexpress genes related to pattern specification (Nakamura et al., 2016). On the other hand, the preference for oxidative phosphorylation over glycolysis is observed in the mouse preimplantation epiblast, but after implantation the cells' metabolism switches to exclusively glycolytic. This can also be observed in naïve mESCs and primed mEpiSCs and hESCs (Zhou et al., 2012; Takashima et al., 2014). Hence, our results from the transcriptome analysis suggested that bc-hESCs could better recapitulate the late post-implantation epiblast of the human embryo, whereas bm-hESCs would correspond to an earlier developmental stage of the embryo. In summary, all these results reinforce the idea that bm-hESCs may be closer to the naïve end of the pluripotency continuum than bc-hESCs, in clear accordance with our findings from the first study.

The GO analysis on the DE genes also revealed a significant overrepresentation of genes associated with the Wnt signalling pathway, which inhibition is associated with primed pluripotency (Xu et al., 2016; Liu et al., 2017a). The results observed suggest that bm-hESCs and bc-hESCs could use different strategies to keep Wnt signalling at low levels. Moreover, these differences in Wnt signalling inhibition may account for the observed differences in their pluripotency state.

Taken together, the results from our transcriptional study indicate that, although bm-hESCs and bc-hESCs lines clustered together in the PCA, subtle differences in their transcriptomic profiles exist. Moreover, the biological processes that are differentially expressed are in line with the results obtained in our first study regarding the differences in the pluripotency state of bm-hESCs and bc-hESCs.

4. The differentiation potential of bm-hESCs

Finally, we analysed the differentiation potential of bm-hESCs, bc-hESCs and naïve converted hESCs towards the three germ layers by generating EBs. We did not find any significant differences among bm-hESCs, bc-hESCs and naïve hESCs when treated as groups. However, we did observe several differences between different hESC lines, even within the same group. These differences were observed in the expression of pluripotency and lineage markers by both undifferentiated hESCs and by EBs relative to hESC samples. Heterogeneity between hESC lines regarding their differentiation potential has been widely reported (Osafune et al., 2008; Bock et al., 2011; Sun et al., 2018). The differences among hESC lines observed in our study did not match different embryonic origins, therefore we attributed them to be an intrinsic characteristic of hESCs. Additionally, results from the differentiation study did not reflect the observed differences in the transcriptome between bm-hESCs and bc-hESCs since the overexpression of genes related to nervous system development by bc-hESCs did not result in an increased differentiation propensity towards the ectodermal lineage. Nevertheless, we cannot rule out the fact that bm-hESCs and bc-hESCs could show distinct differentiation capacities towards mature terminally-differentiated cell types. Therefore, we do not know whether bc-hESCs would be more prone to differentiate into neuronal precursors, in concordance with the results from the transcriptome analysis.

5. Concluding remarks

In summary, the results obtained in this thesis indicate that subtle differences exist between bm-hESC and bc-hESC lines regarding their pluripotency state, which can probably be attributed to the different developmental stages of the source embryos.

Despite pluripotency was first believed to have only two different states: naïve and primed (Nichols and Smith, 2009), more recently a non-dichotomous model of pluripotency has been proposed. This model considers the existence of a formative pluripotent state that would represent a transitory state from the naïve to the primed pluripotency (Smith, 2017). This first hypothesis has been lately supported by other studies that described various intermediate pluripotent states in mouse and human (Neagu et al., 2020; Kinoshita et al., 2021; Yu et al., 2021b). Probably, the different protocols that claim to have established naïve hESCs displaying different characteristics actually generated a variety of naïve hESC populations positioned at different points on the developmental continuum between the naïve and primed end states. Considering this continuum, our results suggest that bm-hESCs would be close to the primed end state but, nevertheless, a step closer towards the naïve state than bc-hESCs themselves.

Aside from the particular pluripotency characteristics of the bm-hESCs lines generated from them, 8-cell embryo blastomeres are an interesting choice as a source for hESCs derivation for other reasons. The ultimate goal for hESCs derivation from single blastomeres would be to derive a hESCs line from a biopsied blastomere from a D3 embryo during an assisted reproduction cycle, while the biopsied embryo is cultured to the blastocyst stage and transferred to achieve pregnancy. With this approach, the new-born individual would have a reserve of autologous hESCs which could be eventually used for a regenerative therapy without any risk of immune rejection (Mehta, 2014; Dittrich et al., 2015). Nevertheless, there is still a long way to go, since the hESC derivation

protocols from single blastomeres need to be optimized to increase their efficiency to values high enough to make this approach viable.

On the other hand, reaching a substantial increase in the derivation efficiency of hESCs from single blastomeres could imply a reduction in the number of embryos needed for the establishment of new hESC lines when compared with derivation from the whole blastocysts since, theoretically, each of the blastomeres of an 8-cell embryo could give rise to a hESC line (Vila-Cejudo et al., 2019).

Lastly, the higher single-cell clonogenicity observed in bm-hESCs with respect to bc-hESCs could lead to a faster obtention of large amounts of cells for their uses in drug screening, disease modelling or regenerative therapies (Kumari, 2016).

6. Strengths and weaknesses of the study

The pluripotency state of hESCs is highly influenced by culture conditions, as evidenced by the existence of a wide variety of different hESC populations that are generated by targeting different signalling pathways with small molecules and medium supplements (see section 5.3.2). Accordingly, our naïve-converted hESCs cultured in 2iF medium, despite being derived from a blastocyst-stage embryo, displayed significant differences from our bc-hESCs at the transcriptional level, as well as in their morphology, proliferation capacity, clonogenicity, mitochondrial activity and DNA methylation status, thus indicating a true conversion into the naïve state. On the other hand, in this study, bm-hESCs and bc-hESCs were derived and cultured virtually on the same conditions from the 4-cell stage embryo onwards. Furthermore, our analyses were performed at early culture passages to minimize the effect of culture conditions on the characteristics of bm-hESCs and bc-hESCs lines. This allowed us to ensure that the observed differences between bm-hESCs and bc-hESCs

were caused by their different embryonic origin rather than by any other variable.

Moreover, our work did not focus on a specific indicator, but rather analysed a wide variety of characteristics of hESCs, covering the behaviour of the cells in culture (morphology, growth and survival rates), gene expression (including whole transcriptome sequencing), epigenetics, cell differentiation and metabolic features. This provided us with a fairly broad perspective of the pluripotency state profile of bm-hESCs.

Unfortunately, we were not able to derive any XX hESC line from single blastomeres, which prevented us to assess the XCI status of bm-hESCs. This analysis would definitely have provided relevant information to further increase the robustness of our study. Additionally, as mentioned before, the fact that no XX hESC lines were present in the bm-hESCs group limited our transcriptional study. To avoid sex bias, a DE analysis had to be performed only with XY hESC lines, which implied a decrease of the sample size in the bc-hESCs control group from 4 to 2.

Finally, another weakness of the study is the low efficiency in the derivation of hESC lines from single blastomeres, as well as the limitation of having only one naïve-converted hESC line. A higher number of bm-hESC and naïve hESC lines would certainly have enhanced the robustness of the study. Nonetheless, our sample size is still higher than that of two out of the three transcriptional studies comparing bc-hESCs and bm-hESCs and, all in all, we believe that our sample size is sufficient to draw reliable conclusions.

7. Future perspectives

The results obtained in this thesis have opened the door to further research to better understand the pluripotency state of bm-hESCs and the utility of single blastomeres as a source of hESC lines. First, we observed an overexpression of

mitochondrial genes in bm-hESCs with respect to bc-hESCs. Although we did not observe differences in the mitochondrial membrane potential with TMRE staining, further research using more sensitive approaches is needed to find out whether transcriptional differences between bm-hESCs and bc-hESCs are translated into metabolic changes. Understanding the metabolic needs of bm-hESCs could be useful to optimise their culture conditions and possibly increase the derivation efficiencies, which are still low.

Second, applying terminal differentiation protocols to bm-hESCs and bc-hESCs would be interesting to determine whether bc-hESCs have an increased propensity to differentiate into neural cells, as suggested by the results of the transcriptional analysis. It would also be interesting to determine whether this hypothesized neural propensity of bc-hESCs when compared to bm-hESCs would imply a lower potential to become other mature cell types derived from endodermal or mesodermal lineages. This information would be very valuable with regards to the use of bm-hESCs or bc-hESCs for clinical applications.

Finally, to have a more accurate view of which the *in vivo* counterpart of bm-hESCs is, a comparison of the transcriptome of bm-hESCs with that of human embryos at different developmental stages should be performed. With this experiment, we could unequivocally determine whether bm-hESCs and bc-hESCs truly represent different stages of the human embryonic development.

Conclusions

1. The use of inhibitors of GSK3β and ROCK during embryo culture from the 4-cell stage onward is beneficial for the derivation of hESCs from single blastomeres, since it allows a higher efficiency of outgrowth formation and the establishment of new hESC lines.

2. Single blastomeres, like whole blastocysts, arrange into a post-inner cell mass intermediate during the hESC derivation process. Therefore, the formation of this structure is necessary for the establishment of a hESC line regardless of the development stage of the source embryo.

3. Blastomere-derived hESCs present a primed pluripotentcy state, although slightly closer to the naïve end of the pluripotency continuum than blastocyst-derived hESCs, However, they cannot be considered fully naïve hESCs since there are significant differences in most of the naïve pluripotency indicators when compared with hESCs cultured under naïve conditions.

4. Long-term culture in hESC standard medium causes downregulation of the expression of some naïve pluripotency markers in blastomere-derived hESCs, making them acquire a pluripotency state more similar to that of blastocyst-derived hESCs.

5. The 2iF naïve conditions allow the formation of a post-inner cell mass intermediate during derivation from single blastomeres, but they do not support the derivation of stable hESC lines.

6. The transcriptional profiles of blastocyst- and blastomere-derived hESCs are similar to each other and different from that of the naïve hESCs, although blastocyst-derived hESCs overexpress some genes related to the development of the nervous system, the specification of embryonic patterns and the formation of the embryonic epithelium, whereas

blastomere-derived hESCs overexpress mitochondrial genes associated with cellular respiration.

7. Differences in the transcriptomic profile between blastomere and blastocyst derived hESCs do not translate into differences in the differentiation potential towards the three germ layers.

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