# Expansion of Human Induced Pluripotent Stem Cells on Dissolvable Microcarriers Under Dynamic Culture Conditions

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*For a human being, nothing comes naturally,*
*We have to learn everything we do.*

Philip Pullman

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# ABSTRACT

Human induced pluripotent stem cells (hiPSCs) have been regarded as very promising for applications in the biomedical field, due to their abilities to self-renew and differentiate into all cell types of the human body. hiPSC expansion *in vitro* to large numbers is already possible through xeno-free chemically defined large scale processes, with microcarrier culture systems amongst the most popular. Microcarriers pose disadvantages regarding cell harvesting after expansion: cells are left on the surface of microcarriers or lost during filtration – a process yet to be optimised, particularly when considering large-scale production. Corning Inc. has developed microcarriers based on a digestible pectin matrix, which can be dissolved with pectinase after hiPSC expansion, preventing one of the problems of cell recovery and making hiPSC production more economically viable. Here, hiPSCs expansion was tested under static and dynamic conditions using dissolvable microcarriers (DM) and compared with the results obtained using polystyrene microcarriers (PSM). Adhesion was very similar on both types of microcarriers using similar coatings. Expansion achieved higher cell numbers on a shorter time frame using DM – $4.61\pm0.07 \times 10^7$ cells on Matrigel®-coated DM using E8™ culture medium, and $3.80\pm0.73 \times 10^7$ cells on Synthemax®II DM using mTeSR™1 culture medium, in 5 days of culture. Cell recovery efficiency was similar using both protocols of cell harvesting – 50.8±7.8% with protease and pectinase incubation, and 51.1±9.4% with protease incubation and filtration. Preparation of DM for use was also easier and less time consuming. hiPSC expansion on DM proved to be a very promising alternative to replace PSM.

# RESUMO

Células humanas pluripotentes induzidas (hiPSCs) são promissoras para aplicações biomédicas devido às capacidades de auto-renovação e diferenciação em todos os tipos de células do corpo humano. A produção *in vitro* de hiPSCs atinge elevados números usando processos livres de produtos animais, quimicamente definidos e de larga escala, estando a cultura com *microcarriers* entre os mais populares. *Microcarriers* apresentam desvantagens relativamente à recolha das células após expansão: células são deixadas na sua superfície ou perdidas durante a filtração – um processo não optimizado, particularmente considerando a produção em larga-escala. A Corning Inc. desenvolveu *microcarriers* baseados numa matriz digestível de pectina, que podem ser dissolvidos, usando pectinase, após expansão das hiPSCs, evitando um dos problemas da recolha de células, tornando a sua expansão mais economicamente viável. A expansão de hiPSCs foi testada em condições estáticas e dinâmicas em *microcarriers* dissolúveis (DM) e comparada com resultados em microcarriers de poliestireno (PSM). A adesão de hiPSCs foi semelhante nos dois tipos de microcarriers usando revestimentos iguais. A expansão atingiu valores superiores num tempo de cultura mais curto nos DM – $4.61\pm0.17x10^7$ células em DM com revestimento de Matrigel® em meio E8™, e $3.80\pm0.73x10^7$ células em Synthemax®II DM em meio mTeSR™1, em 5 dias de cultura. A eficiência de recuperação foi semelhante nos dois protocolos de recolha de células – 50.8±7.8% com incubação com protease e pectinase, 51.1±9.4% com incubação com protease e filtração. A preparação dos DM foi também mais simples e rápida. A expansão de hiPSCs em DM mostrou ser uma alternativa promissora aos PSM.

## KEYWORDS

Human Induced Pluripotent Stem Cells

Dissolvable microcarriers

Expansion

Cell harvesting

Spinner-flask

Scale-up

# PALAVRAS-CHAVE

Células Humanas Pluripotentes Induzidas

Microtransportadores dissolúveis

Expansão

Recolha de células

*Spinner-flask*

*Scale-up*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 2D | Two-dimensional |
| 3D | Three-dimensional |
| AKT | Protein kinase B |
| ALK | Anaplastic lymphoma kinase |
| APC | Adenomatous polyposis coli |
| ATP | Adenosine triphosphate |
| AXIN | Axis inhibitor |
| BMP | Bone morphogenic protein |
| BSA | Bovine serum albumin |
| CAM | Cell adhesion molecule |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DM | Dissolvable microcarriers |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| dPBS | Dulbecco's Phosphate-Buffered Saline |
| Dvl | Dishevelled |
| E8 | Essential 8 |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamine tetraacetic acid |
| ERK | Extracellular signal–regulated kinases |
| ESC | Embryonic stem cell |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| GAG | Glycosaminoglycan |
| GMP | Good manufacturing practices |
| GSK-3β | Glycogen synthase kinase-3 beta |
| hESC | Human embryonic stem cell |
| hiPSC | Human induced pluripotent stem cell |
| HMG | High motility group |
| hPSC | Human pluripotent stem cell |
| ICM | Inner cell mass |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IgSF | Immunoglobulin superfamily |
| iPSC | Induced pluripotent stem cell |
| KLF4 | Kruppel-like factor 4 |
| KO-DMEM | Knockout Dulbecco's modified eagle medium |

| | |
|---|---|
| KO-SR | Knockout-serum replacement |
| LEF | Lymphoid enhancer factor |
| MAPK | Mitogen-activated protein kinase |
| Mat | Matrigel |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| MMTV | Mouse mammary tumor virus |
| mRNA | Messenger RNA |
| NGS | Normal goat serum |
| NICD | Notch intracellular domain |
| NMMIIA | Non-muscle myosin IIA |
| OCT4 | Octamer-binding transcription factor 4 |
| PBS | Phosphate-buffered saline |
| PenStrep | Penicillin streptomycin |
| PFA | Paraformaldehyde |
| PGA | Polygalacturonic acid |
| PI3K | Phosphoinositide 3-kinase |
| POU5F1 | POU domain, class 5, transcription factor 1 |
| PSM | Polystyrene microcarriers |
| RNA | Ribonucleic acid |
| ROCK | Rho-associated coiled coil protein kinase |
| ROCKi | ROCK inhibitor |
| RPM | Revolutions per minute |
| SC | Stem cell |
| SII | Synthemax®II |
| siRNA | Small interference RNA |
| Sox2 | SRY (sex determining region Y)-box 2 |
| SSEA | Stage specific embryonic antigen |
| TCF | T-cell factor |
| TGF-β | Transforming growth factor β |
| TRA | Tumour rejection antigens |
| WNT | Wingless-type MMTV integration site family members |

# I. AIM OF STUDIES

Human induced pluripotent stem cells (hiPSCs) have become more and more promising regarding applications in fields such as personalized regenerative medicine, disease modelling and drug development. However, in order for these applications to prove successful, one of the first steps is to be able to achieve clinically meaningful cell numbers, which translates as the need to optimise hiPSC expansion *in vitro*.

Many efforts have been made regarding this optimisation, one of the most important being the transition from two-dimensional monolayer cultures to three-dimensional cultures – with the use of microcarriers as support for cell attachment (amongst other alternatives) – which provides increased surface area for cells to expand. Even though the use of microcarriers within stirred-tank bioreactors was such a great improvement, there are still some limitations regarding the use of these type of systems, mainly regarding the harvesting of the cells after their expansion. During this process, some of the cells are left still attached to the surface of the microcarriers, and some are lost during the filtration step used to separate cells from the microcarriers, which ultimately results in lower expansion yields. Despite the fact that the cells themselves are the product of interest, little focus on the harvesting procedure has been made.

Corning Inc. developed new microcarriers completely based on a digestible matrix, that can be easily dissolved using the appropriate enzyme; by eliminating the microcarriers altogether, the harvesting process would be simplified and less expensive, as the filtration step would be eliminated, which would, in theory, lead to an increase in hiPSC expansion yield, and consequently make hiPSC expansion more economically viable.

The aim of this project is to analyse hiPSC attachment and expansion on the newly developed dissolvable microcarriers, and develop an easy and efficient downstream process for hiPSC recovery, by allowing a simpler and less time-consuming cell harvesting process, and to facilitate cell/microcarrier separation, under chemically-defined and xeno-free conditions.

# II. INTRODUCTION

## II.1. HUMAN STEM CELLS

Stem cells (SCs) possess two very defining characteristics: the ability to self-renew, through mitotic cell division, and to differentiate into several types of mature cells [1, 2]. Stem cells are currently classified according to two features: origin and differentiation potential.

Regarding their origin, SCs are classified as: Embryonic Stem Cells (ESCs) which are obtained from the inner cell mass (ICM) of the blastocyst during the first 4-5 days of embryo development [3]; Foetal SCs exist from the eighth week of embryonic development and can be obtained from foetal blood and tissues, these are believed to have similar properties to adult tissue-derived SCs, and their development potential is more restricted than pluripotent ESCs [4]; Adult Stem Cells (ASCs), that exist in small portions in mature tissues, multiply by mitotic cell division and differentiate in order to replace dying cells and regenerate damaged tissues – these can be of Endodermal, Mesodermal or Ectodermal origin and are usually referred to accordingly to their tissue origin: Pulmonary Epithelial SCs, Gastrointestinal Tract SCs, Pancreatic SCs, Hepatic Oval Cells, Mammary and Prostatic Gland SCs, Ovarian and Testicular SCs, Hematopoietic SCs, Mesenchymal SCs, Bone Marrow SCs, Cardiac SCs, Neural SCs, Skin SCs and Ocular SCs are only a few examples [1, 2]; Cancer SCs are the stem cells within a tumour mass [2]; and induced Pluripotent Stem Cells (iPSCs) which are artificially obtained by reprogramming an adult somatic cells [3].

Concerning their potential for differentiation, stem cells are categorised into: Totipotent SCs, which can originate an entire functional organism including all the extra embryonic tissues necessary for the development of the embryo; Pluripotent SCs, which can give rise to any type of cell that comprises the human body (cells derived from each of the three embryonic germ layers – endoderm, mesoderm, and ectoderm); Multipotent SCs, which are committed to a limited range of cells within a tissue; and Unipotent SCs which are the precursor cells that can only further differentiate into one specific cell type [1, 2].

### II.1.1. Pluripotent stem cells

Human pluripotent stem cells (hPSCs), in addition to being able to give rise to all cell types that comprise the human body, have an unlimited capacity to self-renew while maintaining their undifferentiated state; they exist as *in vitro* artefacts and can be human embryonic stem cells (hESCs) derived from the ICM of the blastocyst during embryonic development, and human induced pluripotent stem cells (hiPSCs) which are reprogrammed from adult somatic cells (**Figure II.1**).

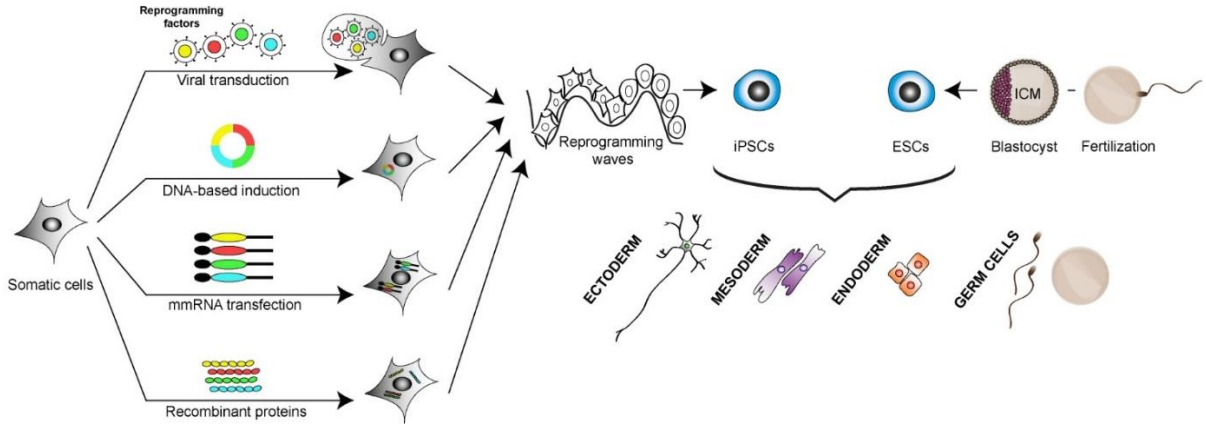



***Figure II.1. Comparison between iPSCs and ESCs.*** *Overview of reprogramming methods of adult somatic cells to induced pluripotent stem cells (iPSCs) and differentiation potential of pluripotent stem cells: iPSCs and embryonic stem cells (ESCs) [5].*

#### *II.1.1.1. Embryonic stem cells*

ESCs are derived from the ICM of the blastocyst [6] which corresponds to one of the early stages of the embryo (4-5 days) [1]. These cells were isolated for the first time in 1998 by James Thomson [6] and, although very promising for therapeutic applications, these implicate important ethical issues regarding the use of human embryos [3], even though these are not embryos themselves - hESCs are cell lines established from embryos obtained through *in vitro* fertilisation of donor eggs [7] –, and there is growing evidence that these cells do not behave *in vitro* in the same way they would in a developing embryo [8] to a point where they should only be considered to be culture artefacts [1]. These cells can also be obtained through somatic cell nuclear transfer [9].

#### *II.1.1.2. Induced pluripotent stem cells*

iPSCs were obtained for the first time in 2006, by Shinya Yamanaka, by reprogramming mice adult fibroblasts using defined factors – Oct3/4, Sox2, Klf4 and c-Myc [10]. Later, in 2007, the same team of researchers announced the successful reprograming of adult human fibroblasts using the same four factors through retroviral transduction, while at the same time James Thomson and his team reported reprogramming using Oct4, Sox2, Lin28 and Nanog to induce somatic cells to become pluripotent, obtaining the first lines of hiPSCs [3, 11]. The reprogrammed cells obtained were similar to hESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity and could differentiate into cell types of the three germ layers *in vitro* and in teratomas [3] – tumours that include cells from all three germ layers as well as undifferentiated stem cells [1, 12]. Recent experiments have demonstrated that the differences between hESCs and hiPSCs can be as little as differences in the expression of forty-nine genes and that difference may be more linked to genetic background variation rather than the difference in cellular origin or alterations due to the reprogramming methods used [13].

The methods used to reprogram adult cells into iPSCs have to be carefully chosen taking into account the type of application that these cells will have. The use of viral technologies to deliver the necessary genes to the cells, while presenting higher reprogramming yields, also implies higher risks regarding the

use in humans since most of the virus integrate the cells' DNA in a non-controllable way; this means that even though there is a possibility of not affecting any important coding regions, it is also possible to have major impact on gene expression and potentially trigger the expression of oncogenes. Currently there are already techniques to remove these oncogenes after reprogramming, making iPSCs safer for human applications [2].

Reprogramming approaches are still evolving and very promising DNA-free methods have been successfully applied, both in mouse and human cells, by delivery of the reprogramming proteins or mRNA directly into cells, rather than their expression from DNA. In the case of proteins, for them to be able to cross the plasma membrane of somatic cells, the carboxyl terminal of the reprogramming factors needs to be fused to a poly-arginine protein transduction domain, achieving an induction efficiency approximately twice as high as when using viral transduction. The use of synthetic mRNA showed some cytotoxicity problems on human somatic cells but achieved high reprogramming efficiencies in shorter time periods [14]. Chemical induction, which is the reprogramming of somatic cells by use of small molecules – by either combining them with transcription factors or using only the small molecules –, has also been successful; these have the advantage of being non-immunogenic, and present the possibility to be more easily administered and standardized; the effects of this process may be reversible and are dose-dependent [14].

#### *II.1.1.3. Pluripotent stem cell niche*

When considering stem cell culture, the cells themselves are the final product one aims to obtain [5, 6]. If ever hiPSCs are to be generally applied into regenerative medicine purposes it will be of the uttermost significance to be able to mimic the cells' niche *in vitro*, using only xeno-free and chemically defined factors and molecules [10]. This means that one must make sure that all the conditions for the optimal growth of the cells are being ensured. *In vitro*, the culture of mammalian cells requires growth medium, extracellular matrices and environmental factors [11].

In order to understand what the optimal growth conditions are, it is wise to look at the cells' niche *in vivo*; this concept was first proposed in 1978 as the regulatory microenvironment surrounding adult hematopoietic SCs [12]. Stem cell niches are defined as specialised local microenvironments that are crucial for the cells' normal function, including stem cell self-renewal, proliferation and differentiation [13] (**Figure II.2**). Within the cells native tissues, the three-dimensional microenvironment offers interactions from different sources that will influence stem cell fate; these include soluble and immobilized signalling factors, interactions between stem cells and the extracellular matrix (ECM), direct cell-cell interactions, the physicochemical environment, and biomechanical forces, which are dynamic through space and time [15] and must be mimicked in culture systems in order to maintain undifferentiated cell expansion or to direct stem cell differentiation to desired fates [1, 13]. The successful application of stem cells depends on the ability to recreate this microenvironment *in vitro* since otherwise stem cells may lose their function, having a deep impact on tissue function and regeneration [1]. The optimisation of pluripotent stem cell culture conditions should also allow the reduction of the selective growth advantage of mutant cells, which are not suitable for therapeutic applications and do not represent the true characteristics of hPSCs [10].

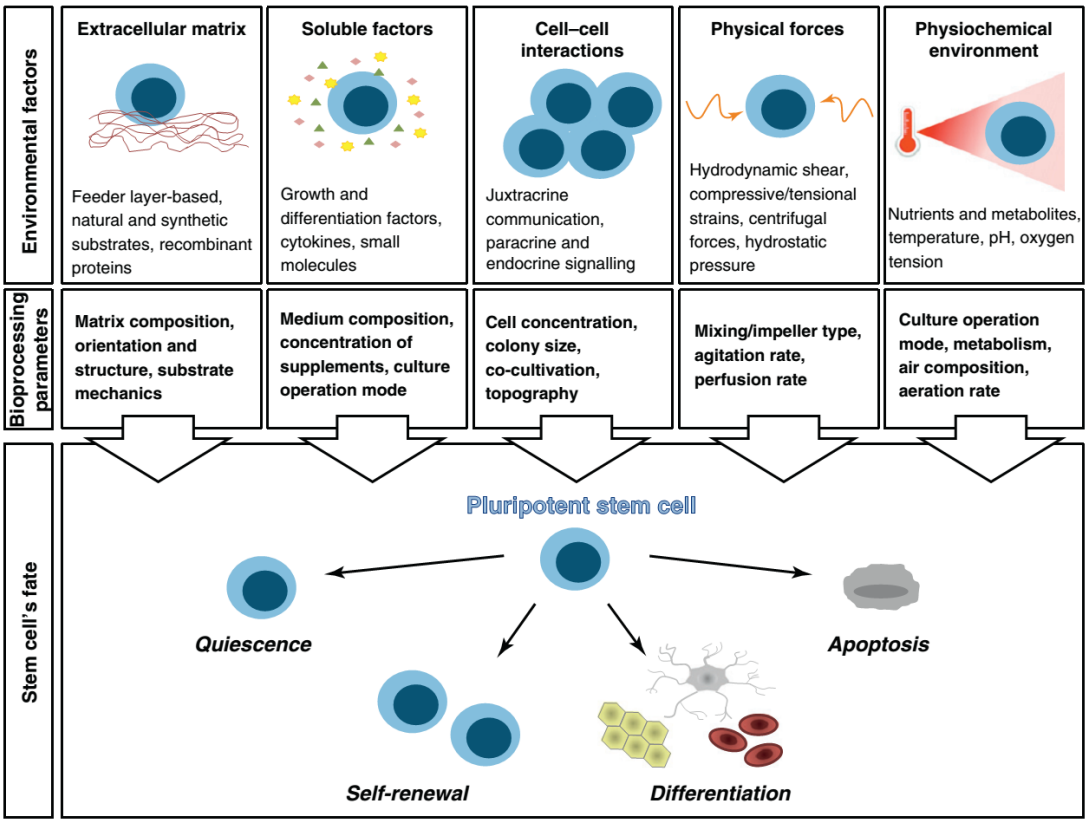



***Figure II.2 Influence of the cell niche on pluripotent stem cell fate**. Environmental factors and bioprocessing parameters that impact on hPSC fate decisions (quiescence, self-renewal, differentiation and apoptosis). The main environmental cues and examples of bioprocessing parameters controlling the fate of stem cells are depicted [16].*

#### II.1.1.3.1. Soluble factors

Soluble factors are small proteins such as growth factors, cytokines and morphogens which are crucial signalling factors that have a major role in the regulation of stem cell function *in vivo* [1, 17, 18]. These molecules have the power to modulate cell fate in a very complex process where concentration, location and even the time of exposure of the cells to the molecules must be thoroughly regulated in order for them to have the desired effect – the same molecule can have a very different effect on a cell depending on these parameters and also according to the type of cell that is subjected to its presence which means that, in order to be able to completely replicate organ and tissue formation *in vitro*, it is essential to understand all the signalling pathways that are being influenced [1, 17]. Small molecules can have long term effects when present in a soluble form, which is what generally happens in culture; however, *in vitro* these factors may also be immobilised in the ECM which alters the concentration that is actually available for the cells to use, since no diffusion occurs [1].

Extrinsic soluble factors – such as developmental morphogen proteins including Wnts, hedgehog proteins, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (see section II.1.1.4.1) – induce signals that are transmitted through intracellular components and regulate the expression of pluripotency factors [1, 19].

#### II.1.1.3.2. Extracellular matrix

*In vivo*, the ECM is the physical scaffold synthesized by cells [20]. Because of cell-to-ECM interaction induced signalling, which is a critical determinant of cell behaviour, the ECM is one of the main components of the stem cell niche [10]. It can be either a two-dimensional structure or a three-dimensional one, and is mostly composed of structural proteins such as collagen, elastin, laminin and fibronectin, which are secreted by specialised cells in the niche, and proteoglycans, which include glycosaminoglycans (GAGs) [1, 13]. Its main function is to provide structural and biochemical support to the cells, acting as a substrate for cell migration, regulation of cell morphology, development and metabolic functions [13], but it is also important in integrin-mediated activation (as it is the connection between the niche and the internal cytoskeleton of the cells) and downstream signalling events [1, 18].

While during embryogenesis the niche where the ICM develops comprises the enveloping extraembryonic tissues, which provide the necessary interactions to direct stem cell differentiation [21], for *in vitro* expansion of hPSCs, the goal is to maintain the cells in an undifferentiated state. *In vitro* studies allowed to determine that ECM composition, and therefore its mechanical properties, can impact stem cell fate; one of the most studied phenomena is the direct lineage specification of ESCs (see section II.1.1.3.5), obtained through manipulation of ECM rigidity, which can be achieved by alteration of ECM composition, cross-linking and integrin-binding site orientation [20].

The structure and function of tissues and organs depends on the relative proportions of the constituent molecules; the tissues that need to endure larger tensional forces will be richer in fibrillary collagens and elastin, while tissues that are exposed to compressive forces will contain higher levels of proteoglycans [13]. *In vitro*, hPSCs can be cultured on Matrigel™ which is a complex mixture of growth factors and matrix molecules that includes Laminin and Collagen IV [10]. Laminin is a complex adhesion protein that is part of the basement membrane and is responsible for the mediation of cell adhesion, migration, growth and differentiation. Fibrillar collagens are the most abundant and widely spread ECM proteins in mammals and have important roles in the biomechanical stability of tissues, cell adhesion and migration during growth, differentiation, morphogenesis and wound healing [13]. As for elastin, it allows the tissues to return to their shape after stretching or contracting, particularly due to its hydrophobic character [13]. Fibronectin is a major ECM glycoprotein that plays an important part in cell adhesion and migration, and normal embryonic development of vascular structures; it binds to integrins, as well as to other ECM components including collagens, heparin, fibrin and proteoglycans [13]. Integrins are transmembrane receptors that act as the bridges for cell-cell and cell-to-ECM interactions; they are crucial for the transmission of many extracellular signals to the stem cells and when triggered they will in turn trigger chemical pathways to the interior of the cells because of their direct connection between the niche's ECM and the internal cytoskeleton of the stem and progenitor cells. When mechanical forces are applied to the ECM, the integrins are affected and can trigger the activation of various signalling pathways and influence stem cell fate [13].

#### II.1.1.3.3. Cell-cell interactions

Cell-cell interactions – either between stem cells or between stem cells and specialised cells in the niche – are mediated either through adherence or gap junctions. This direct physical interaction can regulate cell anchorage to the niche, stem cell fate modulation, and mobilization to and from the niche [1]. This contact is also important for cellular organisation and morphology and to determine cell density and colony morphology [22] through contact inhibition, which prevents further proliferation of the stem cells after a certain cell density has been achieved.

Cell adhesion molecule (CAM)-mediated cohesive interaction among hPSCs and between the cells and their neighbouring support cells and extracellular matrix are a major contribution to the self-renewal and the pluripotent state of hPSCs. CAMs are the proteins on the surface of mammalian cells that contribute to juxtacrine cell-cell binding or cell-to-ECM binding; they take part in functions such as mechanical support, target recognition, cell differentiation, initiation and regulation of signalling platforms, amongst others. Some of these proteins have already been identified as being present on the surface of hPSCs with a role in the regulation of their self-renewal and pluripotency [23]. The main classes of CAMs are the cadherins, integrins, selectins and immunoglobulin superfamily (IgSF), being E-cadherins very highly expressed in undifferentiated PSCs and the most important in this type of contact since they are coupled to the cells' actin-myosin cytoskeleton through a protein complex that includes p120-catenin, β-catenin, α-catenin, vinculin and non-muscle myosin IIA (NMMIIA). E-cadherin signalling may also affect the lineage commitments of hPSC [24].

#### II.1.1.3.4. Physicochemical environment

The physicochemical environment includes oxygen tension ($pO_2$) and pH values and is also important in the regulation of stem cell fate and viability.

It is difficult to measure the exact $pO_2$ for each tissue *in vivo*, however, it is easy to understand that different tissues are subjected to different oxygen levels. It has been suggested that low oxygen tensions (hypoxic environments) are beneficial when culturing hPSCs [1] since the development of the embryo *in vivo* occurs under hypoxic conditions; however, hPSC culture is usually performed under normoxia (≈21% $O_2$). Studies on the influence of oxygen tension for stem cell proliferation have shown that a lower (closer to physiological) oxygen tension (≈ 2-3% $O_2$) prevents spontaneous differentiation of the stem cells [25, 26].

There are other physical and chemical parameters that influence hPSC growth, such as temperature, humidity, osmolarity and acidity, cell density, gas diffusion exchange, and modes of multicellular association [11].

#### II.1.1.3.5. Biomechanical forces

Important biomechanical characteristics in the cell niche include stiffness, shear force and cyclic strain [1]. The stiffness of organs and tissues varies according to the differences in their ECM composition (as previously mentioned), in the density of cross-linking, and the degree of mineralization, which are static

biophysical properties. These mechanical cues are communicated to cells *via* their interaction with the ECM [24].

The stiffness of the substrate where hPSCs are grown affects cell attachment and proliferation, with cells achieving higher values for both parameters when they grow on more rigid substrates. There is evidence that stiff substrates may promote PSC differentiation; data indicates that hESC-derived EB cells seeded into scaffolds with low elastic modulus (<0.1 MPa) express higher levels of ectodermal markers, whereas cells grown on scaffolds with intermediate moduli (0.1 -1 MPa) express higher levels of endodermal markers. Therefore, intermediate and higher elastic modulus scaffolds promote mesodermal differentiation [24].

Shear stress plays a very important role in cell regulation, in particular those of the vascular and circulation system, in which cells are subjected to the flowing of blood. When in culture, hPSCs should be protected from higher shear stress rates at risk of down-regulation of pluripotency markers Oct4, Nanog and Sox2 (see section II.1.1.4.2); but if the goal is to differentiate the cells, a combination of shear stress and specific soluble factors can be used to direct differentiation of hPSCs that have already started to commit to a specific lineage [24].

#### *II.1.1.4. Pluripotency of stem cells*

Even though extracellular signals are crucial for the maintenance of the PSC phenotype [27], making the niche a very important part of the cell fate regulation, these are just the cues that will lead to alterations at the gene expression level; the regulatory machinery of hPSCs is a central piece of the pluripotency maintenance and self-renewal.

##### II.1.1.4.1. Signalling pathways

In mice, the maintenance of the pluripotency state has already been described in great detail [1]; in humans, although the knowledge about the signalling pathways and transcriptional circuits that regulate PSC self-renewal and differentiation has greatly improved over the past few years, there are still some mysteries regarding how the cell niche – the molecular and cellular interactions – influence stem cell fate [10]. Knowledge about these interactions could shed light on the intricacies of early human development, tissue regeneration and repair as well as allow researchers to improve the processes of culture, expansion and differentiation of PSCs *in vitro*.

Pluripotency is the result of the sum of all extracellular signalling pathways and intracellular regulations that control gene expression [10]; it is a state under the tight control of specific signalling pathways – namely TGF-β/Activin/Nodal, FGF and Wnt – which, in their turn, control a core transcriptional network that includes Oct4, Sox2 and Nanog [28] (**Figure II.3**).

The predominant signalling pathway that regulates pluripotency is the Transforming Growth Factor beta (TGF-β); the TGF-β superfamily is divided in two branches: the first one includes factors like activin, nodal, myostatin, lefty and TGF-β while the other one contains BMPs [29]. In hPSCs, TGF-β regulates pluripotency through the activation of Smad2/3/4 via ALK4/5/7 [28, 30]; when Smad2/3 is activated it is phosphorylated in the cytoplasm and then translocated into the nucleus of the cell where SMAD proteins

activate undifferentiation-associated genes such as Nanog, by acting as transcriptional factors. On the other hand, BMP ligands lead to SMAD1/4/8 phosphorylation through ALK2/3/6 binding, resulting in SMADs proteins being translocated into the nucleus in a process that will induce hPSC differentiation, which is why the use of Noggin and FGF as suppressors of BMP signalling allows for long-term self-renewal of hPSCs [29, 31]. The role of BMP4 on the regulation of hPSC pluripotency is still not completely understood; the existing information is very contradicting and points towards influence on differentiation to extra-embryonic lineages or mesoderm, that could be inhibited by adding Noggin to the culture conditions [19].

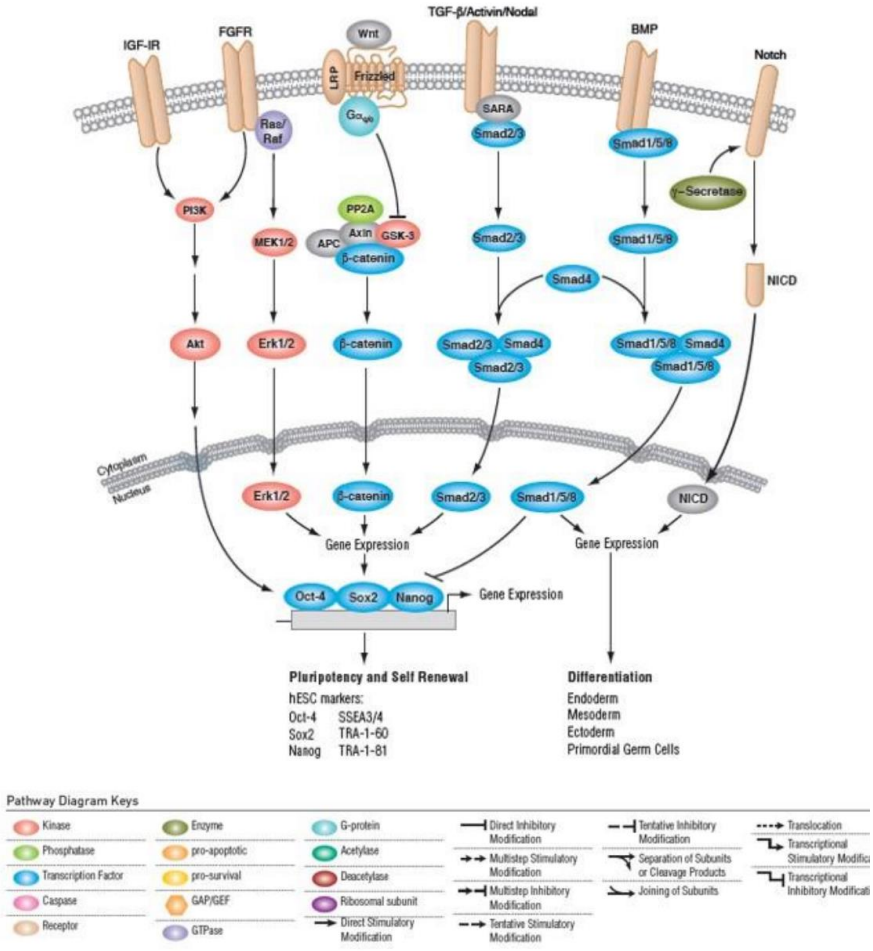



***Figure II.3 hESC Pluripotency and Differentiation Signalling Pathways.*** *From Cell Signalling Technology, Inc [30].*

Fibroblast Growth Factor Receptors (FGFR), which activate the Protein kinase B (AKT) and the Mitogen-activated protein kinase (MAPK) pathways, are also involved in pluripotency maintenance [30]. The signalling cascade activated through Phosphoinositide 3-kinase (PI3K) ultimately results in AKT activation and in its translocation to the plasma membrane where it promotes cell proliferation, survival, growth and motility [19, 32]. The mechanisms through which MAPK controls pluripotency are still not fully understood. When cultured, hPSCs require bFGF to maintain their undifferentiated state; the exogenously added bFGF binds to FGF receptors and activates ERK1/2 signalling, which is responsible

for pluripotency maintenance in these cells, while at the same time inhibiting the spontaneous differentiation to extra-embryonic lineage or neural-induction [19].

The Wingless-Type Mouse mammary tumor virus (MMTV) Integration Site Family Members (Wnt) influence pluripotency through a non-canonical mechanism involving a balance between the transcriptional activator, T-cell factor 1 (TCF1), and the repressor TCF3 [30]; this signalling promotes β-catenin stabilisation, accumulation and translocation into the nucleus through glycogen synthase kinase-3 beta (GSK-3β) inhibition. WNT ligands bind to the Frizzled receptor, activating Dishevelled (Dvl) which in turn prevents the association of GSK-3β to Axis inhibitor (AXIN) or Adenomatous Polyposis Coli (APC), ultimately preventing β-catenin degradation. Inside the nucleus, β-catenin associates itself with TCF and Lymphoid Enhancer Factor (LEF) proteins which will activate the expression of target genes [33, 34]. There is evidence showing that Wnt/β-catenin signalling is inactive in the self-renewal of hPSCs and that during that process, Oct4 (or POU5F1) reportedly represses β-catenin signalling. Higher expression of Wnt leans differentiation towards endodermal and cardiac fates whereas hPSCs expressing lower levels of Wnt generate cells from neuro-ectodermal lineage [19].

Because of their particular properties, hPSCs can be differentiated into derivatives of the three primary germ layers – endoderm, mesoderm and ectoderm; much like the maintenance of the pluripotent state, differentiation is achieved by influencing specific signalling pathways. One of the primary pathways responsible for the process of differentiation is the BMP pathway, which uses Smad1/5/9 to promote differentiation by inhibiting expression of Nanog while also activating the expression of differentiation-specific genes; Notch also influences the differentiation process through the Notch intracellular domain (NICD). As differentiation proceeds, cells leave the primary germ layer state and further differentiate along lineage-specific pathways [30]. Self-renewal of hPSCs requires inhibition of neuro-ectoderm [35] as well as inhibition of mesodermal and endodermal induction [10].

The balance between all these signals will determine the fate of the cells, which is why when a stem cell divides, three different scenarios can happen: the stem cell can give rise to two copies of itself, one copy of the cell and one cell that is committed to a specific lineage, or no copies of the original cell which means two new more differentiated cells; this results in maintenance, expansion or depletion of the stem cell pool, respectively [36].

#### II.1.1.4.2. Core pluripotency maintenance transcription factors

The sum of the signalling events that occur through these pathways supports the pluripotent state, which essentially depends on three key transcription factors: Oct-4, Sox2, and Nanog, which function through feedback regulatory circuit positively regulating their own genes [19] – these three are part of a group that has been known as Yamanaka Factors since their use for the reprogramming of cells to obtain iPSCs [37]. In their turn, these transcription factors activate ESC-specific genes, while regulating their own expression, supressing genes involved in differentiation, and can also be used as hPSC intracellular markers [30].

One of the core transcription factors for pluripotency maintenance is Oct4 (octamer-binding transcription factor 4) which has also been called POU5F1 (POU domain, class 5, transcription factor 1); an embryo

without this factor will survive the morula stage but will not give rise to the ICM *in vivo* or PSC colonies *in vitro*. This transcription factor binds to the octamer motif 5'-ATGCAAAT-3' of DNA to control the expression of genes involved in pluripotency maintenance and often collaborates with Sox2 in that role [38]. Oct4 expression level is particularly important: too much or too little expression causes PSCs to start differentiating.

Sox2, is part of the Sox family of transcription factors which have a highly conserved high-mobility-group (HMG) DNA-binding domain. It is expressed throughout the whole developing embryo; Sox2-null embryos do not survive after implantation. This transcription factor is essential for PSC self-renewal and pluripotency and its absence leads to differentiation. Together with Oct4, Sox2 regulates the transcription of genes such as Fgf4, Nanog, Lefty1, as well as Oct4 and Sox2 themselves [38].

Nanog is a homeodomain-containing protein that works together with Oct4 and Sox2 in establishing the PSC identity. In human cells, overexpression of Nanog allows feeder-free propagation for multiple passages. In the case of Nanog-null embryos, the initial development seems to give rise to pluripotent stem cells but these cells start differentiating straightaway, into the extra-embryonic endoderm lineage [38]. Nanog knockdown assays lead to a negative regulation on primitive endoderm inducer Gata6, both in mouse and human cells. Genome wide mapping of this transcription factor binding sites allowed the identification of pluripotency related genes such as Esrrb, Rif1, Foxd3 and REST. But the derivation of Nanog-/- ESCs and iPSCs from Nanog-/- somatic cells has shown that Nanog is not necessarily required for the maintenance or establishment of pluripotency [38, 39].

#### II.1.1.4.3. Human pluripotent stem cell surface markers

Cell surface glycolipid Stage Specific Embryonic Antigen (SSEA) 3/4 and glycoproteins Tumour Rejection Antigen (TRA)-1-60 and TRA 1-81 are surface markers for hPSCs [30] and can be used to identify and characterise cells in culture, and for purification of the desired cells after expansion.

The purification steps are rather important since, either after reprogramming of somatic cells to obtain hiPSCs or after hiPSC expansion *in vitro*, the population of cells obtained will consist only on the cells that were desired. This is particularly evident after the reprogramming process: due to its low efficiency rates the cells obtained are a mixture of somatic cells that were not reprogrammed, cells that were partially or incorrectly reprogrammed, and only a few cells are fully reprogrammed hiPSCs which need to be purified in order to be used [1].

## II.1.2. Clinical applications of human pluripotent stem cells

There have always been some concerns regarding the use of hESCs for clinical, pharmacological and developmental research purposes, due to the lack of knowledge on all the intricacies that regulate these cells, but also due to the origin of the cells which are isolated from human embryos. This is one of the main reasons why the successful reprogramming of somatic cells into iPSCs was a major breakthrough and has put hiPSCs at the forefront of potential regenerative medicine therapies; hiPSCs could also be used as an unlimited source of cell lines for pharmaceutical applications, in automated, high-throughput methods for synthesizing and screening libraries of biomaterials, and also as a way to monitor local

microenvironments of soluble factors, such as small molecules, siRNAs and other signalling molecules [1] (**Figure II.4**).

Regenerative medicine aims to repair injured or degenerated tissues by means of generating those tissues in the laboratory from hiPSCs and then transplanting them to the affected site within the patient. When an organ fails it can be replaced by a "new" one, from a donor but in order for transplantation to be possible there needs to be a well-matched donor available which often is not the case and many people die waiting for a compatible solution; hiPSCs offer the possibility to obtain cells from the patient that can be differentiated into the necessary cell type and used for an autologous transplant. There is, however, still the need for standardised protocols for these procedures [40].

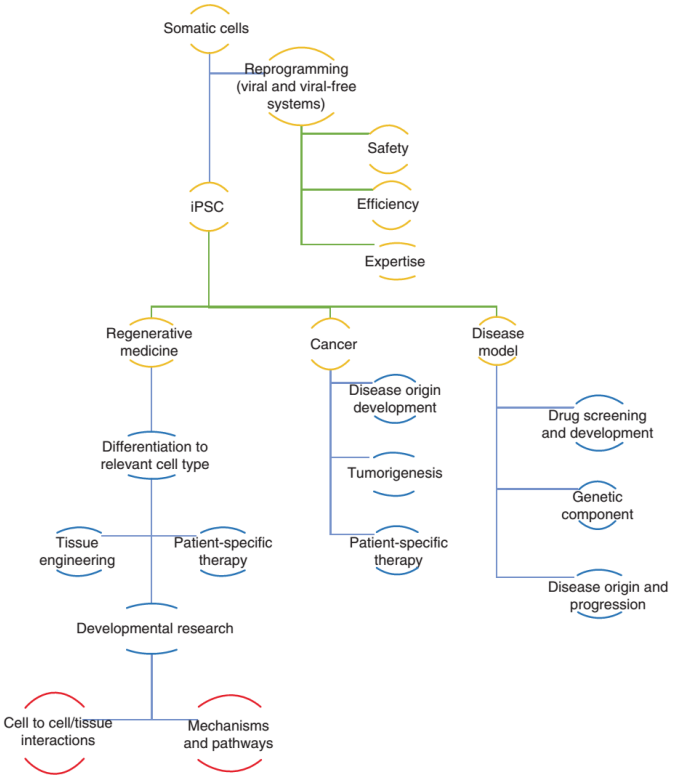



***Figure II.4 . From generation to possible applications of hiPSC.*** *From Rosa et al, 2014 [40].*

Still, the ability to treat different diseases always depends on the knowledge about the mechanisms that influence the disease progression; the possibility of modelling diseases could help on the development of appropriate treatments. Animal models are still widely used for these experiments because they are capable of mimicking human cellular microenvironment and metabolism to some extent; however, the evolution on the hiPSC field has allowed hiPSCs to start replacing those models for therapeutic purposes and disease modelling. This application takes advantage of the cells' abilities to self-renew and differentiate, which makes it possible to obtain different disease models directly from the patients that are affected, meaning that the cell lines established will have the perfect genetic mutations to be studied. The generation of patient specific iPSCs may also turn the idea of personalised medicine into a reality, making it possible to adapt each therapeutic course to each specific patient [41] (**Figure II.5**).

However, many cell types present limitations such as cell collection from the patient, viability, proliferation, variability, and problems related with the disease they carry. Also, human primary cell cultures usually achieve low numbers and tend to present high batch-to-batch variability, which makes them not suitable to study diseases in a controlled way. New data [42] indicates that the generation of hiPSCs in compliance with Good Manufacturing Practices (GMP) is already possible (although the processes currently employed still need to be approved by the regulatory authorities), through a robust and reproducible process that allows the generation of hiPSCs that completely fulfil PSC characteristics using the standard assays for this validation; furthermore, the cells obtained can be used for gene editing to repair genetic defects. This means that hiPSCs may soon be generally available for pre-clinical and clinical applications which represents a revolution on the study of pathogenesis and mechanisms underlying the phenotype expression, improving drug screening in human-specific normal and diseased cells and tissues [40, 42].

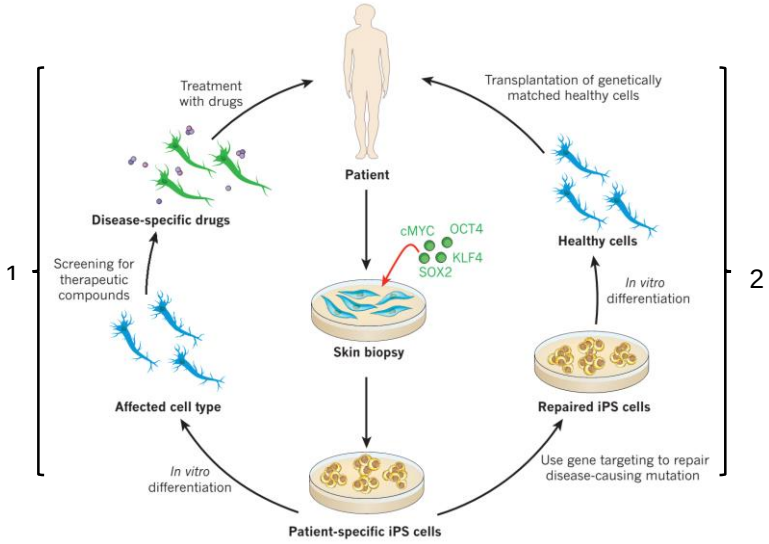



***Figure II.5. Medical applications of hiPS cells.*** *Reprogramming technology and iPS cells have the potential to be used to model and treat human disease. This image takes as an example a patient with a neurodegenerative disorder. Patient-specific hiPS cells — in this case derived by ectopic co-expression of transcription factors in cells isolated from a skin biopsy — can be used in one of two pathways. (Pathway 1 – on the left) Directed differentiation of the patient-specific iPSCs into the affected neuronal subtype will allow the patient's disease to be modelled in vitro, and potential drugs can be screened, aiding in the discovery of novel therapeutic compounds. (Pathway 2 – on the right) If the disease causing mutation is known, gene targeting could be used to repair the DNA sequence. The gene-corrected patient-specific iPSCs would then undergo directed differentiation into the affected neuronal subtype and be transplanted into the patient. Adapted from Robinton and Daley, 2012 [43].*

The development of new drugs includes a long process of *in vitro* and *in vivo* research before human trials can begin, and only after the full process the drugs may or may not be approved by the regulatory agencies. The overall process is rather expensive which is aggravated by the fact that many drugs are abandoned during trials due to shortage of efficacy or safety issues, and that for the development of a single drug, thousands are tested during the preliminary phases, amounting to great costs on animal models and cells lines. Many times, biocompatibility studies are performed on animal models but these are limited in terms of the human physiological conditions that they can mimic and many drugs can be toxic to animals but not to humans and vice-versa. These studies can also be performed on cell culture

systems but this alternative presents some drawbacks: immortalized cells can lose specific markers that may influence the cellular response to drugs, cells can present altered cytomorphology, and can contain metabolic abnormalities and chromosomal instability due to their derivation, which means that the models may become too divergent from the initial cells [40, 41]

By using healthy donor-derived hiPSCs it would be possible to evaluate toxic effects of drugs, materials and devices; and the use of patient-derived iPSCs would allow scientists to gather genetic information and phenotype features for each case, offering a more realistic scenario where it is possible to make the disease related phenotype regress to the non-disease state [40, 41]. Either way, animal model testing cannot yet be excluded altogether since it is part of the mandatory testing for drug approval.

## II.2. EXPANSION OF HUMAN INDUCED PLURIPOTENT STEM CELLS *IN VITRO*

Culture systems optimised for *in vitro* hESC culture promote expansion of the cells without loss of pluripotency characteristics [1]. hPSCs can be expanded under different types of culture systems – two-dimensional and three-dimensional – and according to different culture methods, ranging from adherent cultures, to cell aggregates, attached to microcarrier surfaces or encapsulated in different biomaterials.

The choice of a specific culture format for hPSC growth would depend on individual research aims, and pharmaceutical or clinical applications [11].

### II.2.1. hPSC passaging in culture

#### *II.2.1.1. Anoikis mechanisms*

hPSCs are anchorage dependant cells, which means these cells require cell-cell and cell-to-ECM interactions to survive; these interactions are mostly mediated by cadherins, integrins and cytoskeleton components such as actin [1, 18, 24].

Actin-myosin contraction is a downstream target of ROCK regulation in cloning and survival; there has been demonstrated a central role of actin-myosin contraction in the death of dissociated hESCs. Actin-myosin motors comprise actin filaments and non-muscle myosin II heavy chains (MYHs); the actin filaments are usually anchored on integrins and E-cadherins through focal-adhesion and a/b catenin complexes, respectively. The ability of MYH ATPase to hydrolyse ATP allows for the generation of energy and causes the MYHs to slide along actin filaments, resulting in their contraction; this contraction is triggered by the binding of myosin light chain (MLC), which is activated by phosphorylation through kinases – such as Rho-associated kinase (ROCK) or MLC kinase (MLCK) (**Figure II.6**). High levels of Rho- or ROCK-mediated actin contractility are incompatible with cell-cell junctions so, for that reason, and in order to maintain a normal cellular function and morphology, there must be a balance between actin-myosin contraction forces and the opposing anchoring forces. Cell death during passaging usually occurs because this balance is broken and there is excessive actin-myosin contraction. Cell survival increases with direct cell-cell contact, which depends on E-cadherin.

Cell-to-ECM interactions provide the necessary anchoring forces needed to balance the actin-myosin contracting forces; whenever cells are separated from the ECM actin-myosin is free to contract,

generating altered phenotypes, and ultimately resulting in cell death. Studies have shown that the disruption of actin-myosin contraction in isolated hPSCs greatly improves the survival of the cells [44], which can be achieved by using ROCK inhibitors – such as Y-27632; but these should only be used during the first 24h of culture after passaging, since its presence prevents the formation of colonies.

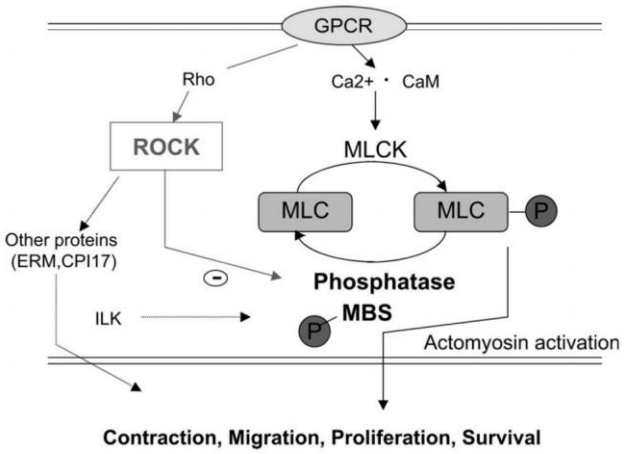



***Figure II.6. Regulation of cellular function by ROCK.*** *Stimulation of G-protein-coupled receptors (GPCR) leads to an increase in intracellular calcium/calmodulin (CaM)-mediated activation of myosin light chain kinase (MLCK). MLCK phosphorylates MLC, leading to actin-myosin interaction and cellular contraction, migration, proliferation, and survival. Stimulation of GPCR also leads to ROCK activation via Rho guanine exchange factor (GEF). Activated ROCK, mediated through, phosphorylates various downstream targets, such as ezrin-radixin-moesin (ERM), a 17-kDa PKC-potentiated inhibitory protein of protein phosphatase-1 (CPI17), and the myosinbinding subunit (MBS) of MLC phosphatase. Phosphorylation of MBS inhibits MLC phosphatase activity leading to increase MLC phosphorylation and actomyosin activation. ILK, integrin-linked kinase. From Liao et al, 2007 [45].*

#### *II.2.1.2. EDTA cell passaging*

As previously mentioned, cell death after individualisation is mostly related to myosin-actin dependent contraction, and cell-cell interactions through direct contact promote cell survival through its inhibition. In order to avoid hiPSC death due to the individualisation of the cells, it is desirable to maintain cell-cell contact during passaging by allowing cells to keep within small aggregates; by using EDTA treatment (an enzyme-free method) to perform the cell passaging, cells are able to dissociate and survive without the need for a ROCK inhibitor in the culture medium (**Figure II.7**).

EDTA is a chelating agent that sequesters a variety of divalent cations [46] – such as $Mg^{2+}$ and $Ca^{2+}$ – which are essential in establishing cell-cell and cell-to-ECM bounds through cadherins and integrins. The cell aggregates provide enough interactions for cell survival while being small enough for growth factors to reach all the cells. Due to the efficiency of this method, EDTA passaging can be used in long-term culture expansion of hPSCs with different feeder-free media, as a cost and time effective dissociation protocol [47].

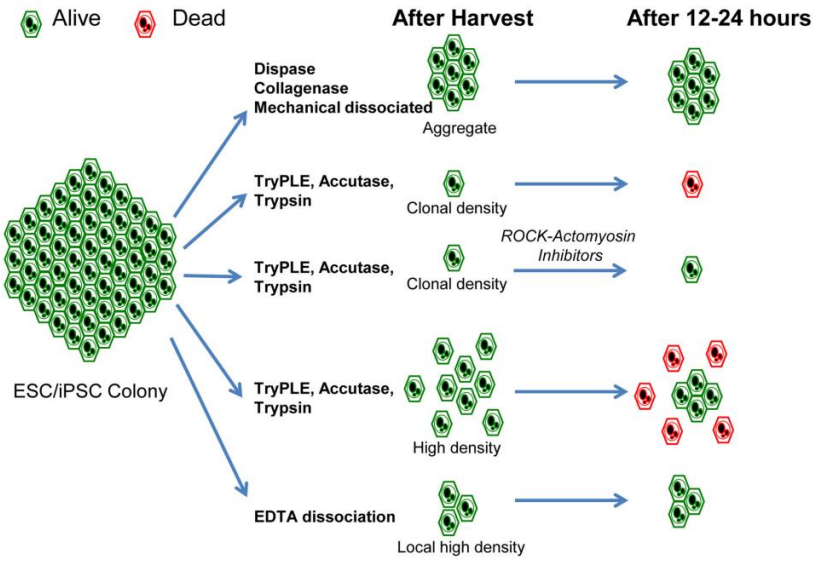



***Figure II.7. EDTA dissociation is an effective method for pluripotent stem cell passaging.*** *Summary of cell survival efficiency by different methods. After dissociation, enzyme/ mechanic-generated large aggregates survive efficiently; individualized cells die at clonal density, but survive in the presence of inhibitors or high loading density; EDTA-generated small aggregates survive efficiently by high local density without the help of drug treatment. From Beers et al, 2012 [47].*

### II.2.2. Two dimensional culture systems

Traditionally, and because hPSCs are adherent cells, these have been expanded on feeder layers, as two-dimensional adherent colonies, using culture medium formulations that contains animal-derived serum or human or animal serum albumin [27, 48, 49].

Mouse embryonic fibroblasts were the first type of feeder layer used in this type of culture and these cells supported hPSC expansion by secretion of growth factors, cytokines and ECM molecules such as TGFβ, Activin A, laminin-511 and vitronectin [50]. However, even though these cells were irradiated to inhibit their proliferation, the use of mouse feeder layers presented serious risks of contamination with mouse viruses and a limited lifespan in culture up to nine passages before cells entered a state of senescence [51]. In order to obtain cells that could be used in clinical applications it was imperative to culture them under conditions that met the requirements of clinical or pre-clinical utilization – this meant that no animal products could be included on both substrate and media formulations, in order to prevent not only batch to batch variability but also the risk of cross-contamination with xenogeneic pathogens [48]. This led to the use of human feeder layers for hPSC expansion which varied from adult marrow cells, to new-born foreskin fibroblasts, foetal tissues and adult fallopian tubal fibroblasts; these allowed to extend the lifespan of the feeder layers to 42 passages and eliminated the risky exposure to other animal cells while retaining the problem of the batch to batch variability because of the inability to standardise the source of feeders as well as the complexity of a co-culture system [51].

With the intention of overcoming these risks, feeder-free cultures were developed which meant that all necessary soluble factors had to be added to the culture medium and the ECM molecules had to be provided through surface coatings in order to guarantee the expansion of viable hPSCs, which were still

able to self-renew and maintain their pluripotency potential. Biological substrates were developed, which could be used as coating matrices applied to culture plates, such as Matrigel™ (originally from BD Biosciences®) and Geltrex® (ThermoFisher Scientific); these consist mainly of laminin, collagen IV, heparin sulphate proteoglycans, entactin and growth factors, obtained from Engelbreth-Holm-Swarm mouse sarcomas [52] and are still widely used for hPSC expansion. Still, because of their xenogeneic origin, these presented significant batch-to-batch variability, as well as the risk of cross-contamination, which rendered them as not the most appropriate for hPSC expansion for clinical applications. The use of recombinant human (rh) proteins – such as rh laminin-511 and rh vitronectin and rh E-cadherin – is regarded as the leading example of defined and xeno-free surface that allow an efficient and GMP-compliant expansion of hPSC [53–55], but biological substrates pose problems regarding their difficult isolation and the high costs of their manufacture which means that they are not the most suitable for large scale expansion of hPSCs.

More recently, commercial synthetic peptide-acrylate surfaces – such as Synthemax® (Corning®) a synthetic, xeno-free substrate composed of a biologically active peptide derived from vitronectin [56] – have been established to fully support hPSC expansion *in vitro*, under fully defined conditions; these are a conjugation of acrylate and biologically active peptides, such as vitronectin, fibronectin and laminin [57]. These commercially available products can be established using complete proteins, small peptides or a combination of both, polymers or polymers conjugated with biomolecules. Because these synthetic surfaces are obtained from defined processes and sources, there are no risks of xenogeneic contamination, and there is little batch-to-batch variation which translates to an easier scaling-up of the expansion process [58].

Regarding culture media, one of the first alternatives was the use of feeder conditioned medium – this meant that the feeder-layer cells were cultured in standard culture medium and that medium was reused to culture hPSCs, because it already contained the soluble factors needed [59]. This option, however, still maintained the problem of the variability and the lack of knowledge on the culture medium composition. In more recent years, the development of chemically defined culture medium formulations was achieved through the increased knowledge of the stem cells' niche and some of the signalling events that occur *in vivo*, allowing for the replacement of xenogeneic elements in the traditional formulations [11, 60]. Culture medium composition was ultimately optimised by James Thomson [61] with the creation of the Essential 8™ culture medium which is xeno-free, chemically defined and has the simplest formulation amongst the currently used culture media for hPSC culture.

### II.2.3. Three-dimensional culture systems

The major limitation of the two-dimensional culture systems traditionally used is the surface area available for cells to adhere and proliferate. In order for hiPSCs to be used in all of their potential, in regenerative medicine and biotechnology research applications, is it imperative that they are produced in clinically relevant numbers, which ultimately means that the expansion approaches needed to be optimised. hPSC culture had to be transitioned from small and static culture systems – such as multi-well plates – to larger scale dynamic culture systems.

Due to their *in vivo* microenvironment, hPSCs naturally form aggregates of different sizes when cultured in suspension and can be expanded using this natural conformation; but this can leave cells more susceptible to cellular damage and spontaneous differentiation, which can be a response to hydrodynamic shear stress in the culture. Aggregates, if allowed to grow too large, also pose a risk of apoptosis and spontaneous differentiation and even necrosis of the cells in the inner most part of the aggregate [11].

Encapsulation of cells within biomaterials is an alternative that allows the customisation of the scaffold environment, the use of different biomaterials – such as alginate, poly lactic-co-glycolic acid, poly L-lactic acid, hyaluronic acid – making it possible to control the cells microenvironment and making it ideal for stem cell self-renewal or for directing their differentiation; encapsulation also offers protection of the cells from shear stress during the culture period [11].

The focus of this project lies with a third option: hiPSC expansion using microcarriers as a support for the growth of adherent cells.

#### *II.2.3.1. Microcarrier culture systems*

Within 3D culture system options, hPSC culture on microcarriers may be the solution for a scalable way to control cell aggregation in suspension [11]. Microcarriers act as the surface for the growth of cells that are dependent of adhesion, enhancing the available surface area per volume for cell growth, allowing for a reduction in the consumption of culture medium and growth factors [1].

The development of microcarriers as a support for anchorage-dependent cell culture began in 1967, with the studies of van Wezel using Dextran particles [62]. Since then, many different types of microcarriers were developed, varying from porous and non-porous, with or without functional coating, made from plastics (polystyrene, polyethylene, polyester and polypropylene), glass, acrylamide, silica, cellulose, dextran, collagen (gelatine) and GAGs.

The existing data suggests that most of the commercially available microcarriers, used in mammalian cell culture, are not suitable for long term expansion of undifferentiated hPSCs, without any surface modification [63, 64], which once again demonstrates the relevance of using appropriate biological or synthetic substrates specifically developed for hiPSCs. In recent years, hPSC expansion on microcarriers has made great improvements and there are currently many reports on hPSC expansion yields using different types of microcarriers with diverse coatings, and the cell numbers obtained seems to be ever increasing (**Table II.1**), which shows that optimisation of the culture system is on the right track.

Microcarrier culture optimisation has to take into consideration parameters such as surface charge, microcarrier diameter, microcarrier density, and seeding density of the cultured cells [65], in order to promote efficient cell adhesion and a robust cell proliferation without differentiation, but also allow efficient and technically simple cell harvesting [66].

Nevertheless, cell expansion on microcarriers still poses some disadvantages. Because hPSCs expand as multilayers on the microcarrier surface, large microcarrier aggregates are formed [66], making the

process of cell harvesting – which has yet to be fully characterised and monitored – rather difficult and not as efficient as one would prefer. A variable amount of cells is left still attached to the surface of the microcarriers and some cells are lost during the filtration step used to separate the detached cells from the microcarriers; the use of proteases to detach the cells from the surface of the microcarriers often leads to cleavage of cell surface proteins, which can in turn lead to dysregulation of the cell functions [67] and also affect the efficiency of cell sorting techniques that are based on the identification of surface proteins. Optimisation of the cell recovery step could improve the cell recovery yields. Furthermore, due to the formation of these microcarrier agglomerates, quantification of cells attached to the microcarriers becomes very challenging.

***Table II.1 Propagation of hPSC in microcarrier cultures.*** *Chronological maximum hPSC expansion achieved using commercially available microcarriers, using different coatings, culture media, and under different types of culture – static culture (SC), spinner-flask (SF), and stirred tank bioreactor (STB); these values were obtained using different hPSC lines. CM – conditioned medium; E8 – Essential 8™. Adapted from Badenes et al. (2016) [66].*

| Microcarriers/Coating | Culture Media | Type of reactor | Maximum hPSC expansion | Reference |
|---|---|---|---|---|
| HILLEX® II | CM | SC | $0.7x10^5$ cells/mL (3 fold) | Phillips et al. (2008) [68] |
| Cytodex 3/Feeder cells or Matrigel™ | CM | SC | $2.7/1.7x10^6$ cells/mL (5/3 fold) | Nie et al. (2009) [69] |
| DE-53/Matrigel™ | CM | SF | $3.5x10^6$ cells/mL (6 fold) | Oh et al. (2009) [70] |
| Cytodex® 3 | CM | SF | $1.5x10^6$ cells/mL (6 fold) | Fernandes et al. (2009) [71] |
| Collagen-coated HyQSphere/Matrigel™ | CM | SF | $2.0x10^6$ cells/mL (10 fold) | Lock and Tzanakakis (2009) [72] |
| Cytodex® 3 / Matrigel™ | CM | STB | $2.3x10^6$ cells/mL (5 fold) | Serra et al. (2010) [73] |
| Collagen-coated HyQSphere /Matrigel™ | CM | SF | $0.4x10^6$ hiPSCs/mL (7 fold) | Kehoe et al. (2010) [6] |
| DE-53 and Cytodex® 1/Matrigel™ or laminin | CM | SF | $3.5/2.0x10^6$ cells/mL (9/5 fold) | Chen et al. (2011) [64] |
| Polystyrene microcarriers/Laminin or vitronectin | StemPro® | SC | $1.5/1.4x10^6$ cells/mL (8 fold) | Heng et al. (2012) [74] |
| DE-53/Matrigel™ | mTeSR™1 | SF | $3.0x10^6$ cells/mL (10 fold) | Bardy et al. (2013) [75] |
| Cytodex® 1/Matrigel™ | mTeSR™1 | SF | $2.9x10^6$ cells/mL (7 fold) | Ting et al. (2014) [76] |
| Polystyrene microcarriers/PLL+murine laminin | mTeSR™1 | SF | $3.0x10^6$ cells/mL (15 fold) | Lam et al. (2014) [77] |
| Polystyrene microcarriers/PLL+vitronectin | TeSR™2 | SF | $1.6x10^6$ cells/mL (19 fold) | Fan et al. (2014) [78] |
| Synthemax®II microcarriers | mTeSR™1 | SF | Not available | Badenes et al. (2015) [79] |
| Synthemax®II microcarriers | mTeSR™1 | SF | $2.1x10^6$ cells/mL (7 fold) | Silva et al. (2015) [80] |
| Polystyrene microcarriers/Human vitronectin | E8™ | SF | $1.4x10^6$ cells/mL (3.5 fold) | Badenes et al. (2016) [81] |

#### II.2.3.1.1. Dissolvable microcarriers

After conducting the cell harvesting procedures on microcarriers, a variable number of viable cells is usually left behind, not having been properly separated from the microcarrier surface; in order to prevent the loss of these cells, and in turn increase the yield of the cell expansion, it is possible to use microcarriers based on a completely digestible matrix, which eliminates the need to perform separation protocols by dissolving the microcarriers altogether [82, 83]. This type of microcarriers have been

described for many decades now but were never fully developed and although they had been tested with animal cells [82], hiPSC expansion had not been tried before.

Following the principle of dextran beads used for chondrocyte culture, which were digested at the end of culture using dextranase to separate the cells from the microcarriers [84], and starch coated microcarriers that can be digested by amylase [85], Corning Inc.'s dissolvable microcarriers are made up of a ionically cross-linked polysaccharide – pectic acid or polygalacturonic acid (PGA), which is a gelatinous water soluble acid – and can be fully digested using a harvesting pectinase/EDTA solution. These microcarriers can be functionalised with cell adhesion promoting peptides, preferably by coating with a synthetic polymer bearing adhesion peptides, which enable bio-specific adhesion of the cultured cells [83].

Cell recovery from these microcarriers can be achieved without using a protease, but rather pectinase and a chelating agent – in this case, EDTA; the pectinase will dissolve the PGA microcarrier and EDTA will help dissociate cells, resulting in a single cell suspension which can then be further processed (**Figure II.8**). Nevertheless, this process has yet to be optimised; since the cells covering the surface of the microcarrier do not allow for the harvesting solution to reach the microcarrier and thus dissolve it, the use of a protease is still necessary. Cell recovery without complete digestion of the microcarriers has also been tested; in this case cells must be separated from the microcarriers using a physical process such as filtration, decantation, centrifugation, just as one would do with, for example, polystyrene microcarriers [83].

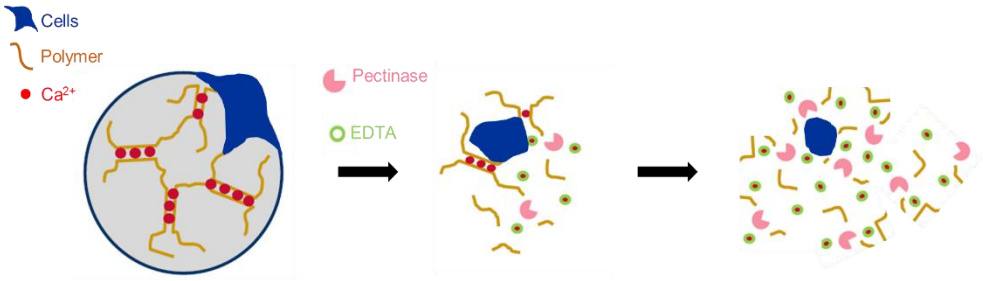



***Figure II.8. Digestion of dissolvable microcarriers (Corning®).*** *The new ionically cross-linked polysaccharide microcarriers can be digested after hPSC expansion using a harvesting solution made up of pectinase and EDTA, which fully dissociates the matrix that comprises the microcarriers, leaving cells in suspension. From Corning Inc.*

### II.2.4. Scale up of stem cell expansion

The culture of hESCs in suspension bioreactors was first reported in 2004, by Gerecht-Nir et al [80]. The choice of bioreactor configuration is mainly dependent on the cultivation method chosen – surface adherent cultures (as monolayers or on scaffolds), aggregates, microcarriers, cell encapsulation – or the one that best suits the final requirements. Currently, there are four main bioreactor configurations that are used for hPSC culture: roller bottles, rotating wall vessels, wave bioreactors and stirred bioreactors.

Roller bottles were first described by George Gey in 1993 [87] and are one of the simplest solutions for adherent cell culture under dynamic conditions consisting only of cylindrical vessels that slowly turn on

their own axis, alternately exposing the cells – which are attached to the inner surface of the vessel – to culture medium and gas phase, and thus allowing for a more efficient oxygenation. However, these are not easily monitored and controlled and can only be scale out and not scaled up, which leads to batch-to-batch variability.

Other example of a bioreactor conformation is the rotating wall vessels, which were originally developed by NASA; cells are grown under a microgravity environment inside culture medium filled cylindrical chambers that contain scaffolds were the cells attach and expand. The gravitational force ensures that the scaffolds remain in suspension and gas transfer is rather efficient but this system is not appropriate for scaling up and therefore not suitable to culture cells for clinical applications [88].

Wave bioreactors are an alternative that is particularly suitable for good manufacturing practices (GMP) operation, mainly because it is composed by a disposable bag that contains the culture medium where cells are inoculated and the remainder of the bag is filled with air, to ensure gas transfer to the cells; during cell culture the bag is placed on a platform that agitates its contents, creating waves that ensure efficient mixing of the culture medium and gas transfer. Since the bags are disposable they can be previously sterilised and applied to single use operations, reducing the risks of contamination. Other advantages include the possibility for monitoring and control of the process through the use of probes and the possibility for scaling up, even though it is a costly procedure [89].

Stirred vessels include spinner flasks and stirred-suspension bioreactors (SSBs) and are the most widely used and easy to operate amongst these options while also being very promising in terms of scalability [6].

#### *II.2.4.1. Spinner flasks*

Spinner-flasks are laboratory scale stirred culture systems, which were specifically designed for suspension cell culture – where they can be regarded as very versatile culture platforms since hPSCs can be cultured as aggregates, adherent to scaffolds or microcarriers –, allowing for superior gas exchange, homogeneous concentration of nutrients in the flask, and the culture of higher volumes of cells and are commonly used to perform the first steps of the scaling-up process before stepping on to large volume cultures in SSBs [6]. In these, culture medium is usually agitated by using a horizontal impeller with a magnetic stirrer, and a magnetic stir plate underneath; there are many types of paddles commercially available that have to be chosen to best suit the culture conditions and the cell type.

Spinner-flasks have been widely used for hPSC expansion at laboratory scale for research purposes, but still offer some disadvantages: first, cell culture must be performed inside a $CO_2$ incubator because spinner-flasks are not prepared for real-time monitoring and control of culture parameters such as pH level and $pO_2$ [66]; other important limitation is directly linked to the agitation of the culture medium which may cause high levels of shear stress and significantly affect cell growth – there is also information showing that the effect of agitation on cell differentiation is dependent on the cell line used [64] –, meaning that the selection of optimal stirring speed and paddle conformation is a critical step [6].

# III. MATERIALS AND METHODS

## III.1. CELLS AND MEDIUM

### III.1.1. Cell line

The human induced pluripotent stem cell line F002.1.13, derived from a healthy donor (46, XX) at the company TCLab, was used as the model system throughout this work. This cell line was reprogrammed from fibroblasts through retroviral transduction of the human genes OCT4, SOX2, C-MYC and KLF4 [3].

### III.1.2. Culture media

#### *III.1.2.1. Culture media for hiPSC expansion*

III.1.2.1.1. Essential 8™ culture medium

Essential 8™ (E8™) culture medium (Life Technologies™) is a xeno-free, feeder-free, commercially available culture medium formulated to contain only the eight absolutely essential components for hPSC expansion [12]. E8™ culture medium is prepared (according to manufacturer's instructions) by thawing the Essential 8™ supplement at room temperature, adding it to the Essential 8™ basal medium and homogenising the solution obtained.

III.1.2.1.2. mTeSR™1 culture medium

mTeSR™1 (StemCell Technologies™) is a fully defined, serum-free, commercially available medium used for hiPSC and hESC expansion in vitro. The composition of mTeSR™1 was formulated to maintain stem cells grown in the medium in an undifferentiated state through multiple culture passages [13]. mTeSR™1 is prepared (according to manufacturer's instructions) by thawing the mTeSR™1 5X supplement overnight at 4$^{o}$C, adding it to the mTeSR™1 basal medium and homogenising the solution obtained; from the total volume, 40 mL aliquots are prepared, kept at -20$^{o}$C and thawed at room temperature when needed.

III.1.2.1.3. Adaptation of hiPSCs from Essential 8™ culture medium to mTeSR™1

Cells cultured in E8™ culture medium can be adapted to growth in mTeSR™1. On the day previous to a cell passaging, E8™ culture medium is replaced with mTeSR™1; the following day, a normal cell passage is performed, using mTeSR™1 instead of E8™ culture medium. Cells should be passaged twice before being used to perform any experiments.

#### *III.1.2.2. Culture media for hiPSC differentiation*

III.1.2.2.1. Embryoid body differentiation medium

Embryoid Body differentiation medium is used for *in vitro* differentiation of hiPSCs, according to the procedure described in III.3.3; the medium is prepared in the laboratory using Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with Foetal Bovine Serum (FBS, Sigma-Aldrich),

Minimum essential medium (MEM)-non essential amino acids (aa, Gibco), Sodium pyruvate (Gibco), β-Mercaptoethanol (Sigma-Aldrich), and Penicillin-Streptomycin (PenStrep, Gibco), according to the quantitative composition described below (**Table III.1**):

***Table III.1. Embryoid body differentiation medium composition.*** *List of all the components used in the preparation of Embryoid body differentiation medium, their concentrations and volumes needed to prepare a total volume of 250 mL.*

| Component | Concentration | Volume in mL (for a total ≈250mL) |
|---|---|---|
| **DMEM** | 76.9% (v/v) | 192.25 |
| **FBS** | 20% (v/v) | 50 |
| **MEM-non essential aa** | 1% (v/v) | 2.5 |
| **Sodium pyruvate** | 1 mM | 2.5 |
| **B-Mercaptoethanol** | 0.1 mM | 0.250 |
| **Pen Strep** | 1% (v/v) | 2.5 |

#### *III.1.2.3. Culture media for analysis*

III.1.2.3.1. Washing medium

Washing medium is used when processing cells in suspension; it is prepared in the laboratory using as base Knock-out DMEM (KO-DMEM, Gibco) culture medium, supplemented with Knockout-Serum Replacement (KO-SR, Gibco), MEM-non essential aa, L-glutamine (Gibco) and PenStrep, according to the quantitative composition described below (**Table III.2**):

***Table III.2. Washing medium composition.*** *List of all the components used in the preparation of washing medium, their concentrations and volumes needed to prepare a total volume of 250 mL.*

| Component | Concentration | Volume in mL (for a total ≈250mL) |
|---|---|---|
| **KO-DMEM** | 87.5% (v/v) | 218.5 |
| **KO-SR** | 10% (v/v) | 25 |
| **MEM-non essential aa** | 1% (v/v) | 2.5 |
| **L-glutamine** | 10mM | 1.25 |
| **P/S** | 1% (v/v) | 2.5 |

### III.1.3. Cell storage

#### *III.1.3.1. hiPSCs cryopreservation*

Cells are cryopreserved within cryogenic vials (Cryovials, Thermo Scientific™) with at least 1 million cells per vial. Firstly, all culture medium is removed from the wells where cells are cultured; each well is washed two times with 1 mL of EDTA and then incubated with 1.5 mL of EDTA for 5 minutes. Cells are recovered with washing medium using a micropipette and added to a 15 mL-tube where they are centrifuged for 3 min at 1000 rpm. After centrifugation the supernatant is discarded and cells are

resuspended using KO-SR containing 10% dimethylsulfoxide (DMSO; Gibco), in a total volume so that each cryogenic vial contains 250 µL of cell suspension. Cryogenic vials are stored overnight at -80ºC and later moved to liquid nitrogen storage.

#### *III.1.3.2. Thawing of hiPSCs*

hiPSCs are kept in cryogenic vials stored in liquid nitrogen. The desired vial is removed from storage and immersed in a bath at 37ºC for a swift thawing process. In a sterile environment, 1 mL of washing medium is used to thaw the remaining cell suspension and to homogenise the content until completely thawed; the total suspension is transferred to a 15 mL-tube with 4 mL of washing medium, homogenised and centrifuged at 1000 rpm for 3 min. After centrifugation the supernatant is discarded and the pellet is resuspended in culture medium to a total of 1 mL of cell suspension and homogenised. The cell suspension is plated in one culture well previously coated with Matrigel™ and with 0.5 mL of culture medium and kept at 37ºC, 5% $CO_2$ and 20% $O_2$ inside a $CO_2$ incubator (Memmert).

## III.2. *EX-VIVO* EXPANSION OF HIPSCS

### III.2.1. Static culture systems

#### *III.2.1.1. Monolayer culture systems*

III.2.1.1.1. Substrate: Matrigel™ Coating

In order for cells to grow on multi-well tissue culture plates (Falcon®), these must be coated with the appropriate extracellular matrix components. Matrigel™ (Corning®) is a complex mixture of growth factors and matrix molecules that includes Laminin and Collagen IV [6], and is extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma.

Matrigel™ is stored in aliquots at -20ºC, with one 60 µL aliquot corresponding to the necessary quantity for a 6-well plate. To prepare multi-well plates for the expansion of hiPSCs, the necessary Matrigel™ is thawed in ice and diluted 10x with the appropriate volume of DMEM-12 medium (Gibco), the mixture is distributed to each well (1mL) and left for 1.5 h at room temperature, or stored at -4ºC for up to two weeks. Plates should be brought up to room temperature, and the medium used for the coating must be removed before plating any cells.

III.2.1.1.2. Culture medium change

For hiPSCs, culture medium change must be performed every day.

For cells cultured in coated 6-well plates all the medium is aspirated from each well and 1.5 mL of fresh culture medium is added to each well; plates are then gently shaken to assure homogenous culture medium distribution.

III.2.1.1.3. Cell passaging using EDTA dissociation solution

All culture medium is removed from the wells containing the cell culture. All wells are washed two times with 1 mL sterile EDTA 0.05 mM solution and then incubated with 1.5 mL of the same EDTA solution for 5 min, which is completely removed afterwards. In each well, 1 mL of culture medium is used to detach and recover the cells by using a 1000 µL pipette to a maximum of 5 flushes per well and the cell suspension is then transferred to a 15 mL-tube. The total cell suspension is recovered, homogenised and plated onto a new multi-well plate, previously coated with Matrigel™ and homogenised on the plate to assure an even distribution of the cells (which should be in small aggregates and never single cells). The plates are incubated at 37ºC with 5% $CO_2$ in a humidified atmosphere.

EDTA dissociation solution

The ethylenediaminetetraacetic acid (EDTA) solution used for cell dissociation is prepared by adding 0.9 g of Sodium Chloride (NaCl, Sigma-Aldrich) and 500 µL of EDTA 0.5 mM (Invitrogen™) to 500 mL of phosphate buffered saline (PBS, Gibco) and filtering the solution through a sterile filter into a sterile glass bottle for storage.

***III.2.1.2. Microcarrier culture systems***

III.2.1.2.1. Microcarrier preparation

III.2.1.2.1.1. Sterilisation of polystyrene microcarriers

Polystyrene microcarriers (PSM, Solo Hill®) and high concentration Synthemax®II microcarriers (polystyrene-based microcarriers from Corning®; these microcarriers are gamma irradiated but sterilisation of the product had been compromised) are sterilised using the same procedure: microcarriers are weighted into a 15mL-tube and incubated in ethanol 70% for 1 h under agitation. After incubation the ethanol is removed and the microcarriers are washed three times with sterile PBS, agitated and left to settle completely before removing the PBS solution, and are stored at room temperature.

These microcarriers have a surface area of 360 $cm^2$ per gram.

III.2.1.2.1.2. Hydration of dissolvable microcarriers

Dissolvable microcarriers were prepared according to manufacturer's instructions: according to the necessary surface area, in a laminar hood, the appropriate amount of microcarriers should be weighted in a sterile glass bottle. Microcarriers are hydrated by adding 150 mL of sterile water (Corning®) per gram of microcarriers, swirling the suspension to ensure homogeneous hydration, and left to hydrate for 1 h, after which a 500 µL sample should be collected to assess if the hydration has been completed, if not, microcarriers should be left to hydrate for an additional 30 min. Before use, microcarriers should be allowed to settle in order to remove the water used for the hydration and replace it with culture medium.

These microcarriers have a surface area of 5000 $cm^2$ per gram and all manipulations should be performed using glass vials.

III.2.1.2.2. Coating with Matrigel™

Matrigel™ coating is performed by thawing 200 µL of Matrigel™ and adding it to 6 mL of medium; microcarriers are then incubated in the Matrigel solution for 1.5 h at room temperature, under agitation.

III.2.1.2.3. hiPSC seeding on microcarriers

III.2.1.2.3.1. Cells recovered using EDTA

Cells are recovered using EDTA, as if for a normal passaging procedure, using culture medium supplemented with PenStrep (0.5%) and 10 µM Rho associated protein kinase inhibitor (ROCKi) Y-27632 (STEMCELL Technologies) – this inhibitor is used to increase cell survival during passaging procedures and allows cells to maintain their viability as single cells [90] – 10 µL of the cell suspension obtained are added to 40 µL of Trypan Blue (Gibco), in a 1:5 dilution, and 10 µL of that solution are used in a Neubauer chamber to count the cells under the microscope. The necessary amount of cell suspension is used for inoculation, according to the desired cell density.

III.2.1.2.3.2. Single cell inoculation

Culture medium is replaced with washing medium supplemented with ROCKi, and cells are incubated for 1 h at 37ºC. After incubation the medium is removed and each well is washed once with PBS before adding 1 mL Accutase® (Sigma Aldrich) and incubated for 5 min. Cells are then flushed, using a micropipette, in order to loosen them and allow their recovery; washing medium supplemented with ROCKi is added to each well to stop Accutase reaction and the total volume is transferred into 15mL-vials. Washing medium supplemented with ROCKi is used to wash the wells after recovery of the cells and transferred to the same vial, to be centrifuged for 3 min at 1000 rpm. After centrifugation, supernatant is discarded and cells are resuspended in culture medium supplemented with PenStrep and ROCKi. 10 µL of the cell suspension obtained are added to 40 µL of Trypan Blue, in a 1:5 dilution, and 10 µL of that solution are used in a Neubauer chamber to count the cells under the microscope. The necessary amount of cell suspension is used for inoculation, according to the desired cell density.

III.2.1.2.4. Culture medium change

For cell culture on microcarriers, ultra-low adhesion multi-well plates (Corning®) are used. The plates are tilted in order to allow for the microcarriers to settle to the bottom of the wells and 80% of the culture medium volume is removed from each well and replaced with the same volume of fresh culture medium; plates are then shaken to assure homogenous culture medium distribution.

III.2.1.2.5. Cell recovery

III.2.1.2.5.1. Harvesting from polystyrene microcarriers

Culture medium is removed from each well which is then washed with 500 µL of PBS. 300 µL of Accutase are added and the plate is incubated for 5 min at 37 ºC. Each well is flushed using a micropipette in order to detach cells from each other and from the microcarriers and 500 µL of washing medium are added to each well to stop Accutase reaction. All the contents of the wells are recovered

and the suspension containing polystyrene microcarriers is sifted through a strainer with a 100 µm mesh, into a 50mL-tube.

III.2.1.2.5.2. Recovery from dissolvable microcarriers

This process consists on the digestion of the dissolvable microcarriers. Culture medium is removed from each well which is then washed with 500 µL of PBS. 300 µL of Accutase and 200 µL of pectinase/EDTA solution (50 U/mL pectinase (Sigma-Aldrich) and 5 mM EDTA (Life Technologies) pH 8 in PBS) are added and the plate is incubated for 5 min at 37ºC. Each well is flushed using a micropipette in order to detach cells from each other and 500 µL of washing medium are added to each well to stop Accutase reaction. All the contents of the wells are transferred into 15 mL-tubes.

## III.2.2. Dynamic culture systems

For hiPSC culture on polystyrene microcarriers in spinners, cells are expanded using 1 g/L of microcarriers, which translates into 600 mg PSM to a total culture volume of 30 mL, the equivalent to 216 $cm^2$ of surface area on the microcarriers. This surface area corresponds to 43 mg of dissolvable microcarriers, for the same volume. The optimal hiPSC density for the inoculation of spinners using microcarriers has been defined as 55 000 cells/$cm^2$ [81], which translates as $1.2x10^7$ cells.

### *III.2.2.1. Inoculation of a spinner-flask*

Spinner-flask's inner surface is siliconized using SIGMACOTE® (SigmaAldrich) according to manufacturer's instructions: the surface is covered in undiluted SIGMACOTE® and left to air dry overnight in a hood; the solution is washed out using water to remove any HCl by-products. After siliconization, the spinner-flask is sterilised in an autoclave (with enough distilled water inside the flask to cover the paddle); before inoculation, the water is removed and 10 mL of the culture medium are added to the spinner-flask to remove any water droplets that were left behind, this culture medium is discarded. Spinner-flasks are inoculated using cells previously expanded on monolayer culture, recovered using EDTA (section III.2.1.2.3.1) and counted, in order to know the amount of cell suspension to be used.

Spinner-flasks are inoculated using half the final volume that will be used for cell expansion; in this case, spinner-flasks were inoculated using a total volume of 15 mL. This volume includes the cell suspension needed, the previously prepared microcarrier suspension, and the necessary culture media (supplemented with PenSrep and ROCKi) to make up the total volume.

### *III.2.2.2. Culture medium change*

After 24 h of culture time, microcarriers are left to settle to the bottom of the vessel, and exhausted media is removed until only 20% of the volume (6 mL) that will be used for expansion is left. To obtain the total final volume, 24 mL of fresh culture media (supplemented only with PenStrep) is added.

For remaining culture time, 80% of exhausted media is replaced with the same volume of fresh culture media (supplemented with PenStrep).

#### *III.2.2.3. Monitoring*

Every 24 h, two 500 µL samples are obtained from the spinner flask; for this, agitation is stopped and the samples are recovered immediately, by using a 2 mL pipette. The samples are processed using the cell quantification protocols described in section III.2.4.1 and section III.2.4.2.

#### *III.2.2.4. Cell recovery*

After expansion, the volume within the spinner flask is transferred to a 50mL-tube, where microcarriers and cells are allowed to settle; the exhausted medium is carefully removed and discarded, and the remaining cells and microcarriers are incubated for 1 h in washing medium supplemented with ROCKi; after incubation, the total volume is homogenised and half the volume transferred to a separate 50mL-tube. The two halves are processed separately.

The recovery of the cells from the microcarriers after expansion is performed using two different protocols (**Figure III.1** and **Figure III.2**); the enzymes needed are used according to the volumes suggested by the manufacturer on the protocol for cell expansion on dissolvable microcarriers, which states the following:

- Add 250mL of protease (in this case, Accutase was used) per gram of microcarriers, in a concentration appropriate for the cell type;
- Add pectinase (Sigma-Aldrich) and EDTA (Life Technologies) directly to the protease solution, ensuring a final pectinase concentration of 100 U/mL and EDTA concentration of 10 mM.

##### III.2.2.4.1. Cell recovery using protease treatment followed by filtration

In the first half of the cell suspension, cells and microcarriers are allowed to settle to the bottom of the tube, washing medium is carefully removed, the cells are washed using PBS solution, left to settle once more, and the PBS is carefully removed. The appropriate volume of protease is added to the cells/microcarriers mixture, transferred back to the spinner flask, and incubated at 37$^{o}$C, under 35 rpm agitation; after 10 min, cells are flushed using a micropipette to help loosen the cells from the microcarriers, and incubated for 10 more minutes. At the end of the incubation time, washing medium is added to the contents of the spinner-flask (usually twice the volume of protease used), the total volume is filtered through a 100 µm mesh strainer into a new 50mL-tube, and centrifuged for 10 min at 1500 rpm. The supernatant is discarded, cells are resuspended using either washing medium or culture medium supplemented with ROCKi (if cells are to be re-plated), and a sample is used to quantify the amount of cells recovered, in order to calculate the efficiency of the process.

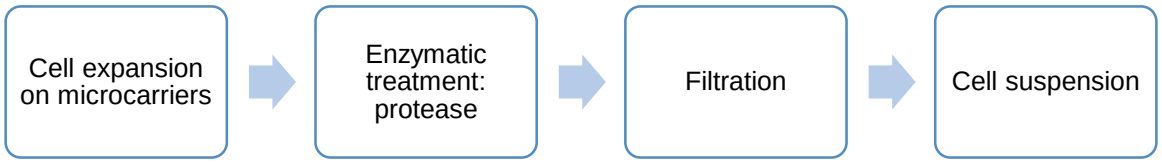



***Figure III.1 Simplified process of cell recovery from microcarriers.*** *After expansion of the cells on the surface of the microcarriers, cells must be detached from both the microcarriers and other cells using a protease (in this case, Accutase® is used); the resulting suspension of cells and microcarriers is then filtered to remove all the microcarriers, allowing to obtain of the desired cell suspension.*

III.2.2.4.2. Cell recovery using protease and harvesting solution

This protocol is only performed when working with dissolvable microcarriers. For the second half of the cell suspension, cells and microcarriers are allowed to settle to the bottom of the tube, washing medium is carefully removed, the cells are washed using PBS solution, left to settle once more, and the PBS is carefully removed. The appropriate volume of protease is added to the cells/microcarriers suspension, transferred back to the spinner-flask, and incubated for 10 min at 37ºC, under 35 rpm agitation; after the first incubation, cells are flushed using a micropipette to help loosen the cells from the microcarriers, the appropriate volume of harvesting solution is added to the contents of the spinner flask and the whole volume is incubated for 10 more minutes. At the end of the incubation time, washing medium is added to the contents of the spinner-flask (usually twice the volume of enzyme/protease used), the total volume transferred into a new 50mL-tube, and centrifuged for 10 min at 1500 rpm. The supernatant is discarded, cells are resuspended using either washing medium or culture medium supplemented with ROCKi (if cells are to be re-plated), and a sample is used to quantify the amount of cells recovered, in order to calculate the efficiency of the process.

Harvesting solution

The harvesting solution is prepared according to manufacturer's instructions by adding 1.3 mL of pectinase and 1 mL od EDTA to 97.7 mL of Dulbecco's Phosphate-Buffered Saline (dPBS, Gibco), obtaining a final solution with 50 U/mL pectinase and 5 mM EDTA pH8 in dPBS.

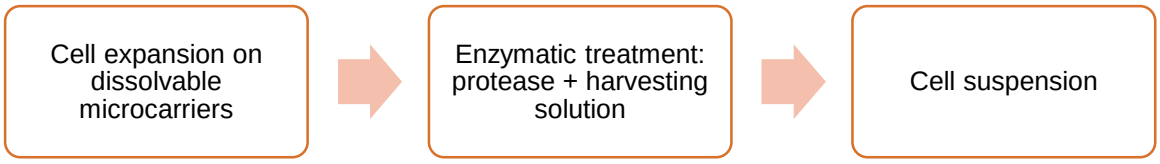



***Figure III.2 Simplified process of cell recovery from dissolvable microcarriers.*** *After expansion of the cells on the surface of dissolvable microcarriers, the microcarriers need to be dissolved using the necessary enzyme (the pectinase contained in the harvesting solution), cells need to be detached from the microcarriers beforehand (using a protease, in this case Accutase® is used) in order for the pectinase to reach the microcarriers and successfully dissolve them, after which the result is the desired cell suspension.*

## III.2.3. Re-plating

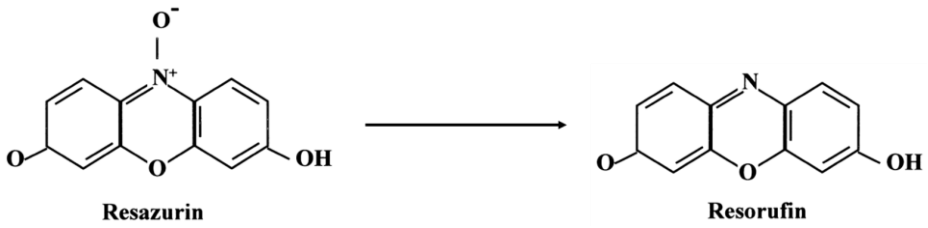
Re-plating consists on recovering the cells cultured on microcarriers and using them to inoculate new multi-well plates; the procedure starts with a 1 h incubation with culture medium supplemented with

ROCKi, followed by a cell recovery protocol used for cell counting, using culture medium supplemented with ROCKi for that recovery and the cells recovered are used to inoculate multi-well plates previously coated with Matrigel™.

## III.2.4. Cell quantifications

### *III.2.4.1. Indirect method*

Alamar Blue (Invitrogen®) is widely used in studies of cell viability by monitoring the reducing environment of the living cells; its active compound is a blue non-fluorescent dye commonly known as resazurin – a non-toxic compound, permeable through cell membranes and compatible with culture medium composition – that is reduced to resorufin, a pink-coloured highly fluorescent compound (**Figure III.3**) and its fluorescence can be measured and translated into cell numbers [91].




Resazurin Resorufin

***Figure III.3 AlamarBlue® mechanism as an indicator of cell viability****. Resazurin, a non-fluorescent indicator dye, which is converted to bright red–fluorescent resorufin via the reduction reactions of metabolically active cells. The amount of fluorescence produced is proportional to the number of living cells. From O'Brien et al, 2000 [92].*

#### III.2.4.1.1. Establishing a calibration curve

In order to be able to determine cell numbers from fluorescence readings, a calibration curve was established by measuring the fluorescence of pre-determined cell numbers – no cells, 20,000, 40,000, 60,000 80,000 and 100,000 cells – and plotting the results in three different conditions: cells on Matrigel™-coated plates, on Matrigel™-coated polystyrene microcarriers and on Matrigel™-coated dissolvable microcarriers, both on ultra-low attachment plates, all three conditions using E8™ culture medium. Triplicates were prepared for each condition and for each number of cells desired, after inoculation with the cells all plates were incubated for 2 h at 37 ºC; after this first incubation, 25 µL of Alamar Blue were added to each well and all plates were incubated for 1 h at 37 ºC. After the second incubation, 380 µL of the culture medium were recovered from each well, without carrying any microcarriers, and centrifuged in Eppendorf vials for 3 min at 1000 rpm; on a 96-well plate, duplicates of each condition were added using 170 µL in each well and the plate was analysed on a multimode microplate reader (Infinite® 200 Pro, Tecan) to measure the fluorescence intensity, which is monitored at 560 nm excitation wavelength and 590 nm emission wavelength; optimal gain was measured from one of the wells containing 100,000 cells.

The same protocol was used to establish a calibration curve using Synthemax®II dissolvable microcarriers on mTeSR™1 culture medium.

III.2.4.1.1. Indirect cell quantification

For 500 µL samples, in each well, 80% of the medium is removed and the same volume of fresh culture medium is added; 25 µL of Alamar Blue are added to each well that will be analysed and the plates are incubated for 1 h at 37ºC. At the same time, two wells containing only culture medium, without cells, are incubated with 25 µL of Alamar Blue and will be used as the blank measure in order to take into account the influence of culture medium interference in the fluorescence measurement. After incubation, 350 µL of the culture medium are recovered from each well, without carrying any microcarriers, and centrifuged in Eppendorf vials for 3 min at 1000 rpm; the supernatant is used to obtain dilutions – in the same culture medium used for the culture medium change – in order for the measurements to fit in the calibration curves established. On a 96-well plate, duplicates of each dilution are added using 170 µL in each well, and the plate is analysed on a multimode microplate reader (Infinite® 200 Pro, Tecan) to measure the fluorescence intensity.

#### *III.2.4.2. Direct cell quantification*

After the indirect cell quantification procedure, cells are harvested according to the appropriate protocol (section III.2.1.2.5). The contents of the tubes are centrifuged for 5 min at 1000 rpm After centrifugation, supernatant is discarded and each tube is grinded against the grid of the chamber in order to loosen the pellet, which is then resuspended in 730 µL of washing medium – assuming a final volume of 750µL; from the suspensions obtained, 10 µL are diluted in a 1:2 proportion with Trypan Blue, 10 µL of the final solution are added to Neubauer chambers and the cells are counted under the microscope.

## III.3. HIPSC CHARACTERISATION

### III.3.1. Immunocytochemistry

Immunocytochemistry comprises highly sensitive and specific techniques that can be used to detect specific proteins or antigens within cells by resorting to specific antibodies [93]. Specific primary antibodies can be used to find pluripotency markers expressed by hiPSCs and secondary antibodies, which emit fluorescent signals, can be used to detect the primary antibodies using fluorescence microscopy.

#### *III.3.1.1. Extracellular staining*

The chosen pluripotency surface markers were TRA-1-60 and SSEA-4. The culture medium is removed from all the wells which are then washed three times with 500 µL of PBS and then incubated with 300 µL of primary antibody solution (**Table III.3**) – the dilution of the antibodies is made using washing medium – for 30 min at 37ºC. Afterwards, each well is washed three times with 500 µL of PBS and incubated with 300 µL of secondary antibody solution for 30-45 min, at 37ºC in the dark. The wells are washed two times with 500 µL of PBS and 500 µL of PBS are added to each well.

***Table III.3 Dilutions regarding the preparation of antibody solutions for surface markers detection.*** *Dilutions for each antibody solution, respective secondary antibodies and their dilutions.*

| Marker | Primary Antibodies | | Secondary Antibodies | |
|---|---|---|---|---|
| | **Antibody** | **Dilution** | **Antibody** | **Dilution** |
| ***TRA-1-60*** | Mouse IgM (Stemgent) | 1:135 | Goat anti-mouse IgM (Invitrogen) | 1:500 |
| ***SSEA-4*** | Mouse IgG (Stemgent) | 1:135 | Goat anti-mouse IgG (Invitrogen) | 1:500 |

#### *III.3.1.2. Intracellular staining*

The chosen intracellular pluripotency markers were OCT4 and NANOG. Firstly, the culture medium is removed and each well is washed with PBS; 4% Paraformaldehyde (PFA, Sigma-Aldrich) in PBS solution is added and incubated for 10-15 min at room temperature. After incubation, each well is washed with PBS and incubated with 300 µL of Blocking solution (10% Normal Goat Serum (NGS, Sigma-Aldrich), 0.1% Triton-X (Sigma-Aldrich), in PBS) for 1 h at room temperature. Blocking solution is removed and 300 µL of primary antibody solution (**Table III.4**) is added to each well – the dilution of the primary antibody is made using Staining buffer (5% NGS, 0.1% Triton-X, in PBS) – and incubated at 4ºC, overnight.

Each well is washed three times using 500 µL of PBS and incubated using 300 µL of the secondary antibody solution – the dilution of the secondary antibody is made using staining buffer – for 1h at room temperature, in the dark. Each well is once again washed three times with 500 µL of PBS and incubated with DAPI solution for 3 min at room temperature, in the dark. Each well is washed two times with 500 µL of PBS to remove DAPI crystals and 500 µL of PBS are added to each well.

***Table III.4 Dilutions regarding the preparation of antibody solutions for intracellular staining.*** *Dilutions for each antibody solution, respective secondary antibodies and their dilutions.*

| Marker | Primary Antibodies | | Secondary Antibodies | |
|---|---|---|---|---|
| | **Antibody** | **Dilution** | **Antibody** | **Dilution** |
| ***NANOG*** | Rabbit IgG (Merck Millipore) | 1:5000 | Goat anti-rabbit IgG (Invitrogen) | 1:500 |
| ***OCT4*** | Mouse IgG (Merck Millipore) | 1:750 | Goat anti-mouse IgG (Invitrogen) | 1:500 |

### III.3.2. Flow cytometry

Flow cytometry is a widely used technique that allows for the analysis of cell characteristics, such as size, granularity, and presence of a fluorescence molecule – which can be an antibody or a dye; as cells and particles in a liquid stream move through a laser or light beam, the relative light-scattering, the colour of the dye, or the fluorescence, can be measured [94].

Flow cytometry was performed to quantify the percentage of cells that maintained the expression of their extracellular and intracellular pluripotency markers – SSEA4 and TRA-1-60, and OCT4, respectively – after expansion on dissolvable microcarriers under dynamic conditions, and the percentage of cells that started differentiating by analysing early differentiation marker SSEA1.

#### *III.3.2.1. Extracellular staining*

Cells kept in 2% PFA solution are centrifuged for 3 min at 1000 rpm and each pellet is resupended in 100 µL of FACS buffer (4% foetal bovine serum (FBS; Sigma Aldrich) in PBS) for each analysis that is to be performed. 100 µL of cell suspension is transferred to a FACS vial, 10 µL of phycoerythrin (PE) conjugated antibody solution – SSEA4 (Miltenyi Biotec), TRA-1-60 (Miltenyi Biotec) and SSEA1 (Miltenyi Biotec) – are added to each vial and incubated for 15 min at room temperature, in the dark. After incubation, 2 mL of PBS solution are added to each vial, which is then centrifuged for 3 min at 1000 rpm; supernatant is discarded and 2 mL of PBS are added to each vial, which are again centrifuged and the supernatant discarded. 300 µL of PBS are used to resuspend the pellet and the cell suspension obtained is then analysed in a FACScalibur (Becton Dickinson) flow cytometer, using CellQuest™ software (Becton Dickinson) for data acquisition.

#### *III.3.2.2. Intracellular staining*

The Eppendorf vials to be used in this procedure are coated with 400 µL of BSA solution for 15 min at room temperature. Meanwhile, samples kept in 2% PFA solution are centrifuged for 5 min at 1000 rpm, washed twice with 5 mL of 3% NGS solution, and centrifuged for 5 min at 1000 rpm, each time. The supernatant is discarded and cells are resuspended in 500 µL of 3% NGS solution for each analysis to be performed with that same sample. BSA solution is removed from the Eppendorf vials, and 500 µL of cell suspension is added to each one. Vials are centrifuged for 3 min at 1000 rpm, supernatant is removed, and the pellet is resuspended in 150 µL of 3% NGS solution and 150 µL of saponin (Sigma Aldrich) and incubated for 15 min at room temperature. After incubation the vials are centrifuged for 3 min at 1000 rpm, the supernatant is removed, and the pellet is resuspended in 300 µL of NGS solution and incubated for 15 min at room temperature. After incubation the vials are centrifuged for 3 min at 1000 rpm. The supernatant is removed, the pellet is resuspended in the appropriate primary antibody solutions – Anti-OCT4 (mouse IgG, Merck Millipore) antibody is used in a 1:300 proportion – and incubated for 1.5 h in the dark.

After incubation vials are centrifuged for 3 min at 1000 rpm, pellet is washed twice with 1% NGS solution, and centrifuged for 3 min at 1000 rpm, each time. The supernatant is removed, all samples are resuspended in 300 µL of secondary antibody solution – Alexa 488 anti-mouse IgG antibody (Invitrogen) in a 1:300 proportion –, and incubated for 45 min in the dark. After incubation, vials are centrifuged for 3 min at 1000 rpm, pellet is washed twice with 1% NGS solution, and centrifuged for 3 min at 1000 rpm, each time. Supernatant is removed, the pellet is resuspended in 500 µL of PBS solution, cell suspension is transferred to FACS vials and analysed in a FACScalibur flow cytometer, using CellQuest™ software for data acquisition.

## III.3.3. Embryoid body formation

Embryoid bodies (EBs) are three-dimensional aggregates of pluripotent stem cells; when differentiating in suspension *in vitro*, PSCs in EBs acquire molecular markers specific to the three embryonic germ layers [95].

### *III.3.3.1. Formation of aggregates*

In order to obtain aggregates of hiPSCs, cells are recovered as if for a normal passage using EDTA, counted, and plated on ultra-low attachment 6-well plates, at a density of $1x10^6$ cells per well, using 2 mL of expansion culture medium (either E8™ or mTeSR™1) supplemented with PenStrep and ROCKi. After 24 h, 80% of the volume of culture medium is replaced with fresh medium not supplemented with ROCKi.

### *III.3.3.2. EB differentiation*

After 2 days of cell expansion, 80% of spent culture medium is replaced with EB differentiation medium (medium composition in section III.1.2.2.1) every other day for 28 days.

### *III.3.3.3. Re-plating of cells from EBs*

In order to re-plate the cells from the EBs, the necessary wells in a 24-well plate are coated with poly-ornithine (200 µL per well) for 1 h at 37ºC, followed by an incubation with laminin solution (1 µL of laminin stock solution per well on 200 µL per well of PBS solution), for 2 h at 37ºC.

On the 29th day of differentiation, aggregates are dissociated. Differentiation medium is removed, aggregates are washed twice using 2 mL of PBS solution per well, and incubated with 1 mL of Trypsin per well for 5 min at 37ºC; after incubation, 1 mL of EB differentiation medium is added to each well to stop the reaction, cells are flushed using a micropipette, cell suspension is transferred to a 15mL-tube and centrifuged. After centrifugation, the supernatant is discarded. Cells are resuspended in 2 mL of EB differentiation medium, and 500 µL of cell suspension are re-plated in each well of a previously coated 24-well plate.

### *III.3.3.4. Analysis by immunocytochemistry*

On the 7th day after re-plating, cells were fixed using a PFA 4% solution, and marked with antibodies for each one of the germ layers, in order to understand if all three germ layers were present, as well as for a pluripotency marker, to establish whether or not there were still pluripotent cells remaining after the differentiation. Immunocytochemistry analysis is performed as described in section III.3.1.2. using the antibodies listed below (**Table III.5**):

***Table III.5 Dilutions regarding the preparation of antibody solutions for embryoid bodies analysis.*** *Dilutions for each antibody solution, respective secondary antibodies and their dilutions, used to analyse the presence of each of the three germ layers – endoderm, mesoderm, and ectoderm, using Sox17, Tuj1, and SMA, respectively – as well as pluripotent stem cells – OCT4.*

| Marker | Germ layer /Pluripotent cells | Primary Antibodies | | Secondary Antibodies | |
|---|---|---|---|---|---|
| | | Antibody | Dilution | Antibody | Dilution |
| ***OCT4*** | Pluripotency | Mouse IgG | 1:150 | Goat anti-mouse IgG | 1:500 |
| ***Sox17*** | Endoderm | Mouse IgG (R&D Systems) | 1:1 000 | Goat anti-mouse IgG | 1:500 |
| ***Tuj1*** | Ectoderm | Mouse IgG (Covance) | 1:20 000 | Goat anti-mouse IgG | 1:500 |
| ***α-SMA*** | Mesoderm | Mouse IgG(Dako) | 1:1 000 | Goat anti-mouse IgG | 1:500 |

## III.4. DATA ANALYSIS

The values obtained from all the cell quantifications were used to calculate:

- Difference (in percentage) in cell numbers 24h after inoculation, through direct and indirect cell quantification methods, relatively to the number of cells initially inoculated;
- Fold increase, which corresponds to the ratio between the highest cell numbers achieved and the number of cells after 24h, both obtained through direct cell quantifications;
- Seeding yield which corresponds to the ratio between the highest cell numbers achieved and the number of cells inoculated, through values obtained through direct cell quantifications.
- Standard deviation values are always presented and are calculated from the measurements obtained.

# IV. RESULTS AND DISCUSSION

## IV.1. OUTLINE

In order to assess hiPSC adhesion and expansion on dissolvable microcarriers (DM), and the efficacy of cell recovery from these microcarriers, cells were first cultured on the surface of different types of microcarriers – dissolvable and polystyrene microcarriers (PSM) –, with different types of surfaces – Matrigel™ (Mat) coating (performed in the laboratory) and Synthemax®II (SII) surface (treatment to enhance cell attachment to the surface of the microcarriers, performed by the manufacturer) –, under static culture conditions, and using different types of culture media – Essential 8™ (E8™) and mTeSR™1; the goal was to be able to establish a comparison between hiPSC culture on polystyrene microcarrier systems – which had already been successfully established for hESCs [70, 96] – and on the new dissolvable microcarriers.

In order to obtain preliminary results pertaining to all these features, cells were cultured on both types of microcarriers, under the same conditions – microcarrier static culture, in ultra-low attachment 24-well plates – and all results obtained for cell adhesion and expansion were compared. From the results obtained in static expansion, some of the conditions tested were chosen to be scaled-up to microcarrier dynamic culture on spinner-flask, with a working volume of 30 mL, in order to verify if cells remained attached to the microcarriers when subjected to dynamic culture conditions, and to determine cell growth on dissolvable microcarriers in a scalable stirred suspension system, and, once again, compare all the results obtained with the results from previously established systems.

The hypothesis being tested was that, if cell adhesion and expansion proved to be similar on both types of microcarriers, the percentage of cell recovery should be higher for the new dissolvable microcarriers due to the differences on the harvesting protocols. This would not only result in higher hiPSC expansion yields, due to fewer losses during the recovery, but also in a simpler and less time consuming cell recovery protocol, to replace the standard harvesting protocols currently in place – particularly important when regarding large-scale expansion of hiPSCs.

Cells were characterised after expansion: immunocytochemistry assays were performed to detect pluripotency surface – SSEA4 and TRA-1-60 – and intracellular – OCT4 and NANOG – markers, as well as early differentiation marker SSEA1. Characterisation was also performed after recovery from the microcarriers: flow cytometry was used to compare the cell populations before and after expansion, regarding their expression of SSEA4, TRA-1-60, and OCT4; Embryoid Body formation was used to establish whether or not the cells maintained their ability to differentiate into cells of all three germ layers, the recovered cells were re-plated and new immunocytochemistry assays were performed, to guarantee that the changes in the harvesting protocol did not affect the expression of pluripotency markers OCT4 and NANOG.

## IV.2. HIPSC EXPANSION AS MONOLAYERS UNDER STATIC CULTURE CONDITIONS

hiPSCs were first expanded as monolayers on Matrigel™-coated multi-well plates, using either E8™ or mTeSR™1 culture media. When cultured *in vitro*, using feeder-layers or serum supplemented media, hiPSC colonies' morphology is typically round with defined borders; however, when using a feeder-free serum-free culture system, cells present themselves as flat densely packed colonies, with defined borders and irregular shapes (**Figure IV.1**).

Cells were cultured until they reached 80% confluence, when they were passaged as small aggregates using the EDTA passaging protocol, onto new Matrigel™-coated multi-well plates, or otherwise recovered and used to inoculate microcarriers, and thus beginning a new experiment.

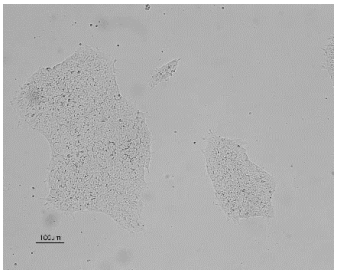


***Figure IV.1 . hiPSC colony morphology.*** *hiPSC colonies formed in a two-dimensional cell culture with mTeSR™1 culture medium on Matrigel™-coated plates, 2 days after inoculation. Bar scale: 100µm.*

## IV.3. ALAMAR BLUE CALIBRATION FOR INDIRECT CELL QUANTIFICATION

The first step towards getting information about the efficiency of cell recovery from microcarriers was to establish a way to quantify the cells attached to the microcarriers' surface before the harvesting protocol; this way it would be possible to compare the cell numbers obtained before and after recovering the cells from the microcarriers and determine the amount of cells that were being lost.

An indirect method for cell quantification was chosen: Alamar Blue is a commercially available blue dye that is reduced to a pink fluorescent compound by cellular metabolism; this principle can be applied to correlate the number of cells in culture with the intensity of the fluorescence measured. To establish the calibration curves (**Figure IV.2**) six cell densities were used, three independent wells were inoculated for each cell density, and each well's fluorescence was measured twice.

From the raw data obtained (not shown) it was possible to understand that the wells that contained no cells showed high fluorescence intensity. Alamar blue protocols specifically state that phenol red (present in both E8™ and mTeSR™1 and responsible for their colours) is not responsible for interference in this type of assay, and mention that components such as BSA (present in mTeSR™1) and FBS can interfere with fluorescence measurement due to quenching of fluorescence [91]. In order to prevent any type of interference, the fluorescence intensity values from the wells with no cells were subtracted from every measurement (both for the calibration curves and in every measurement after that), so that the results obtained only referred to the number of cells.

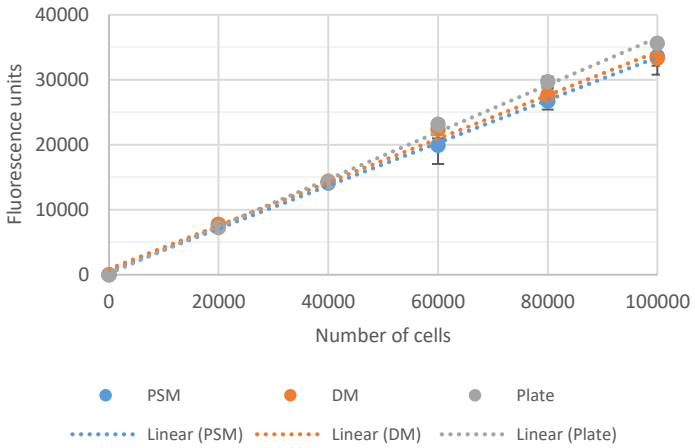



***Figure IV.2. Alamar Blue calibration curves for hiPSCs in Essential 8™ culture medium.*** *The graphic shows the fluorescence intensity measured in relation to hiPSC numbers on Matrigel™-coated polystyrene microcarriers (PSM), dissolvable microcarriers (DM) and on two-dimensional plates (plate), and the linearization of the experimental fluorescence values measured.*

The equations for the calibration curves were obtained through linear regression of the average fluorescence measured in each condition, by using the fluorescence intensity values with the corrections to exclude the culture media interference, and are represented below for hiPSCs on Matrigel™-coated polystyrene microcarriers **(Equation 1**), on Matrigel™-coated dissolvable microcarriers **(Equation 2**), and on Matrigel™-coated plates **(Equation 3**), using E8™ culture media. In these equations x stands for the number of cells, and y stands for the fluorescence intensity measured.

$$y = 0.3295x + 507.08\ (R^2 = 0.9987)$$

***(Equation 1)***

$$y = 0.3344x + 834.08\ (R^2 = 0.9951)$$

***(Equation 2)***

$$y = 0.3631x + 152.73\ (R^2 = 0.9971)$$

***(Equation 3)***

The same procedure was used to establish a calibration curve for mTeSR™1 medium and Synthemax®II-coated dissolvable microcarriers (**Figure IV.3**), resulting in the equation below **(Equation 4)**.

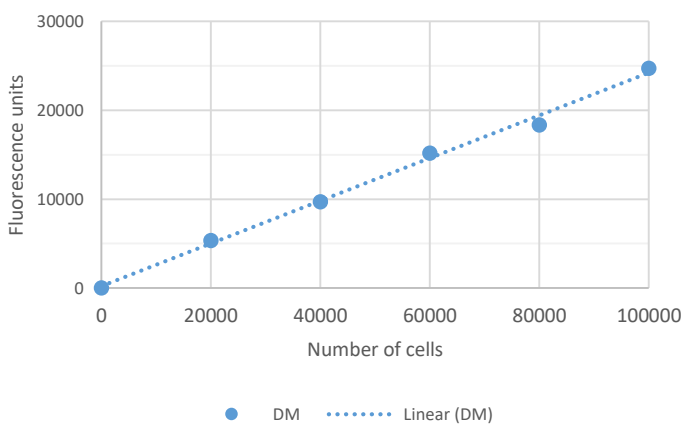



***Figure IV.3. Alamar Blue calibration curve for hiPSCs in mTeSR™1 culture medium.*** *The graphic shows the fluorescence intensity measured in relation to hiPSC numbers on Synthemax®II dissolvable microcarriers (DM), and the linearization of the experimental fluorescence values measured.*

$$y = 0.2401x + 206.01\ (R^2 = 0.9955)$$

***(Equation 4)***

The use of Alamar blue for indirect cell quantifications proved to work only to the point before cells started to form large aggregates; beyond that, the cell numbers obtained with this technique were always below the values obtained through direct quantification of the samples recovered, not allowing to perform reliable indirect cell quantifications until the end of culture.

## IV.4. HIPSC EXPANSION ON MICROCARRIERS UNDER STATIC CONDITIONS

Dissolvable microcarriers were developed for use with any cell type; however, these had only been tested for Mesenchymal Stem Cell (MSC) culture [83] and had not been used for hiPSC expansion before. Therefore, it was important to begin testing those microcarriers by analysing hiPSC adhesion to their surface, monitoring cell expansion and distribution over the available surface area, and comparing cell functionality before and after being cultured on these dissolvable microcarriers.

hiPSCs were inoculated on six different combinations of microcarriers – polystyrene or dissolvable –, coating surfaces – Matrigel™ or Synthemax®II –, and culture media – Essential 8™ or mTeSR™1. Cells were cultured for 4 days (cells cultured on E8™ medium) or 5 days (cells cultured on mTeSR™1), and cell expansion was monitored everyday through direct and indirect cell quantifications; the cell numbers obtained were used to determine the number of cells 24 h after inoculation (to determine cell adhesion to the microcarriers), the cell fold increase (ratio between the highest cell numbers achieved and the number of cells after 24h, from values obtained through direct cell quantifications), and the cell seeding yield (ratio between the highest cell numbers achieved and the number of cells inoculated, from values obtained through direct cell quantifications).

***Table IV.1 hiPSC expansion in static three-dimensional culture on microcarriers.*** *Values obtained for: difference in hiPSCs numbers 24h after inoculation of the microcarriers, determined through indirect (Alamar Blue) and direct cell quantification methods, and culture time at which highest hiPSC expansion was achieved, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™ and Synthemax®II surfaces, for cultures using Essential 8™ and mTeSR™1 culture media. All values were calculated from duplicate samples collected during each experiment (n=1).*

| Static 3D culture – hiPSC expansion on microcarriers | | | | | |
|---|---|---|---|---|---|
| **Culture media** | **Surface type** | **Microcarrier type** | **After 24h** | | **Highest expansion** |
| | | | **Alamar Blue** | **Direct Counts** | |
| **Essential 8™** | Matrigel™ | PSM | 98.5±12.6% | 32.2±1.1% | Day 3 |
| | | DM | 128.6±12.8% | 48.3±17.2% | Day 3 |
| **mTeSR™1** | Matrigel™ | PSM | 92.5±20.4% | 41.7±8.9% | Day 4 |
| | | DM | 112.9±33.5% | 61.1±5.0% | Day 5 |
| | Synthemax®II | PSM | 48.3±2.1% | 29.7±1.4% | Day 5 |
| | | DM | 75.3±24.0% | 48.1±0.3% | Day 5 |

The first experiments were performed using E8™ culture medium. When comparing polystyrene and dissolvable microcarriers with the same Matrigel™ coating, cell quantification 24h after inoculation showed higher cell numbers on dissolvable microcarriers, both through direct and indirect cell quantifications – 48.3 ± 17.2% and 128.6 ± 12.8%, respectively – than on the polystyrene microcarriers – 32.2 ± 1.1% and 98.5 ± 12.6%, respectively –, suggesting that dissolvable microcarriers allow for a better adhesion of hiPSCs to their surface; the same tendency was observed when replacing E8™ medium with mTeSR™1, with direct and indirect cell quantifications 24 after inoculation, showing 61.1 ± 5.0% and 112.9 ± 33.5% for the dissolvable microcarriers, and 41.7 ± 8.9% and 92.5 ± 20.4% for polystyrene microcarriers. This difference was also observed when inoculating cells on microcarriers with Synthemax®II surface and culturing them on mTeSR™1 medium, where direct and indirect cell quantifications 24 h after inoculation indicated percentages of cell adhesion of 48.1 ± 0.3% and 75.3 ± 24.0% for dissolvable microcarriers, and 29.7 ± 1.4% and 48.3 ± 2.1% on polystyrene microcarriers, these two conditions having the lowest cell adhesion of all six (**Table IV.1**).

On the other hand, regarding cell expansion efficiency (**Figure IV.4**), the use of mTeSR™1 proved to yield better results for hiPSC expansion, when compared to E8™ culture medium, except when culturing hiPSCs on Matrigel™-coated polystyrene microcarriers – only achieving a fold increase of 2.7 ± 0.3 and a seeding yield of 1.1 ± 0.1, after 4 days of culture; regarding these two parameters, the best hiPSC expansion was achieved using Matrigel™-coated dissolvable microcarriers on mTeSR™1 culture medium – achieving a fold increase of 7.3 ± 0.5 and a seeding yield of 4.4 ± 0.3, after 5 days of culture –, closely followed by a combination of Synthemax®II dissolvable microcarriers on mTeSR™1 culture medium – achieving a fold increase of 7.1 ± 0.0 and a seeding yield of 3.4 ± 0.0, after 5 days of culture –, being the difference in seeding yield due to the better adhesion observed for the first system, when compared to the second. The use of either Matrigel™-coated or Synthemax®II dissolvable microcarriers in mTeSR™1 did not seem to have an impact in hiPSC fold increase – 7.3 ± 0.5 and 7.1 ± 0.0,

respectively –, the seeding yields obtained were higher when using a Matrigel™ coating (4.4 ± 0.3) than when using Synthemax®II (3.4 ± 0.0) due to fewer cell losses during the first 24h.

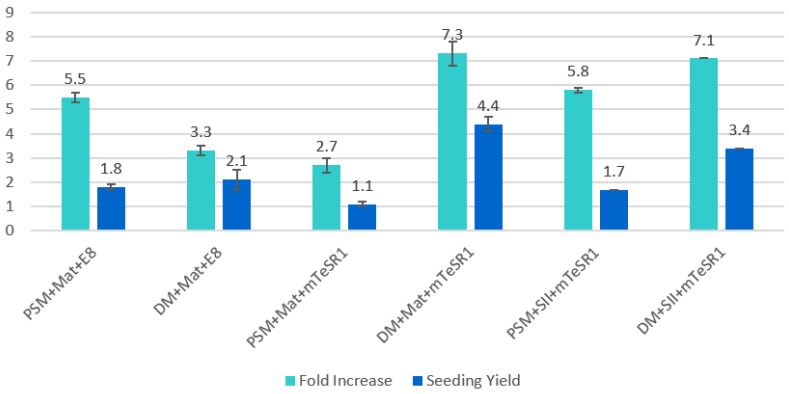



***Figure IV.4 hiPSC expansion in static three-dimensional culture.*** *Values obtained for: fold increase and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™ (Mat) and Synthemax®II (SII) surfaces, for cultures using Essential 8™ (E8) and mTeSR™1 culture media. All values were calculated from duplicate samples collected during each experiment (n=1).*

Daily visual monitoring (through optical microscopy) was used to keep track of cell distribution throughout the available surface area (**Figure IV.5** and **Figure IV.6**). Through this monitoring it was possible to understand that microcarrier aggregates start to appear as early as 24 h after inoculation; these aggregates become larger throughout culture time and may be a great source of concern regarding the diffusion of nutrients and gases, especially if hiPSCs are to be cultured for long periods of time. The formation of aggregates was aggravated by the use of Matrigel™ coating, due to its nature being very similar to that of gelatine which naturally promotes aggregation, even of empty microcarriers; in comparison, the use of Synthemax®II surface helps reducing aggregation but does not completely prevent it. The composition of the microcarriers does not appear to have any impact on aggregation.

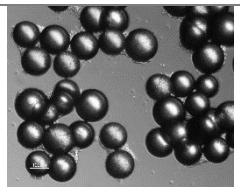
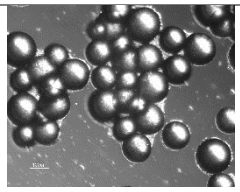
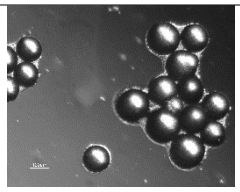
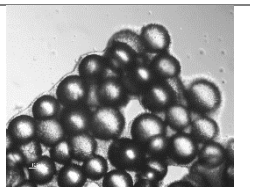
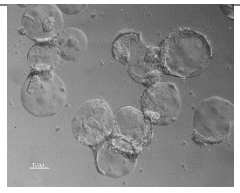
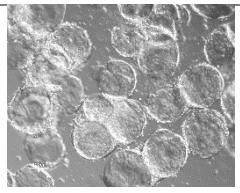
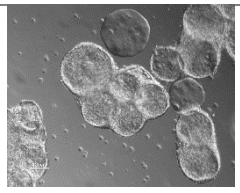
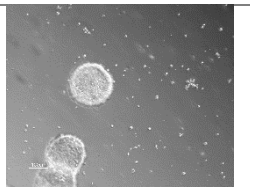
| Static 3D culture – hiPSC expansion on microcarriers | | | | |
|---|---|---|---|---|
| **E8™** | **Day 1** | **Day 2** | **Day 3** | **Day 4** |
| **Matrigel™-coated polystyrene microcarriers** |  |  |  |  |
| **Matrigel™-coated dissolvable microcarriers** |  |  |  |  |

***Figure IV.5 Bright field microscopy of hiPSC cultured on Matrigel™-coated polystyrene and dissolvable microcarriers with Essential 8™ culture medium, throughout 4 days of culture.*** *The images show an increase on the cell density throughout the culture time, and the distribution of the hiPSCs throughout the surface area available for cell adhesion and expansion. Bar scale: 100µm.*

The overall distribution of cells throughout the available microcarriers shows that some microcarriers were completely covered with cells while others were left with no cells which indicates that initial cell distribution throughout the microcarriers surface was not as homogeneous as it would be desirable.

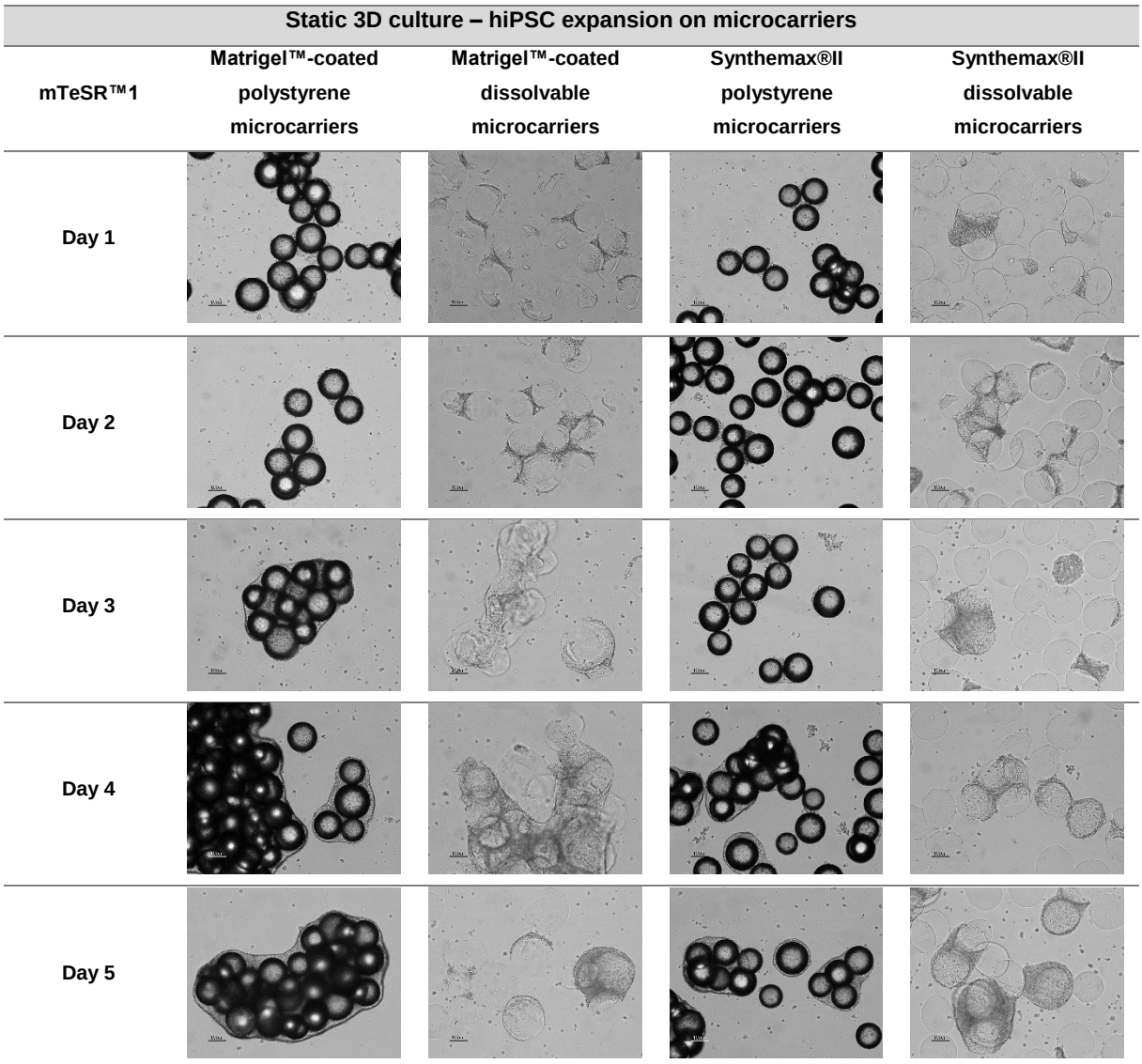



***Figure IV.6 Bright field microscopy of hiPSC cultured on polystyrene and dissolvable microcarriers with Matrigel and Synthemax®II surfaces on mTeSR™1 culture medium, throughout 5 days of culture.*** *The images show an increase on the cell density throughout the culture time, and the distribution of the hiPSCs throughout the surface area available for cell adhesion and expansion. Bar scale: 100µm.*

Experiments using a combination of Synthemax®II dissolvable microcarriers in Essential 8™ culture medium were also performed; when using the standard protocol of inoculation using small hiPSC aggregates at a density of 50,000 cells/cm$^2$ only a few of the cells adhered to the surface of the microcarriers, instead starting to grow as cell aggregates, only partially attached to the surface of the dissolvable microcarriers. When performing a single-cell inoculation protocol, using the same cell density, even less cells adhered to the surface of the microcarriers, instead forming many small cell aggregates. In both cases, before the end of the 5 days of culture, no cells were left attached to the microcarriers, and most were lost during daily culture media change. Small progress was achieved when

performing a single-cell inoculation protocol using half the standard cell density – 25,000 cells/cm$^2$; the idea behind this was to provide less chances for hiPSCs to adhere to each other, leading them to attach to the surface or the dissolvable microcarriers. Even though there were still some cell aggregates, many microcarriers showed small colonies forming on their surface after 24 h of culture, and by the end of the 5 days of culture time, very few of the dissolvable microcarriers were completely covered with cells (**Figure IV.7**).

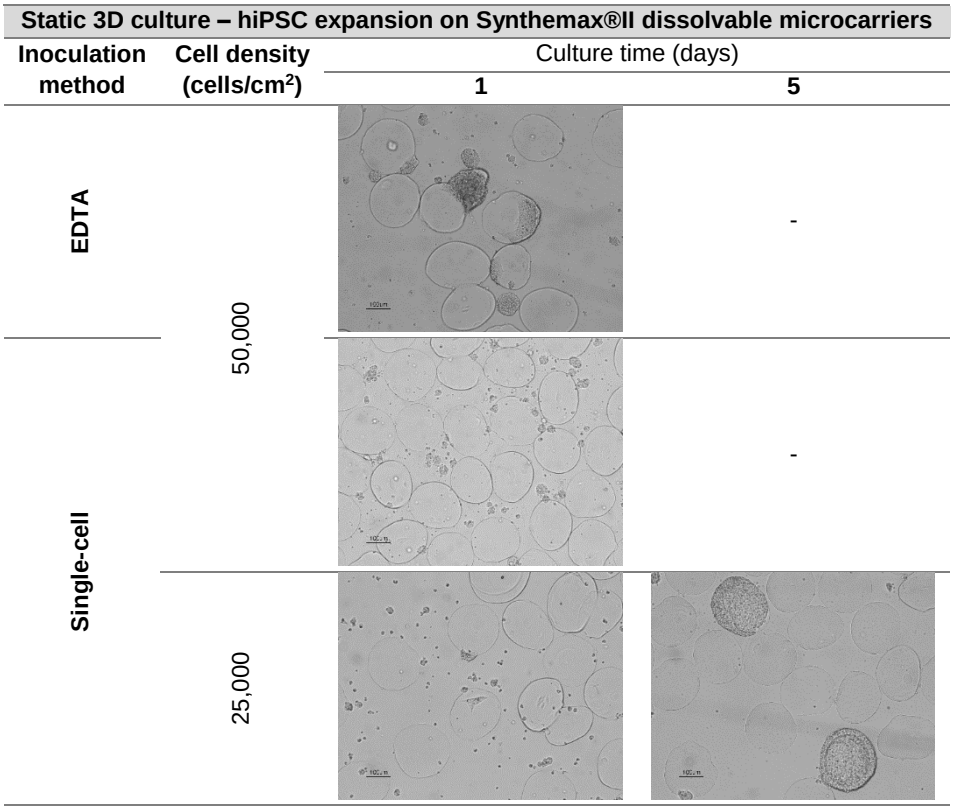



***Figure IV.7 Bright field microscopy of hiPSC cultured on Synthemax®II dissolvable microcarriers with Essential 8™ culture medium, throughout 5 days of culture.*** *The images show a comparison between inoculation of the microcarriers using small hiPSC aggregates or using single cell inoculation – at normal 50,000 cells/cm$^2$ compared to 25,000 cells/cm2. Instead of adhering to the surface of the microcarriers cells instead expand as aggregates, unless a lower cell density is used. Bar scale: 100µm.*

Characterisation of the cells after expansion was performed by immunocytochemistry to detect pluripotency markers SSEA4 and OCT4 on cells attached to the microcarriers at the final day of culture; the results were positive for every condition tested (results not shown) which suggested that cells were maintaining their pluripotency characteristics during expansion.

The global analysis of all the results obtained indicates that microcarrier culture systems, under static conditions, are able to support hiPSC expansion, as long as there is surface area available for cell adhesion; also, regarding the dissolvable microcarriers under analysis, it is safe to state that they are very promising for hiPSC expansion, showing better results than those obtained with polystyrene microcarriers, under the culture conditions used in these experiments.

## IV.5. HIPSC EXPANSION ON MICROCARRIERS UNDER DYNAMIC CONDITIONS

In the case of dynamic culture, the use of Alamar Blue for indirect cell counting was not an accurate method; we hypothesise that because microcarriers start forming aggregates amongst themselves, cells that grow within the aggregates are not reached by the compound, therefore not reducing it, and leading to cell numbers below the ones obtained through direct cell quantification. Taking this into consideration, indirect cell quantifications were only performed 24 h after inoculation in order to assess cell adhesion to the microcarriers, and determine whether or not hiPSCs began expansion during that time.

### IV.5.1. Adhesion protocols

The first experiment performed under dynamic culture conditions was performed using dissolvable microcarriers with Matrigel™ coating and Essential 8™ culture medium, combining a widely tested matrix substrate and a xeno-free chemically defined culture medium.

The vessels used for hiPSC culture were spinner-flasks which are specifically designed for suspension cell culture. The spinner-flask chosen had a vertical impeller for agitation and a maximum working volume of 50 mL. Nevertheless, the experiments were performed with a total volume of 30 mL. The optimal microcarrier density for hiPSC expansion on polystyrene microcarriers within spinner flasks is recommended by the manufacturer as 20 g of microcarriers per litre of culture media; in a working volume of 30 mL, this translates to 216 $cm^2$ of surface area available for cell growth, which was the surface area considered for all experiments performed. The optimal number of cells for inoculation of microcarriers had been established as 55,000 cells/$cm^2$ [81], which for the surface area available was rounded up to $1.2x10^7$ cells.

Indirect cell quantifications after 24 h showed 100% adhesion of the cells seeded, and cell proliferation during that time frame – with 115.5% ± 2.0% cells comparing to the number of cells used for initial seeding.

In order to optimise cell adhesion, three different protocols were used, which differed in terms of the agitation regimes employed during the first 48h:

- A1 – 24 h static, 24 h intermittent agitation – 3 min at 25 rpm every 2 h –, continuous agitation at 25 rpm until end of culture;
- A2 – 2 h static, intermittent agitation – 3 min at 25 rpm every 2 h – until 24 h, continuous agitation at 25 rpm until end of culture;
- A3 – 24 h static, continuous agitation at 25 rpm until end of culture.

All protocols tested showed complete adhesion (100%) of the cells used for seeding, however, protocol A2 showed higher expansion within the first 24h when compared with the other two (**Table IV.2**).

***Table IV.2. Adhesion protocols of hiPSCs on Matrigel™-coated dissolvable microcarriers.*** *Values obtained for: difference in hiPSC numbers 24h after inoculation of the microcarriers, determined through indirect (Alamar Blue) and direct cell quantification methods, fold increase and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on Matrigel™-coated dissolvable microcarriers, using Essential 8™ and culture medium. All values were calculated from duplicate samples collected during each experiment (n=1).*

| Dynamic 3D Culture (30mL) – Adhesion Protocols on Dissolvable Microcarriers | | | | | |
|---|---|---|---|---|---|
| Culture media | Surface type | Adhesion Protocol | After 24h | | Highest expansion |
| | | | Alamar Blue | Direct Counts | |
| Essential 8™ | Matrigel™ | A1 | 115.5±2.0% | 66.5±4.8% | Day 5 |
| | | A2 | 139.2±21.7% | 91.0±9.4% | Day 4 |
| | | A3 | 113.4±7.7% | 84.7±12.5% | Day 6 |

On the other hand, when considering the average cell numbers, neither protocol A2 nor A3 achieved cell numbers as high as those obtained using protocol A1, (**Figure IV.8**) if the error bars are not considered and assuming that the samples used for quantification were as representative as possible.

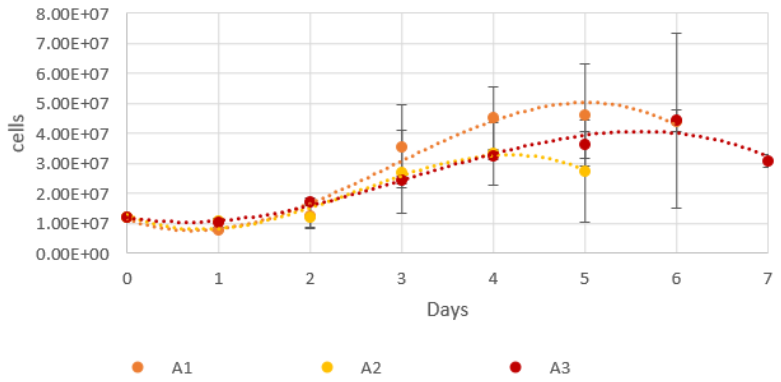



***Figure IV.8 Adhesion protocols of hiPSCs on Matrigel™-coated dissolvable microcarriers in Essential 8™ culture medium.*** *The graphic represents the number of cells present in the culture vessel at each day of culture time, regarding each adhesion protocol tested using hiPSCs on Matrigel™-coated dissolvable micarocarriers in E8™ culture medium. Each curve represents a polynomial regression of the values obtained for hiPSC expansion using different types of agitation during the first 48h of culture: A1 (orange) – 24h static, 24h intermittent agitation – 3min at 25rpm every 2h –, continuous agitation until end of culture; A2 (yellow) – 2h static, intermittent agitation – 3min at 25rpm every 2h – until 24h, continuous agitation until end of culture; A3 (red) – 24h static, continuous agitation at 25rpm until end of culture. All values were calculated from duplicate samples collected during each experiment (n=1).*

When considering fold increase and seeding yield numbers (**Figure IV.9**), protocol A1 showed the best results from all three experiments compared – with a fold increase of 5.8 ± 1.7 at day 5 when compared to the number of cells present after 24 h, and a seeding yield of 3.2 ± 1.2 at day 5 when compared to the initial cell numbers used for seeding, which was only bested by the seeding yield of protocol A3 with a value of 3.7 ± 0.1 at day 6.

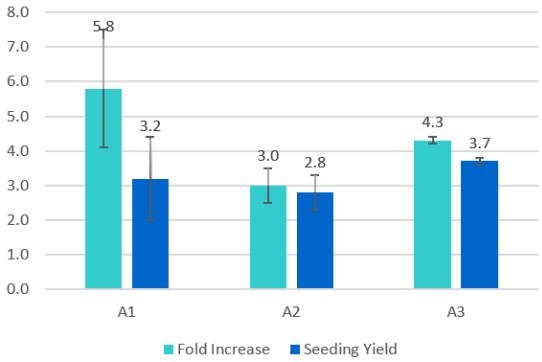



***Figure IV.9. Adhesion protocols of hiPSCs on Matrigel™-coated dissolvable microcarriers.*** *Values obtained for: fold increase and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on Matrigel™-coated dissolvable microcarriers, using Essential 8™ and culture medium. All values were calculated from duplicate samples collected during each experiment (n=1).*

In terms of cell distribution over the surface area available for expansion, which was monitored daily through microscopic visualisation, it was possible to observe that hiPSCs covered an area close to the maximum offered by the dissolvable microcarriers, although distribution seemed more even when using adhesion protocol A2, and more surface area seemed to remain available when using adhesion protocol A1. Regarding microcarrier aggregation, by the end of culture time, large microcarrier aggregates were visible in all three experiments, however, when using adhesion protocols A1 and A3, the aggregates obtained appeared to be larger than those formed when using protocol A2 (**Figure IV.10**); nevertheless, these conclusions were obtained through visual interpretation alone and are only as representative as the samples recovered for microscopy and cell quantification.

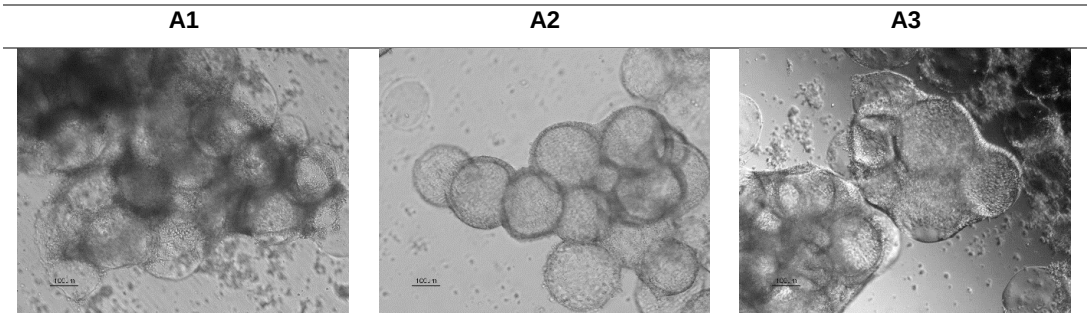



***Figure IV.10 Cell distribution and microcarrier aggregation during hiPSC expansion.*** *The images show the distribution of hiPSCs over the available microcarrier surface and aggregation of Matrigel™-coated dissolvable microcarriers at the final day of hiPSC expansion, for each of the adhesion protocols tested: A1 (left) – 24h static, 24h intermittent agitation – 3min at 25rpm every 2h –, continuous agitation until end of culture; A2 (centre) – 2h static, intermittent agitation – 3min at 25rpm every 2h – until 24h, continuous agitation until end of culture; A3 (right) – 24h static, continuous agitation at 25rpm until end of culture. Bar scale: 100µm.*

Considering all the results obtained, which showed that protocol A1 allowed to achieve higher cell numbers and obtain the highest fold increase in cell expansion, protocol A1 was kept as the protocol of choice for the rest of the experiments performed, keeping in mind that agitation could still be improved to prevent formation of large microcarrier aggregates and allow for a better cell distribution.

## IV.5.2. Scale-up of hiPSC expansion using dissolvable microcarriers

Once the adhesion protocol was established, different combinations of microcarriers, types of surfaces, and culture media were evaluated for expansion of hiPSCs under dynamic conditions.

In terms of cell adhesion (**Table IV.3**), cells used for inoculation adhere completely to the microcarriers available, and begin expansion within the first 24h of culture, with the exception of hiPSC expansion on Synthemax®II dissolvable microcarriers using mTeSR™1; in this case, cell quantifications after 24 h were between 49.0 ± 2.3% and 52.7 ± 5.6% with direct quantifications, and between 48.3 ± 0.8% and 59.6 ± 5.0% with indirect cell quantifications, These results are in accordance to those obtained in static experiments, where adhesion to Matrigel™-coated microcarriers was also higher than adhesion to Synthemax®II microcarriers. Adhesion to Matrigel™-coated dissolvable microcarriers in mTeSR™1 appears to be the highest – 187.5 ± 0.0% and 228.1% through direct and indirect cell quantifications, respectively – however, due to the gelatinous nature of Matrigel™, microcarriers in these systems formed very large aggregates within the first 24h of culture, making it virtually impossible to retrieve homogenous and representative samples for hiPSC quantifications, meaning that the values obtained after 24 h were highly overestimated and should not be taken into account without considering these problems.

***Table IV.3 hiPSC expansion in dynamic three-dimensional culture**. Values obtained for: difference in hiPSCs numbers 24h after inoculation of the microcarriers, determined through indirect (Alamar Blue) and direct cell quantification methods, fold increase and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™ and Synthemax®II surfaces, for cultures using Essential 8™ and mTeSR™1 culture media. All values were calculated from duplicate samples collected during each experiment (n=1).*

| Dynamic 3D Culture (30mL) – hiPSC expansion | | | | | | |
|---|---|---|---|---|---|---|
| Culture media | Coating | Microcarrier Type | After 24h | | Maximum cells | Maximum expansion |
| | | | Alamar Blue | Direct quantification | | |
| Essential 8™ | Matrigel™ | DM | 115.5 ± 2.0% | 66.5 ± 4.8% | 4.61 ± 0.17 x$10^7$ | Day 5 |
| | | PSM | 117.6 ± 33.5% | 55.3 ± 3.6% | 5.68 ± 0.78 x$10^7$ | Day 9 |
| mTeSR™1 | Matrigel™ | DM | 228.1% | 187.5 ± 0.0% | 3.22 ± 0.06 x$10^7$ | Day 5 |
| | Synthemax®II | PSM | 132.9 ± 39.1% | 88.6 ± 1.9% | 4.68 ± 0.07 x$10^7$ | Day 10 |
| | | DM | 48.3 ± 0.8% | 49.0 ± 2.3% | 3.80 ± 0.73 x$10^7$ | Day 5 |
| | | | 59.6 ± 5.0% | 52.7 ± 5.6% | 3.26 ± 0.27 x$10^7$ | Day 6 |

Regarding hiPSC expansion, in order to establish a base for comparison, hiPSCs were expanded on polystyrene microcarriers with Matrigel coating on Essential 8™ culture medium (PSM+Mat+E8) and on polystyrene microcarriers with Synthemax®II surface on mTeSR™1 medium (PSM+SII+mTeSR™1), systems that had been previously tested in the laboratory. hiPSC expansion in each of these systems yielded 5.68 ± 0.78x$10^7$ cells after 9 days of culture time, and 4.68 ± 0.07 x$10^7$ cells after 10 days of culture time, respectively (**Table IV.3**).

Even though none of the experiments performed on dissolvable microcarriers achieved a number of cells as high as any of the systems using polystyrene microcarriers – which were used as a basis for comparison –, the results obtained are still very promising. Although hiPSC expansion using PSM+Mat+E8 and PSM+SII+mTeSR1 allowed to achieve higher final cell numbers – 5.68 ± 0.78 x$10^7$ cell in 9 days and 4.68 ± 0.07 x$10^7$ cells in 10 days, respectively – hiPSC expansion on Matrigel™-coated dissolvable microcarriers using Essential 8™ culture medium (DM+Mat+E8) allowed for a faster expansion of the cells, achieving higher cell numbers – 4.61 ± 1.71x$10^7$ cells – after 5 days of culture. The higher efficiency of this system is even more evident when considering that at 5 days of culture time, hiPSC expansion on PSM+Mat+E8 had achieved 2.38 ± 0.07x$10^7$ cells, and on PSM+SII+mTeSR1 only 1.15 ± 0.04x$10^7$ cells.

Looking at hiPSC expansion on Synthemax®II dissolvable microcarriers (which would be the most interesting when considering establishing a xeno-free culture system) using mTeSR™1 culture medium (DM+SII+mTeSR1), which achieved their highest of 3.80 ± 0.72x$10^7$ cells (purple) and 3.26 ± 0.27x$10^7$ cells (pink) between days 5 and 6, respectively, the results obtained, while not as high as those obtained with expansion on DM+Mat+E8, are still higher than the number of cells obtained through expansion on polystyrene microcarriers within that culture time (**Figure IV.11**).

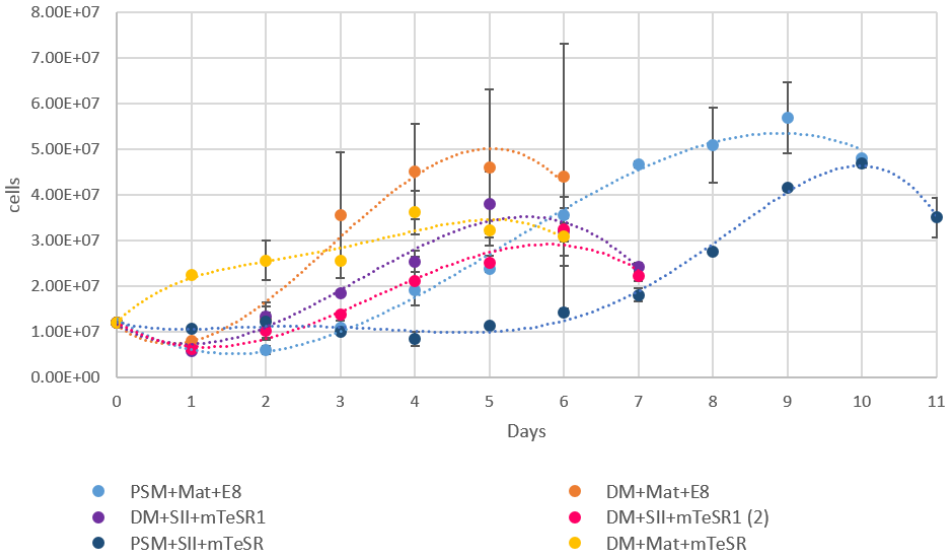



***Figure IV.11 hiPSCs expansion in 3D dynamic culture conditions in spinner-flasks (V=30mL).*** *The graphic represents the number of cells present in the culture vessel at each day of culture time, regarding each condition used for the experiments; the curves used as basis for comparison of expansion are represented in light blue (PSM+Mat+E8) and dark blue (PSM+SII+mTeSR1), the remaining curves show hiPSC expansion on dissolvable microcarriers, in different culture conditions: in orange DM+Mat+E8, in purple and pink DM+SII+mTeSR1, and in yellow DM+Mat+mTeSR1. The dotted curves represent polynomial regressions of the values obtained All values were calculated from duplicate samples collected during each experiment (n=1). Key: PSM – polystyrene microcarriers; DM – dissolvable microcarriers; Mat – Matrigel™; SII – Synthemax®II; E8 – Essential 8™ culture medium; mTeSR1 – mTeSR™1 culture medium.*

Considering the differences between the number of hiPSCs used to inoculate the spinners, and the number of cells achieved by the end of culture, hiPSC expansion achieved higher fold increase and seeding yield when cells were cultured on PSM+Mat+E8 (8.6 ± 1.2 and 4.7 ± 0.6, respectively). hiPSC expansion on DM+Mat+E8 achieved 5.8 ± 1.7 and 3.2 ± 1.2, fold increase and seeding yield,

respectively and expansion on DM+SII+mTeSR1 reached 6.5 ± 1.2 and 3.2 ± 0.6, on the first experiment, and 5.2 ± 0.4 and 2.7 ± 0.2 on the second experiment, for those same parameters. Cell expansion on DM+Mat+mTeSR1 proved to be the least productive (**Figure IV.12**).

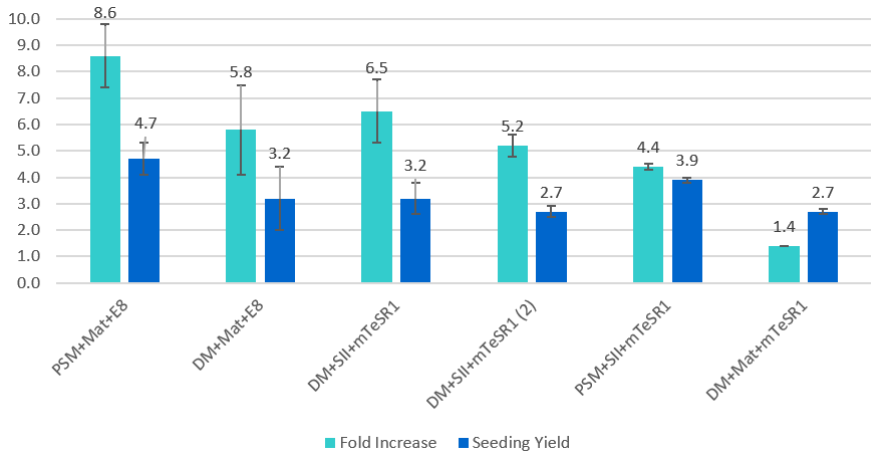



***Figure IV.12 hiPSC expansion in dynamic three-dimensional culture**. Values obtained for: fold increase and seeding yield (calculated from the highest values obtained from direct cell quantifications) considering only the cells that were recovered, on polystyrene (PSM) and dissolvable (DM) microcarriers, with Matrigel™(Mat) and Synthemax®II (SII) surfaces, for cultures using Essential 8™ (E8) and mTeSR™1 culture media. All values were calculated from duplicate samples collected during each experiment (n=1).*

It is important to keep in mind that the conditions – cell density and microcarrier surface area – used in these experiments, had been optimised for expansion on polystyrene microcarriers, therefore, hiPSC expansion on dissolvable microcarriers still has room for optimisation, and could achieve even better results.

Regarding cell distribution over the microcarriers' surface area, it is possible to observe that 24 h after inoculation on dissolvable microcarriers cells start to expand between the microcarriers, which begins formation of small microcarrier aggregates; on polystyrene microcarriers cell distribution appears to be more homogeneous. At the final day of culture, it was still possible to observe some empty microcarriers in all situations but large aggregates completely covered with hiPSCs were larger on cultures using dissolvable microcarriers than on those using polystyrene microcarriers (**Figure IV.13**). Once again, these conclusions were obtained through visual interpretation alone and are only as representative as the samples recovered for microscopy and cell quantification.

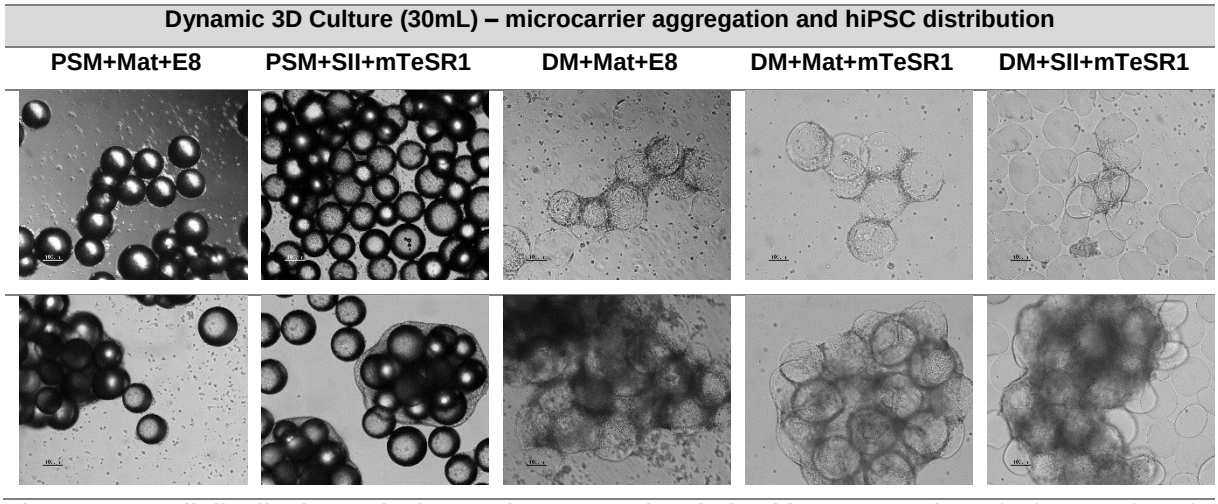



***Figure IV.13 Cell distribution and microcarrier aggregation during hiPSC expansion.*** *The images show the distribution of hiPSCs over the available microcarrier surface and aggregation 24h after inoculation (above) and at the final day of culture time (below). Bar scale: 100µm.*

Overall, by allowing to achieve higher cell numbers within a shorter time frame, hiPSC expansion on dissolvable microcarriers seem to be the most cost effective choice, making dissolvable microcarriers a very promising product, as long as hiPSCs were conserving all their characteristics.

#### *IV.5.2.1. hiPSC characterisation after expansion on 3D dynamic culture systems*

After expansion of hiPSCs in each of the experiments performed, samples of cells attached to the surface of the microcarriers were recovered from the spinner-flasks and characterised in order to guarantee that cells retained their main features after being subjected to shear stress and other stimuli provided during time in culture, including different types of microcarriers cores and coatings.

##### IV.5.2.1.1. Immunocytochemistry analysis

Following each experiment, cells attached to the surface of the microcarriers were analysed through immunocytochemistry, to detect known pluripotency markers, namely OCT4, SSEA4 (**Figure IV.14**), and TRA-1-60 (results not shown), as well as the early differentiation marker SSEA1 (results not shown).

The immunostaining analysis of the cells with DAPI counterstaining after culture on polystyrene and dissolvable microcarriers, showed that, in all cases, 90-100% of the cells showed a positive result regarding the pluripotency markers analysed and an even distribution of those throughout all the cells, while at the same time, the analysis of SSEA1 marker was completely negative, which is a good indication that hiPSCs do maintain their phenotype and pluripotency characteristics when cultured under these conditions and that no cells had started to differentiate.

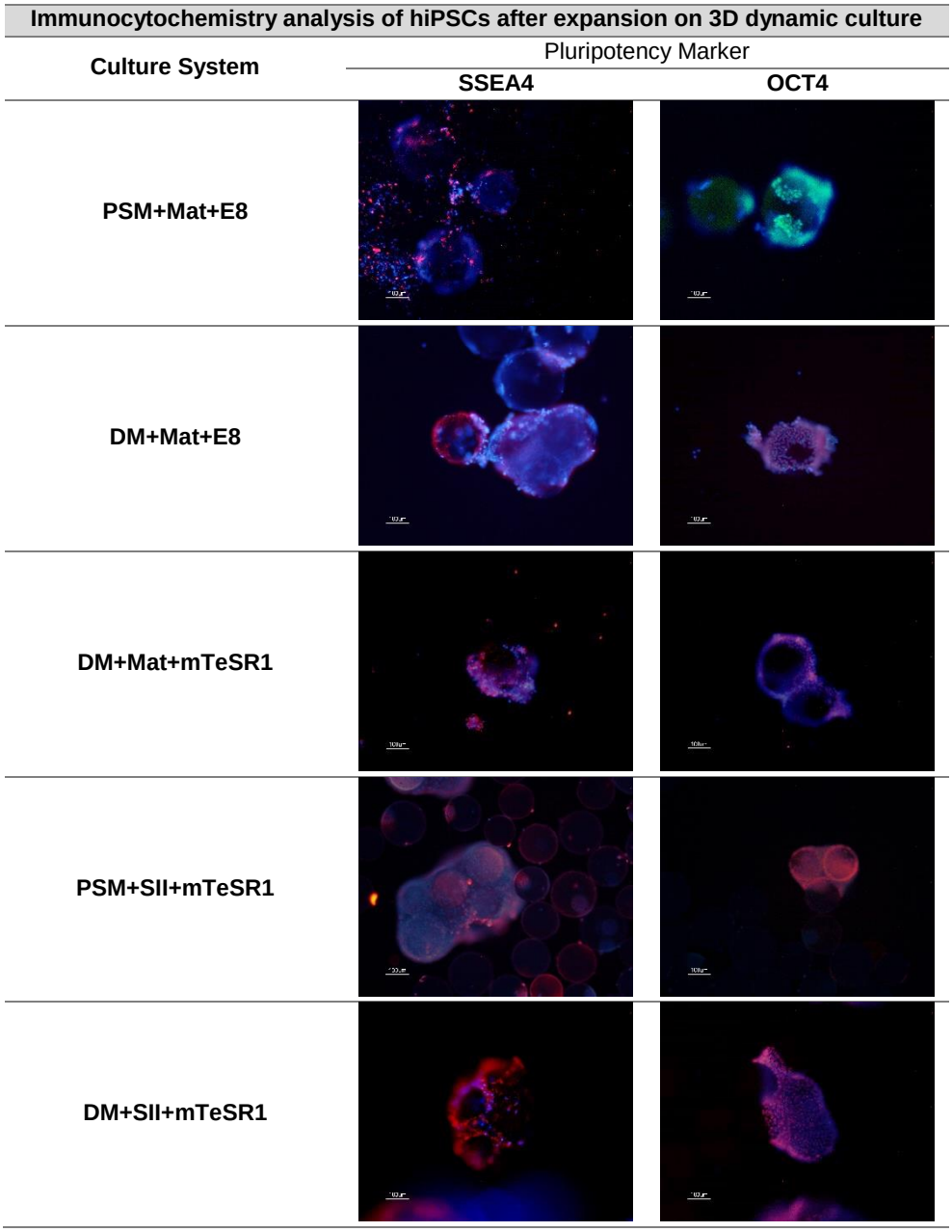



***Figure IV.14 Immunocytochemistry analysis of hiPSCs after expansion on 3D dynamic culture.*** *Immunocytochemistry results obtained after merging immunofluorescence imaging (red and green) of pluripotency markers SSEA4 and OCT4 and DAPI counterstaining imaging (blue). Images were obtained from cell/microcarrier samples obtained on the final day of expansion in 3D dynamic culture. Bar scale: 100µm.*

### IV.5.3. hiPSC recovery efficiency

After being cultured within the spinner-flasks, hiPSCs were recovered using different harvesting protocols in order to compare the efficiency of each one and perform characterisation of the cells after recovery. Following the application of the recovery protocols, cells were re-plated onto Matrigel™-coated multi-well plates in order to understand if they retained their ability to expand as colonies and to characterise those cells through immunocytochemistry assays to establish whether cells still expressed pluripotency surface and intracellular markers.

In order to be able to establish a direct comparison with polystyrene microcarriers, cell recovery was conducted using two different harvesting protocols:

- A – Using Accutase and a 100µm mesh cell strainer
- B – Using Accutase and pectinase/EDTA harvesting solution

The goal was to understand whether or not cell loss during harvesting from the microcarriers could be reduced and to determine if the use of the harvesting solution would have any impact on cell viability after cell recovery.

In terms of recovery efficiency, the results obtained (**Table IV.4**) are still inconclusive since the cell harvesting protocol is yet to be optimised and these are only preliminary results, but the recovery efficiencies from both protocols were almost identical. The average cell recovery obtained using harvesting protocol A (protease + strainer) is 51.1 ± 9.4%. The lowest recovery obtained with this protocol (18.1%) was attained using dissolvable microcarriers – this low efficiency may be due to prolonged exposure to the protease but was at first attributed to dissolvable microcarriers being able to pass through the strainer and contaminate the cell suspension; however, dissolvable microcarriers are moderately rigid, and have a narrow size distribution (200-300 µm), which should prevent any microcarriers from passing through a 100 µm mesh strainer, and therefore, should not interfere with cell recovery. When using protocol B (protease + harvesting solution) the average cell recovery is 50.8 ± 7.8%. Regarding cell viability, the use of harvesting protocol A has a viability average of 86.5 ± 5.4%, while protocol B shows, on average, a cell viability of 95.4 ± 2.4%, slightly higher than the first one.

***Table IV.4 hiPSC recovery efficiency after expansion on microcarriers**. Values obtained for cell recovery efficiency after expansion of hiPSCs under 3D dynamic culture conditions, using different combinations of polystyrene (PSM) and dissolvable (DM) microcarriers, Matrigel™ (Mat) and Synthemax®II (SII) surfaces, and Essential 8™ (E8) and mTeSR™1 culture media, and corresponding viability of the cells recovered, comparing two different harvesting protocols: A - use of protease and a 100µm mesh cell strainer; B – use of protease and pectinase/EDTA harvesting solution.*

| **Cell recovery efficiency after expansion** | | |
|---|---|---|
| **hiPSC expansion conditions** | Cell recovery Efficiency % (cell viability %) | |
| | **Harvesting Protocol** | |
| | **A** | **B** |
| **PSM+Mat+E8** | 56.0 | - |
| **PSM+SII+mTeSR1** | 41.4 (78.9) | - |
| **DM+Mat+mTeSR1** | 18.1 (89.8) | 42.2 (93.0) |
| **DM+SII+mTeSR1** | 42.9 | 61.0 |
| **DM+SII+mTeSR1 (2)** | 64.0 (90.9) | 49.3 (97.8) |

These experiments are only the preliminary testing regarding cell recovery from dissolvable microcarriers, and recovery protocol B has yet to be optimised. The ultimate goal would be to be able to recover cells without the use of a protease. As it is, protocol B already allows for easier cell recovery, with the benefit of avoiding the risk of microcarriers debris on cell suspension (broken microcarriers that

get through the filters). Scale-up of this protocol would also be easier to perform, and more cost effective, as there would be no need for a large-scale filtration step.

#### *IV.5.3.1. hiPSC characterisation after recovery from the microcarriers*

After recovery of the cells from the microcarriers, further characterisation was performed, to guarantee that cells retained their main features after expansion on 3D dynamic systems and after being subjected to different cell harvesting protocols.

##### IV.5.3.1.1. hiPSC re-plating

hiPSCs were re-plated on Matrigel-coated multi-well plates (**Figure IV.15**); re-plating of the cells allowed to confirm that hiPSCs did not lose their characteristic colony morphology after being expanded in 3D dynamic culture conditions, and were able to expand normally as 2D monolayers.

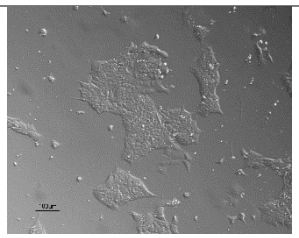
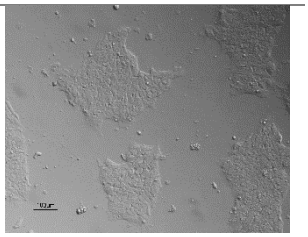
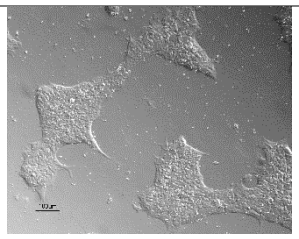
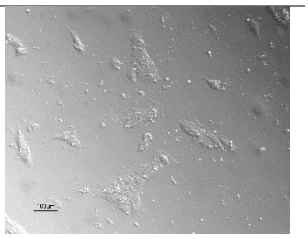
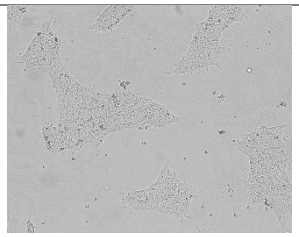
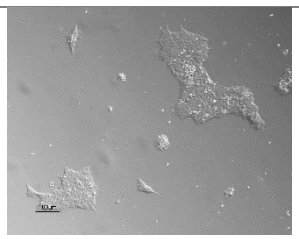
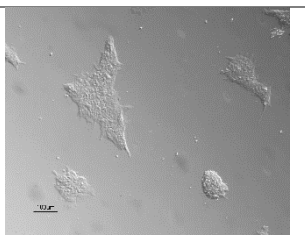
| Re-plated hiPSCs | | |
|---|---|---|
| **Culture System** | **Harvesting Protocol** | |
| | **A** | **B** |
| **PSM+Mat+E8** |  | Not applicable |
| **DM+Mat+E8** | Not tested |  |
| **DM+Mat+mTeSR1** |  |  |
| **PSM+SII+mTeSR1** |  | Not applicable |
| **DM+SII+mTeSR1** |  |  |

***Figure IV.15 Bright field microscopy of re-plated hiPSCs recovered after expansion under 3D dynamic culture conditions.*** *On the right (A) hiPSCs recovered using protease treatment followed by filtration with a strainer, on the left (B) hiPSCs recovered using protease treatment and harvesting solution. Bar 100µm.*

#### IV.5.3.1.2. Immunocytochemistry analysis of re-plated hiPSCs

When colonies reached the appropriate size, immunocytochemistry analysis was performed in order to characterise the cells obtained and make sure cells were expressing pluripotency markers and not showing signs of differentiation.

The immunocytochemistry analysis of the colonies obtained showed that all cells maintained normal expression of pluripotency markers OCT4 and NANOG (**Figure IV.16**), as well as SSEA4 and TRA-1-

60 (results not shown), and there was no expression of early differentiation marker SSEA1 (results not shown), which allows the conclusion that neither of the harvesting protocols used affect hiPSCs in any of the characteristics analysed.

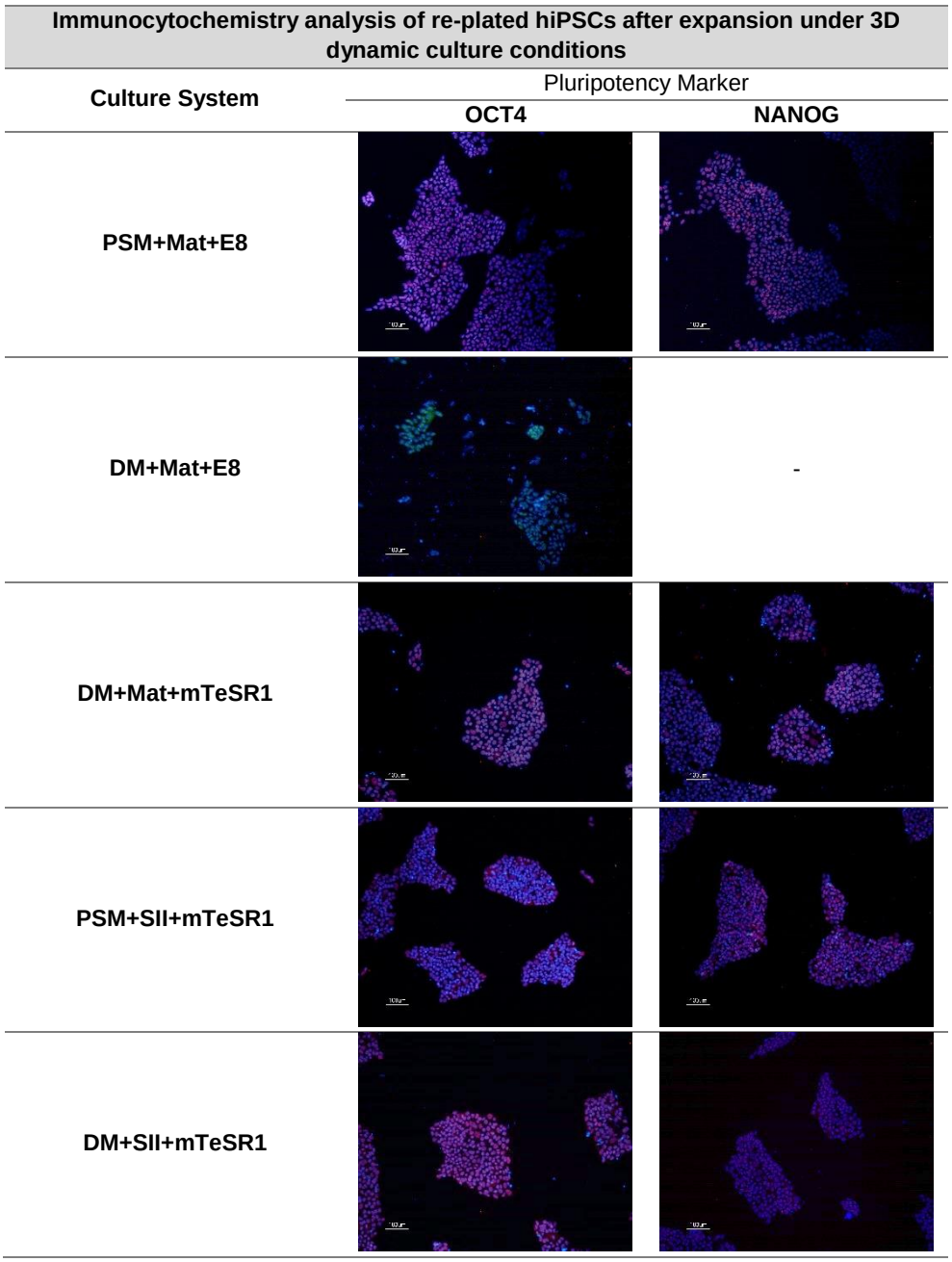



***Figure IV.16 Immunocytochemistry analysis of re-plated hiPSCs recovered after expansion under 3D dynamic culture conditions.*** *Merging of immunofluorescence imaging (red or green) of intracellular pluripotency markers OCT4 and NANOG, with DAPI counterstaining imaging (blue), on days 2-3 after re-plating on Matrigel-coated multi-well plates. Bar scale: 100µm.*

#### IV.5.3.1.3. Flow cytometry

For the characterisation of cell populations used to inoculate the microcarriers, and the resulting populations after expansion under stirred culture conditions, samples of both cell populations were analysed through flow cytometry. This analysis was performed in order to quantify cells expressing

pluripotency markers and the alterations in that expression after expansion; the goal was to determine the percentage of cells expressing pluripotency markers and verify if those numbers were maintained at the end of culture.

According to the values obtained (**Table IV.5**), in hiPSCs cultured on dissolvable microcarriers, expression of pluripotency marker OCT4 decreased only when cells were expanded on Matrigel™-coated dissolvable microcarriers on Essential 8™ culture medium (DM+Mat+E8) (2.8% less expression of OCT4 after 5 days of culture), which may be attributed to the transition from a 2D static culture system to a 3D dynamic culture system. Expression of SSEA4 and TRA-1-60 never decreased when compared to the expression of those same pluripotency markers on the respective hiPSC population used to inoculate the spinner-flask, with expression values increasing 0.5 to 5.5%. In all cases, the differences observed are not very significant – therefore it is possible to consider that no alterations in expression of pluripotency markers occurred. Regarding cells expanded on polystyrene microcarriers, OCT4 and TRA-1-60 expression dropped 9.9 and 17.4% respectively when cells were cultured on PSM+Mat+E8, which could be due to the prolonged time of cell culture, however, no significant alterations were obtained on hiPSCs cultured on PSM+SII+mTeSR1 which could indicate that prolonged culture time on its own is not a decisive factor, and only affects cells cultured on a specific substrate (Matrigel™) or with a specific culture medium (E8™).

***Table IV.5 Flow cytometry analysis of hiPSCs obtained after expansion under three-dimensional dynamic culture conditions.*** *Percentage of cells expressing the pluripotency markers; cells were analysed at day 0 (inoculation) of each experiment, and on the final day of each expansion in order to quantify the cells expressing surface – TRA-1-60 and SSEA4 – and intracellular – OCT4 – pluripotency markers. DM – dissolvable microcarriers; PSM – polystyrene microcarriers; Mat – Matrigel™; SII – Synthemax®II; E8 – Essential 8™; mTeSR1 – mTeSR™1; A1 – adhesion protocol 1; A2 – adhesion protocol 2; A3 – adhesion protocol 3.*

| **hiPSC characterisation after expansion on 3D dynamic culture - Flow cytometry analysis** | | | | |
|---|---|---|---|---|
| **Culture Conditions (Adhesion Protocol)** | **Day** | **% of cells expressing the pluripotency marker** | | |
| | | **OCT4** | **TRA-1-60** | **SSEA4** |
| **DM+Mat+E8 (A2)** | 0 | 97.7 | 96.2 | 97.9 |
| | 5 | 94.9 | 96.7 | 99.7 |
| **DM+Mat+E8 (A3)** | 0 | 95.5 | 90.5 | - |
| | 7 | 96.6 | 96.0 | - |
| **DM+SII+mTeSR1 (A1)** | 0 | 95.5 | - | 99.0 |
| | 7 | 98.4 | - | 91.8 |
| **DM+Mat+mTeSR1 (A1)** | 0 | 94.8 | - | 95.3 |
| | 6 | 98.5 | - | 99.7 |
| **PSM+Mat+E8 (A1)** | 0 | 98.3 | 92.5 | - |
| | 10 | 88.4 | 75.1 | - |
| **PSM+SII+mTeSR1 (A1)** | 0 | 99.5 | - | 96.6 |
| | 11 | 99.0 | - | 89.8 |

Overall, hiPSC expansion on dissolvable microcarriers seems to have no negative effect on pluripotency markers expression.

#### IV.5.3.1.4. Embryoid body formation

In order to verify if cells obtained after expansion on dissolvable microcarriers under dynamic culture conditions retained their ability to differentiate into cell types derived from the three germ layers of embryogenesis, some of the cells recovered after expansion were used for embryoid body formation assays.

Two independent assays were performed, using cells cultured on dissolvable microcarriers with Matrigel™ or Synthemax®II surfaces. On both experiments, the cells obtained at the end of the assay showed positive results for markers of cell types from each of the three germ layers (**Figure IV.17**) as well as the morphology expected, and negative results for the pluripotency marker tested (OCT4, results not shown), showing that even after being subjected to different stimuli during expansion under 3D dynamic culture conditions, hiPSCs maintain their ability to differentiate into cell types from the three germ layers of embryonic development, which is one of their two main characteristics.

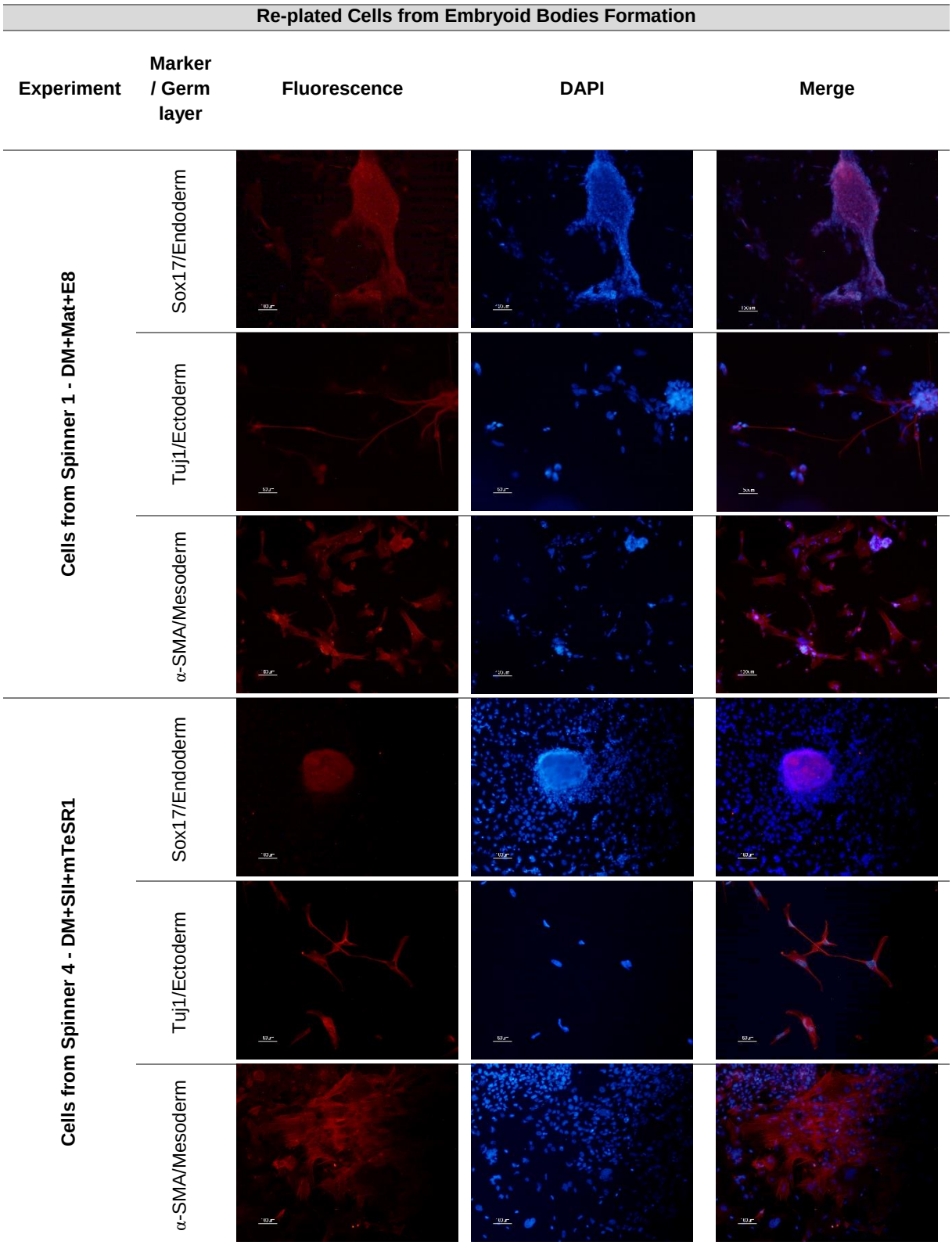



***Figure IV.17 Immunocytochemistry analysis of re-plated cells derived from hiPSCs through embryoid bodies differentiation****. On the left, immunofluorescence imaging (red) of differentiation markers SOX17, TUJ1, and α-SMA; at the centre, DAPI counterstaining imaging (blue); on the right, merging of both images to show relative positioning of the nuclei. Analysis performed 7 days re-plating of the cells from embryoid body differentiation on laminin-coated multi-well plates.*

# V. CONCLUSIONS

hiPSC culture *in vitro* is a rather challenging process, but many progresses have been achieved in recent years. In the development of new supports for hiPSC expansion is it very important to take into account the need to comply with GMP, the development of chemically-defined and xeno-free systems – which in this case include both the microcarriers and surface coating –, the possibility of scaling up the system, the downstream processing, and ultimately, the cost effectiveness of the whole process.

Regarding hiPSC adhesion and expansion on dissolvable microcarriers in 3D culture systems, overall, adhesion to dissolvable microcarriers was comparable to adhesion to polystyrene microcarriers when using Matrigel™ coating, and although adhesion was lower when using Synthemax®II dissolvable microcarriers, the fold increase obtained at the end of the expansion was higher when compared to Synthemax®II polystyrene microcarriers.

hiPSC expansion under 3D dynamic culture conditions allowed to achieve higher cell numbers – 4.61 ± 0.17x$10^7$ cells on Matrigel™-coated dissolvable microcarriers using Essential 8™ culture medium, and 3.80 ± 0.73x$10^7$ cells on Synthemax®II dissolvable microcarriers using mTeSR™1 culture medium – within a shorter time frame (5 days), than the cell numbers obtained after expansion on polystyrene microcarriers, which took 9 days to achieve maximum yield; meaning hiPSC expansion on dissolvable microcarriers is a more cost effective choice when directly compared to polystyrene microcarriers, and making dissolvable microcarriers a very promising product for hiPSC expansion.

Concerning cell recovery, efficiency and cell viability results obtained using two different cell harvesting protocols were very similar – both on harvesting from polystyrene microcarriers using a protease and a strainer (51.1 ± 9.4% cell recovery with 86.5 ± 5.4% cell viability), and on recovery from dissolvable microcarriers using a pectinase/EDTA harvesting solution (50.8 ± 7.8% cell recovery with 95.4 ± 2.4%,); Nevertheless, cell recovery protocol from dissolvable microcarriers is simpler, easier, and requires less manipulation of the cells, requiring only one additional reagent that can be used together with the protease used for cell harvesting from polystyrene microcarriers.

Since both cell recovery efficiency and cell viability after recovery are very similar, and there is no alteration of the characteristics of the cells after re-plating, the choice between polystyrene and dissolvable microcarriers has to be made taking into account not only hiPSC expansion, but also microcarrier preparation and manipulation, and the differences between the harvesting protocols.

Due to their composition, dissolvable microcarriers adhere very easily to polystyrene surfaces, which means that all manipulations should be performed using glass materials and failing to do so will result in extensive losses of microcarriers during manipulation, especially in the presence of culture media. Also, due to their transparent appearance, dissolvable microcarriers are very difficult to identify in culture media when they are not covered in cells – which could be easily overcome by adding some colour to the microcarriers. Nevertheless, because packaged dissolvable microcarriers are completely sterilised through γ-radiation and, in some cases, their surface is functionalised with small peptides during production (Synthemax®II dissolvable microcarriers), preparation time for these microcarriers requires

only that they are fully hydrated before being used, which widely shortens preparation time and, therefore, the time needed to begin an experiment. Moreover, regardless of cell recovery efficiency, the recovery protocol itself – using a combination of a protease and a pectinase/EDTA harvesting solution – requires less mechanical manipulation of the cells which makes it a less time consuming and more practical approach to cell recovery than the traditional protocol of protease incubation and filtration step, especially when considering large scale production. Furthermore, the translucent appearance that makes it so challenging to work with dissolvable microcarriers is also one of their best characteristics; because these microcarriers are completely transparent they are much easier to observe through microscopic analysis, allowing for better visualisation of the cells on the surface of the microcarriers.

Taking all this into consideration, although it may present some challenges, working with dissolvable microcarriers allows a faster and easier preparation of the microcarriers, a simpler and faster recovery of the cells after expansion, and, in general, a better monitoring of cell growth throughout expansion.

Overall, taking into consideration all the results obtained, the use of dissolvable microcarriers for *in vitro* expansion of hiPSCs seems like a very promising solution for effective cell culture, easier recovery of the cells obtained – both in terms of time and manipulation –, and regarding the costs associated with the process.

# VI. FUTURE WORK

The experiments described throughout this report represent only the initial studies of hiPSC expansion on dissolvable microcarriers.

The next steps should include further optimisation of hiPSC expansion under dynamic conditions using laboratory scale spinner flasks, and further testing and optimisation of cell recovery after said expansion. All experiments should be replicated for this cell line and confirmed using a different hiPSC line to properly validate all the results obtained so far. In addition to proper validation it would be important to perform a complete characterisation of the cells recovered after expansion on dissolvable microcarriers to complement the characterisation that was described here; the full characterisation panel should include: immunostaining for detection of pluripotency markers, alkaline phosphatase staining, flow cytometry analysis, RT-PCR, analysis of Embryoid Bodies (EBs) formation and spontaneous differentiation into cell types from the three germ layers, karyotype analysis, and, ultimately, the formation of teratomas on immunocompromised mice.

Another very important step will be a parallel cost analysis in order to compare the cost of hiPSC *in vitro* production using polystyrene microcarriers and a harvesting protocol that includes the use of a protease and a strainer, with the production of the same amount of cells on dissolvable microcarriers using a recovery protocol that requires a protease and the appropriate harvesting solution.

If hiPSC expansion on dissolvable microcarriers and respective recovery of those cells proves to be successful, the final outcome from these experiments will be the establishment of a new protocol for hiPSC expansion under chemically-defined and xeno-free conditions, and a downstream cell recovery process which does not require the filtration step to separate cells from the microcarriers, but rather relies on the dissolution of the microcarriers to recover the cells without damage or loss. Further optimisation of cell recovery would ideally lead to a protease-free recovery protocol, but at this time, this has yet to be accomplished. Ultimately, the protocol established ought to comply with all GMP guidelines and should be an important step towards an integrated process of hPSC expansion – with or without controlled differentiation of the cells after expansion – and downstream processing within a closed system.

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