Scalable Expansion of Human Induced Pluripotent Stem Cells (hiPSC) under Xeno-free Conditions

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Thesis to obtain the Master of Science Degree in Biological Engineering

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O sonho é ver as formas invisíveis
Da distância imprecisa, e, com sensíveis
Movimentos da esp’rança e da vontade,
Buscar na linha fria do horizonte
A árvore, a praia, a flor, a ave, a fonte –
Os beijos merecidos da Verdade.

Fernando Pessoa, Mensagem, “Horizonte”

The dream consists in seeing the invisible shapes
Of the hazy distance, and, with perceptible
Moviments of hope and will,
Search out in the cold line of the horizon
The tree, the beach, the flower, the bird, the spring –
The well deserved kisses of Truth.

Fernando Pessoa, Message, “Horizon”
ABSTRACT

Human induced pluripotent stem cells (hiPSCs) represent a very promising tool in biomedical research field by being capable of making multiple copies of themselves for long periods in culture and generating cells from the three embryonic germ layers upon differentiation. Thus and to fully realize their potential for disease modelling, drug screening and cell therapy, it is mandatory the conception of chemically defined culture systems for large-scale production of hiPSCs, under good manufacturing practices (GMP), free of xenogeneic components and without batch-to-batch variability. In this work, an optimized xeno- and serum-free culture system is proposed for the maintenance of hiPSCs using a microcarrier-based system in spinner-flasks. A multifactorial design was used to identify the operation conditions that maximize the expansion index of hiPSCs in culture. Cells were seeded at 55000 cells/cm² in vitronectin-coated beads, after an EDTA treatment with ROCK inhibitor, and cultured with xeno-free E8 medium. An agitation speed of 44 rpm was used for a proper homogenization of the medium. Under this optimized protocol, an expansion index of $4.4 \pm 0.7$ was achieved after 10-13 days of culture. Cells were further characterized in terms of pluripotency by intracellular immunostaining for markers Oct4 and Nanog and flow cytometry. Cells were $92 \pm 2\%$ positive for Oct4, $97 \pm 1\%$ for Sox2 and $98 \pm 1\%$ for Nanog. The proposed culture system provides an efficient and cost effective platform for the expansion of hiPSCs for numerous applications, from basic biological research to clinical development.

Key words: hiPSCs, vitronectin, E8 medium, microcarriers, spinner-flask
RESUMO

As células humanas pluripotentes induzidas (hiPSCs) constituem um instrumento promissor na investigação biomédica. Visando tirar o máximo partido do seu potencial na modelação de doenças, rastreio de fármacos e terapia celular, é indispensável a concepção de um sistema de cultura quimicamente definido para a produção em massa de hiPSCs, sob condições GMP, livre de componentes de origem animal e sem variabilidade entre produções. Neste trabalho, é proposto um sistema optimizado, livre de soro e de componentes xenogénicos, para a manutenção de hiPSCs em microcarriers cultivadas em spinner-flasks. Uma análise multifactorial foi usada para identificar as condições de operação que maximizam o índice de expansão de hiPSCs em cultura. Foi usada uma densidade de 55000 células/cm² para a inoculação celular, após tratamento com EDTA e inibidor ROCK, em microcarriers revestidas com vitronectina e cultivadas com meio E8. Uma agitação de 44 rpm foi igualmente usada para uma correcta homogeneização do meio. Sob estas condições optimizadas foi obtido um índice de expansão de 4.4 ± 0.7 após 10-13 dias em cultura. As células geradas foram, posteriormente, caracterizadas em termos de pluripotência por imunofluorescência intracelular, aos marcadores Oct4 e Nanog, e por citometria de fluxo. As células apresentaram-se positivas em 92 ± 2% para Oct4, 97 ± 1% para Sox2 e 98 ± 1% para Nanog. O proposto sistema de cultura representa uma plataforma eficiente e económica para a expansão de hiPSCs para diversas aplicações, desde investigação biológica básica a desenvolvimento clínico.

Palavras-Chave: hiPSCs, vitronectina, meio E8, microcarriers, spinner-flasks
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<th>Description</th>
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<tbody>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALKs</td>
<td>activin receptor-like kinases</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>AXIN</td>
<td>axis inhibitor</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dsh</td>
<td>dishevelled</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ESCs</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FC-CD</td>
<td>face-centered composite design</td>
</tr>
<tr>
<td>Fi</td>
<td>fold increase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practices</td>
</tr>
<tr>
<td>GSK3-β</td>
<td>glycogen synthase kinase-3 beta</td>
</tr>
<tr>
<td>GT</td>
<td>Geltrex</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HARV</td>
<td>high aspect ratio vessel</td>
</tr>
<tr>
<td>hESCs</td>
<td>human embryonic stem cells</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>human induced pluripotent stem cells</td>
</tr>
<tr>
<td>hPSCs</td>
<td>human pluripotent stem cells</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Klf4</td>
<td>kruppel-like factor 4</td>
</tr>
<tr>
<td>KO-DMEM</td>
<td>knockout Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>LEF</td>
<td>lymphoid enhancer factor</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MYHs</td>
<td>myosin II heavy chains</td>
</tr>
<tr>
<td>NASA</td>
<td>national aeronautics and space administration</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>Oct4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>OOC</td>
<td>optimal operational condition</td>
</tr>
<tr>
<td>PAS</td>
<td>peptide-acrylate surface</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDK-1</td>
<td>phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol (3, 4)-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3, 4)-triphosphate</td>
</tr>
<tr>
<td>PMEDSAH</td>
<td>poly [2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide]</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>rho-associated coiled coil protein kinase</td>
</tr>
<tr>
<td>ROCKi</td>
<td>ROCK inhibitor</td>
</tr>
<tr>
<td>RPH</td>
<td>revolutions per hour</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SC</td>
<td>single-cells</td>
</tr>
<tr>
<td>SMAD</td>
<td>smad and mothers against decapentaplegic related protein</td>
</tr>
<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>SSEA</td>
<td>stage specific embryonic antigens</td>
</tr>
<tr>
<td>SSB</td>
<td>stirred-suspension bioreactor</td>
</tr>
<tr>
<td>STLV</td>
<td>slow turning lateral vessel</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TRA</td>
<td>tumor rejection antigens</td>
</tr>
<tr>
<td>VTN-N</td>
<td>vitronectin</td>
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<tr>
<td>WNT</td>
<td>wingless-type mmtv integration site family members</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
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<tr>
<td>3D</td>
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I. **AIM OF STUDIES**

To fulfill all the clinical potential of human induced pluripotent stem cells, robust and reproducible culture systems must be developed for the production of large quantities of cells under GMP conditions. These systems should be totally free of contaminants and chemically defined, to ensure all the required clinical safety, and capable in maintaining iPSCs self-renewal as well as their pluripotency and differentiation potential.

In the last decades, significant progress has been made towards the development of both xenogeneic free and chemically defined culture systems for human pluripotent stem cells (hPSCs). The early culture conditions for hPSCs were established by effectively following the developed methods for mouse embryonic stem cells, particularly using feeder-cell layers. Over time, these cultures were replaced by feeder-free systems, using for example biological substrates, coupled with serum-based or serum-free media.

This work aims to establish an optimized and scalable culture system for the expansion of hiPSCs, under xeno-free and chemically defined conditions, using human vitronectin-coated microcarriers in spinner-flasks supplied with serum- and xeno-free Essential 8™ medium. For this purpose, the influence of three culture parameters will be evaluated, namely cell dissociation method, initial hiPSCs density and agitation speed, and a feeder-free hiPSC line will be used as model. Generated cells will be further characterized concerning their pluripotency through intracellular immunostaining and flow cytometry.
II. INTRODUCTION

II.1 Human Stem Cells

Human stem cells are unspecialized cells with a remarkable ability for self-renewal and differentiation into functional and specific tissue cells under defined and controlled conditions. Human adult stem cells, as being the precursors of all cells in the human body, can only be found in particular anatomic regions, known as stem-cell niches, like bone marrow, umbilical cord blood, intestine, hair follicle, adipose tissue, brain and muscle (1) (2) (3).

Owing to their properties, they have been identified as a potential source and starting point for the development of cell-based therapies for regenerative medicine and gene therapy, as well as the main instrument for drug screening and disease modeling. Therefore, and in the last few decades, many and intensive studies have been performed in order to fully characterize and comprehend stem cells cellular biology, in particular, signals and conditions regulating their survival, self-renewal and differentiation.

According to their differentiation potential, stem cells can be classified into totipotent, pluripotent or multipotent stem cells. Both totipotent and pluripotent cells are capable of giving rise to all cell types in a living organism, but only the first class has the additional ability to specialize into extra embryonic tissues. Multipotent stem cells are more differentiated thus less versatile being only capable of generating a limited number of cell types (4).

II.1.1 Human Pluripotent Stem Cells

A single human pluripotent stem cell (hPSCs) is capable of giving rise to all cells derived from the three germ layers, known as mesoderm, endoderm and ectoderm, hence all cells of the human body (Figure II-1).

Mesoderm gives rise to bone, cartilage, muscle and connective tissues, while endoderm is responsible for the formation of endocrine glands, liver, pancreas, as well as gastrointestinal and respiratory tracts. Lastly, ectoderm generates skin, hair, lens of the eye and nervous system.

The most studied human pluripotent stem cells are human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).
Human pluripotent stem cells can give rise to all cells derived from the three embryonic germ layers, namely mesoderm, endoderm and ectoderm (5).

II.1.1.1 Human Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst, an early stage of the mammalian embryo, and they were first isolated from a human embryo in 1998 by James Thompson and his co-workers at University of Wisconsin (6). Human ESCs can be isolated from blastocysts, obtained, for example, from in vitro fertilized eggs donated for research purposes, and then cultured under appropriate conditions (Figure II-2).

Figure II-2 Embryonic stem cells are isolated from blastocysts after an in vitro fertilization (7).
Despite their enormous potential due to their pluripotency, long-term self-renewal capacity and relatively easy isolation and maintenance in culture, there are some ethical concerns regarding the sacrifice of human embryos for hESCs generation that may limit and compromise their use in research studies and cell-based therapies.

II.1.1.2 Induced Pluripotent Stem Cells

In 2006, a significant breakthrough in stem cell research field was established when Takahashi and Yamanaka surprised the scientific community by showing that enforced expression of four key transcription factors, Oct4, Sox2, Klf4 and c-Myc, in mouse somatic cells was enough to reprogram them to a pluripotency-like state (8). One year later, the team from Kyoto University reported a successful reprogramming of human somatic cells to pluripotency by transduction of the same four transcription factors. These so called human induced pluripotent stem cells were similar to human embryonic stem cells in terms of morphology, proliferation, feeder dependence, surface antigens, gene expression profile, telomerase activity, and were also able to differentiate in vitro into all cell types of the three germ layers (Figure II-3) (9).

![Figure II-3](image)

Figure II-3 Generation of hiPSCs through enforced expression of four transcription factors (Oct-4/3, Sox2, Klf4 and c-Myc) in human somatic cells (10).
Therefore, hiPSCs can potentially overcome the ethical concerns associated with hESCs, since their generation does not involve the manipulation and destruction of human embryos, and potentially circumvent immune rejection in patients when an autologous transplantation of hiPSC-derived cells is performed.

As a result of their pluripotency, both hESCs and hiPSCs cannot be directly used for transplantation because they can form teratomas, rare tumours usually containing remnants of all three germ layers, after implantation. Thus, and for clinical purposes, human pluripotent stem cells need to be previously differentiated into functional and specific tissue cells.

II.1.1.2.1 Generation of Human Induced Pluripotent Stem Cells

The first generation of human induced pluripotent stem cells was established in 2007 by retroviral transfection of four transcription factors, Oct3/4, Sox2, Klf4 and c-Myc, in adult human dermal fibroblasts (9).

Both Oct-3/4 and Sox2 are identified as being suppressors of differentiation-associated genes in hESCs (9). Klf4 is responsible for gene expression of Nanog, a key factor in maintaining hESCs pluripotency and promoting its self-renewal, by directly binding to Nanog proximal promoter region (11). Finally, c-Myc plays a crucial role in hiPSCs generation by controlling histone acetylation, directly related to chromatin modification, but can lead to tumour formation due to its oncogenic property (12). Thankfully, its use is non-essential for somatic cells reprogramming and an efficient generation of hiPSCs without c-Myc has already been reported (13).

Since their discovery, several and intensive studies have been performed to significantly improve hiPSCs generation in terms of reprogramming efficiency and clinical safety through the use of non-integrative or DNA-free systems for gene delivery and new selection of transgenes. Methods like plasmids, adenovirus, proteins and RNAs have already been tested, but still the reprogramming efficiency is very low, typically less than1% of transfected fibroblasts (14).

Although most, if not all, somatic cells have the potential to generate hiPSCs, the type of somatic cell used is as well determinant for reprogramming efficiency. For example, human hepatocytes can be reprogrammed to pluripotency at much higher frequency and faster speed than fibroblasts (15). Nevertheless and while several and important issues are not completely addressed, hiPSCs can currently be used for disease modeling and drug screening.
II.1.1.3 Signalling Pathways Controlling Pluripotency

Human pluripotent stem cells are characterized by being capable of making multiple copies of themselves for long periods in culture and generating cells from the three germ layers upon differentiation. These two essential properties, known as self-renewal and pluripotency, respectively, are under control of specific signalling pathways, which converge towards the activation of a core transcriptional network involving Oct4, Sox2 and Nanog (Figure II-4). Among them, three signalling pathways can be highlighted, namely TGF-β/Activin/Nodal, FGF and Wnt.

Figure II-4 Signalling pathways controlling pluripotency and self-renewal of hPSCs (16).
The TGF-β superfamily members can be divided into two different branches. Factors like Activin, Nodal and TGF-β are grouped in one branch and BMPs are clustered into a distinct one. The TGF-β/Activin/Nodal signalling pathway plays a major role in maintaining hPSCs self-renewal and pluripotency through the activation of SMAD2/3 via ALK4/5/7 (17). While activated, SMAD2/3 phosphorylates within the cytoplasm and then translocates into the nucleus, where SMAD proteins act as transcriptional factors to activate undifferentiation-associated genes, like Nanog.

In contrast, BMP ligands lead to SMAD1/4/8 phosphorylation through ALK2/3/6 binding. The resulting SMADs proteins are then translocated into the nucleus to induce and promote hPSCs differentiation (18). Nevertheless, the suppression of BMP signalling by Noggin and FGF can support long term self-renewal of human PSCs (19). For both braches, a common SMAD4 protein supports receptor-activated SMADs translocation into the nucleus.

FGFs (Fibroblast Growth Factors) are also involved in pluripotency maintenance of hPSCs via intracellular activation of PI3K/AKT and/or Ras-Ref-MEK/ERK downstream pathways. PI3K catalyzes the conversion of PIP2 (Phosphatidylinositol (3,4)-bisphosphate) into PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate) through phosphorylation. The resulting PIP3 activates PDK1 (Phosphoinositide-Dependent Kinase-1) phosphorylation and, consequently, AKT activation and translocation to the plasma membrane. Activated AKT promotes cell proliferation, survival, growth and motility (20). The Ras-Ref-MEK/ERK signalling, also known as MAPK/ERK, is believed to cooperate with PI13/AKT pathway in maintaining hPSCs pluripotency although its mechanism of action remains unclear (21).

WNT (Wingless-Type MMTV Integration Site Family Members) signalling also plays an important role in controlling stem cell pluripotency. Canonical WTN signalling promotes β-catenin stabilization and its further accumulation and translocation into the nucleus through GSK-3β inhibition. When WNT ligands bind to the Frizzled receptor, Dsh (Dishevelled) is activated preventing the association of GSK-3β with AXIN (Axis Inhibitor) / APC (Adenomatous Polyposis Coli) and, subsequently, β-catenin degradation. In the nucleus, β-catenin associates with TCF (T-Cell Factor) and LEF (Lymphoid Enhancer Factor) proteins to activate target genes expression (22).

Apart from the above-mentioned signalling pathways, epigenetics factors also play a vital role in maintaining stem cell pluripotency. In particular, mechanisms like histones modifications, DNA methylation/acetylation and small RNAs are, as well, involved in the expression of genes responsible for hPSCs pluripotency and self-renewal (23).
II.2 Expansion of Human Pluripotent Stem Cells

In the last few years, significant progress has been made towards the development of scalable and robust culture systems for hPSCs expansion that ensure the maintenance of their pluripotency and self-renewal properties. In particular, and to fully realize their potential for disease modelling, drug screening and clinical application, it is mandatory the conception of chemically defined culture systems for the production of increased cell quantities, under good manufacturing practices (GMP), free of contaminants and without batch-to-batch variability.

II.2.1 Towards Xeno-Free and Chemically Defined Culture Systems

II.2.1.1 Adherent Cultures: From Feeder-Cell Layers to Synthetic Coatings

II.2.1.1.1 Feeder-Cell Cultures

The early culture conditions for human pluripotent stem cells were established by effectively following the developed methods for mouse ESCs, in particular the use of irradiated mouse embryonic fibroblasts (MEFs) as feeder-cell layer. MEFs support hPSCs self-renewal through secretion of essential growth factors, cytokines and extracellular matrices (ECM) such as TGFβ, Activin A, laminin-511 and vitronectin. However, the γ-irradiation of MEFs not only inhibits their proliferation but also induces apoptosis and, subsequently, modifies the secretion of soluble factors and ECM deposition, which leads to an inconsistent microenvironment for hPSCs culture. Moreover, feeder-cells and their products, even of human origin, can be as well a source of pathogens which may induce immune response upon transplantation of hPSCs derivatives (24).

Thus, and in order to overcome the aforementioned concerns, feeder-free cultures have been developed as alternatives to feeder-dependent hPSCs cultures. Without all the factors secreted by feeder-cells, it is now required to provide them through supplemented culture medium and/or in coating matrices to ensure hPSCs self-renewal and pluripotency in culture.

II.2.1.1.2 Feeder-free Cultures: Biological Substrates

One of the first examples of feeder-free cultures are Matrigel™ (BD Biosciences) and its equivalent Geltrex™ (Life Technologies), both coating matrices composed mainly of laminin, collagen IV, heparin sulphate proteoglycans, entactin and growth factors (25) (26). However, they are xenogeneic substrates, derived from Engelbreth-Holm-Swarm mouse sarcomas, and exhibit batch-to-batch
variability, thus not being ideal for hPSCs culture. Nevertheless, Matrigel™ remains one of the most commonly used substrates for both hESCs and hiPSCs expansion, at least for research purposes.

Each component of Matrigel™ exhibits a different level of support for human pluripotent stem cells proliferation and survival. Laminin, in opposition to fibronectin and collagen IV, has been reported to effectively and by itself support hESCs growth (27). Additionally, it has also been stated that the specific laminin isoforms -111, -332 and -511 support the adhesion and proliferation of hESCs due to their affinity to the expressed integrin α6β1, while isoforms -211 and -411 do not (28).

Since integrins are the main cell-surface receptor that mediate cell-ECM adhesion and signalling (Figure II-5), the identification of integrins in human pluripotent stem cells led to the development and use of other supporting ECM proteins for hPSCs maintenance in culture, in particular vitronectin and laminin -511.

Integrins are heterodimeric transmembrane molecules formed by two chains, named α and β subunits. Vitronectin has been stated to support hESCs self-renewal by binding integrin αVβ5, while integrins α5β1 and α6β1 are responsible for hESCs adhesion to fibronectin and laminin+entactin matrices, respectively (29). E-cadherin, which mediates cell-cell interactions thus being involved in hESCs colony formation and self-renewal, has been used as well as a substrate for long-term culture of hPSCs (30).

The use of recombinant human (rh) proteins, such as (rh) laminin -511 and rh vitronectin and rh E-cadherin, represents a significant breakthrough in the culture of hPSCs as being the leading examples of defined and xenogeneic-free substrates that efficiently support hPSCs self-renewal in culture (28) (29) (30). However, biological substrates do possess some weaknesses, such as difficult isolation and expensive manufacture, which must be overcome prior to their use in large-scale expansion of hPSCs under chemically-defined conditions (24).
II.2.1.3 Feeder-free Cultures: Synthetic Substrates

As alternatives, synthetic substrates have been designed to support hPSCs proliferation and maintenance in vitro. Several materials and material combinations have already been tested, mostly peptide or protein-based systems, polymers or polymers coupled with biomolecules.

One example of a polymer-based substrate is PMEDSAH (poly [2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide]), a fully defined synthetic polymer coating that efficiently sustain long-term hESCs expansion in combination with chemically defined culture media (31).

Likewise, synthetic peptide-acrylate surfaces (PAS), like Synthemax™ (Corning®), have recently been developed to support human pluripotent stem cells in culture. These surfaces are made of acrylate conjugated to biologically active peptides, such as vitronectin, fibronectin and laminin (32).

Both approaches PMEDSAH and PAS, have shown to be compatible to common sterilization procedures, in particular γ-radiation, which is significantly important when considering their use as biomedical products (33).

II.2.1.2 Culture Media Composition

The generation of robust and scalable culture systems for hPSCs expansion, under clinically acceptable GMP, involves the combination of both xeno-free and chemically defined substrate and culture medium (Figure II-6). Therefore and over the past years, significant progress has also been made toward the identification of molecular factors that sustain hPSCs self-renewal and pluripotency for the conception of effective and defined culture media.

![Figure II-6](image-url) Developments in culture systems for human pluripotent stem cell culture (24).
Although the nutritional requirements for animal cells are very stringent and vary according to the cell type, there is a set of essential components that can be defined when culturing animal cells, particularly carbon/energy source (e.g. glucose and glutamine), nitrogen source (e.g. glutamine), vitamins and growth factors.

Additionally, there are chemical and physical parameters that need to be strictly monitored since they also influence cell growth, such as metabolic by-products (e.g. lactase and ammonia) concentration, dissolved oxygen, pH, temperature and osmolality. Antibiotics, like penicillin and streptomycin, are commonly used in media formulation to avoid adverse contaminations with bacteria.

The early hESCs culture media comprehended the use of FBS (fetal bovine serum) which contains important growth and adhesion factors, minerals, lipids and hormones (6). Later, FBS was, by most of the research teams, replaced by alternatives sera as those of human origin. However, all of them represent complex mixtures of unknown compounds offering batch-to-batch variability, risks of contamination and undesirable cell differentiation events, thus not being ideal for hPSCs culture.

Therefore, and to avoid the above-mentioned concerns, serum-free formulations using undefined serum replacement (KnockOut Serum Replacement™, Invitrogen™) supplemented with basic fibroblast growth factors (bFGF or FGF2) have been designed as alternatives (34). Although providing more standardized culture conditions compared to serum-based media, they are still not ideal for hPSCs culture since they contain animal proteins.

Nevertheless, serum-free media have been over the years optimized and reformulated and there are currently available serum- and xeno-free culture media for the long-term maintenance of hPSCs expansion in vitro, namely Essential 8™ Medium (Life Technologies) and TeSR™2 (StemCell™ Technologies).

Essential 8™ (E8) medium is a xeno- and feeder-free medium specifically formulated for the growth and expansion of hPSCs and originally developed by Guokai Chen and co-workers at James Thomson’s laboratory (35). It has been extensively tested and proven to support several hPSC lines growth along with the maintenance of their pluripotency (36) (37).

The simplicity of Essential 8™ medium, which merely comprises eight components (L-ascorbic acid, selenium, transferrin, NaHCO₃, insulin, FGF2, TGFβ1 and DMEM/F12), reduces both manufacture cost and lot-to-lot variability and simplifies quality control. Because it is highly defined, E8 should significantly help facilitate the transfer of basic research on hPSCs to the clinic (35).

II.2.2 Passaging of hPSCs

A common procedure during anchorage-dependent cells expansion, like hPSCs, is cell subculturing also known as cell passaging. It basically involves the total or partial individualization of cells and further
transfer from a previous culture to a fresh culture system. Each cycle of subculturing the cells is referred to as a passage.

Traditional protocols for hPSCs passaging involve enzymatic dissociation, mostly leading to full individualization of cells. Because hPSCs are more sensitive to treatments and prone to cell death as single-cells, small molecules, such as Rho-associated protein kinase (ROCK) inhibitors, must be used to increase cell survival during passaging procedures (38).

Therefore and since all these methods require specific reagents and tools, the long-term expansion of human PSCs becomes too expensive. Moreover, the quality of enzymes from batch to batch usually affects the consistency of enzymatic dissociation. Bearing these concerns in mind, it is highly desirable to develop a dissociation approach that is both cost effective and chemically defined and with low lot-to-lot variability (39).

II.2.2.1 EDTA-based Cell Passaging

It has already been reported that cell death after individualization is mainly related to myosin-actin-dependent contraction and that cell-cell adhesions promote cell survival through its inhibition. As a result, cells survive in the first few hours after dissociation by reforming small aggregates in subculture (40).

![Figure II-7 hPSC survival efficiency after individualization by different dissociation methods: the higher the cell density after harvesting, the better the reassociation efficiency between adjacent cells (39).](image-url)
EDTA treatment is an enzyme-free method that allows the dissociation of human pluripotent stem cells with high survival rates without ROCK inhibitor support. EDTA captures metal ions like Mg$^{2+}$ and Ca$^{2+}$ responsible for cell-cell and cell-ECM bounds through cadherins and integrins. EDTA-based passaging partially dissociates hPSCs by forming small cellular aggregates strong enough to survive but small enough to be accessed by growth factors (Figure II-7).

Due to its efficiency in cell survival, EDTA passaging can be used in long-term culture expansion of hPSCs with different feeder-free media, including the serum- and xeno-free Essential 8™ Medium (Life Technologies), as both cost and time effective dissociation protocol (39).

II.2.2.2 Myosin-Actin Contraction

Human pluripotent stem cells grow in culture as adherent colonies thus depending for survival on cell-cell and cell-ECM interactions mediated mostly by cadherins, integrins and cytoskeleton components, like actin.

The actin-myosin based cytoskeleton is a dynamic system crucial for contraction, motility and tissue organization. Actin-myosin motors comprise actin filaments and non-muscle MYHs (myosin II heavy chains). Hydrolysis of ATP through MYH ATPase, besides generating energy, causes the MYHs to slide along actin filaments resulting in contraction. The process is triggered by the binding of MLC (myosin light chain), which is activated through phosphorylation by kinases, such as ROCK (40).

ROCK is a downstream target of GTP-binding protein Rho located in the cytoplasm. Rho has different effects on cell adhesion depending on the downstream effector that binds, while activated thought GTP binding. Rho signalling through Dia induces actin reorganization in a manner that strengthens cell-cell adhesion via cadherins, whereas signalling through ROCK increases actin contractility which promotes cell-cell and cell-ECM adhesion via cadherins and integrins, respectively (41). However, high levels of Rho- or ROCK-mediated actin contractility is incompatible with cell-cell junctions (Figure II-8).

Therefore and in order to maintain a normal cellular function and morphology, a balance between actin-myoosin contraction forces and the opposing anchoring forces has to be achieved. When cells are harvested and individualized during cell passaging, this balance is normally broken leading to cell death by the excessive actin-myoosin contraction (40).

The use of ROCK inhibitors (e.g. Y-27632) effectively supports passaging of hPSCs by disrupting actin-myoosin contraction through reduction of MLC phosphorylation (38). However, it should only be used on the first 24h of subculture since its presence impedes colonies formation.
Figure II-8 Roles of active Rho by GTP binding on cell adhesion: 1 - Rho signalling through Dia reinforces cell-cell adhesion, 2 - whereas signalling through ROCK increases actin contractility promoting cell-cell and cell-ECM adhesion; 3 - high levels of Rho- or ROCK-mediated actin contraction can demote cell-cell interactions (41).

II.2.3 Platforms for Large-Scale Expansion of hPSCs

The use of hPSCs either for clinical applications or drug screening requires the generation of a large number of viable cells. Culture systems like multiwell plates or Petri dish are neither reasonable nor suitable for the ex-vivo scale-up of hPSCs, thus the transition from small and static to large and dynamic culture units is mandatory (Figure II-9).

Figure II-9 Large expansion of hPSCs requires the use of large volume culture units, like bioreactors. The first scale-up step for cell expansion usually relies on the use of spinner flasks.
II.2.3.1 Bioreactors

*Ex-vivo* scaling-up culture systems aim to combine the simulation of the *in vivo* biological and physicochemical conditions required for a proper expansion and maintenance of cells with real-time monitoring of culture parameters. These parameters include pH, temperature, dissolved oxygen, agitation, key nutrients, growth factors and metabolites, among others. Currently, different bioreactors configurations are available for the scale-up of human stem cells and choice usually relies on the cell cultivation method applied (i.e. surface cultures, cell aggregates, microcarriers, cell encapsulation) (Figure II-10).

![Cell cultivation methods applicable for expansion of hPSCs in different bioreactor configurations.](image)

**II.2.3.1.1 Roller Bottles**

One of the simplest forms of cultivating adherent cells under dynamic conditions is provided by roller bottles (Figure II-11A). They consist of cylindrical vessels (250-2000 mL) that revolve slowly, at rotational speeds between 5 and 60 rph, whose inner surface is used for cell attachment and growth. The gentle agitation provided prevents gradients from forming within the medium that may undesirably affect cells growth. Since roller bottles are not completely filled with medium (medium/air volume ratio between 1:5 and 1:10), cells are normally covered by a thin layer during culture. Thus, the resulting alternating exposure of cells to medium and gas phase allows a more efficient oxygenation.

One to hundreds of roller bottles can be accommodated in a single rotating apparatus. Nevertheless, the use of racks with multiple roller bottles leads to variability and heterogeneity among batches. Moreover, a straightforward monitoring and control of physicochemical parameters is not allowed.
II.2.3.1.2 Rotating Wall Vessels

Interestingly, the principle of growing cells as rotating cultures was first developed in 1993, by George Gey at Johns Hopkins University, through the conception of special culture tube for the growth of tissue cells in its entire inner surface (42).

Rotating wall vessels (Figure II-11C) represent another example of rotating culture and were originally developed by NASA, in order to minimize shear stress and turbulence in mammalian cell cultivation during space shuttle take-off and landing. In the proposed bioreactor, cells grow in a microgravity environment under two possible configurations: the High Aspect Ratio Vessel (HARV) and the Slow Turning Lateral Vessel (STLV) (43) (44).

They consist of cylindrical chambers filled with media where scaffolds containing cells are free to move. Both outer and inner vessel walls are capable of rotating at a constant angular speed, together or separately. A balance between downward gravitational force and upward hydrodynamic drag force acting on each scaffold need to be reached to ensure its remaining in suspension. Therefore, as mass increases in the bioreactor during cell growth, the rotational speed must be increased to balance the gravitational force. Gas transfer efficiently occurs through a gas exchange membrane. However, scaling up these systems is not an easy task due to its complexity.

II.2.3.1.3 Wave Bioreactors

Wave bioreactor (Figure II-11D) represents a very attractive alternative to the aforementioned bioreactors, especially suitable for GMP operations. It comprises a disposable bag, partly filled with media and inoculated with cells, with the remainder inflated with air. The culture bag is positioned on a special platform responsible for the generation of waves in the culture fluid through rocking motion. The resulting agitation provides an efficient and continuous mixing and gas transfer.

Culture bags are pre-sterilized and designed for a single-use to reduce contamination issues. Sampling, monitoring and control are possible although less simple than in other systems. Scaling up is easy but costly (45).

II.2.3.1.4 Stirred Vessels

Stirred vessels, such as spinner flasks and stirred-suspension bioreactors (SSBs), are widely used, easy to operate and can be scaled-up.

Spinner flasks (Figure II-11B) have been used to expand cells in suspension for over 50 years. Medium agitation is usually provided by a vertical impeller coupled with a magnetic stirrer on top of a magnetic stir plate. The total culture volume should never exceed half of the indicated volume of the spinner flask for a better and proper aeration. Many paddle designs are commercially available for medium mixing.
These culture systems are often used as a first scale-up step to optimize the operating protocol in stirred bioreactors due to the similarity and comparable simplicity and smaller scale.

Stirred-suspension bioreactors provide a homogeneous growth environment for human stem cells and can be completely equipped with real-time monitoring instrumentation for control of culture conditions. Different agitation systems are commercially available. Still, shear stress caused by mechanical agitation is a major limitation since it can significantly affect cell growth. Therefore, selection of optimal stirring speed and paddle design is a critical task.

Nevertheless, SSBs represent one of the most attractive solutions for large-scale expansion and long-term culture of hiPSCs. Concentrations between $10^6$ to $10^7$ mammalian cells/mL are actually common in conventional stirred-suspension bioreactors. Thus, the production of $10^9 - 10^{10}$ hiPSC-derived cells for clinical applications would only require working volumes of a few hundred millimeters to a few liters (46). SSBs also provide a flexible culture platform since human pluripotent stem cells can be cultured as cell aggregates, adherent to scaffolds or microcarriers.

**Figure II-11** Schematic representation of different bioreactors used for stem cell cultivation: roller bottle (A), spinner flask (B), rotating wall vessel (C) and wave bioreactor (D).
II.2.3.2 Microcarrier-based Cultivation Systems

Human pluripotent stem cells have a natural tendency to form aggregates under cultivation. In fact, cell aggregates allow a better recapitulation of the *in vivo* microenvironment which may enable hPSCs to more efficiently differentiate into particular phenotypes.

However, cells cultured as aggregates are more susceptible to cellular damage and spontaneous differentiation caused by hydrodynamic shear-force. Moreover, variability in sizes of aggregates represents another drawback. As cell aggregates increase in size and in irregularity, they may induce apoptosis and spontaneous differentiation. Bearing this in mind, microcarrier-based cultivation system might be an attractive alternative for hPSCs expansion in bioreactors (47).

Microcarriers are a simple, scalable and flexible culture system. They consist of small spheres, (ideally with 100-300 μm in size) which afford the flexibility of culturing stem cells either on porous or nonporous beads, with or without customization with synthetic peptides or extracellular matrix molecules. These culture systems are characterized by high surface-to-volume ratio, thus accommodating higher cell densities compared to those in static cultures. Additionally, the available area for cell attachment and growth can be easily adjusted by varying the amount of microcarriers (46) (48).

In microcarrier-based cultures, cells grow as monolayers on the surface of small compact (Figure II-12A) or microporous beads or inside the pores of macroporous spheres (Figure II-12B) suspended in a culture medium.

![Figure II-12 SEM images of compact (A) and macroporous (B) microcarriers. Optical microscope image of an agglomerate of microcarriers (C) (49) (50) (51).](image)

When cultivated in nonporous microcarriers, cells are directly exposed to bulk medium, which facilitates nutrient supply and by-product removal due to the shorter diffusion paths. Microporous microcarriers have small pores, with diameters smaller than 1 μm, in which cells cannot enter. Nevertheless, the microporosity facilitates nutrients and growth factors supply to all sides of cells in culture. Lastly, macroporous microcarriers have pores usually with 30-40 μm in diameter allowing the entrance and
anchorage of cells (mean cell diameter of single cells in suspension about 10 μm), thus protecting them against shear stress caused by mechanical agitation (48).

A wide variety of materials have been used to produce microcarriers: plastics (polystyrene, polyethylene, polyester and polypropylene), glass, acrylamide, silica, cellulose, dextran, collagen (gelatin) and glycosaminoglycans. They influence microcarrier toxicity, hydrophobicity, microporosity, mechanical stability, shape, permeability and diffusion of oxygen and nutrients, etc. (48)

Although microcarrier-based systems represent one of the most attractive and effective methods to culture anchorage-dependence cells, they also possess some weaknesses. In particular, cell harvesting and counting becomes a complex and difficult task due to the formation of agglomerates of microcarriers (Figure II-12C), mainly later in culture. Nevertheless, they are one of the most used and efficient systems for large-scale expansion of human stem cells.
III. MATERIALS AND METHODS

III.1 Expansion of Human Induced Pluripotent Stem Cells

III.1.1 Cell Culture

III.1.1.1 Cell Line

The human induced pluripotent stem cell line used in all studies, Gibco® Episomal hiPSC line, was gently provided by Life Technologies for the purpose of this study. The Gibco® Episomal hiPSC line is a zero-footprint, viral-integration-free human iPSC line, adapted to feeder-free and serum-free culture conditions. These cells were reprogrammed from human cord blood-derived CD34+ progenitors with seven episomally expressed factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, and SV40 T) (52).

III.1.1.2 Thawing of hiPSCs

The vial containing human iPSCs was removed from liquid nitrogen storage and immersed in a 37°C water bath for a few seconds, without submerging the cap, until most of the content was thawed. Then, and under sterile conditions, 1 mL of washing media was added drop-wise to cells. After resuspending cells, the entire vial content was transferred to a 15-mL tube and mixed with 3 mL of washing media. The suspension was centrifuged (Hermle Z-400k) at 1000 rpm for 5 minutes and the supernatant discarded. Lastly, the cell pellet was resuspended in 1 mL of Essential 8™ Medium and plated in one culture well, previously coated with Matrigel™ and containing 0.5 mL of Essential 8™ Medium. The cells were transferred into a humidified incubator at 37°C, 5% CO2. The culture medium was replaced daily.

III.1.2 Culture Media Preparation

Washing Media

Washing media comprises a base of Knock-Out DMEM (KO-DMEM; Gibco®) supplemented with Knockout-Serum Replacement (KO-SR; Invitrogen™), MEM-non essential amino acids (Gibco®), L-glutamine (Gibco®), β-Mercaptoethanol (Sigma®) and Penicillin-Streptomycin (P/S, Life Technologies). The quantitative composition of the medium is described in Table III-1.
### Table III-1 Composition of washing medium.

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO-DMEM</td>
<td>~ 87.4% (v/v)</td>
</tr>
<tr>
<td>KO-SR</td>
<td>~ 10% (v/v)</td>
</tr>
<tr>
<td>MEM-non essential amino acids</td>
<td>~ 1% (v/v)</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1 mM</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Penicillin Streptomycin (P/S)</td>
<td>~ 1% (v/v)</td>
</tr>
</tbody>
</table>

**Essential 8™ Medium**

Essential 8™ medium (E8, Life Technologies) is a xeno-free and feeder-free medium specially formulated for the growth and expansion of human pluripotent stem cells. The medium was prepared according to the manufacturer’s instructions. The Essential 8™ supplement (10 mL) was thawed overnight in the fridge (2–8°C). After homogenization, the supplement content was aseptically mixed with Essential 8™ Basal Medium (490 mL).

**III.1.3 Culture on Feeder-Free Layers**

**III.1.3.1 Substrate Preparation**

**Matrigel™**

An aliquot of Matrigel™ (BD Biosciences) stock solution was thawed overnight on ice, at 4°C. A coating solution was prepared at a proportion of 1:30 (v/v) in ice cold Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12; Gibco®) and distributed in culture tissues plates. After at least two hours of incubation at room temperature, Matrigel™ solution was removed prior to cell seeding.

**Vitronectin**

Vitronectin (VTN-N, Life Technologies) is a recombinant, truncated human protein, corresponding to the amino acid fragment 62–478 of human vitronectin expressed in *E. coli* and purified from inclusion bodies and refolded for use as a substrate for the feeder-free culture of human PSCs.

A vitronectin stock solution aliquot was thawed at room temperature and then used to prepare a coating solution in phosphate-buffered saline (PBS; Gibco®) at a proportion of 1:100 (v/v). The resulting dilution was distributed in a 6-well culture plate and incubated at room temperature for one hour. Before cell plating, vitronectin coating solution was fully removed.
III.1.3.2 hiPSCs Passaging: EDTA Treatment

Culture medium from a 6-well plate containing human iPSCs was fully removed. Cells were washed twice with sterile EDTA 0.5 mM (1mL/well) and incubated at room temperature with EDTA solution (1.5 mL/well) for 5 minutes, to remove all magnesium and calcium. EDTA solution was then replaced with Essential 8™ medium (1 mL/well). Cells were detached and harvested with a 1000 μL pipette (maximum of 5 flush per well) and transferred to a 15-mL tube. After ensuring that the cell suspension was well mixed, cells were directly plated into a newly Matrigel™ or VTN-N coated 6-well plate. Plate was shaken back and forth and side to side to equally distribute cells and placed in a humidified incubator at 37°C, 5% CO₂.

III.1.4 Microcarrier-based Culture Systems

III.1.4.1 Microcarriers Preparation

III.1.4.1.1 Sterilization

Xeno-free plastic microcarriers (SoloHill®) were preweighted in 15-mL or 50-mL tube and incubated with ethanol 70%, for 1 hour at room temperature, with mixing (10 rpm).

III.1.4.1.2 Coating

After sterilization with ethanol, beads were washed three times with sterile PBS and incubated for coating during 2 hours at room temperature, with mixing (20 rpm). Later, the coating solution was removed and beads were incubated at 37°C with E8 medium (0.5% P/S) for at least 30 minutes. Before culture, medium was replaced with a defined volume of E8 medium (0.5% P/S) with or without ROCK inhibitor.

Vitronectin Coating Solution

A vitronectin stock solution aliquot was thawed at room temperature and then used to prepare a coating solution in sterile PBS (1% P/S) at a proportion of 1:100 (v/v).

Geltrex Coating Solution

A Geltrex® (GT, Life Technologies) stock solution aliquot was thawed at room temperature and then used to prepare a coating solution at a proportion of 1:60 (v/v) in DMEM-F12 (1% P/S).

III.1.4.2 hiPSCs Inoculation
III.1.4.2.1 EDTA Inoculation

Cells were harvested from 6-well plates according to the aforementioned protocol for human iPSCs passaging with EDTA treatment. A defined volume of E8 medium (0.5% P/S) was used to flush cells from wells (typically, 1mL to flush 3 wells). For EDTA inoculation with ROCK inhibitor (Y-27632; StemGent®), cells were flushed with E8 medium (0.5% P/S) containing ROCK inhibitor (10 μM). Cell counting by trypan blue staining was performed before cell inoculation.

Static Conditions

Cells were inoculated in an ultra-low attachment 24-well plate (beads surface area: 3 cm²/well; beads density: 360 cm²/g). For that purpose, cell suspensions with concentrations of 30000, 50000 and 70000 cells/cm² were prepared in E8 medium (0.5% P/S, 10 μM of ROCK inhibitor) and added to the beads as 250 μL/well. Pre-coated beads were already suspended in E8 medium (0.5% P/S, 10 μM of ROCK inhibitor) and placed as 250 μL/well. A total culture volume of 500 μL/well was used for inoculation. The plate was carefully placed in humidified incubator at 37ºC, 5% CO₂. E8 medium without ROCK inhibitor was daily replaced (500 μL/well). Cell harvesting and counting was done at Day 5.

Dynamic Conditions

For inoculation in a StemSpan™ Spinner Flask (StemCell™ Technologies) a cell suspension of 55,000 cells/cm² was prepared in E8 medium (0.5% P/S, 10 μM of ROCK inhibitor) and added to VTN-N pre-coated beads (beads concentration: 20 g/L) suspended in E8 medium (0.5% P/S, 10 μM of ROCK inhibitor). A total volume of 25 mL was used for cell inoculation, at Day 0, and cells were incubated with ROCK inhibitor for 24 hours in a humidified incubator at 37ºC, 5% CO₂, without mechanical agitation. Intermittent agitation (stirring at 40-50 rpm during 3 minutes, every 2 hours) was used to promote cell adaptation to shear stress at Day 1. From Day 2, inclusive, continuous agitation (44 rpm) was applied for mixing. Daily medium change was performed by replacing 80% of the medium with fresh E8 medium without ROCK inhibitor (working volume of 50 mL). Cell counting and viability evaluation was daily performed, as well.

III.1.4.2.2 Single-Cell Inoculation

Culture medium from wells was replaced with pre-warmed medium containing 10 μM of ROCK inhibitor (1mL/well). After an incubation with ROCK inhibitor for 1 hour at 37ºC, cells were washed twice with sterile PBS (1mL/well) and incubated with Accutase (Sigma®) for 5-10 minutes at 37ºC, until colonies break into single cells. Cells were then flushed twice from wells with washing medium (1mL/well),
transferred into a 15-mL tube and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant and resuspending the pellet in 1 mL of E8 medium, cells were counted by trypan blue staining.

For inoculation in 24-well plate, a cell suspension of 50000 cells/cm\(^2\) was prepared with E8 medium (0.5% P/S, 10 μM of ROCK inhibitor) and added to beads as 250 μL/well. Pre-coated beads were already suspended in E8 medium (0.5% P/S, 10 μM of ROCK inhibitor) and placed as 250 μL/well. A total culture volume of 500 μL/well was used for inoculation. The plate was carefully placed in humidified incubator at 37°C, 5% CO\(_2\). E8 medium without ROCK inhibitor was daily replaced (500 μL/well). Cell harvesting and counting was done at Day 5.

### III.1.4.3 Cell Counting

#### Static Conditions

At Day 5, culture medium was carefully removed from wells and cells were washed with sterile PBS (500 μL/well). PBS was then replaced by Accutase (300 μL/well) and cells on beads were collected from wells to different FACS tubes. More 300 μL/well of Accutase was added to collect any remaining cells to the same tubes. FACS tubes were incubated at a Thermomixer\(^®\) (Eppendorf) at 37°C and 750 rpm, for 30 minutes. At the end, cells were flushed with a 1000 μL pipette and 600 μL/tube of washing media was added to separate cells from microcarriers by filtration through cell strainer, using a 2 mL pipette. After centrifugation at 1000 rpm for 5 minutes, the pellet was resuspended in 500 μL or 1 mL of washing media and cells were count by trypan blue staining.

#### Dynamic Conditions

Two samples of 700 μL were daily collected from spinner-flasks and transferred to different FACS tubes. Culture medium was removed from each tube and 1 mL/tube of PBS was added. After washing, PBS was replaced by 1 mL/tube of 0.05% Trypsin-EDTA (Life Technologies) and FACS tubes were incubated at a Thermomixer\(^®\) at 37°C and 750 rpm, for 10 minutes. At the end, 1 mL/tube of washing media was added, cells were flushed with a 1000 μL pipette and separated from beads by filtration through cell strainer, using a 2 mL pipette. After centrifugation at 1000 rpm for 5 minutes, the pellet was resuspended in washing media (0.5 - 1 mL) and cell counting was performed by trypan blue staining.

### III.1.4.4 Cell Harvesting

#### Dynamic Conditions

At the end of cell growth, the entire spinner-flask content was carefully transferred to a 50-mL tube and culture medium was replaced by 5 mL of washing media with ROCK inhibitor (10 μM). After incubation
with ROCK inhibitor at 37°C for 1 hour, beads containing cells were washed twice with 5 mL of PBS and incubated with 5 mL of Accutase at a Thermomixer® (37°C, 750 rpm) for 20 minutes. Then, 5 mL of washing media (10 μM of ROCK inhibitor) was added to the tube and cells were separated from microcarriers by filtration through cell strainer, using a 5 mL pipette. After centrifugation at 1000 rpm for 5 minutes, the pellet was resuspended in medium (0.5% P/S, 10 μM of ROCK inhibitor) and cells counted by trypan blue staining.

### III.2 Characterization of hiPSCs

#### III.2.1 Immunofluorescence

To assess the pluripotency marker expression of generated cells, immunostaining was performed using specific fluorescently labeled antibodies for human PSCs markers. These antibodies bind to specific surface or intracellular antigens expressed by PSCs and emit a fluorescent signal upon binding.

#### III.2.1.1 Surface Marker Staining

The analyzed surface markers were Tra-1-60, Tra-1-81 and SSEA-4. The medium from wells was removed, replaced by washing medium containing the primary antibody (Table III-2) and incubated for 30 minutes at 37°C. Cells were then washed three times with 1 mL of washing media to remove any excess of primary antibody. Medium containing the secondary antibody was placed and left to incubate during 30 minutes, at 37°C. Finally, cells were once more washed three times, with washing media, examined using a fluorescence microscope.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Dilution from Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tra-1-60</td>
<td>Mouse IgM</td>
<td>Goat anti-mouse IgM</td>
<td>1:135</td>
</tr>
<tr>
<td>Tra-1-81</td>
<td>Mouse IgM</td>
<td>Goat anti-mouse IgM</td>
<td>1:1000</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>Mouse IgG</td>
<td>Goat anti-mouse IgG</td>
<td></td>
</tr>
</tbody>
</table>

*Table III-2 Antibody concentrations and dilutions for use in surface staining of pluripotency markers. Note: the primary antibodies are from StemGent, while the secondary antibodies are from Invitrogen™.*
III.2.1.2 Intracellular Staining

Culture medium from wells was aspirated and replaced with 4% paraformaldehyde (PFA; Gibco®) for cell fixation, for 10 minutes at room temperature. Cells were then washed twice with PBS (Phosphate Buffered Saline) and incubated for 30-60 minutes, at room temperature, with Blocking Solution (10% NGS, 0.1% Triton in PBS), in order to reduce background staining. After blocking solution removal, cells were incubated with the primary antibody in staining solution (5% NGS, 0.1% Triton in PBS), according to dilutions presented in Table III-3, overnight at 4°C.

Table III-3 Antibody dilutions for use in intracellular staining of pluripotency markers. The secondary antibodies were Alexa fluor ® 488- or 546-conjugated from Invitrogen™. Note: the primary antibodies Oct4 and Nanog are from Millipore™ and Sox2 is from R&D Systems®.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primary Antibody</th>
<th>Dilution from Stock</th>
<th>Secondary Antibody</th>
<th>Dilution from Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4</td>
<td>Mouse IgG</td>
<td>1:400</td>
<td>Goat anti-mouse IgG</td>
<td></td>
</tr>
<tr>
<td>Sox2</td>
<td>Mouse IgG</td>
<td>1:200</td>
<td>Goat anti-mouse IgG</td>
<td>1:1000 (546)</td>
</tr>
<tr>
<td>Nanog</td>
<td>Rabbit IgG</td>
<td>1:5000</td>
<td>Goat anti-rabbit IgG</td>
<td>1:500 (488)</td>
</tr>
</tbody>
</table>

After three washes with PBS, cells were incubated with secondary antibody diluted in staining solution (Table III-3) for 1 hour in the dark at room temperature. Later on, cells were washed once with PBS to remove the excess of secondary antibody and incubated with diluted 4',6-diamidino-2-phenylindole (DAPI, 1:10000 in PBS), during 2 minutes at room temperature, for nuclear-staining. Finally, the cells were washed twice with PBS to remove any remaining DAPI crystals and left with PBS for further examination under a fluorescence microscope.

For the analysis of cells anchored on beads (Oct4 and Nanog), the immunostaining procedure was slightly different. A sample from the spinner-flask was transferred to a 15-mL tube and culture medium was replaced with 4% PFA. After cell fixation, PFA was removed and 1 mL of blocking solution was added. The total volume was equally distributed in two wells, of a 12-well plate, and cells were incubated for 1 hour at room temperature. Later, blocking solution was replaced with 400 μL/well of a primary antibody solution (1:400 for Oct4; 1:2000 for Nanog) and left overnight at 4°C. After incubation with primary antibody, cells were washed three times with PBS (1 mL/ well) and incubated with secondary antibody solution (1:400 (546) in staining solution) for 1 hour, in the dark. Cells were then washed three times with PBS and 500 μL/well of DAPI solution was added. Finally, two washes with PBS were performed and cells were left with PBS for further examination under a fluorescence microscope.
III.2.2 Flow Cytometry

Flow cytometry is a commonly used technique that simultaneously measures the size and analyses the intracellular and/or surface markers of single cells, as they flow in a fluid stream through a beam of light. In this work, flow cytometry technology was used to quantify the expression of pluripotency markers for characterization purposes of human iPSCs after expansion under both static and dynamic culture conditions.

III.2.2.1 Intracellular Staining

Cells were fixed with 2% PFA, centrifuged at 1250 rpm, for 5 minutes, and washed twice with 1% NGS, centrifuging each time at 1250 rpm, for 5 minutes. Then, cells were resuspended in 3% of NGS and equally distributed in Eppendorf tubes, previously coated with 1% Bovine Serum Albumin (BSA, Invitrogen™). After centrifuging at 1000 g, for 3 minutes, the supernatant was discarded and the pellet resuspended in a 1:1 solution of 3% NGS and 1% Saponin (Sigma®). Cells were left to incubate for 15 minutes, at room temperature, for membrane permeabilization. Following another centrifugation, cells were washed with 3% NGS, incubated at room temperature, for 15 minutes, and centrifuged once again. The obtained pellet was resuspended in primary antibody solution (Table III-4) in 3% NGS and incubated in the dark, for 1 hour and 30 minutes, at room temperature.

Table III-4 Antibody dilutions for use in intracellular staining for flow cytometry analysis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primary Antibody</th>
<th>Dilution from Stock</th>
<th>Secondary Antibody</th>
<th>Dilution from Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4</td>
<td>Mouse IgG</td>
<td>1:150</td>
<td>Goat anti-mouse IgG</td>
<td>1:300</td>
</tr>
<tr>
<td>Sox2</td>
<td>Mouse IgG</td>
<td>1:150</td>
<td>Goat anti-mouse IgG</td>
<td></td>
</tr>
<tr>
<td>Nanog</td>
<td>Rabbit IgG</td>
<td>1:3000</td>
<td>Goat anti-rabbit IgG</td>
<td></td>
</tr>
</tbody>
</table>

After another centrifugation step, cells were washed twice with 1% NGS, to remove any remaining primary antibody solution, centrifuging each time at 1000 g for 3 minutes. The pellet was incubated for 45 minutes, in the dark, with the secondary antibody diluted in 3% NGS. At last, and after washing twice with 1% NGS, cells were resuspended in PBS and transferred to FACS tubes for further analysis in the flow cytometer (Becton Dickinson®).
IV. RESULTS AND DISCUSSION

IV.1 Maintenance of hiPSCs on 2D Surfaces

In order to assess the efficiency of vitronectin (VTN-N) as a xeno-free and chemically defined substrate for the feeder-free culture of human pluripotent stem cells, human iPSCs were initially cultured and expanded in E8 medium as a monolayer in tissue culture plates coated with VTN-N.

After expansion under these conditions for 3 passages, hiPSCs presented a typical morphology characterized by flat and densely packed colonies with defined borders and round shape (Figure IV-1) (53).

![Figure IV-1 Optical microscope image of a colony of human iPSCs cultured on VTN-N coated wells (scale bar – 100 μm).](image)

Cells were routinely passaged as small clumps using EDTA when ~85% confluency was reached, typically every 3-4 days. At each cell passage, cell counting was performed by trypan blue staining and the expansion index determined according to the average fold increase per passage ($F_i$), given by the Equation IV-1. According to the results (Figure IV-2), cells cultured on VTN-N-coated plates in E8 medium present an expansion index of $4.3 \pm 0.4$.

$$F_i = \frac{N_i}{N_0} = \frac{\text{Total number of generated cells}}{\text{Number of plated cells}}$$

Equation IV-1
Cultured cells were characterized in terms of pluripotency by intracellular and surface antigens immunostaining for the expression of the pluripotency markers Oct4, Sox2 and Nanog, and Tra-1-60, Tra-1-81 and SSEA4, respectively (Figure IV-3). The results for the intracellular staining were merged with DAPI nuclei counterstaining which binds strongly to Adenine-Thymine rich regions of DNA (54).

Although it has been confirmed the presence of pluripotency markers throughout the entire area of the colonies by immunostaining, flow cytometry was additionally performed to quantify the Oct4, Sox2 and Nanog intracellular markers. Results indicate that 95 ± 1% of the analysed cells were positive for Oct4, 92 ± 1% for Sox2 and 89 ± 3% for Nanog (Figure IV-4).
Figure IV-4 Flow cytometry analysis of intracellular pluripotency markers Oct4, Sox2 and Nanog, in hiPSCs expanded in VTN-N with E8 medium. Area under green curve represents the negative control and the purple area corresponds to the analysed samples. The percentage of analysed cells expressing each pluripotent marker is shown above bars.

Taking into account the results presented above related to colony morphology, expansion index, presence and quantification of pluripotency markers, it was confirmed the efficiency of vitronectin and Essential 8™ medium combination as a robust xeno- and serum-free culture system for the maintenance of human induced pluripotent stem cells.

IV.2 Expansion of hiPSCs in Microcarrier-based Culture

As previously mentioned, the purpose of this work is to develop a scalable system for the expansion of human iPSCs under xeno-free and chemically defined conditions using a microcarrier-based culture in spinner-flasks.

Therefore, preliminary studies were initially performed under static conditions to evaluate once more the efficiency of human vitronectin as a substrate to support in vitro expansion of human iPSCs, now anchored on xeno-free compact microcarriers, and to identify the optimal conditions for cell seeding, namely dissociation method and initial cell density. For both studies, cells were expanded on Matrigel™ coated 6-well plates prior to inoculation on microcarriers.

IV.2.1 Static Conditions

IV.2.1.1 Vitronectin Efficiency and Optimal Dissociation Protocol

To evaluate the optimal dissociation method for inoculation on compact microcarriers, 50000 cells/cm² were seeded separately to vitronectin (VTN-N) and Geltrex® (GT) pre-coated beads, in an ultra-low attachment 24-well plate. Cells were inoculated as small clumps using EDTA treatment, with E8
medium, with or without ROCK inhibitor (ROCKi), and as single-cells (SC) using enzymatic dissociation with Accutase and ROCKi. Three replicates were performed for each condition (Figure IV-5).

Geltrex® was deliberately used as a control in order to compare and assess the efficiency of VTN-N in promoting and maintaining human iPSCs expansion for being a multi-component, undefined and xenogeneic substrate, in opposition to vitronectin.

At day 5, cells were harvested and counted and the expansion index for each condition was determined using Equation IV-1. According to the results (Figure IV-6), when cells were inoculated in E8 medium with EDTA, EDTA with ROCKi and as single-cells with ROCKi present an expansion index of 0.76, 4.43 and 3.50, on VTN-N coated beads, and 0.97, 4.30 and 4.80, on GT coated beads, respectively.
As the above results suggest, EDTA treatment with ROCK inhibitor is the most promising and efficient inoculation protocol as it presents the higher average expansion index for cell seeding on VTN-N coated beads.

To be noted that the use of ROCK inhibitor has been reported as not required when cultivating human induced pluripotent stem cells as a monolayer culture after an EDTA treatment. Nevertheless, the achieved results demonstrate that its use is crucial and mandatory for cell inoculation with EDTA on microcarriers, independently of beads coating, at least with the particular hiPSC line used in this work.

This might be related to the difficulty of cells to establish an immediate interaction with the substrate, and thus re-establishing cell-ECM interactions which are also crucial for cell survival, when inoculated on the surface of 3D structures, as it is the case of compact beads. The use of ROCK inhibitor increases the survival time of cells and, consequently, the adherence efficiency to beads. In fact, cell-ECM interaction is more easily restored in a 2D monolayer culture since gravity promotes sedimentation of cells on the top of coated culture plates.

By analyzing Figure IV-6, it is still possible to note that results are relatively similar for VTN-N and GT, being the only observed variation related to inoculation as single-cells with ROCK inhibitor. Nevertheless, this observation does not invalidate EDTA treatment with ROCKi as the most efficient method.

**IV.2.1.2 Optimal Initial Cell Density**

After establishing the efficacy of VTN-N & E8 system for the maintenance of human induced pluripotent stem cells, and EDTA inoculation with subsequent incubation with ROCK inhibitor as the most suitable method for cell dissociation and seeding, the assessment of the optimal initial cell density was then performed. For the purpose, three cell densities were tested, 30000, 50000 and 70000 cells/cm², for cell inoculation on VTN-N coated beads (Figure IV-7).

Once more, at day 5 cells were harvested and counted and the expansion index for each condition was determined according to Equation IV-1. By looking at the results (Figure IV-8), it is clearly evident that inoculation with 50000 cells/cm² is preferable to the other assessed options. Cells when inoculated in E8 medium on VTN-N coated beads present an expansion index of 3.5 ± 0.3, 4.4 ± 0.5 and 2.2 ± 0.2, after seeding of 30000, 50000 and 70000 cells/cm², respectively.
Figure IV-7 Evaluation of the optimal initial cell density for cell inoculation on VTN-N coated beads on an ultra-low attachment 24-well plate. Three cell densities were tested: 30000, 50000 and 70000 cells/cm². Cells were seeded by EDTA inoculation with ROCK inhibitor.

Figure IV-8 Expansion index of human iPSCs culture in E8 medium after cell inoculation with EDTA and ROCK inhibitor of 30000, 50000 and 70000 cells/cm² on VTN-N coated beads (n=3).

At days 1 and 4 following inoculation, optical microscope images were taken to VTN-N-coated beads with iPSCs anchored, for each cell seeding condition (Figure IV-9). At day 1, is noticeable the variance in the rate of cell adhesion which increases with initial cell density. However at day 4, prior to cell harvesting and counting, this difference is no longer visible in cells growing on the surface of microcarriers, especially for conditions of 50000 and 70000 cells seeded initially per cm².
Figure IV-9 Optical microscope image iPSCs cultured in E8 medium at days 1 and 4, after cell seeding of 30000, 50000 and 70000 cells/cm² on VTN-N coated beads (scale bars – 100 μm).

Therefore, and taking into account the results from the assays performed under static conditions, it is expected that the system resulting from iPSCs seeded with close to 50000 cells/cm² in VTN-N coated microcarriers, after an EDTA treatment with ROCK inhibitor, is the most suitable for large scale expansion and maintenance of human induced pluripotent stem cells.

IV.2.2 Dynamic Conditions

A Face-Centered Composite Design (FC-CD) was performed to identify the optimal operating condition (OOC) for the expansion of human iPSCs on VTN-N coated beads in a spinner-flask system supplied with E8 medium, after EDTA inoculation with ROCK inhibitor.

In this factorial design, two parameters were used as input, namely initial cell density (30000, 50000 or 70000 cells/cm²) and agitation speed (30, 50 or 70 rpm), to maximize fold increase (output). Therefore, nine different conditions were tested resulting from the combination in pairs of these two parameters.

The experimental data used in this multifactorial design, as well as the plots resulting from its analysis, are presented at Appendix (Table A. 1 and Figure A. 1, respectively). The optimal condition given by the model was 55000 cells/cm² and 44 rpm, as initial cell density and agitation speed, respectively, with a predicted fold increase of 3.5. Interestingly, the optimal cell density given by the model is similar to the one predicted in assays under static conditions.
Therefore, two spinner flasks were performed to validate the optimal operating condition (Figure IV-10). Prior to inoculation, cells were expanded on Matrigel™ coated 6-well plates. Cell counting was performed daily, in order to monitor cell growth, and cells were maintained in culture until death phase was reached. The resulting growth curves are presented in Figure IV-11.

**Figure IV-10** Validation of the optimal operational condition for the expansion of human iPSCs on VTN-N coated beads in a spinner-flask system, supplied with E8 medium.

**Figure IV-11** Growth curves of human iPSCs cultured in a spinner-flask system at optimal operating conditions determined by multifactorial design (n=1) (Note: \(N_i\) = total number of cells at day \(i\)).
According to these results, the rate of cell adhesion to VTN-N coated microcarriers is 41 ± 1%. As observed in both growth curves, stationary phase takes more than 7 days to be reached. Cells when cultured in a spinner-flask system at optimal operating condition present an expansion index of 4.4 ± 0.7. The average value obtained from experimental data is 27% higher than the one predicted by the model (Figure IV-12).

Despite the observed difference between expansion indexes, it cannot be unequivocally concluded that the model was not efficient in predicting fold increase value for OOC, since only two validations were performed, thus not being statistically relevant. Nevertheless, it can be clearly stated that the model allowed finding an operation condition which maximizes expansion index of hiPSCs in culture.

Still with the purpose of controlling the proposed culture system, samples from cells anchored on VTN-N coated beads were taken at day 6 and visualized at optical microscope. As expected, aggregates of microcarriers were observed resulting from cell growth on the surface of beads (Figure IV-13).

Figure IV-12 Expansion indexes of human iPSCs in a spinner-flask system at optimal operating conditions: experimental (n=2) (blue) and expected (grey) values.

Figure IV-13 Optical microscope image of human iPSCs cultured on VTN-N coated beads at day 6 in a spinner-flask culture system operated at optimum condition (scale bar – 100 μm).
Prior to harvesting, hiPSCs were characterized in terms of pluripotency by intracellular immunostaining for the expression of the pluripotency markers Nanog and Oct4. DAPI nuclei counterstaining was performed, as well. Results confirm that the majority of the cells attached to the microcarriers expresses both pluripotency markers (Figure IV-14).

![Immunostaining of human iPSCs cultured on VTN-N coated beads in a spinner-flask culture system operated at optimum conditions. Cells were stained for pluripotency markers Nanog and Oct4, and nuclei counterstained with DAPI (scale bar – 100 μm).](image)

**Figure IV-14** Immunostaining of human iPSCs cultured on VTN-N coated beads in a spinner-flask culture system operated at optimum conditions. Cells were stained for pluripotency markers Nanog and Oct4, and nuclei counterstained with DAPI (scale bar – 100 μm).

Flow cytometry was performed after cell harvesting to quantify the percentage of cells expressing Oct4, Sox2 and Nanog intracellular pluripotency markers. Results indicate that 92 ± 2% of the analysed cells were positive for Oct4, 97 ± 1% for Sox2 and 98 ± 1% for Nanog (Figure IV-15).
Figure IV-15 Flow cytometry analysis of intracellular pluripotency markers Oct4, Sox2 and Nanog, in human iPSCs cultured for 10-13 days on VTN-N coated beads in a spinner-flask culture system operated at optimum condition. Area under green curve represents the negative control and the purple area corresponds to the analysed samples. The percentage of analysed cells expressing each pluripotent marker is shown above bars.

Taking into account the results presented above related to rate of cell adhesion, expansion index, presence and quantification of intracellular pluripotency markers, it can be clearly stated the efficiency of the optimized spinner-flask culture system for expansion and maintenance of human induced pluripotent stem cells. The proposed microcarrier-based culture is both xeno- and serum-free and perfectly suitable for large-scale expansion of human induced pluripotent stem cells under GMP conditions.
V. CONCLUSIONS

Culture systems for large-scale production of viable hiPSCs by reproducible, clinically safe and cost effective methods still need to be develop to fulfill all their biomedical potential. In order to reduce variability in hiPSC cultures and efficiently avoid immune rejection upon transplantation of hiPSC-derived cells, these systems must be chemically defined and completely free of xenogeneic components.

In this work, a scalable culture system was established for the expansion and maintenance of hiPSCs. Xeno- and serum-free Essential 8™ (E8) medium was used as a culture medium for daily supply of all crucial nutrients and growth factors. Xeno-free microcarriers coated with human recombinant vitronectin (VTN-N) were used as well to provide a feeder-free adherent culture for hiPSCs expansion in spinner-flasks. This system is the first to combine the use of a microcarrier-based culture with E8 medium.

Preliminary assays were performed under static conditions to assess the efficiency of the combination of VTN-N & E8 system and to predict the optimal conditions for cell seeding on microcarriers, particularly dissociation method and initial cell density.

Results revealed EDTA treatment with ROCK inhibitor as the most promising and efficient inoculation protocol for cell seeding on VTN-N coated beads, with an expansion index of 4.4 at day 5. Moreover, results for each dissociation method were relatively similar between VTN-N and GT, which reinforces the possibility of using simple and xeno-free substrates in opposition to multi-component and xenogeneic ones in hiPSCs culture. Furthermore, an initial cell density around of 50000 cells/cm² was identified as the most suitable for hiPSCs inoculation of VTN-N coated beads, with an expansion index of 4.4 ± 0.5.

Bearing in mind these findings, hiPSCs expansion was transposed to a dynamic spinner-flask culture. A factorial design was initially performed to maximize fold increase (Fi) by adjusting two culture parameters, particularly initial cell density and agitation speed. The optimal operational condition found by the model, 55000 cells/cm² and 44 rpm, with a predicted Fi of 3.5, was validated twice.

Under this optimized spinner-flask culture system, cells presented an adhesion efficiency of 41 ± 1% and an expansion index of 4.4 ± 0.7, 27% higher than the one predicted by the model. The expression of pluripotency markers was evaluated by intracellular immunostaining and flow cytometry. Cells were 92 ± 2% positive for Oct4, 97 ± 1% for Sox2 and 98 ± 1% for Nanog.

In sum, the established culture system allows an efficient scale-up of hiPSCs expansion under fully xeno-free and chemically defined conditions. The simplicity of Essential 8™ medium dramatically reduces cost and lot-to-lot variability thus representing a practical alternative to other serum-free cultures. Furthermore, the use of EDTA treatment with ROCK inhibitor as cell dissociation method reduces time consumption and variability between batches.
VI. FUTURE WORK

Despite the promising results presented above, some future work is still required towards the development and establishment of a culture system for hiPSCs expansion that aims to meet the demand of practical research and clinical applications.

Future approaches will consist in testing at first the system robustness through the use of other feeder-free hiPSC lines at optimal operational condition. Furthermore, and when transposing and up-scaling the proposed culture system from spinner-flasks to stirred-suspension bioreactors, there are other parameters that can be optimized and adjusted in order to maximize fold increase, such as osmolarity, dissolved oxygen tension and hydrodynamic shear stress.

Additionally, it would also be interesting and useful to optimize cell yield by increasing the rate of cell adhesion to microcarriers. The proposed culture system allows an adhesion efficiency of 41 ± 1%, which is actually common for inoculation of hiPSCs on microcarriers. However, and particularly when comparing to other human stem cells, this is still a sub-optimal value. Moreover, the development of a bioprocess combining expansion and differentiation of hiPSCs with purification step would also be desirable and valuable.
BIBLIOGRAPHY

Consulted Bibliography


References


5. The European Bioinformatics Institute. [Online] [Cited: 24 06 2014.] http://www.ebi.ac.uk/biomodels/ModelMonth/2010-06/fig1.png.


**APPENDIX**

**Table A.1** Expansion of human iPSCs on VTN-N coated beads in spinner flasks supplied with E8 medium (experimental data from Dr. Sara Badenes).

<table>
<thead>
<tr>
<th>Initial Cell Density (cells/cm²)</th>
<th>Agitation (rpm)</th>
<th>Fold Increase (Yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30000</td>
<td>30</td>
<td>3.11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>0.55</td>
</tr>
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<td>500000</td>
<td>30</td>
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<tr>
<td></td>
<td>50</td>
<td>3.36</td>
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<td></td>
<td>70</td>
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<td>3.96</td>
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<td></td>
<td>50</td>
<td>3.96</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Figure A.1** Factorial design model relating initial cell density and agitation speed with fold increase (yield). Input values from experimental data were converted prior to multifactorial analysis. For initial cell density: 30000 cells/cm² (-1 level), 50000 cells/cm² (0 level) and 70000 cells/cm² (1 level). For agitation: 30 rpm (-1 level), 50 rpm (0 level) and 70 rpm (1 level).