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Faculdade de Ciências e Tecnologia

Departamento de Química

Microencapsulation technology: a powerful tool for human embryonic stem cells expansion and cryopreservation

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Dissertation presented to obtain a Master degree in Biotechnology at the Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia

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Monte de Caparica, 2010

Acknowledgements

I would like to acknowledge all the people directly or indirectly involved in this thesis.

To Dr. Paula Alves, for giving me the opportunity to do my master thesis at the Animal Cell Technology Unit at ITQB/IBET, for the good working conditions offered and for being a strong example of leadership. Also, I would like to thank the chance of attending the international symposium "Stem cells in biology and disease" as well as the iPS Cells Technology course that positively contributed to my scientific formation.

To Prof. Susana Barreiros for always being available to help during the entire Master degree.

To Prof. António Laires for accepting to be jury of my thesis.

To Dr. Heiko Zimmermann and his group at Fraunhoffer-IBMT for kindly providing the cGMP grade ultra high viscous alginate.

To Margarida Serra for having taught me the majority of what I learnt this year and for always being there to help me. A deep thanks for your confidence, support, constant encouragement and friendship. It is a pleasure to work with you and to be your friend.

To all the TCA colleagues for the good working environment. A special acknowledgment to: Eng. Marcos Sousa and Dr. Catarina Brito, for always being available to help in anything; Rui Tostões for the help with confocal images; Rita Malpique for helpful discussions on cryopreservation studies; Carina Silva for the initial formation.

A todos os amigos que fiz durante os meus cinco anos académicos na FCT, pelos bons momentos passados dentro e fora desta instituição. Um agradecimento especial à Joana, à Cris, à Lúcia, ao Hélio, ao André e à Vera pela amizade e por me fazerem sempre rir.

Aos amigos de longa data, dos quais nunca me esqueço.

À minha cara-metade por estar sempre presente, por todo o apoio durante estes últimos anos e por ter partilhado comigo alguns dos momentos mais felizes da minha vida.

À minha família, em particular aos meus pais a quem devo tudo. Obrigada por me terem transmitido os vossos valores e por me terem ajudado e apoiado sempre em todos os momentos. À minha querida avó, de quem tanto gosto, por ajudar a cuidar de nós. À minha sis, por ser quem é, para mim um exemplo de coragem e de perseverança, e por nunca se esquecer de mim.

Preface

This work was performed in the scope of the project - Integrated strategy for expansion, neuronal differentiation and cryopreservation of human embryonic stem cells (PTDC/BIO/72755/2006) funded by FCT (Fundação para a Ciência e Tecnologia). The main aims of this project are:

i) Scale-up of undifferentiated hESCs production, with the goal of obtaining large expansion of healthy undifferentiated hESCs.

ii) Optimize scalable protocols to direct and control differentiation of hESCs into neuronal lineages.

iii) Improvement of cryopreservation protocols to successful storage and transport of viable and genetically stable stocks of both undifferentiated and differentiated hESCs.

This thesis contributed to achieve the objectives i) and iii).

This work led to the following article:

Serra, M., Correia, C., Malpique, R., Brito, C., Bjorquist, P., Carrondo, M.J.T., and Alves, P.M. Microencapsulation technology: a powerful tool to integrate expansion and cryopreservation of pluripotent human embryonic stem cells. Accepted in PLoS One.

Abstract

Human embryonic stem cells (hESCs) are known by their ability to either self-renewal and differentiate into any adult cell type. These properties confer to hESCs a huge applicability for cell therapy, tissue engineering and drug screening. However, successful implementation of hESCs-based technologies requires the production of large numbers of well characterized cells and their efficient long-term storage. In this study, alginate microencapsulation technology was used in order to develop an efficient, scalable and integrated 3D culture system for expansion and cryopreservation of pluripotent hESCs. Three strategies were outlined: microencapsulation of hESCs as single cells, cell aggregates and cells immobilized on microcarriers.

Encapsulation of hESCs immobilized on microcarriers was the best strategy to expand and cryopreserve pluripotent hESCs. The culture of encapsulated hESCs-microcarriers in spinner vessels assured an approximately 20-fold increase in cell concentration. Moreover, this strategy improved twice cell survival after cryopreservation by a slow-freezing rate procedure, comparatively with non-encapsulated culture. Microencapsulation also protected hESC aggregates from damage caused by stirring, allowed the control of aggregates size and the maintenance of cells pluripotency for two weeks.

This work demonstrates that microencapsulation technology is a powerful tool to enhance growth and post-thawing recovery of pluripotent hESCs. The 3D culture systems developed herein represent a promising vehicle to assist the transition of hESCs to the clinical and industrial fields.

Key Words: Microencapsulation, hESCs, expansion, cryopreservation, 3D culture

Resumo

As células estaminais embrionárias humanas (hESCs) são conhecidas pelas capacidades de proliferar indefinidamente em cultura e de diferenciar em qualquer tipo de célula adulta. Estas propriedades conferem às hESCs uma enorme aplicabilidade em terapia celular, engenharia de tecidos e no desenvolvimento de novas drogas. No entanto, para que a implementação de tecnologias baseadas em hESCs seja bem-sucedida, é necessário assegurar a produção de um elevado número de células bem caracterizadas e o seu armazenamento adequado a longo prazo. Neste estudo, a tecnologia de microencapsulação em alginato foi utilizada para desenvolver um sistema de cultura 3D eficiente, escalonável e integrado para a expansão e criopreservação de hESCs pluripotentes. Três estratégias foram delineadas: encapsulação de hESCs como células individuais, encapsulação de agregados de hESCs e encapsulação de hESCs imobilizadas em microsuportes.

A encapsulação de hESCs imobilizadas em microsuportes revelou ser a melhor estratégia para expandir e criopreservar hESCs pluripotentes. A cultura de hESCs-microsuportes encapsulados em vasos agitados permitiu um aumento de aproximadamente 20 vezes na concentração celular. Além disso, com esta estratégia de cultura 3D, conseguiu-se duplicar a percentagem de sobrevivência das células, imediatamente após criopreservação por um processo de congelamento lento, em relação à cultura não-encapsulada. É importante realçar que a microencapsulação permitiu proteger os agregados de hESCs dos danos causados pela agitação, controlar o tamanho dos mesmos e manter o estado pluripotente das células por duas semanas.

Em suma, este trabalho demonstra que aplicando a tecnologia de microencapsulação é possível melhorar o crescimento de hESCs pluripotentes e a recuperação das células após os processos de congelamento/descongelamento. Desta forma, os sistemas de cultura 3D aqui desenvolvidos constituem uma estratégia promissora para acelerar a transição de hESCs para a área clínica e industrial.

Palavras-Chave: Microencapsulação, hESCs, expansão, criopreservação, cultura 3D

Abbreviations list

ASC adult stem cells
ASMA α-smooth muscle actin
bFGF basic fibroblast growth factor
DAPI 4,6-diamidino-2-phenylindole
DMEM Dulbecco's minimum essential medium
DMSO dimethyl sulfoxide
CPA cryoprotective agent
EB embryoid bodies
ECM extracellular matrix
EG ethylene glycol
FBS fetal bovine serum
FDA fluoresceíne diacetate
FOXA2 forkheadbox A2
HSA human serum albumin
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESCs human embryonic stem cells
ESCs embryonic stem cells
hFFs human foreskin fibroblasts
ICM inner cell mass
IgG immunoglobulin G
IgM immunoglobulin M
IVF *in vitro* fertilization
KO-SR knockout serum replacement
LN2 liquid nitrogen
mEFs mouse embryonic fibroblasts
mEFs-CM conditioned media in mEFs
mESCs mouse embryonic stem cells
NEAA non-essential aminoacids
non-CM non-conditioned media
PBS phosphate buffered saline
Pen/Strep Penicillin/Streptomycin
PI propidium iodide
PFA paraformaldehyde
Oct-4 transcription factor octamer-4
OPS open pulled straw
ROCKi Rho-associated kinase (ROCK) inhibitor, Y-27632
SCED single cell enzymatic dissociation
SD standard deviation
SSEA-1 stage-specific embryonic antigen-1
SSEA-4 stage-specific embryonic antigen-4
TRA-1-60 tumour rejection antigen-1-60
TX-100 Triton X-100
UHV ultra-high viscous
VS vitrification solution
WS warming solution
2D two dimensional
3D three dimensional

Contents

1. Introduction

1.1. Human embryonic stem cells (hESCs)

Human embryonic stem cells (hESCs) have an enormous potential for cell-based therapy, due to their ability to self-renew indefinitely and to differentiate into all mature cell types of the human body (pluripotent nature). The first reports describing hESCs potential were published in 1984[1] and 1994[2], but it was only in 1998 that Thomson and co-workers described the isolation of hESCs from blastocysts and the creation of the first permanent and characterized hESC lines for research[3].

hESCs are derived from the inner cell mass (ICM) of blastocysts[3] (**Fig. 1.1**). A blastocyst is a pre-implantation embryo that develops 5 days after the fertilization. It contains all the material necessary for the development of a complete human being. In normal development, the blastocyst would implant in the wall of the uterus to become the embryo and continue developing into a mature organism. The outer cells (trophoblast) would begin to form the placenta and the cells from the ICM would begin to differentiate into the progressively more specialized cell types of the body.

Today, more than 1000 hESC lines are described in the literature[4]. Some of these cell lines are well characterized and organized in international stem cell banks, for example, the hESCreg (www.hescreg.eu), the UK stem cell bank (www.ukstemcellbank.org.uk), and the National stem cell bank (www.nationalstemcellbank.org)[5].

hESC lines can be identify by the presence of surface marker antigens (Tra series, SSEA series, GCT series, HLA, and CD markers) and transcriptional factors (Oct4, Nanog), by the ability to differentiate into tissues originating from all three germ layers *in vivo* (via teratoma formation) and *in vitro* (via embryoid body differentiation), by the chromossomal stability with serial culture, alkaline phosphatase positiveness and high telomerase activity[6, 7].

1.1.1. Sources of pluripotent stem cells

As mentioned above, hESCs are derived from the ICM of blastocysts. In brief, ICM is isolated mechanically[3] or by immunosurgery[8] and plated in a culture dish with feeder cell layers (inactivated fibroblasts) and a nutrient-rich media where they give rise to embryonic stem cells (**Fig. 1.1**).

The main suppliers of blastocysts for stem cell research are *in vitro* fertilization (IVF) clinics. The process of IVF requires the retrieval of a woman's eggs via a surgical procedure after undergoing an intensive regimen of "fertility drugs". When IVF is used for reproductive purposes, all of the donated eggs are fertilized in order to maximize their chance of producing a viable blastocyst that can be implanted. Because not all the fertilized eggs are implanted, this has resulted in a large bank of excess blastocysts that are currently stored for use in medical research. Importantly, the unused blastocysts can only be utilized for research purposes with the written informed consent of the donors.

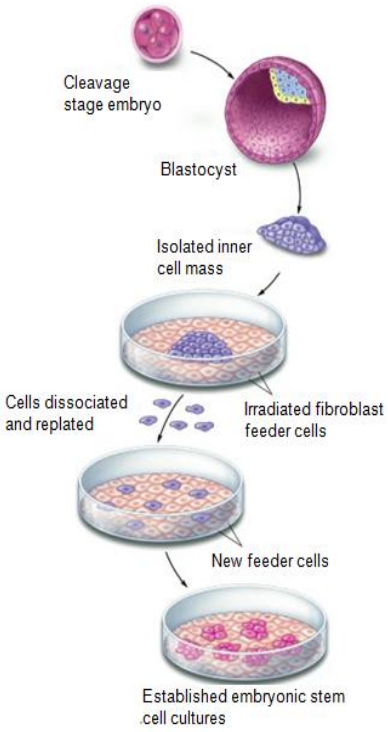

Fig. 1.1 - **hESC line derivation.** Main steps of the protocol for establishment of hESCs lines[9].

The creation of stem cells specifically for research using IVF rises ethical issues because it involves the destruction of human embryos. Accordingly, other strategies are still being developed with different methods and cell sources to obtain pluripotent stem cells (**Fig. 1.2**). For example, single-cell biopsy of the embryo using a procedure similar to that used in preimplantation genetic diagnosis[10] is one approach. The process called nuclear transfer or nuclear reprogramming offers another potential way to produce pluripotent stem cells. This procedure consists on inserting the nucleus of a somatic cell (which is diploid, 2n) into an enucleated oocyte. In the environment of the oocyte, the somatic cell nucleus is reprogrammed so that the cells derived from it are pluripotent. From this oocyte, a blastocyst is generated, from which embryonic stem cell lines are derived in tissue culture[11]. The resulting cells are genetically identical with the cell donor. However, somatic cell nuclear transfer has not yet been successfully performed in humans and the difficulty in obtaining human oocytes remains a major limitation[12]. Recent advances in reprogramming have been developed, making possible the generation of induced pluripotent stem cells (iPSCs) from somatic cells. The first iPSCs were reported in 2006 and were obtained from murine fibroblasts[13]. In general, iPSCs are generated from differentiated cells that have been reprogrammed to acquire a pluripotent state through overexpression of a core transcription factors known to be required for maintenance of pluripotency and proliferation of ESCs (*Oct4*, *Sox2*, and either *c-Myc* and *Klf4* or *Nanog* and *Lin28*)[10, 13, 14]. iPSCs

exhibit similar features to embryonic stem cells, including cell morphology, cell-surface markers, growth properties, telomerase activity expression, and epigenetic marks of pluripotent cell–specific genes[13, 14] and can give rise to cells derived from all three germ layers *in vitro* and *in vivo*. This technology can be used to generate patient-specific cell types[11, 15], opening the door to "personalized" cell-based therapy. However, generation of iPSCs still suffers from low efficiency and high cost[10]. Furthermore the viral expression vectors used to obtain iPSCs[16], the potential for insertional mutagenis[11] and the recent knowledge that hiPSCs expresses cancer hallmarks[17] have raised additional concerns regarding the safety of these cells for clinical applications.

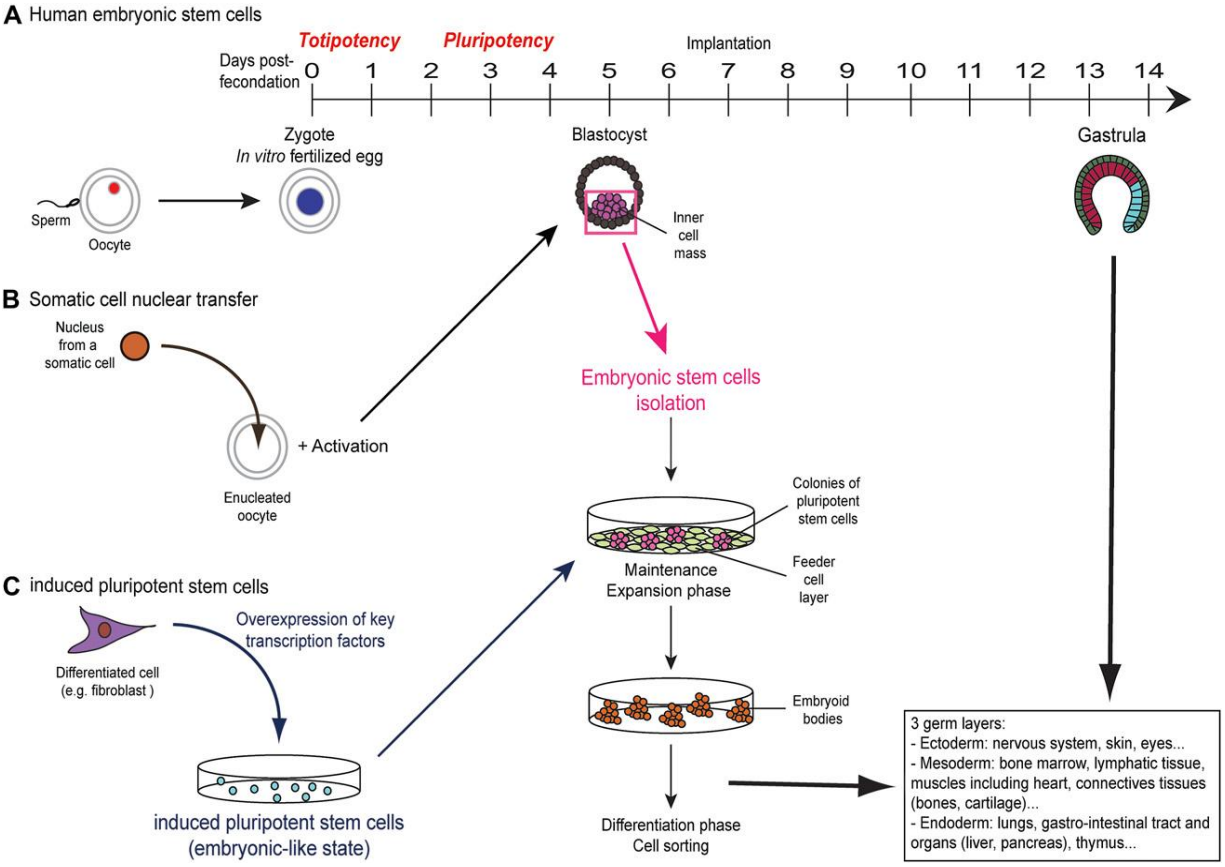

Fig. 1.2 – Isolation/generation, culture and differentiation of pluripotent stem cells. The development of pluripotent stem cells by **A)** *in vitro* fertilization, **B)** somatic cell nuclear transfer and **C)** generation of induced pluripotent stem cells is illustrated. Typically pluripotent cells are expanded in culture on feeder cell layers. When removed from feeders and transferred to suspension cultures, hESCs begin to form 3D aggregates of differentiated and undifferentiated cells that resemble early post-implantation embryos, named embryoid bodies. Plated cultures of embryoid bodies spontaneously display a variety of cellular types from the 3 germ lineages at various differentiation stages. Progenitors of differentiated cells can then be identified and sorted by specific markers, expression of reporter genes and characteristic morphology. Enriched culture in progenitor cells should then be cultured in a way that allowed their differentiation into more mature cell types, normally in the presence of specific growth factors. (Adapted from Brignier *et al.* [10]).

1.1.2. Advantages of using hESCs instead ASCs or primary cells

Another type of stem cells is also currently used in research, particularly adult stem cells (ASCs) found in specialized tissues of the body, including the brain, bone marrow, liver, skin and gastrointestinal tract[10]. However these cells are less flexible than hESCs, because their differentiation potential is limited. ASCs form only a restricted number of cell types, usually only the cell types of the lineage of their origins, thus they are classified as multipotent stem cells. Moreover, hESCs are also generally more easier to isolate, purify and maintain *in vitro* than ASCs (with the relative exception of hematopoietic stem cells)[18] (**Tab. 1.1**). Therefore, ASCs have a limited usefulness for tissue engineering or regenerative medicine compared with hESCs. In addition primary cells suffer from having an even lower expansion and differentiation potential (**Tab. 1.1**). Nevertheless, hESCs are difficult to control in what concerns their stem cell fate, requiring more complex and robust bioprocesses (**Tab. 1.1**).

Tab. 1.1 - Comparison between human embryonic stem cells (ESCs), adult stem cells (ASCs) and primary cells types. Legend: **+** low, **++** medium, **+++** high. (Adapted from Polak *et al.* [19]).

Cell type	Expansion potential	Differentiation potential	Cell availability	Ease of regulation	Bioprocess complexity
ESCs	+++	+++	+++	+	+++
ASCs	+	++	++	++	++
Primary cells	-	-	+	+++	+

1.1.3. hESCs applications

The unlimited self-renewal ability and pluripotency of hESCs gives them limitless applications (**Fig. 1.3**). Indeed hESCs are excellent candidates to cure diseases by repairing or replacing damaged cells and tissues (cell therapy or regenerative medicine), since they could be used to generate an infinite quantity of cells with a clinical interest, including retina cells[21], dopaminergic neurons[22], motor neurons[23], OPCs (oligodendrocyte progenitor cells)[24],

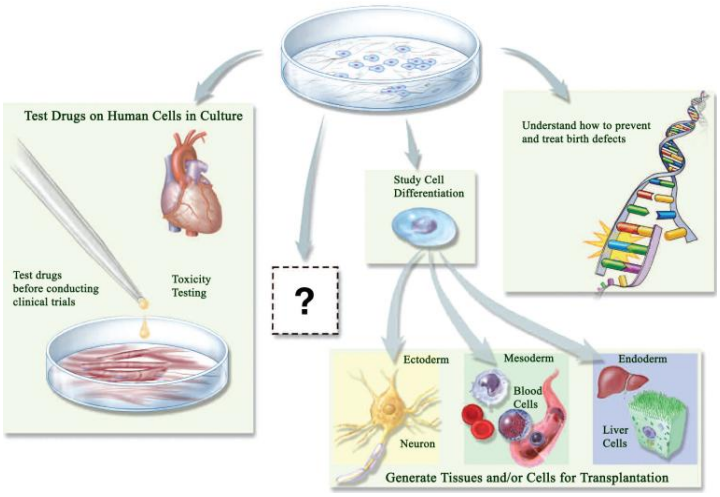

Fig. 1.3 - Human embryonic stem cells applications [20]

cardiomyocytes[25], pancreatic β-cells[26] and hepatocytes[27]. Moreover, the US Food and Drug Administration recently approved the world's first phase I clinical trial using hESCs derivatives (oligodendrocyte progenitor cells) in patients with spinal cord injury[10].

Another interesting application of hESCs is to serve as pharmacological and cytotoxicity screening platforms, supporting the discovery of new drugs for therapeutic use[28]. Given the high costs spending to bring a new drug to market, there are great advantages of having access to large numbers of biologically relevant human cells for early testing and screening. For example, pure cultures of hepatocytes, cardiomyocytes and neuronal cells derived from hESCs would provide robust cell-based *in vitro* assays for toxicity measurements and for drugs being development for cardiovascular or neurodegenerative disorders, respectively[5].

ESCs are also valuable models for scientific research. They can lead to a better understanding of the basic biology of the human body, embryonic development, pathogenesis of congenital defects and cancer formation[6]. In fact, it is possible to derive disease-specific hESCs from embryos with diagnosed mutations by preimplantation genetic diagnosis[29]. For instance hESC lines derived from embryos with Fanconi anemia-A mutation and fragile X mutation have already been established[29]. These hESC lines will provide *in vitro* models for study the phenotype of these mutations, allowing the identification of new treatments for these diseases.

1.1.4. Using hESCs in the clinic: critical issues

Even though hESCs hold great promise for the cure of various human disorders, there are three significant issues that need to be addressed prior these cells being approved for clinical and industrial applications: the right quality, quantity and purity of hESCs and/or their derivatives have to be achieved (**Fig. 1.4**).

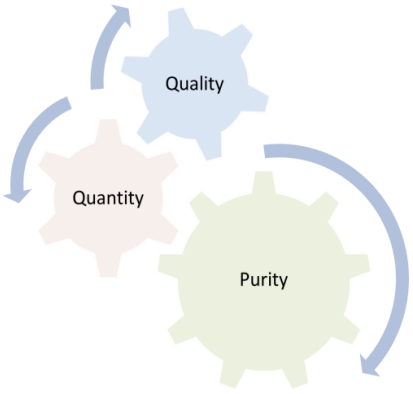

Fig. 1.4 – Criteria that have to be addressed before hESCs approval for clinical use.

1.1.4.1. Quality

Initial methods to culture hESCs were based on techniques originally developed to culture mouse ESCs[3], that involved the culture on a layer of mitotically inactivated mouse embryonic fibroblasts (mEFs) and medium supplemented with 20% fetal bovine serum (FBS). In these conditions, hESC lines could be propagated indefinitely with retention of their

pluripotent properties[3]. However, it was realized that the continuous use of mEFs and animal-derived components in hESCs cultures would hinder the development of clinical applications due to: the presence of non-human sialo proteins in culture, which are immunogenic for humans; the risk of transmitting animal virus or prion material; and difficulty with quality control of these undefined components[30-32]. Subsequently, improvements to these procedures have largely focused on removing the undefined and non-human components.

Serum can be replaced by Knock-Out Serum Replacement (KO-SR), commercially available serum substitute with less batch-to-batch variability than serum. However, KO-SR is still not yet a fully defined product[33] and has animal-derived components, namely AlbuMAX, a lipid-rich albumin fraction of bovine serum and bovine transferrin[32], but in spite of that it has been validated for culture of clinical grade cells[34].

Regarding the use of mEFs, it is not entirely understood the purpose of growing hESCs on feeder cell layer. However it has been suggested that feeder cells provide a suitable attachment substrate and important soluble factors for the maintenance of undifferentiated hESCs[31]. Although, irradiated or treated with mitomycin C, feeder cells are still capable of stimulate ESC growth and also inhibit their differentiation through the secretion of specific growth factors and cytokines[35]. Since the exposure to animal components presents a serious risk to the patients, mEFs have been replaced by human fibroblasts. These feeders equally support hESCs growth and maintenance[36-38]. Nevertheless, due to the drawbacks associated to the use of co-culture systems, several attempts to establish feeder-free systems for hESCs propagation have been performed. A successful feeder-free hESC culture system was developed in which undifferentiated cells were maintained long term on a Matrigel layer in mEFs-conditioned medium[39]. Matrigel is a soluble basement membrane extract from mouse sarcoma which contains extracellular molecules, such as laminin, collagen IV, growth factors and other unknown components[31]. Moreover, a single extracellular matrix (ECM) component such as fibronectin, has also been successfully used to support undifferentiated growth of hESCs in non-conditioned medium supplemented with KO-SR and various growth factors[40, 41]. Basic fibroblast growth factor (bFGF) is one example, it plays a key role in sustaining hESC self-renewal[42], and is included in nearly all the reported medium formulations for hESC propagation[30].

It should be noted that, to establish hESC lines with clinical quality, procedures and compounds (namely isolation of the ICM, derivation and handling of the feeder cells and

culture matrices, culture media, hESCs passaging and cryopreservation) have to minutely follow the FDA (Food and Drug Administration) regulations[43]. Importantly, cell phenotype and function should be characterized and evaluated during culture. Undifferentiated hESCs have to maintain their pluripotency and karyotype stability after expansion while hESCs derivatives must express markers of the specific cell lineage and be fully functional after differentiation.

1.1.4.2. Quantity

Another important challenge is to achieve sufficient numbers of stem cell for an effective therapy. For example, $1x10^9$ to $2x10^9$ cardiomyocytes are required to replace damaged cardiac tissue after myocardial infarction[44] and $1.3x10^9$ insulin producing β-cells per 70-kg patient[45] are needed for insulin independence after islet transplantation. To achieve these high cell numbers scalable bioprocesses need to be developed.

The requirement of two-dimensional (2D) surfaces, such as well-plates and tissue-culture flasks for cell attachment is the first hurdle to limit large stem cell production and clinical applications[19]. Indeed there are several drawbacks related with the 2D culture systems namely: i) low reproducibility due to the uncontrolled culture conditions, ii) achievement of low cell yields, iii) limitations in scaling-up, iv) labor-intensive procedures, v) inability to support complex cellular growth configurations[19]. Therefore, the establishment of novel three-dimensional (3D) culture systems with a high available surface area for cellular attachment and growth that resemble the *in vivo* conditions by accounting for the cell–cell, cell–matrix and cell–growth factor interactions (see **section 1.2**) became a priority.

In addition, in order to improve cell density or the expansion rate of the 3D systems cultures, bioreactors or stirred vessels that can accommodate dynamic culture conditions have been used[46, 47]. Stirred suspension bioreactors makes possible to overcome the mass transport limitations of static cultures due to the constant agitation rate promoted for example by an impeller. However, they require careful impeller design and the delineation of the optimum stirring speed for each culture. Distinct cell types have different sensitivities/necessities in terms of the shear stress (force exerted over the cells due to the flow of the media)[46]. Consequently, an inappropriate agitation could cause unsuitable shear stress that can damage the cells.

Moreover, bioreactors allow the production of high cell numbers in a well-regulated environment, with controlled oxygen, pH, temperature and nutrition supply. They are hidrodynamically well characterized, can be easily scaled-up and the risk of contamination is

low[46, 48]. So far, fully controlled stirred tank bioreactors appear as promising candidates for the expansion of hESCs since at least a 10-fold increase in cell density is achieved when compared with the traditional 2D culture systems[47].

Another worth mentioning aspect is that clonal efficiency of hESC is extremely low. These cells are very sensitive to single-cell dissociation and recover poorly when plated at clonal density after a passage[30], which hinders cell passage from 2D to 3D culture system. In fact, cell-cell interactions seem critical for efficient hESCs propagation, since it was demonstrated that the loss of gap junctions between hESCs can increase cell apoptosis and inhibit colony growth[49]. For these reasons, hESCs have been routinely passaged in cell clumps. In order to facilitate up-scaling of hESCs culture, it was developed a new single-cell enzymatic dissociation (SCED) culture system using recombinant protein-based TrypLE Select[50]. Nevertheless, clonal survival of hESCs can also be enhanced by culturing in the presence of Rho-associated kinase inhibitor (ROCKi)[51].

1.1.4.3. Purity

The tumorigenic potential of pluripotent cells is other important hurdle in the safety utilization of these cells. At present, protocols for the differentiation of hESCs are generally inefficient, resulting in low differentiated cell yields and contamination by other cell types. Of greater concern is the persistence of undifferentiated hESCs and the possibility of these cells form malignant tumors when transplanted in the host[52]. Therefore the use of robust methods for i) differentiation, ii) selection of pure populations of specialized cells and iii) demonstration of their genetic and epigenetic stability will be essential before these cells being used clinically[10].

1.2. 3D models for hESCs culture

The proliferation and differentiation of stem cells depends largely on cell adhesion and the provision of a three-dimensional growth environment that mimic the physiological (*in vivo*) *milieu* in developing and adult tissues[47, 53]. *In vivo* cells microenvironment is composed by the extra cellular matrix (ECM), soluble growth factors, cell-cell interactions and mechanical stimuli[54, 55] (**Fig. 1.5**). ECM provides structural support and physical environmental for cells[56]. The architecture of the ECM can guide morphological changes and cellular organization[53]. Specific signaling molecules on the ECM itself can affect cell behavior and direct cell differentiation into a particular lineage[57]. ECM proteins bind to specific integrin cell surface receptors, activating intracellular signaling pathways that control gene

expression, cytoskeletal organization and cell morphology[47]. Besides that, cells constantly remodel local ECM by degrading or synthesizing new ECM elements[58].

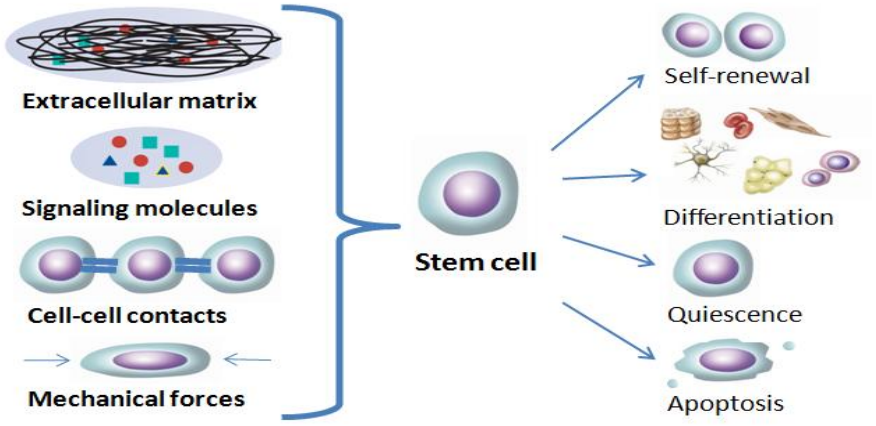

Fig. 1.5 – Components of the cellular *in vivo* microenvironment. The synergy of biochemical (signaling molecules), biophysical (ECM or substrates) and mechanical (hemodynamic forces or shear forces) factors with cell-cell interactions determine the fate of pluripotent stem cells. (Adapted from Abraham *et al.* [55])

Thus, culturing stem cells in 3D microstructures that closely mimic stem cells native microenvironment is imperative to enhance cells performance and fully exploit cells potential.

hESCs are highly anchorage dependent and need a surface to attach and proliferate. Therefore, growth of these cells in a 3D configuration (**Fig. 1.6**)[59] requires normally either the use of a support system, such as microcarriers, or the formation of small aggregates. Moreover, polymeric matrices like alginate hydrogels can also be a good option for hESCs cultivation since this type of matrices better represents the geometry, chemistry and signaling of *in vivo* environment than 2D cultures[60] (**see section 1.5.**).

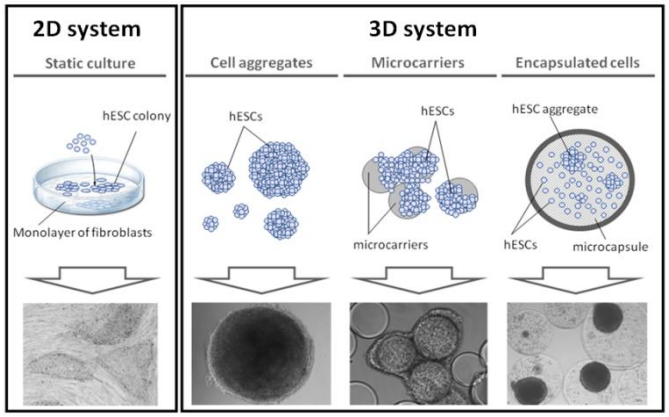

Fig. 1.6 – 2D and 3D systems for cultivation of human embryonic stem cells. (Adapted from Serra *et al.* [59])

1.2.1. Culture of hESCs as aggregates

The first attempts to grow ESCs in scalable suspension cultures were based on the tendency of undifferentiated stem cells to form embryoid bodies in a feeder free or non-adherent system[61]. The culture of hESCs in suspension bioreactors was first reported by Gerecht-Nir *et al.*[62]. This group cultured EBs of hESCs in slow-turning lateral vessels (STLVs).

The cells grew 70-fold, reaching $3.6x10^7$ cell/mL after 28 days and gave rise to cells of the three germ layers.

hESCs differentiate extensively when cultured as aggregates[61], therefore is difficult to control the expansion of undifferentiated hESCs and their further directed differentiation into specific cell types. Indeed, there are only a reduced number of studies that reported the expansion of undifferentiated hESCs as aggregates (see **Tab. 1.2**; Page 22).

1.2.2. Culture of hESCs on microcarriers

Microcarriers are spherical particles, composed of various materials including cellulose, glass, plastic, and polyester. They have a typical diameter of 100-250 µm and can be compact or porous[63]. Currently many types of microcarriers are commercial available and they are used for the growth of several adherent cell types. Indeed, the attributes of microcarriers make them also attractive for culture and directed differentiation of hESCs. First, they allow the translation of anchorage-dependent hESCs from 2D culture into suspension culture by providing a high attachment surface. Also, microcarrier cultures are characterized by high surface-to-volume ratio, which allows higher cell densities than 2D cultures. More important, the available area for cell growth can be adjusted easily by changing the amount, porosity and size of microcarriers[61, 63].

Normally microcarriers have to be customized, for example, by attaching synthetic peptides or extracellular matrix molecules (collagen, Matrigel, fibronectin…) in order to improve the adhesion of hESCs. In fact, different types of microcarriers were already tested to culture hESCs and some distinct results were obtained. For example, Nie *et al.* reported that Cytodex 3 beads appeared to promote better attachment and viability of hESCs[63], whereas others observed that hESCs on these beads exhibited the poorest growth with little or no recovery of viable cells after 48–72 hours[64]. Such discrepancies may be due to the use of different hESC lines. Nevertheless, the adhesion efficiency of hESCs increased when the beads were coated with Matrigel or seeded with mouse embryonic fibroblasts (mEFs)[63]. For instance, our group recently demonstrates a 12-fold in yield of hESCs cultured in Cytodex3 microcarriers coated with Matrigel in 300mL perfused bioreactors fully controlled, operating with pO_2 at 30% air saturation[65]. See **Tab. 1.2** (Page 22) for more examples.

1.3. hESCs cryopreservation

To fully exploit the potential of hESCs in medicine and research, freezing, cryostorage and thawing technology is also of major importance.

Cryopreservation is traditionally defined as the maintenance of biologics at temperatures typically bellow the glass transition of pure water (-132°C), at which biological metabolism is dramatically diminished[66].

An effective method for hESCs cryopreservation is critical for their use in clinical and research applications. A suitable cryopreservation enables a good storage and transportation of the cells between the sites of collection, processing and clinical administration[67]. This makes possible the exchange of cells between research centers, which promotes scientific collaboration and facilitates widespread use of hESCs. Also, the ability to preserve cells permits the banking of stem cells until later use. An inefficient hESCs cryopreservation increases time between cell storage and use in experimental or clinical settings, because of an extended lag in establishing a viable highly populated culture following thawing. Even though hESCs are self-renewable, aging cultures can acquire chromosomal abnormalities and lose their differentiation potential, thus efficient procedures to preserve hESCs in low passage numbers are indispensable.

1.3.1. Cryopreservation methodology

Typically, cryopreservation procedure involves the steps described in **Fig. 1.7**. First cells are subjected to a pre-freezing treatment in order to leave the cells in the state that they will be frozen (e.g. single-cell suspension, cell aggregates, adherent cell monolayers) and the cell viability is evaluated. Second cells are transferred into the cryovessel on which they will be frozen (e.g. vial, well-plate, straw). Before freezing, samples are loaded with a cryoprotective agent (CPA) like DMSO or glycerol to help minimizing the injury to cells during freezing and thawing. The cells are then frozen at the desired cooling rate to the storage temperature at which they will be stored. After thawing CPAs are removed from the sample by dilution. At this time post-thaw cell recovery is evaluated.

CPAs are usually classified based on their ability to diffuse across the plasm membrane of cell in penetrating (e.g. DMSO, glycerol and 1,2-propanediol) or non-penetrating CPAs (hydroxyethyl starch, sucrose and polyssaccharides).

The exact mechanisms by which penetrating CPAs are able to protect cells from cryo injury are not fully understood. However, it was proposed that penetrating CPAs reduce colligatively the concentration of damaging electrolytes at a given subzero temperature[68] and reduce the extent of the cell volume change during slow-rate freezing and thawing[69].

Non-penetrating CPAs are generally relatively high molecular weight, long chain polymers that are soluble in water and can only be taken up by cells through endocytosis or induced processes. They are thought to act by dehydrating the cells before freezing, thereby reducing the amount of water that the cell needs to lose to remain close to the osmotic equilibrium during freezing[70]. On the other hand, trehalose and other polysaccharides protect membranes and proteins against the destructive effects of dehydration by serving as a substitute for structural water associated with their surface[71].

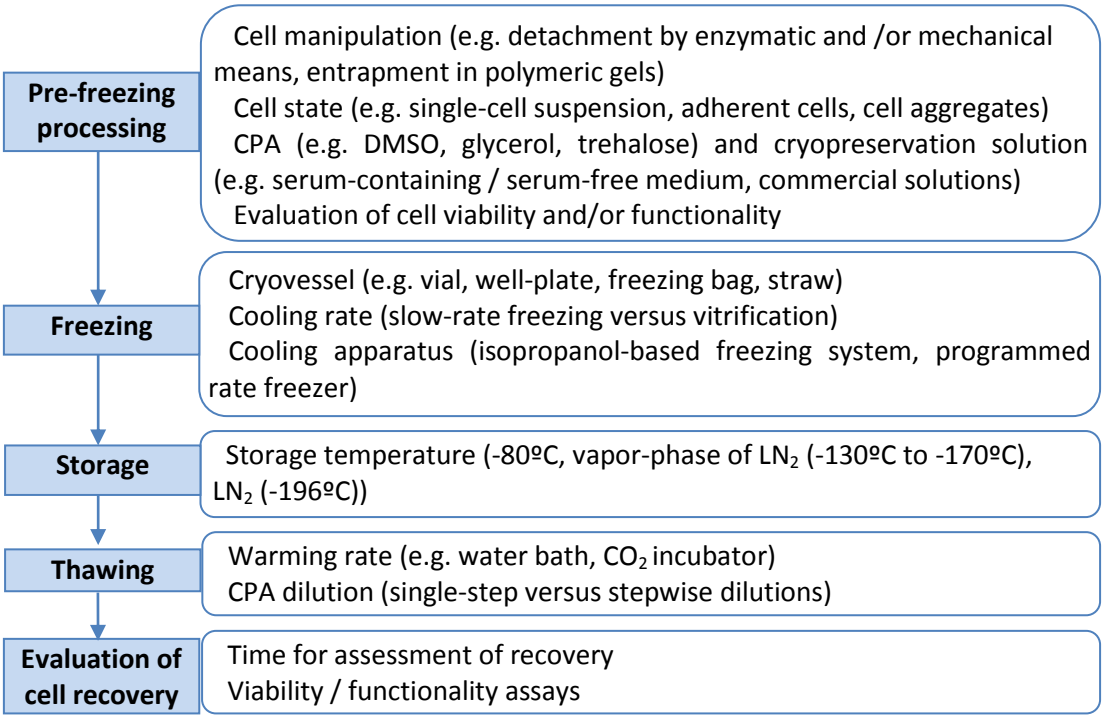

Fig. 1.7 – Main steps composing of a cryopreservation procedure for mammalian cells. For each step the most relevant parameters are listed

1.3.2. Techniques to cryopreserve hESCs

Two different methods have generally been used to preserve hESCs, namely slow freezing-rapid thawing (for larger numbers of cells) and vitrification (for smaller number of cells)[72]. These procedures are based on well-established protocols developed for mESCs and embryos, respectively[73].

1.3.2.1. Slow freezing rate

The conventional slow-freezing rate consists in freezing the samples in the presence of a CPA at a slower cooling rate to minimize the probability of intracellular ice formation, which is likely to damage the cells. Slow cooling leads to a better cell dehydration during the cryopreservation process[74]. Briefly, hESCs colonies or single cell suspension are exposed to the CPA solution (normally 5-20% dimethyl sulfoxide (DMSO) in culture medium or serum)

and placed in a programmed rate freezer or in an isopropanol bath intended to cool the sample at 1°C/min. Slow cooling protocols are effective for the preservation of mESCs, however, applying these to hESCs, has met serious difficulties. Normally these procedures result in low post-thaw survival, low plating efficiencies, high differentiation rates and loss of pluripotency, presumably due to ice crystal formation that disrupts cell-cell adhesion[75-77]. For instance, in early studies colony recovery after thawing were very low: 16%[77], 23%[78] and 30%[79]. Trying to improve post thaw recovery it has been tested the additions of other components to the cooling solution. For instance, extracellular matrix molecules (human type IV collagen or laminin) and trehalose were found to improve post thaw recovery and reduce differentiation of the colonies[80, 81].

In all referred studies, hESCs were cryopreserved in small clumps in order to prevent cell loss from apoptosis after single cell dissociation. However, recently it was reported the cryopreservation of hESCs as single cells suspension by slow freezing rate using ROCKi. Cell survival increased from 30.5±5.2% (absence of ROCKi) to 56.4±7.2% (presence of ROCKi)[82].

1.3.2.2. Vitrification

Vitrification procedures aim at preventing ice formation throughout the sample by applying extremely high cooling rates (>10^4 °C/min) together with high concentrations of CPAs (6 - 8M)[83]. Increasing the concentration of non-penetrating CPAs that interact strongly with water prevents water molecules from interacting to form ice. Moreover high cooling rates through the temperature region of potential crystallisation allows reaching the amorphous glassy state before ice crystals have the opportunity to form[84]. Therefore, samples pass directly from a liquid phase to a glassy state without suffering any nucleation. Briefly, the method consist in a brief stepwise exposure of hESCs colonies (100–400 cells) to two vitrification solutions of increasing CPA concentrations, in which the common components are DMSO, EG and sucrose. The colonies are then loaded into open pulled straws and directly plunged into liquid nitrogen. To avoid ice crystallisation during thawing, colonies are rewarmed as rapidly as possible by direct immersion into pre-warmed culture medium solutions containing decreasing concentration of sucrose[73]. High recovery rates (>75%) are described for vitrified undifferentiated colonies[75, 77, 78].

1.3.2.3. Slow freezing rate vs. vitrification of hESCs

Despite vitrification protocols yield higher post thawing recoveries and lower differentiation rates when compared to slow-rate freezing[77, 78, 80], it presents several

limitations. There is an increased risk of microbiological contamination and transmission of infection associated with the use of open/non sterile straws and the direct exposure to liquid nitrogen[85]. Furthermore, the process uses high concentrations of a CPA that is toxic to cells at room temperature and is difficult to apply to bulk quantities of hESCs. Typically, each straw will hold only 8–12 colony fragments which must be prepared, transferred through the vitrification solutions, loaded into a straw and plunged into liquid nitrogen. The process is extremely labor-intensive and operator-dependent, thus is difficult to reproduce[73]. All this makes current vitrification protocols unsuitable for development of a scalable process and enable its use in hESC banks[67].

Using the slow freezing method, large cell numbers can be frozen in one vial, making easy handling of bulk quantities of hESCs. Therefore, an efficient slow freezing of hESCs in suspension (single cells or hESCs growing in 3D), which can easily and efficiently be stored in cryovials seems to be the optimal scalable system to preserve hESCs. Recently, Nie *et al*. developed a scalable method to cryopreserve hESCs in cryovials. They reported that the cryopreservation of hESCs adherent on mEFs coated microcarriers using slow freezing method, improved post thaw recovery when compared to standard cryopreservation of hESCs colonies[63].

1.4. Cells microencapsulation

1.4.1. History and the concept

The concept of enclosing transplantable cells within a semi-permeable polymeric capsule to protect them from immune rejection was proposed by Chang in 1964[86]. Twenty years later encapsulation was successfully used to immobilize xenograft islet to aid in glucose control for diabetes in rats. Diabetic state was corrected for several weeks[87].

Many different encapsulation systems have been studied. The most commonly used is the encapsulation of cells in microcapsules, because of their spherical shape and small size that offers an optimal surface to volume ratio and an appropriate diffusion capacity when compared for instance with macrocapsules or an intravascular implant[88]. Microencapsulation also minimizes the risk of imunoprotection failure by using thousands of microcapsules instead of a single large macrocapsule. Moreover, microcapsules can be injected directly or transplantable with minimal-invasive surgery into the muscle, peritoneal cavity, liver or elsewhere[89].

The polymer microcapsule modulates the bidirectional diffusion of molecules (**Fig. 1.8**). Nutrients, oxygen, waste products and biotherapeutic agents freely diffuse across the microcapsule, whereas high molecular weight molecules, such as antibodies, immunocytes and other immunologic moieties, are excluded[90]. This barrier can thus isolate the transplanted cells from the host's immune response making possible the graft long-term function[91], without the administration of immunosuppressive drugs. Furthermore, this technology allows the controlled and continuous '*de novo*' delivery of therapeutic products to the host[92]. Indeed, cell microencapsulation ensures a higher success than the encapsulation of therapeutic protein. The therapeutic products are produced and secreted *de novo* at a constant rate by the encapsulated cells, giving rise to a more physiological and effective concentration of the drug, over time. This approach corresponds more to the natural behavior of the cells, and can minimize unintentional side effects[93]. In case of capsule damage, the fast release of high protein concentrations that could be toxic for the patient is avoided[92, 93].

Other advantage of this approach is to make possible the transplantation of allogenic (non-patient) or even xenogenic cells (non-human), which could be a mean of overcoming the obstacle of limited supply of donor cells. Cells can also be genetically modified prior to their encapsulation, to produce or secrete a desired protein *in vivo*.

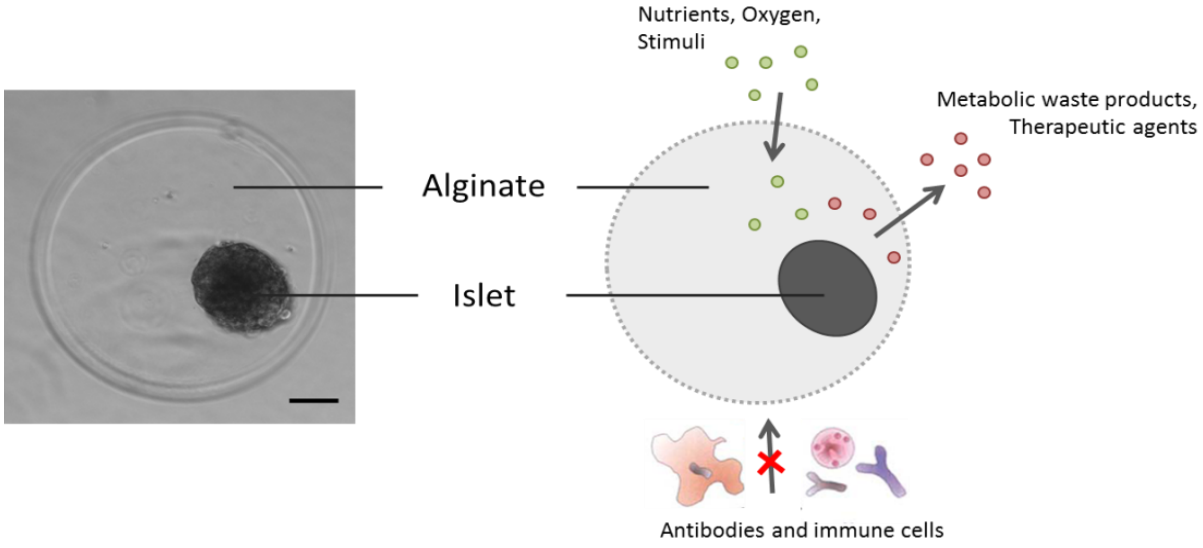

Fig. 1.8 – Concept of cell microencapsulation. This technique consists of enclosing biologically active material within a polymeric matrix (e.g. alginate) that is designed to circumvent immune rejection. The microcapsule membrane allows the bi-directional diffusion of nutrients, oxygen and waste, and the secretion of the therapeutic agents, but prevents immune cells and antibodies from entering the capsule. Scale bar: 200 µm.

In addition, scaffolds used to encapsulate cells guide the formation of tissue from dissociated cells *ex vivo* and *in vivo*, by providing a 3D physical architecture and chemical

environment wherein cells can grow to mimic the *in vivo* process, this makes the microencapsulation technique also very attractive in regenerative medicine[19].

1.4.2. Using cell microencapsulation in the clinic: critical issues

Nowadays an increasing number of biotechnology and pharmaceutical industries have focused their interest on cell microencapsulation for the treatment of endocrine diseases, such as anemia[94], pituitary[95], dwarfism[96], Hemophilia B[97], kidney[98] and liver[99] failure, central nervous system insufficiencies[100] and diabetes mellitus[87]. However, the first clinical trials using cell microencapsulation have demonstrated limited reproducibility. The duration of immunoprotection and/or function of the transplants were subjected to a large variability[89]. The main causes of cell microencapsulation technology failure *in vivo* include hypoxia (due to the great distance between the encapsulated islets and the blood supply), biocompatibility of the encapsulating material and insufficient immune-protective properties of the microcapsules[101, 102]. Indeed, the clinical implementation of cell microencapsulation in humans requires higher levels of quality, efficacy and biosafety. Strict requirements

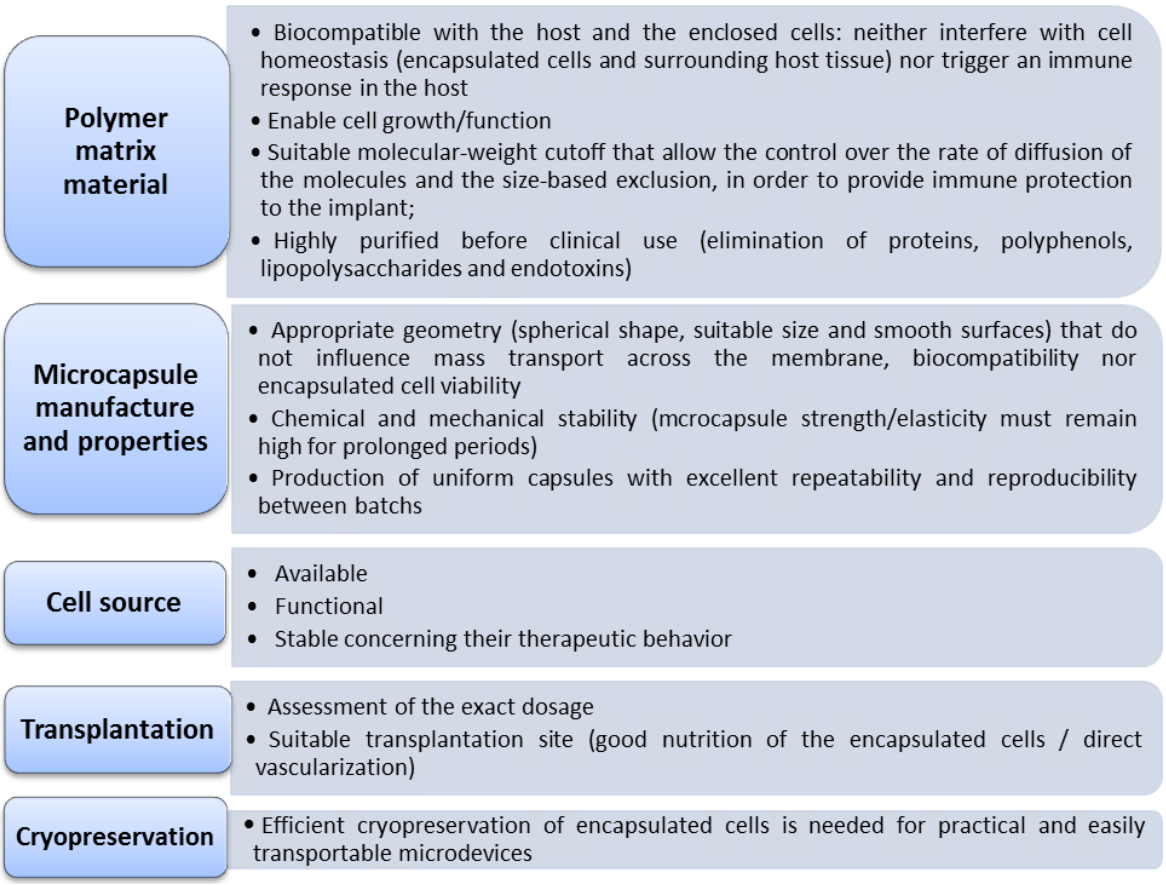

Fig. 1.9 – Requirements that should be addressed to cell microencapsulation technology becomes a real clinical therapeutic strategy.

concerning the exact selection and purity of matrix materials, microcapsule manufacture and final properties, cell source and site of transplantation must be thoroughly fulfill[90, 92, 101, 103-106]. **Fig. 1.9** describes the main requirements for this technology succeeds.

1.4.3. Microcapsule materials

Microcapsules are almost exclusively produced from hydrogels since they provide a highly hydrated microenvironment for embedded cells that guide cellular processes such as differentiation, proliferation and migration[107]. Moreover, their properties can be designed to achieve ideal biocompatibility, degradation and physical characteristics depending on the application[108]. Some materials used for microencapsulation are alginate[87], chitosan[109], agarose[110], poly(hydroxyethylmetacrylate-methyl methacrylate) (HEMA-MMA)[111], copolymers of acrylonitrile (AN69)[112], and polyethylene glycol (PEG)[113].

1.4.3.1. Alginate

Alginate is the most common used polymer for cell microencapsulation, due to the following characteristics: i) has easy gelling properties; ii) allows the processing of the capsules at physiological conditions (room temperature, physiological pH and isotonic solutions)[91]; iii) has an excellent availability, biocompatibility (after purification) and biodegradability; iv) does not interfere with cellular function of the islets[89, 93]. Indeed, it was already reported that alginate-based microcapsules have great potential for transplantation of Langerhans' islets and other factor-secreting cells and tissues[114].

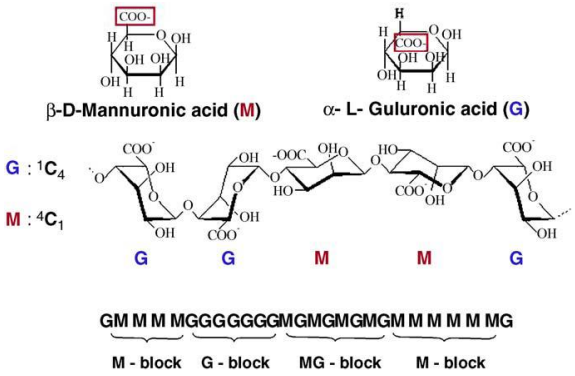

Fig. 1.10 - Structure of alginate. Alginate molecules are linear block copolymers of β-D-mannuronic (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements. (Adapted from de Vos *et al.* [91]).

Alginates are a family of unbranched anionic polysaccharides extracted from brown algae (*Phaeophyta*). They occur extracellularly and intracellularly at approximately 20% to 40% of the dry weight[114]. Alginate molecules are composed by 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers arranged in homopolymeric (GGG blocks and MMM blocks) or heteropolymeric block structures (MGM blocks) (**Fig. 1.10**). The G-blocks cooperatively bind divalent cations and therefore are the main responsible of chain-chain association and gel formation[91].

The proportion and distribution of these two monomers is of paramount importance since they have a significant impact on some of the alginate gel properties (viscosity, permeability, biocompatibility, stability, mechanical resistance, biodegradability)[107, 115]. For instance, stiffness of cross-linked alginates increases as follows: MG blocks < MM blocks < GG blocks, whereas flexibility or elasticity increases in the opposite way[114]. Therefore, the M:G ratio, length of polymeric chains, and the ratio of homologous to heterologous chains must be carefully selected to optimize microcapsules properties. Nevertheless, it should be referred that these properties depend on the source from which the polymer is isolated and also on the harvesting and extraction processes[60].

1.4.3.1.1. UHV Alginate

Ultrahigh viscosity (UHV) alginate (>30 mPa s; 0,1% w/v solution[115]) is a type of alginate designed taking into account the proportion of M/G monomers. It is composed by a 1:1 mixture of alginates from *Lessonia nigrescens* and *Lessonia trabeculata* algae species. Both species grow from central Peru to southern Chile. *L. nigrescens* stipes are very elastic and flexible because of high M alginates (~60%). By contrast, *L. trabeculata* stipes are very stiff due to a high content of G (~90%)[115]. Therefore, UHV_{NT} alginate meet the demands of high stability and flexibility (they are composed by 35% M and 65% G blocks) and has mechanical and elastic properties required for medical, pharmaceutical and biotechnologic applications[114]. It should be noted that this alginate is subject to a detailed purification process, thus the final product fulfills the standards for routine clinical application[115].

1.4.3.1.2. Commercial Alginate

Crude commercial alginates were usually not recommended as materials to use in clinical therapy as they contained many inorganic and organic impurities including polyphenols, proteins and endotoxins[116]. The industrial processes used for extracting alginates from seaweed could introduce further contaminants into the raw alginates. For instance, bacterial contamination is usually treated with formaldehyde, which complicates extraction[114]. The final product normally contains cytotoxic and mitogenic impurities which result in fibrotic overgrowth, inflammatory reactions and ultimately, cell necroses[89, 114]. Furthermore, due to the harvesting and extraction process the viscosity (which is proportional to the molecular weight) of commercial alginates is rather low (1–5 mPa s; 0.1% w/v solution), enhancing the risk of graft failure since the cytotoxicity of polymers increases with decreasing molecular mass[115].

However, some companies have focused on the purification of alginate allowing to guarantee clinical-grade qualities. For instance it is now possible to buy ultrapure alginates with endotoxin levels of less than 100EU/g[91] and lacking immunogenic effects. Pronova UP MVG alginate (Pronova Biomedical) is an example. It is isolated from *Laminaria hyperborea* stipe and has a high G content, a high viscosity (316 mPa s; 1% w/v solution) and a high molecular weight (231 000 g/mole)[117].

1.4.4. Microcapsule formation

Microcapsules are normally produced by forcing the alginate-cells suspension (by using a syringe or a motor-driven prison) through a nozzle with a coaxial air jet to facilitate break-off (**Fig. 2.1**). The resulting droplet is transformed into a rigid bead by gelification in an oppositely charged, di- or trivalent ion solution. The most used cross-linkers of the polymeric chains are Ba^{2+} and Ca^{2+} [88]. Microcapsule size is controlled via the air and alginate flows. Air-jet generators however can create "tails" during break-off that can elicit immunologic reactions, and in some cases small air bubbles can be trapped in the alginate, limiting diffusion and leading to poor long-term stability[114]. Therefore, tails and air bubbles should be avoided during the process.

1.4.5. Microencapsulation of stem cells for clinical application

Although the availability of allogenic or xenogenic mature cells is not a major problem, cell therapy based on encapsulated mature cells still has some drawbacks. The time of secretion of therapeutic proteins is often limited[93]. Still, mature differentiated cells cannot be expanded easily as stem cells. So the combination of stem cells and encapsulation technology has the potential to expand the current application range of this approach. Although most of the published works about stem cells microencapsulation are only *in vitro* approaches, some *in vivo* studies have been developed such as the implantation of encapsulated bone marrow mesenchymal stem cells to improve the formation of the osseous and cartilaginous architecture[118-120]. Moreover, the encapsulation of these cells with hepatocytes improved hepatocyte-specific functions *in vivo*[121].

1.5. Microencapsulation in stem cell bioprocessing

Cell microencapsulation is an attractive tool to achieve stem cell bioprocesses issues like control, reproducibility, validation and safety. In addition, it could be a valuable strategy to integrate expansion, differentiation and cryopreservation of human embryonic stem cells.

1.5.1. Expansion of encapsulated hESCs

Cell encapsulation has been shown to be a good strategy to culture cells since it allows higher cell survival during long-term suspension culture[91]. Cell encapsulation provides a growth support for the islets, prevents excessive aggregation of free cells which can interfere with availability of nutrients and oxygen for the cells in the core of the aggregates[91] and protects cells from shear stress[122]. In fact, 3D cultures are normally highly dependent of the agitation rate. Low stirring speeds result in a few oversized aggregates and/or excessive agglomeration of microcarriers which may cause the formation of necrotic centers. More intense agitation rates induce high shear, compromising cell viability, morphology, gene expression and differentiation potential[123]. Cell encapsulation overcomes this hurdle by protecting the cells from the damage caused by stirring. Moreover, the scaffold environment can be customized by the incorporation of primordial tissue[124], growth factors[125], and small functional groups[126], to design suitable microenvironments for hESCs attachment, growth and/or differentiation. The main studies of culture hESCs encapsulated in alginate are described in **Tab. 1.2** (Page 23).

1.5.2. Cryopreservation of encapsulated cells

The numerous applications of cell microencapsulation make imperative the development of effective cryopreservation protocols for microcapsules that would allow the good storage of encapsulated cells until their use.

The relatively large size (500-600 µm) and fragile semipermeable membrane of the microcapsules makes them particularly prone to cryodamage by ice crystallisation[127]. Nevertheless, same studies have been reported in which encapsulated cells were successfully cryopreserved. Stensvaag *et al*. cryopreserve recombinant human embryonic kidney cells (HEK 293 cells) secreting endostatin, encapsulated in alginate beads, using a slow freezing procedure. Cellular viability, alginate structure, and protein secretion were maintained[128]. Wu *et al.* vitrified hepatocytes encapsulated in 2 types of engineered collagen matrices and achieved post-thaw viabilities of 86%[129]. Heng *et al.* developed an efficient cryopreservation medium for microencapsulated cells (2.8M (20%) DMSO and 0.25M ssucrose) that could give high post-thaw cell viability (>95%)[127]. Malpique *et al.* cryopreserved microencapsulated primary brain neurospheres in ultra-high viscous alginate and enhance post-thaw cell survival and recovery without affecting neurospheres metabolism and differentiation patterns in culture[130].

Although the biophysics associated with the cryopreservation process of microencapsulated cells has not well studied, there are some advantages in using microencapsulation in cryopreservation that could justify the good results described above. Encapsulation of the cells avoids loss of cell-cell and cell-matrix interactions[130] and reduces the fragmentation of islets or other multicellular spheroids[114] which in consequence would lead to a decrease in cell viability after thawing. Also, the prolonged exposure of microencapsulated cells to high cryoprotectant concentrations may not be as detrimental as direct prolonged exposure of non-encapsulated cells, because the semipermeable membrane would slow down or even reduce the exposure of the cells to the cryoprotectant[127]. Therefore, encapsulation of hESCs could be also useful to improve their post-thawing recovery.

Tab. 1.2. Expansion and differentiation of hESCs in 3D culture systems. The main results reported in the literature regarding the expansion and differentiation of hESCs when cultured as aggregates, immobilized on microcarriers or encapsulated in alginate capsules are listed.
Abbreviations: CM, conditioned media; mEFs-CM, media conditioned in mouse embryonic fibroblasts; hFFs-CM, media conditioned in human foreskin fibroblasts; ROCKi, ROCK inhibitor; hESCs, human embryonic stem cells; iPS, induced pluripotent cells.

Culture mode	Culture conditions	Expansion	Differentiation	Reference
Aggregates of hESCs	Inoculum: single cell suspension of hESCs Medium: mTeSR with 10µM ROCKi Heat shock treatment for 30 min after cell dissociation	2-fold expansion per passage in 7 days in 50mL spinner vessels with a bulb-shaped pendulum (>2x10^6 cell/mL)	Cardiomyocyte differentiation	131
	Inoculum: hES and iPS single cell suspension Medium: mTeSR with 10µM ROCKi	6-fold within 4 days in 6 well suspension plates (6.3±0.75x10^5 cell/3mL), beyond day 4 aggregates started to differentiate 4-fold within 4 days in 50mL Erlenmeyer flasks at 50rpm	----	132
	Inoculum: hESCs clumps Medium: DMEM-KO supplemented with 100pg/ml IL6RIL6 chimera	1.66-fold increase of cells *per* spheroid after each passage (5–7 days) in petri dish 25-fold within 10-11 days with two passages of 1:2, in 25mL Erlenmeyer flasks at 90rpm	----	133
	Inoculum: single cell suspension of hESCs Medium: mEFs-CM After dissociation cells were incubated in mEFs-CM with 10% Matrigel (to facilitate initial aggregation) and 10mM ROCKi for 30min	5.6-fold within 7 days in spinner vessels with triangular impeller and glass-etched baffles at 60rpm (~3x10^5 cell/mL)	----	61
	Inocum: single cell suspension of hESCs Medium: mTeSR with 0.1nM Rapamycin Cells were incubated with 10µM ROCKi before and after dissociation for 24 h	25-fold expansion of hESCs over 6 days in 100mL spinner vessels at 100rpm	----	134
hESCs immobilized on microcarriers	Microcarriers: Hyclone coated with Matrigel Medium: mEFs-CM	34- to 45- fold (based on initial hESCs attached onto microcarriers) in 8 days in 50mL spinner vessels (1x10^6 cell/mL)	Differentiation to definitive endoderm (FOXA2$^+$/SOX17$^+$) cells with >80% efficiency	135

Tab. 1.2. Expansion and differentiation of hESCs in 3D culture systems (continuation)

Culture mode	Culture conditions	Expansion	Differentiation	Reference
hESCs immobilized on microcarriers	Microcarriers: non-coated Cytodex3 Medium: mEFs-CM	6.8 fold in 14 days (15.3 x10^5 cell/mL) in 66mL spinner vessels	----	136
	Microcarriers: cellulose microgranular cylindrical shape coated with Matrigel Medium: mEFs-CM	5.8-fold in 5 days (3.5x10^5 cell/mL) in 50mL spinner vessels. Reduction of cell numbers on day 5 onwards	Differentiation to cardiomyocytes precursors	137
	Microcarriers: Cytodex3 coated with Matrigel or mEFs Medium: mEFs-CM	Approximately 3.4 fold in 2.5 days in 60mL spinner vessels	---	63
	Microcarriers: Cytodex3 coated with Matrigel Medium: mEFs-CM with 0.1nM Rapamycin	6.0 fold in 11 days (12.2x10^5 cell/mL) in 100mL spinner vessels 12.5 fold in 11 days (21.1x10^5 cell/mL) in 300mL perfused pO_2-controlled stirred tank bioreactors using 30% air saturation	---	65
hESCs microencapsulated in alginate	Inoculum: 5-7 clusters of hESCs Microcapsules: 1.1% calcium alginate (>1mm diameter) Medium: non-CM	Clumps size increased to 400-500um hESCs maintained their undifferentiated state up to 260 days without passaging	---	138
	Inoculum: single cell suspension of hESCs Microcapsules: 1.1% calcium alginate and 0.1 % gelatin Medium: hFFs-CM 10µM of ROCKi was added 2h before dissociation and encapsulation and also in the first 4 days of culture after encapsulation	Single hESCs maintained good viability (70-80%) throughout 8 days of culture, proliferated and formed clusters	Directed differentiation to definitive endoderm cells	139
	Inoculum: single cell suspension of hESCs Microcapsules: 1.5% pLL-coated alginate (capsules with liquid core) Medium: differentiation medium hESCs were incubated with 10µM ROCKi for 1h prior to their dissociation	Encapsulated hESCs cultured in spinner vessels expanded 8.52±0.89-fold (16x10^4 cell/mL) in 15 days with >85% viability	Differentiation to cardiomyocytes	140

2. Aim of the thesis

The main aim of this thesis was the development of an efficient and scalable methodology to integrate expansion and cryopreservation of human embryonic stem cells. This approach should allow the achievement of high cell yields of pluripotent human embryonic stem cells and, at the same time, high recovery rates of undifferentiated cells after thawing. To achieve this goal, cell microencapsulation in alginate was used and three strategies were outlined: encapsulation of single cells, encapsulation of hESC aggregates and encapsulation of hESCs immobilized on microcarriers.

On a first approach preliminary studies were performed in small scale suspension systems (Erlenmeyer) in order to optimize specific culture parameters. The best conditions were selected and reproduced on a larger scale culture system (spinner vessels), using two different alginate matrices (UHV_{NT} and UP MVG alginates). In parallel, different methods for cryopreservation of 3D hESC cultures were investigated including slow freezing rate and vitrification.

The thesis rational is schematically illustrated in **Fig. 2.1**.

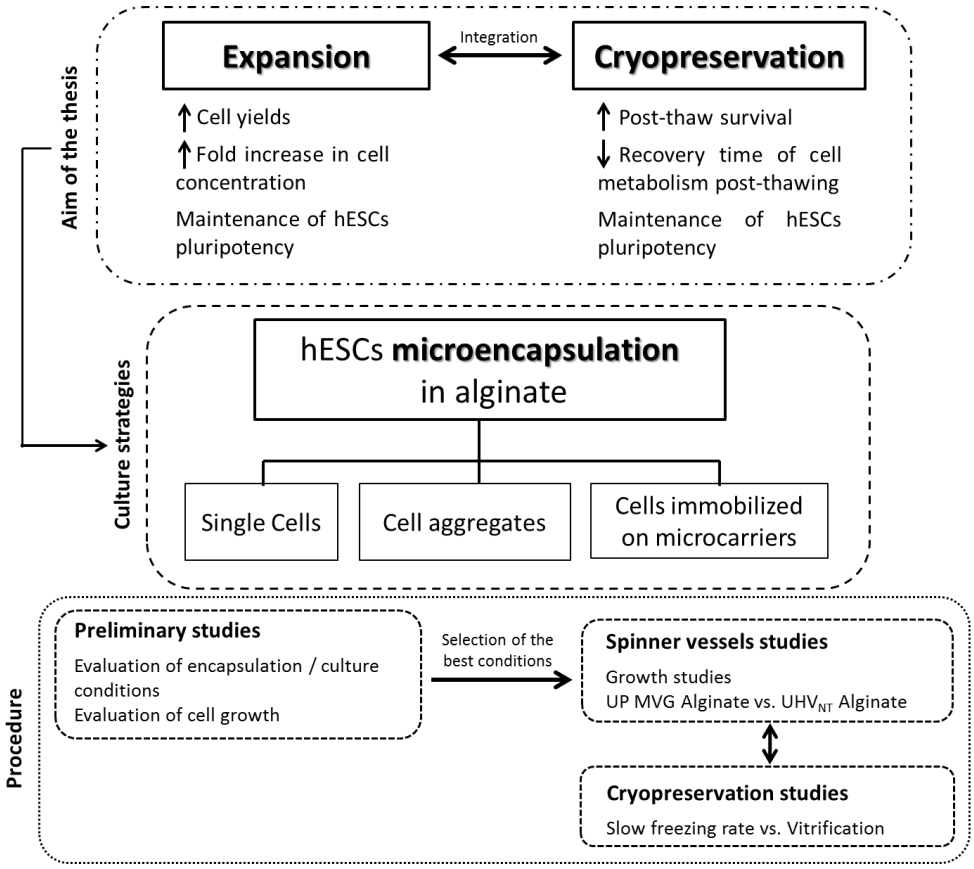

Fig. 2.1. Thesis rational. Aim of the thesis, strategies outlined and procedures performed to achieve the main objectives.

3. Material and Methods

3.1. hESCs culture on feeder layer

hESCs cells (SCED^{TM}461, Cellartis AB) were routinely propagated as colonies in static conditions (6 well-plates) on a feeder layer of human foreskin fibroblasts (ATCC collection, Cat. No. CRL-2429) inactivated with mitomycin C (ihFF) in standard culture medium (DMEM-KO) (DMEM-KO supplemented with 20% (v/v) KO-SR, 1% (v/v) MEM-NEAA, 0.1mM 2-mercaptoethanol, 2mM Glutamax, 1% (v/v) Pen/Strep, 0.5% (v/v) Gentamycin (all from Invitrogen) and 10 ng/mL bFGF (Perprotech), as previously reported[50]. Every 10-12 days, i.e. when the hESC colonies covered approximately 75-85% of the surface area of the culture well, the colonies were digested with TrypLE^{TM} Select (Invitrogen) for 6-8 min, and the resulting single cell suspension was transferred to fresh ihFF feeders (at splitting ratios between 1:4 and 1:24). Culture medium was replaced with fresh medium every 1–3 days.

3.2. Preparation of mEFs conditioned medium

For the production of the conditioned medium (mEFs-CM) for use in 3D hESC cultures, mouse embryonic fibroblast (mEFs, Millipore) were mitotically inactivated and replated on gelatin-coated T-flasks at 5.5x10^4 cell/cm^2 in DMEM-KO medium without bFGF (0.5mL/cm^2). mEFs were cultured at 37ºC with 5% (v/v) CO_2 and conditionated media were collected daily for a total of 10 days per batch. Before feeding hESCs cultures, mEFs-CM was filtered and supplemented with 10 ng/ml bFGF and 0.1 nM Rapamycin (Sigma).

3.3. Encapsulation of human embryonic stem cells

Alginates: A 1:1 mixture of purified UHV alginates from *L. nigrescens* (UHV_N) and *L. trabeculata* (UHV_T) - UHV_{NT} alginate - was used at 0.4% or 0.7% (w/v) in 0.9% NaCl solution[115, 130, 141]. UP MVG alginate (Pronova UP MVG NovaMatrix) was used at 1.1% (w/v) in 0.9% NaCl solution[139].

Microcapsules formation: Microcapsules were formed by passing the alginate-cells mixture using 1 mL syringe through an air-jet generator (**Fig. 3.1;** Page 28), at an air flow rate of 2-3.5 L/min and an air pressure of 1 bar. These encapsulation parameters yielded microcapsules with a diameter of approximately 500-700 µm. For cross-linkage of the UHV_{NT} alginate, microcapsules were dropped directly from the droplet generator's nozzle into a 20 mM $BaCl_2$ solution, adjusted to 290 mOsm using NaCl buffered at pH 7 with 5 mM histidine. For cross-linkage of the UP MVG alginate, a 100 mM $CaCl_2$/10 mM HEPES solution adjusted to pH

7.4 was used. Both alginates microcapsules were washed twice with 0.9% NaCl solution and one time with DMEM-KO medium before being transferred to culture systems.

Alginate dissociation: Ba^{2+}-UHV_{NT} alginate was dissolved by incubating the microcapsules in a 20 mM Na_2SO_4 solution for 20 min at 37°C, in a humidified atmosphere of 5% CO_2 in air[130]. Ca^{2+}-UP MVG alginate was dissolved by incubating the microcapsules with a chelating solution (50 mM EDTA and 10 mM HEPES in PBS) for 5 min at 37ºC[139].

3.4. Three-dimensional hESC cultures

The 3D hESC strategies and culture parameters tested are summarized in **Tab. 3.1** and **3.2**.

3.4.1. Culture of hESCs in Erlenmeyer

Single Cells: Before dissociation from 2D static cultures, hESCs colonies were pre-treated for 1 hour with 5 µM Y-27632, a ROCK inhibitor (ROCKi, Calbiochem). The single cell suspension, resulted from colonies dissociation using Tryple Select, was immediately encapsulated at different concentrations (0.75, 2 and 3 x10^6 cell/mL alginate) using two alginate types (**Tab. 3.1**). In all cultures the final hESCs concentration was 1.5 x10^6 cell/mL. hESCs-microcapsules were then inoculated into 125 mL Erlenmeyer (Corning), cultured in 15 mL mEFs-CM supplemented with 10 µM ROCKi, at 37ºC and 5% CO_2 stirred at 70 rpm using an orbital agitation.

hESC aggregates: hESCs were dissociated from the 2D static cultures and inoculated as single cells at 1.5 $\times 10^5$ cell/mL into 125 mL Erlenmeyer. Cells were cultured in 10 mL mEFs-CM supplemented with 10 µM ROCKi, at 37ºC and 5% CO_2, using an orbital agitation of 70 rpm. Encapsulation was performed at day 2 or 5 (**Tab. 3.1**); aggregates were pre-treated with 5 µM ROCKi for 1 hour and then transferred to 15mL falcon tubes to allow their deposition and culture medium removal. The encapsulation procedure was done after ressuspension of the aggregates with equal volume of alginate solution. Microcapsules were cultured in Erlenmeyers, with 15 mL of mEFs-CM supplemented with 10 µM ROCKi.

hESCs immobilized on microcarriers: hESCs were inoculated at 4.5 $\times 10^5$ cell/mL into 125 mL Erlenmeyer containing 3 g/L (dry weight) Cytodex3 microcarriers (GE Healthcare) coated with Matrigel (BD Biosciences) or human plasma fibronectin (Millipore). The microcarriers were prepared and sterilized according to the manufacture's recommendation and coated with Matrigel and fibronectin as described in the literature[63, 65, 135, 137]. Cells and supports were inoculated in 4 mL mEFs-CM/non-CM supplemented with 10 µM ROCKi and

Erlenmeyers were placed inside an incubator (37ºC, 5%CO_2) under intermittent stirring in order to obtain a homogeneous cell distribution on the supports. After 6h, 2.5 mL of fresh medium was added to cultures and agitation rate set to 70 rpm. By day 3, the volume was adjusted to 15 mL. The encapsulation was performed at different time points (**Tab. 3.2)** using the same procedure described for aggregate cultures.

In all strategies, non-encapsulated cells culture was performed and run in parallel using the same culture conditions. The media were only partially (50%) replaced when necessary.

Tab. 3.1 – Culture parameters of single cells and hESCs aggregates 3D culture strategies

Culture Strategy	Scale of culture	Type of culture	Culture medium	Encapsulation time point	Cell concentration per alginate
Single cells	Erlenmeyer	Encapsulated in UHV_{NT} alginate	mEFs-CM	day 0	$0.75x10^6$ cell/mL Alg.
					$2\ x10^6$ cell/mL Alg.
					$3\ x10^6$ cell/mL Alg.
		Encapsulated in UP MVG alginate	mEFs-CM	day 0	$3\ x10^6$ cell/mL Alg.
hESC aggregates	Erlenmeyer	Non-encapsulated	mEFs-CM	-	---
		Encapsulated in UHV_{NT} alginate	mEFs-CM	day 2	
				day 5	
	Spinner vessels	Non-encapsulated	mEFs-CM	day 2	
		Encapsulated in UHV_{NT} alginate			
		Encapsulated in UP MVG alginate			

3.4.2. Culture of hESCs in spinner vessels

hESC aggregates: hESCs were inoculated at 1.5 $\times10^5$ cell/mL into 300 mL Erlenmeyer (Corning) in 50 mL mEFs-CM supplemented with 10 µM ROCKi. Cells were cultured at 37ºC, 5%CO_2, using an orbital agitation of 70 rpm. Encapsulation was performed at day 2 and then microcapsules were transferred to 125 mL spinner vessels (Wheaton) equipped with paddle impellers and cultured in 100 mL of mEFs-CM at 45 rpm for additional 16 days (**Fig. 3.1**). Culture medium was partially replaced three times a week. This was done by stopping agitation (to induce capsules sedimentation), removing 50% of the medium and feeding with 50% of fresh medium. Non-encapsulated aggregate cultures were also performed and run in parallel. Both cultures were monitored in terms of cell viability, metabolic activity, aggregate size and composition during time. For flow cytometry analysis, aggregates were transferred to gelatin coated surfaces, in mEFs-CM, where cells were able to migrate. After 2-3 days cells were dissociated using TrypLE Select.

hESCs immobilized on microcarriers: hESCs were inoculated at 4.5 $\times10^5$ cell/mL into 125 mL spinner vessels with paddle impellers containing Cytodex3 microcarriers (2 or 3g/L, **Tab. 3.2**)

coated with Matrigel. Cells were cultured in 25mL of mEFs-CM and the spinner vessels were placed at 37ºC, 5% CO_2 under intermittent stirring. After 6h, fresh mEFs-CM was added to cultures and agitation rate set to 24 rpm. By day 3, the volume was completed to 100 mL. The encapsulation was performed at day 6. In some experiments, fresh coated microcarriers (1 or 2 g/L) were added to the cultures 1 hour before encapsulation (**Tab. 3.2**). After encapsulation, hESCs were cultured in the same conditions for additional 13 days (**Fig. 3.1**). Medium was partially (50%) replaced daily. Non-encapsulated cell-microcarrier cultures were also performed and run in parallel. Cultures were monitored in terms of cell viability, cell concentration, metabolic activity and cell characterization during time. For flow cytometry analysis, hESCs were dissociated from the supports after 5 min of incubation with Tryple Select at 37ºC.

After expansion of both cell aggregates and hESC-microcarriers cultures, hESCs were dissociated and plated on a top of a monolayer of ihFFs for further characterization studies.

Tab. 3.2 – Culture parameters of hESCs immobilized on microcarriers culture strategy

Scale of culture	Type of culture	Coating of microcarriers	Culture medium	Encapsulation time point	Concentration of microcarriers added initially	Concentration of microcarriers added at day 6
Erlenmeyer	Non-encapsulated	Matrigel	mEFs-CM	-	3 g/L	-
		Fibronectin	DMEM-KO			
	Encapsulated in UHV_{NT} alginate	Matrigel	mEFs-CM	day 0 (after 8h)	3 g/L	-
				day 1		
				day 3		
				day 6		
Spinner vessels	Non-encapsulated	Matrigel	mEFs-CM	day 6	3 g/L	-
					2 g/L	1 g/L
	Encapsulated in UHV_{NT} alginate	Matrigel	mEFs-CM	day 6	3 g/L	-
					2 g/L	1 g/L
	Encapsulated in UP MVG alginate	Matrigel	mEFs-CM	day 6	2 g/L	1 g/L
					2 g/L	2 g/L

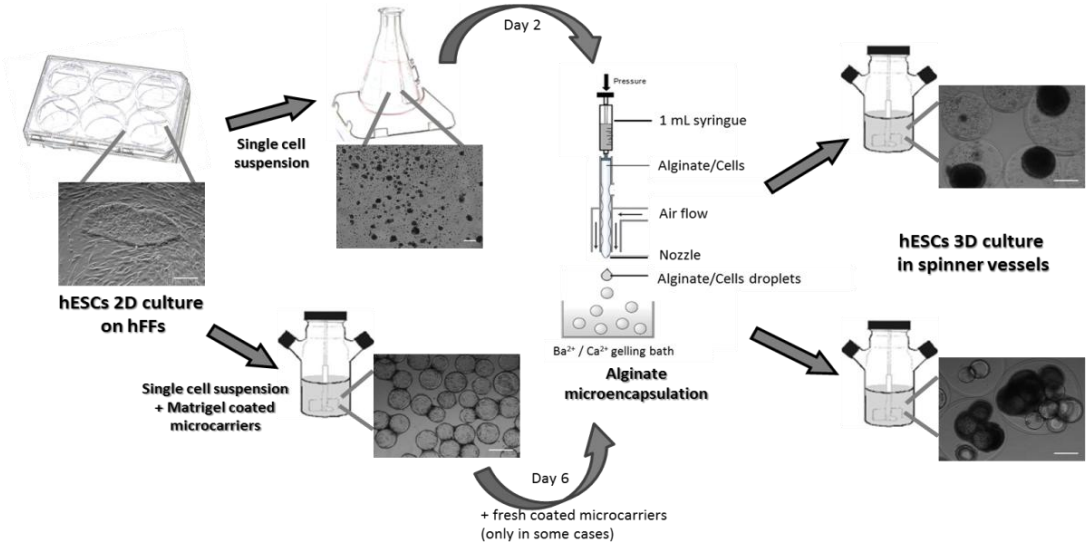

Fig. 3.1 - Schematic illustration of encapsulated hESC cultures in spinner vessels. Both encapsulation of hESCs aggregates and encapsulation of hESCs on microcarriers strategies are illustrated.

3.5. Cell cryopreservation

Cultures of non-encapsulated and encapsulated hESCs (cell aggregates and cell immobilized on microcarriers) were harvested from the spinner vessels at days 14 and 13, respectively, and cryopreserved using two different procedures: slow freezing rate and vitrification. Before cryopreservation, hESCs were pre-treated with 5 μM ROCKi for one hour.

3.5.1. Slow Freezing Rate

Freezing: At the moment of freezing, after deposition of the microcapsules, culture medium was removed and cryopreservation medium (90% KO-SR and 10% (v/v) DMSO (Sigma) supplemented with ROCKi (5μM)) was added. Cell suspension obtained was then transferred to cryovials (1 mL/vial). The cells were allowed to equilibrate in the cryopreservation medium for 20 min at 4ºC. Samples were frozen to -80ºC in an isopropanol-based freezing system, ("Mr. Frosty", Nalgene) at a rate of cooling very close to 1ºC *per* minute, and stored in the gas phase of a liquid nitrogen (LN_2) reservoir until their thawing.

Thawing: Following storage, cells were quickly thawed by placing the cryovials in a 37ºC water bath, a stepwise dilution (1:1, 1:2, 1:4) in mEFs-CM was performed immediately after thawing in order to dilute the DMSO while reducing the osmotic shock. Cells were further cultured for post-thaw studies (cell viability, metabolic activity and differentiation state) in petri-dishes in mEFs-CM suppletemented with 5 μM of ROCKi. Changes of media were performed daily. Whenever possible microcapsules were dissolved, hESCs were dissociated with TrypLE Select, transferred to ihFFs monolayers and maintained in culture for several passages for post-thaw studies of growth and pluripotency.

3.5.2. Vitrification

Freezing: Two serum-free vitrification solutions (VS) were used in the freezing process: VS1 included 10% DMSO and 10% ethyleneglycol (EG); VS2 contained 0.5 M sucrose, 30% DMSO and 30% EG. Cells/capsules were incubated in VS1 for 1 min followed by VS2 for 5 seconds and by 10 seconds in LN_2. Cell strainers of 70 μm (BD) were used to transfer the microcapsules between solutions. As these were preliminary studies the cells were thawed immediately after the cooling process instead of being stored in the vapor-phase of LN_2.

Thawing: Two warming solutions (WS) were used in CPA dilution of the vitrified samples: WS1, consisting of 0.4 M sucrose in VitroHES culture media (Vitrolife) and WS2, consisting of 0.1 M sucrose in VitroHES. Vitrified samples were quickly thawed by an immediate

incubation for 1 min in 2x diluted WS1, followed by 5 min incubation in WS2. Cells were then cultured in mEFs-CM supplemented with 5 μM of ROCKi.

3.5.3. Assessment of hESCs survival after thawing

The percentage of hESCs survival/recovery after thawing was determined by calculating the ratio between the number of viable hESCs after cryopreservation and the number of initially frozen viable hESCs, counted using a Fuchs-Rosenthal haemocytometer chamber (Invitrogen) and the Trypan Blue (Invitrogen) exclusion method.

3.6. Evaluation of cell viability

Cell membrane integrity assay: The qualitative assessment of the cell plasma membrane integrity during culture was done using the enzyme substrate fluorescein diacetate (FDA; Sigma-Aldrich) and propidium iodide (PI; Sigma-Aldrich). Cells were incubated with 20 μg/mL FDA and 10 μg/mL PI in PBS for 2-5 min and then visualized using fluorescence microscopy (Leica Microsystems GmbH). FDA is a non-polar, non-fluorescent fluorescein analogue which enters all cells freely. In viable cells, FDA is converted by intracellular esterases to highly fluorescent fluorescein. Being highly polar, fluorescein becomes trapped within cells that possess membrane integrity (viable cells) and confers to them green fluorescence. PI is a polar, highly fluorescent (red color) compound which can only enter in cells that lack membrane integrity. It intercalates into the major groove of the DNA, therefore nucleus dying/dead cells stain red[142].

Trypan Blue exclusion method: The total number of viable cells was determined by counting the colorless cells in a Fuchs-Rosenthal haemocytometer chamber after incubation with Trypan Blue dye (0.1% (v/v) in PBS). Dead cells with damaged membranes stain blue in the presence of this compound.

Lactate dehydrogenase (LDH) activity: LDH activity from the culture supernatant was dermined spectrophotometrically (at 340 nm) following the rate of oxidation of NADH to NAD^+ coupled with the reduction of pyruvate to lactate. The specific rate of LDH release (q_{LDH}) was calculated for each time interval using the following equaton: $q_{LDH} = \Delta LDH/(\Delta t \times \Delta X_V)$, where ΔLDH is the change in LDH activity over the time period Δt, and ΔX_V is the average of the total cell number during the same period. The cumulative value q_{LDHcum} was estimated by $q_{LDHcum\ i+1} = q_{LDH\ i} + q_{LDH\ i+1}$. This assay is useful to monitor cell lysis

throughout culture since LDH is an intracellular enzyme that is only presented in culture supernatant when the cell membrane is damaged.

3.7. Evaluation of the culture metabolic activity

AlamarBlue™ assay**:** hESCs metabolic activity was assessed using the metabolic indicator alamarBlue following the manufacture's recommendation (Invitrogen). Briefly, 2 mL of hESC culture were incubated over night with fresh medium containing 10% (v/v) alamarBlue. Fluorescence was measured (excitation wavelength: 570 nm, emission wavelength: 585 nm) in 96-well plates using a microwell plate fluorescence reader (MicroMax 384, Horiba Jobin yvon). The active ingredient of alamarBlue (resazurin) is a nontoxic non-fluorescence indicator dye that 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 live and metabolic active cells.

3.8. Evaluation of cell growth

Apparent growth rate (μ): μ was estimated using a simple first-order kinetic model for cell expansion: $dX/dt = \mu X$, where t (day) is the culture time and X (cell) is the value of viable cells for a specific t. The value of μ was estimated using this model applied to the slope of the curves during the exponential phase.

Fold increase in cell expansion (FI): was evaluated based on the ratio X_{MAX}/X_0, where X_{MAX} is the peak of cell density (cell/mL) and X_0 is the lowest cell density (cell/mL).

3.9. Characterization of hESCs

Alkaline Phosphatase (AP) Staining: Undifferentiated embryonic stem cells are characterized by high expression levels of alkaline phosphatase (AP). Cultures were stained using an AP activity detection kit (Millipore) according to the manufacturer's instructions and observed under phase contrast microscopy (Leica Microsystems GmbH). Positively stained hESCs (purple) were considered undifferentiated cells.

Immunocytochemistry: hESC cultures were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 20 min, permeabilized (only for detection of intracellular marker OCT-4) for 5 min in 0.1% (w/v) Triton X-100 solution (Sigma-Aldrich) and subsequently incubated with primary antibody overnight at 4°C. Cells were washed three times in PBS and the secondary antibodies were applied to the cells for 60 min at room

temperature in the dark. After three washes with PBS, cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Cells were visualized using spinning disk confocal (Nikon Eclipse Ti-E, confocal scanner: Yokogawa CSU-x1) and inverted fluorescence microscopy (Leica Microsystems GmbH). In aggregate samples, an additional permeabilization step was performed before the addition of primary antibodies; samples were incubated with 0.2% fish skin gelatine and 0.1% TX-100 in PBS for 2 h at room temperature. Primary antibodies used were: Tra-1-60, SSEA-4, Oct-4 (1:50, all Santa Cruz Biotechnology) and hESCellect^{TM} (1:1000, Cellartis AB). Secondary antibodies used were: goat anti-mouse IgM-AlexaFluor488 and goat anti-mouse IgG-AlexaFluor 488 (1:500, all from Invitrogen). All antibodies were diluted in PBS.

Flow cytometry analysis: cells were dissociated with TrypLE^{TM} Select and the single cell suspension was resuspended in washing buffer (WB) solution (5% (v/v) FBS in PBS). After two washing steps, cells were incubated with primary antibody for 1 hour at 4ºC, washed three times in WB and then incubated with the secondary antibody for additional 30 min at 4ºC. After 2 washing steps with WB, cells were suspended in WB and analyzed in a flow cytometry instrument (CyFlow-space, Partec GmbH). Ten thousand events were registered *per* sample. Primary antibodies used were: Tra-1-60, SSEA-4, SSEA-1 and isotype control antibodies (1:10, all Santa Cruz Biotechnology) and hESCellect^{TM}, hFFCellect^{TM} (1:1000, Cellartis AB). Secondary antibodies used were: goat anti-mouse IgM-AlexaFluor488 and goat anti-mouse IgG-AlexaFluor 488 (1:200, all from Invitrogen). All antibodies were diluted in WB.

3.10. *In vitro* pluripotency test via embryoid bodies (EBs) formation

hESCs were dissociated, transferred to non-adherent petri dishes ($5x10^5$ cell/mL) and cultured in suspension for 1 week in DMEM-KO medium without bFGF. EBs formed during this time were harvested and cultured in gelatin-coated plates for additional 2 weeks (medium was changed three times a week). Cells were then assessed for spontaneous differentiation into derivatives of all three embryonic germ layer. Differentiated cells were identified using immunocytochemistry (protocol described above) with markers for mesoderm (α-smooth muscle actin – ASMA, DAKO 1:200), endoderm (Forkhead box A2 - FOX-A2, Santa Cruz 1:500) and ectoderm (β tubulin type III - βIII-Tub, Chemicon 1:100). Secondary antibodies used were: goat anti-mouse IgG-AlexaFluor488 and donkey anti-goat IgG-AlexaFluor594 (Invitrogen, 1:500).

4. Results

4.1. Evaluation of two different UHV_{NT} alginate concentrations

Different concentrations of Ba^{2+}-UHV_{NT} alginate were evaluated for their ability to support the culture of hESC as aggregates: 0.4% and 0.7% (w/v). After encapsulation hESCs were cultivated in Erlenmeyer and microcapsules integrity was monitored during 3 weeks using phase contrast microscopy (**Fig. 4.1**). The results obtained showed that 0.4% alginate microcapsules were more fragile than 0.7% alginate microcapsules. The formers lost their structural integrity (i.e. capsules broke or disintegrated) more frequently, resulting in the release of encapsulated cells. This fact compromises the usefulness of 0.4% (w/v) Ba^{2+}-UHV_{NT} alginate as 3D matrix. Therefore, 0.7% w/v was the concentration of Ba^{2+}-UHV_{NT} alginate selected for use in the following encapsulation experiments.

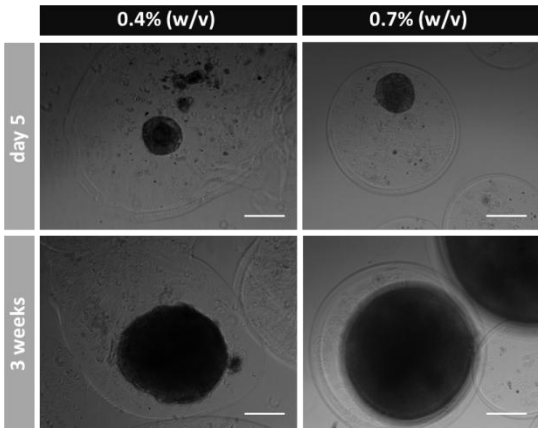

Fig. 4.1 – Evaluation of two concentrations of UHV_{NT} alginate. hESCs aggregates were encapsulated in 0.4% and 0.7% (w/v) alginate. Microcapsules stability "shape and size" after 5 days and 3 weeks of culture post-encapsulation was assessed by phase contrast microscopy.

4.2. Strategy I – Encapsulation of hESCs as single-cells

4.2.1. Culture of encapsulated hESCs as single cells in Erlenmeyer

In a first approach, hESCs were encapsulated immediately after passage from feeders as single cells in 0.7% Ba^{2+}-UHV_{NT} alginate. Different cell concentrations *per* alginate were tested, namely 0.75x, 2x and $3x10^6$ cell/mL. Cells were cultivated in mEFs-CM supplemented with ROCKi, in Erlenmeyer and cell viability was monitored for two weeks. In all cases, cell viability gradually decreased over culture time (**Fig. 4.2**). When a higher cell concentration was used ($3x10^6$ cell/mL alginate), viable cell aggregates were observed in culture from day 7 onwards, indicating that some cells and/or small clusters remained viable after encapsulation and proliferated forming aggregates within the alginate beads (**Fig. 4.2**). However, the frequency of populated microcapsules was low.

In order to investigate if the encapsulation methodology (type of alginate, gelification method with Ba^{2+}, ...) was responsible for the high cell death and inefficient cell proliferation

observed, other alginate microcapsules were tested based on promising results recently reported in the literature[139], 1.1% (w/v) Ca^{2+}-UP MVG alginate.

The viability did not differ significantly between the two encapsulation strategies (**Fig. 4.2**). Formation of more clusters in 1.1% Ca^{2+}-UP MVG alginate were observed, however, cell death after encapsulation was significantly high. These results indicate that the encapsulation of single cells is not a suitable strategy to expand the hESC line used in this study.

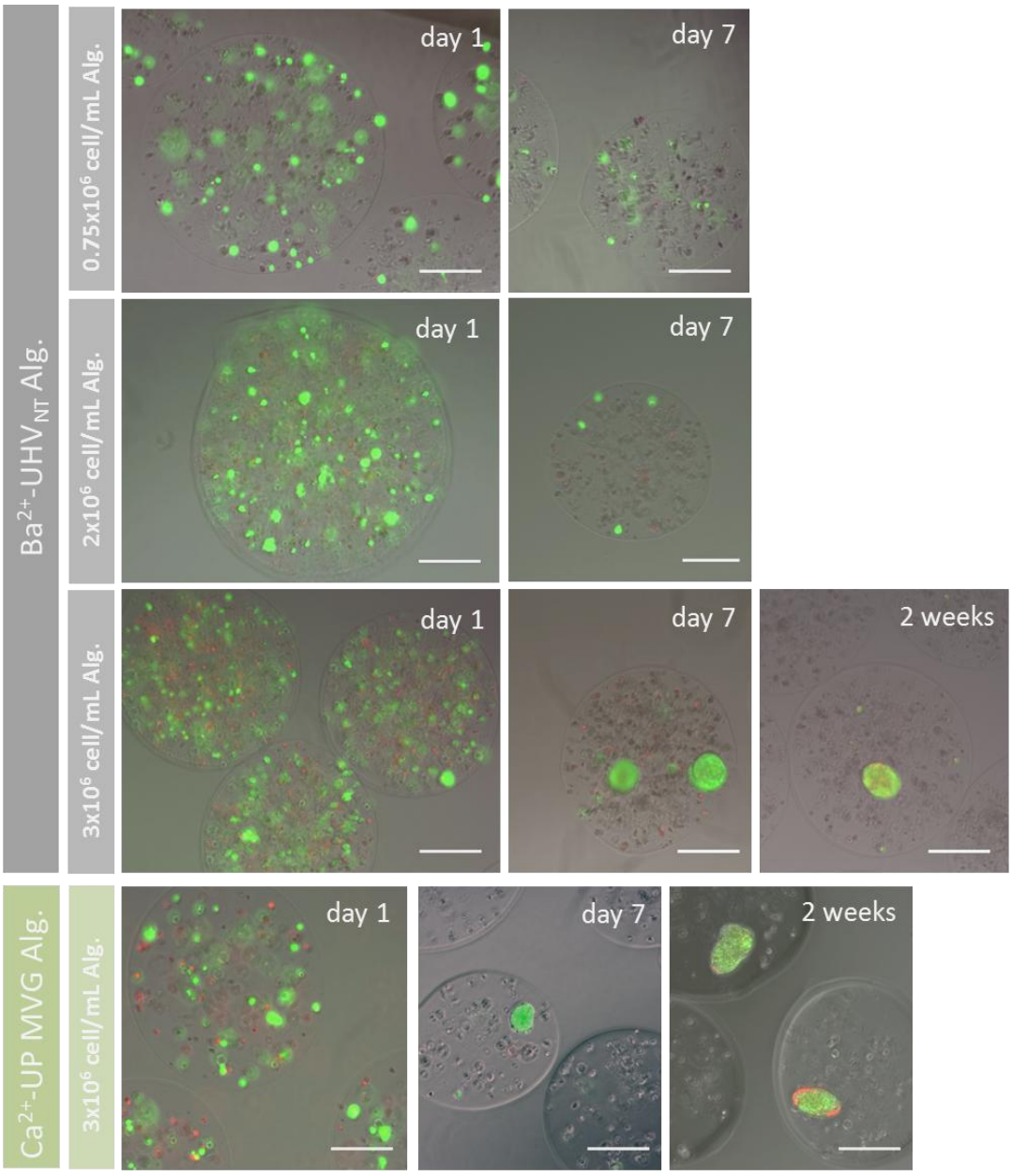

Fig. 4.2 - Effect of cell concentration and alginate matrix on viability of encapsulated single cells. hESCs were encapsulated at different concentrations (0.75x, 2x and $3x10^6$ cell/mL alginate) in 0.7% Ba^{2+}-UHV_{NT} alginate and at $3x10^6$ cell/mL alginate in 1.1% Ca^{2+}-UP MVG alginate. Viability analysis during culture time, based on cell membrane integrity test, is shown: cells stained with fluoresceine diacetate (FDA – live cells, green) and propidium iodide (PI – dead cells, red). Scale bars: 200 µm.

4.3. Strategy II – Encapsulation of hESCs as aggregates

4.3.1. Culture of encapsulated hESCs as aggregates in Erlenmeyer

Encapsulation of hESCs as aggregates was the second evaluated strategy. Cells were induced to form small cell aggregates prior encapsulation by cultivating single cells in suspension conditions using ROCKi supplementation.

hESC aggregates were encapsulated at two culture time points – day 2 and 5. The viability analysis of encapsulated cultures demonstrated that hESCs remained almost viable over culture time (three weeks), even when the aggregates reached larger sizes (approximately 400 µm) (**Fig. 4.3 A**). The fold increase in aggregates size was approximately 4.3 for both times of encapsulation. However it is important to highlight that some aggregates exceed the size of the capsule by the third week in both cultures (data not shown). In general, these large aggregates showed an irregular structure with cystic cavities, resembling embryoid bodies (**Fig. 4.3 B**). For these reasons, the culture time should not be prolonged for more than two weeks.

After two weeks of culture, encapsulated aggregates presented alkaline phosphatase activity (**Fig. 4.3 C**), confirming the undifferentiated phenotype of hESCs.

Overall, the results show no significant differences in the growth of hESC aggregates encapsulated at different culture time points. However, when encapsulation is performed earlier (day 2), cultures start from smaller aggregates (approximately 20 µm less than at day 5) and the possibility of the aggregates in exceeding the size of the capsule (500-700 µm), during the culture, is lower. Therefore, this condition was selected to be used in spinner culture experiments.

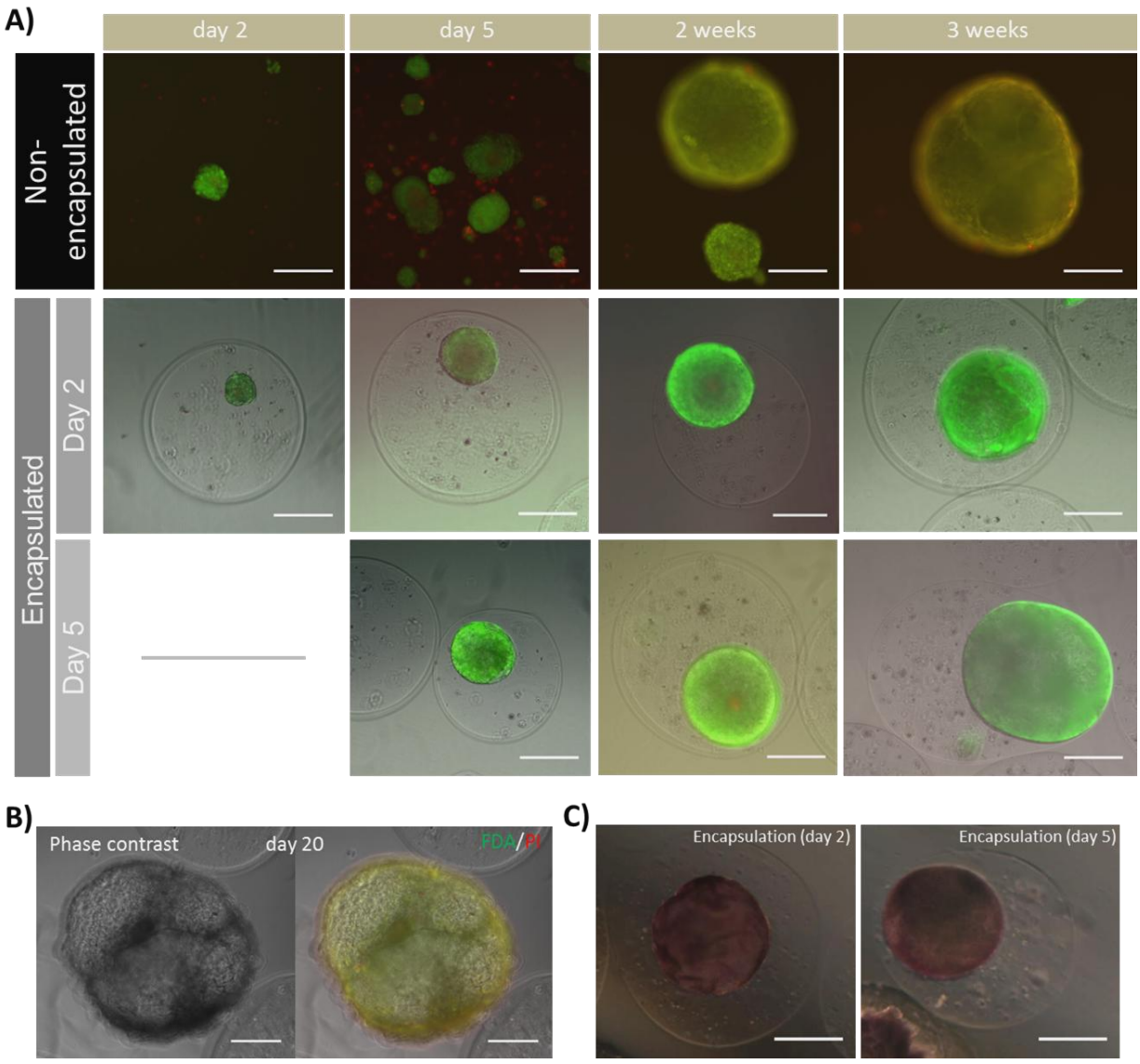

Fig. 4.3 – Impact of the day of encapsulation in the hESC aggregates expansion. Cells were encapsulated at day 2 and 5 of suspension culture in Erlenmeyer. **A)** Viability analysis during culture using FDA (live cells, green) and PI (dead cells, red) for encapsulated and non-encapsulated cultures. **B)** Example of an encapsulated aggregate that exceeded the capsule size in the third week of culture. Its irregular structure with cystic cavities resembles embryoid bodies aggregates. **C)** Phenotypic assessment of alkaline phosphatase activity. Scale bars: 200 µm.

4.3.2. Expansion of encapsulated hESC aggregates in spinner vessels

The encapsulation of hESCs as aggregates was reproduced in spinner vessels with a paddle impeller.

The results clearly showed a marked difference between expansion of encapsulated and non-encapsulated aggregates (**Fig. 4.4**). The viability analysis (**Fig. 4.4 A**) and the high values of cumulative LDH release (**Fig. 4.4 B**) demonstrated that the culture of non-encapsulated aggregates in spinner vessels resulted in an accentuated cell death. The few aggregates that remained in culture clumped together, forming large (> 1mm in size in some cases) and irregular aggregates with necrotic centers (**Fig. 4.4 A**). On the other hand,

encapsulated aggregates were homogeneous, presented a spherical shape and a compact structure throughout time (**Fig. 4.4 A**). The concentration of aggregates was always higher in encapsulated cultures (**Fig. 4.4 C**). However, from day 14 onwards the number of encapsulated aggregates decreased, probably due to the aggregation of small clumps within the same capsule.

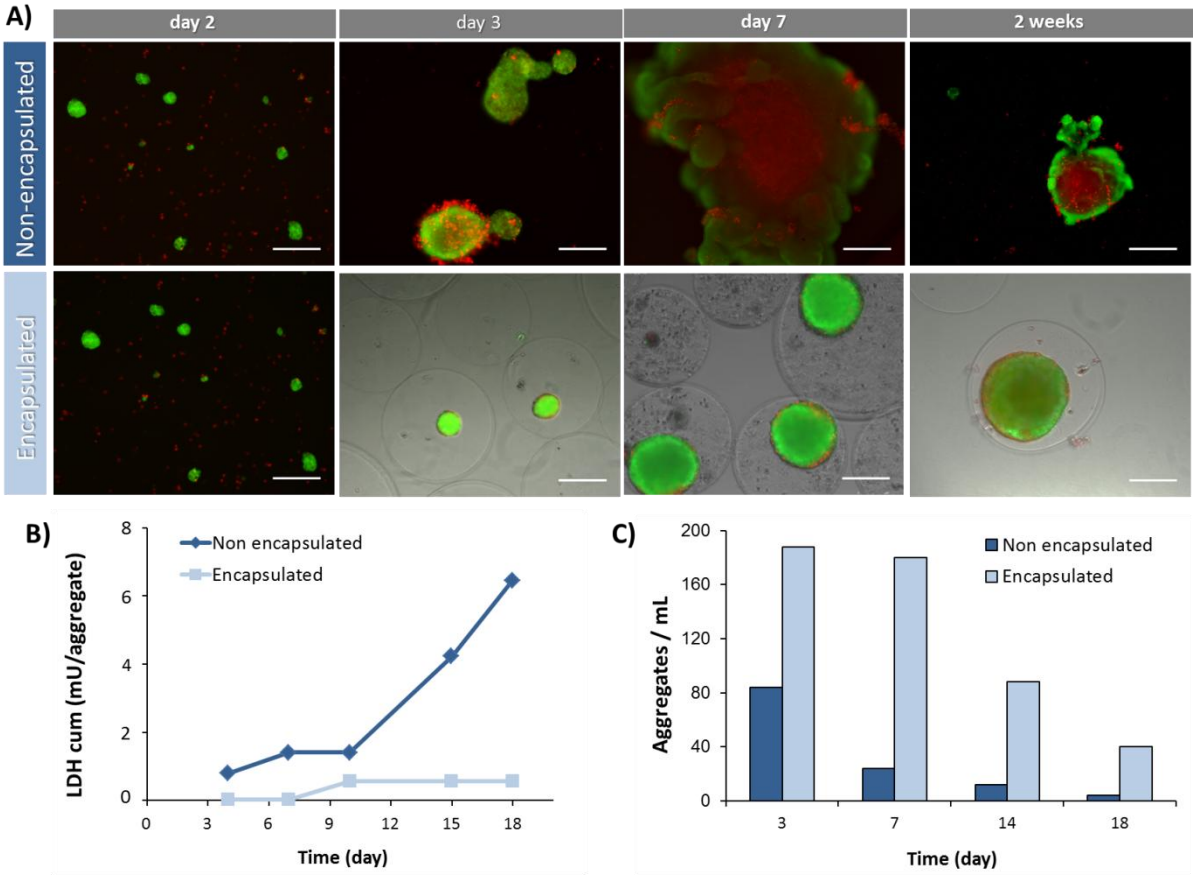

Fig. 4.4 – Effect of alginate microencapsulation on the expansion of hESC aggregates in spinner vessels. Small hESC aggregates were encapsulated at day 2 in 0.7% Ba^{2+}-UHV_{NT} Alginate. **A)** Viability analysis of encapsulated and non-encapsulated aggregates stained with FDA (live cells, green) and PI (dead cells, red). Scale bars: 200 µm **B)** Cumulative values of LDH release per aggregate during time, for encapsulated and non-encapsulated cultures. **C)** Number of encapsulated and non-encapsulated aggregates *per* volume of medium at days 3, 7, 14 and 18 of culture.

4.3.3. Effect of two alginate types in expansion of hESC aggregates in spinner vessels

The proliferation of hESCs aggregates in two types of alginate microcapsules (0.7% Ba^{2+}-UHV_{NT} and 1.1% Ca^{2+}-UP MVG) was investigated. The growth profile of encapsulated hESCs was very similar, both in aggregate size and metabolic activity (**Fig. 4.5 A, B**). The fold increase in aggregates size was 5.3 and 6.8 for aggregates encapsulated in Ba^{2+}-UHV_{NT} and Ca^{2+}-UP MVG alginate, respectively. Metabolic activity, assessed by alamarBlue test (**Fig. 4.5 B**), increased 2 times from the day after encapsulation (day 3) to day 15, in both

encapsulated cultures. In contrast, in non-encapsulated cultures the metabolic activity decreased significantly (**Fig. 4.5 B**). Moreover, hESCs aggregates presented high cell viabilities in both encapsulated cultures (**Fig 4.5 C**).

After microcapsules dissociation, hESCs aggregates recovered from Ba^{2+}-UHV_{NT} alginate beads presented a high percentage of dead cells that compromised aggregate integrity. On the other hand, when the UP MVG alginate was dissolved, aggregates maintained their cohesiveness and viability (**Fig. 4.5 D**), thus assuring an efficient cell recovery and characterization.

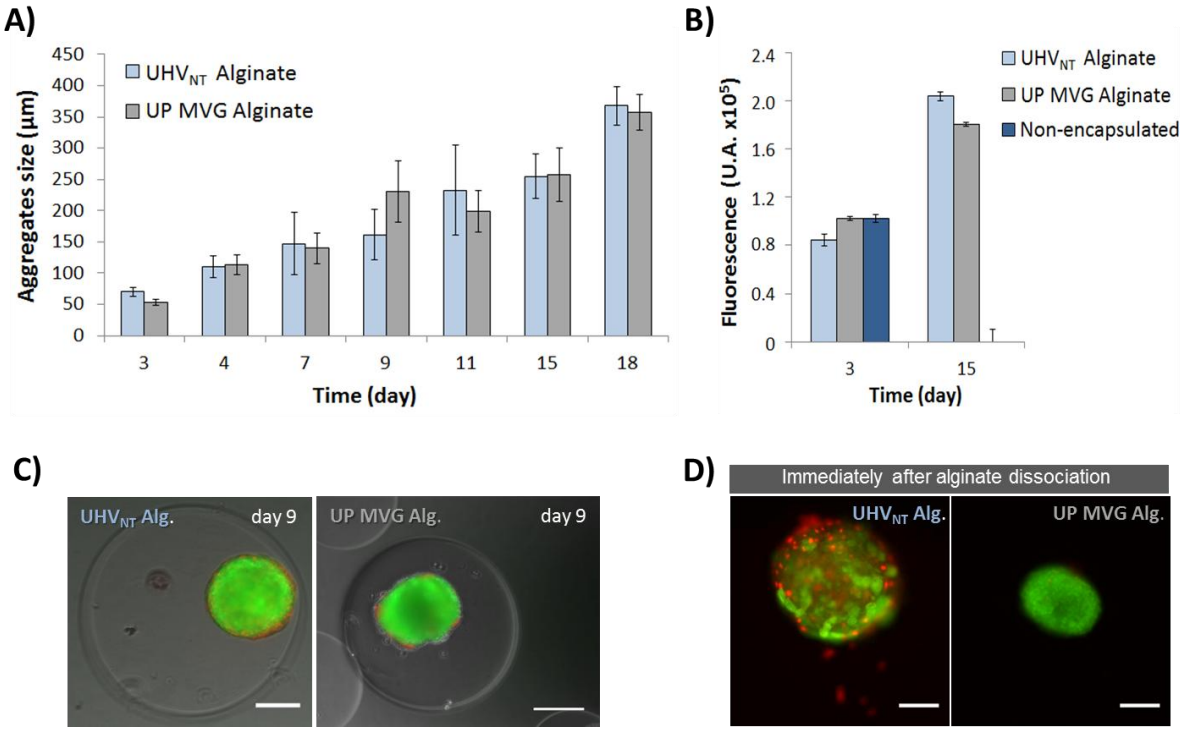

Fig. 4.5 – Impact of alginate type on the expansion of hESC aggregates in spinner vessels. Small aggregates were encapsulated at day 2 in 0.7% Ba^{2+}-UHV_{NT} and in 1.1% Ca^{2+}-UP MVG alginates. **A)** Average size of aggregates encapsulated in both alginates, during time. Error bars denote SD of up to 20 measurements. **B)** AlamarBlue metabolism test of non-encapsulated and encapsulated cultures on the day after encapsulation (day 3) and at day 15. Error bars denote SD of 3 measurements. **C)** Viability analysis of hESCs aggregates at day 9 for both encapsulated cultures. Cells were stained with FDA (live cells, green) and PI (dead cells, red). **D)** Viability test of hESCs aggregates immediately after dissociation. Scale bars: 200 µm

4.3.4. Characterization of expanded hESC aggregates

In order to confirm the undifferentiated state and pluripotency of aggregates during and after culture in spinner vessels, immunofluorescence microscopy, flow cytometry analysis, alkaline phosphatase activity test and embryoid bodies were performed (**Fig. 4.6**).

The results confirmed that hESCs expanded as encapsulated 3D aggregates (in UP MVG alginate) retained their undifferentiated phenotype and pluripotency, evaluated by immunofluorescence microscopy (**Fig. 4.6 A**) and detection of alkaline phosphatase activity.

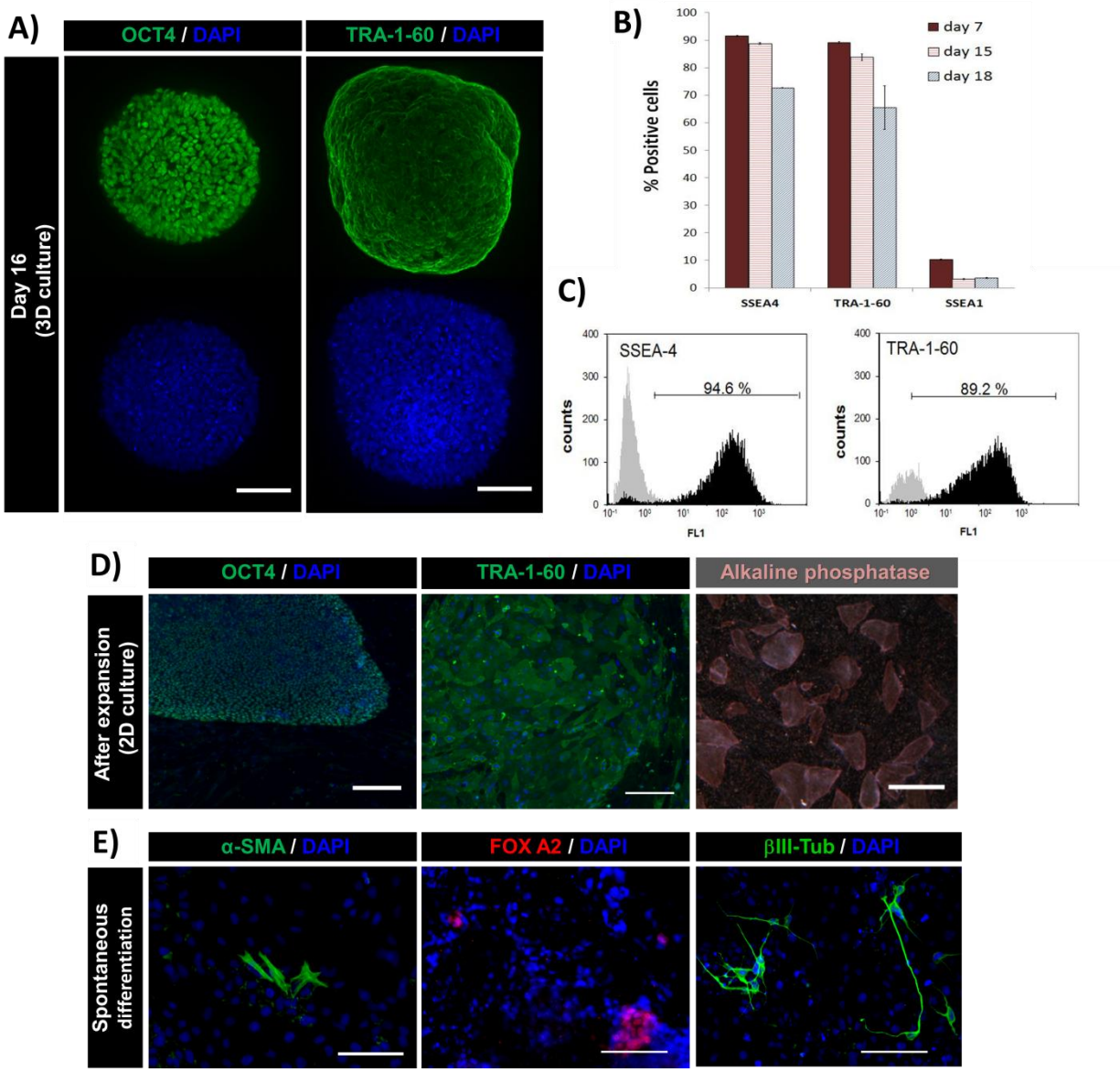

Fig. 4.6 - Characterization of encapsulated hESC aggregates expanded in spinner vessels. **A)** Confocal images of aggregates labeled for stem cell markers (OCT4 and TRA-1-60) at day 16 of 3D culture in UP MVG alginate. Scale bars: 50 µm. **B-C)** Flow cytometry analysis of the expanded population. **B)** Percentage of SSEA-4, TRA-1-60 and SSEA-1 positive cells at days 7, 15 and 18. Error bars represent SD of 2 measurements. **C)** Histograms obtained in flow cytometry analysis of SSEA-4 and TRA-1-60 positive cells at day 7 of culture. **D)** Immunofluorescence images of Oct-4 and TRA-1-60 labeling and phase contrast pictures of alkaline phosphatase (AP) activity, staining after expansion (2D culture). Nuclei were labeled with DAPI (blue). Scale bars: immunofluorescence images - 200 µm, AP image - 1mm**. E)** *In vitro* pluripotency analysis. hESCs derived from expanded aggregates in UP MVG alginate spontaneously differentiated into cells of the three germ layers via embryoid bodies (EBs) formation. Immunofluorescence labeling: FOX-A2 (Forkheadbox A2, endoderm), α-SMA (α smooth muscle actin, mesoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bars: 100 µm.

At day 7 of culture the percentages of SSEA-4 and TRA-1-60 positive cells were high (94.6% and 89.2%, respectively), indicating that almost the entirely population had a pluripotent character (**Fig. 4.6 B, C**). However at day 18, a significant decrease in SSEA-4 and TRA-1-60 positive cells was observed. The presence of embryoid body like structures at this

culture time point (as previously detected in Erlenmeyer studies) suggested that the population of differentiated cells increased from the third week of culture.

For all time points the percentage of SSEA-1 (cell marker for early differentiated cells[143]) positive cells were always ≤ 10% (**Fig. 4.6 B**). The decrease in SSEA-1 positive cells combined with the decrease in the levels of SSEA-4 and TRA-1-60 could be explained by the fact that the early differentiated cells existed at day 7 progressed to a more advanced differentiated state over time. Unfortunately, it was not possible to compare this profile with non-encapsulated cultures due to limitations in biological material. Few aggregates were observed in the culture and those were too large and difficult to dissociate.

In addition, after expansion UP MVG alginate was dissociated, and hESC aggregates were able to form undifferentiated colonies in a monolayer of ihFFs (**Fig. 4.6 D**), indicating that the encapsulation did not compromise hESC ability to grow in traditional 2D culture systems. Moreover, these cells differentiated spontaneously *in vitro* into cells from the three germ layers (**Fig. 4.6 E**), confirming that they maintained their pluripotent potential.

4.3.5. Cryopreservation of encapsulated hESCs aggregates

Since encapsulation technology showed to be a valuable tool to support growth and viability of hESCs as aggregates, the possibility to cryopreserve those encapsulated aggregates was evaluated using two different freezing methods: slow freezing rate and vitrification. The results showed that both techniques were unsuitable for the efficient cryopreservation of hESCs as aggregates.

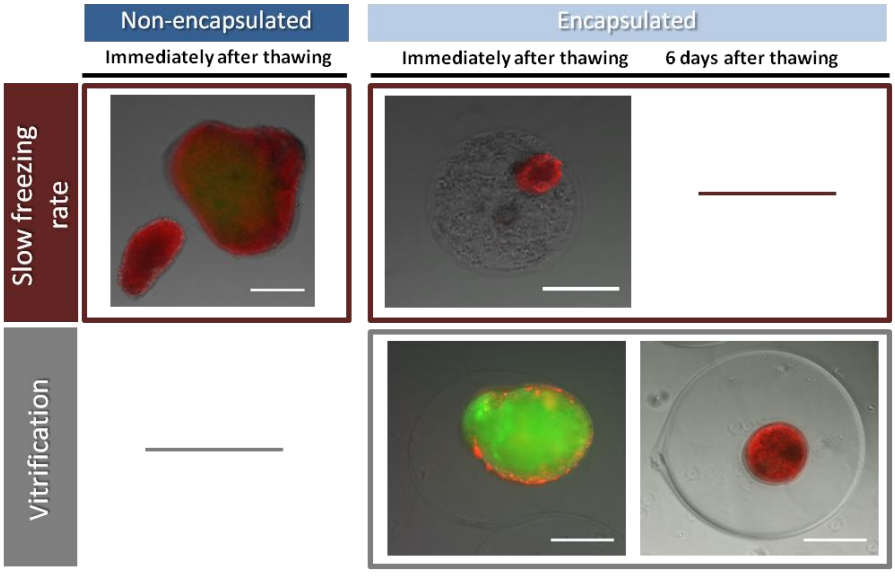

Fig. 4.7 - Cryopreservation of hESCs aggregates by slow freezing rate and vitrification. Viability analysis of encapsulated aggregates immediately after thawing and upon 6 days of cultivation (only in vitrification studies), using FDA (live cells, green) and PI (dead cells, red). Viability results for slow freezing rate of non-encapsulated aggregates are also presented. Scale bars: 200 µm.

In the case of slow freezing rate, cells died immediately after thawing, both in encapsulated and non-encapsulated cultures (**Fig. 4.7**). When encapsulated aggregates were cryopreserved using the vitrification protocol, the percentage of viable cells was high immediately after thawing. Nevertheless, all cells died in the following days (**Fig. 4.7**).

4.4. Strategy III – Encapsulation of hESCs immobilized on microcarriers

4.4.1. Culture of hESCs immobilized on microcarriers in Erlenmeyer

On the third strategy, the impact of encapsulate hESCs immobilized on supports (Cytodex 3 microcarriers) in enhancing the expansion and cryopreservation of hESCs was investigated. Preliminary studies were designed aiming to select specific culture parameters including, the coating matrix, culture medium and encapsulation timepoint. These studies were performed using small scale suspension systems (Erlenmeyer).

4.4.1.1. Evaluation of culture parameters to grow hESCs immobilized on microcarriers

In an effort to reduce the animal origin components present in the culture, the possibility of cultivating hESCs on microcarriers coated with human plasma Fibronectin using non conditioned culture medium (non-CM) was evaluated and compared with the already established culture conditions (Matrigel + mEFs-CM) [65, 144].

The results demonstrated that hESCs growth was faster in Matrigel + mEFs-CM than in Fibronectin + non-CM (apparent growth rate was approximately 2.5 times higher in Matrigel + mEFs-CM conditions) (**Fig. 4.8 A, B**). However, similar expansion ratios were obtained in both culture conditions, the fold increase in cell concentration was 5.26 and 4.46 for Matrigel + mEFs-CM and Fibronectin + non-CM, respectively. Nevertheless, in Fibronectin + non-CM culture, the highest cell concentration was obtained only after 5 weeks of cultivation (**Fig. 4.8 B**).

Since higher cell yields were obtained faster in Matrigel + mEFs-CM, these conditions were selected to be used in the following experiments.

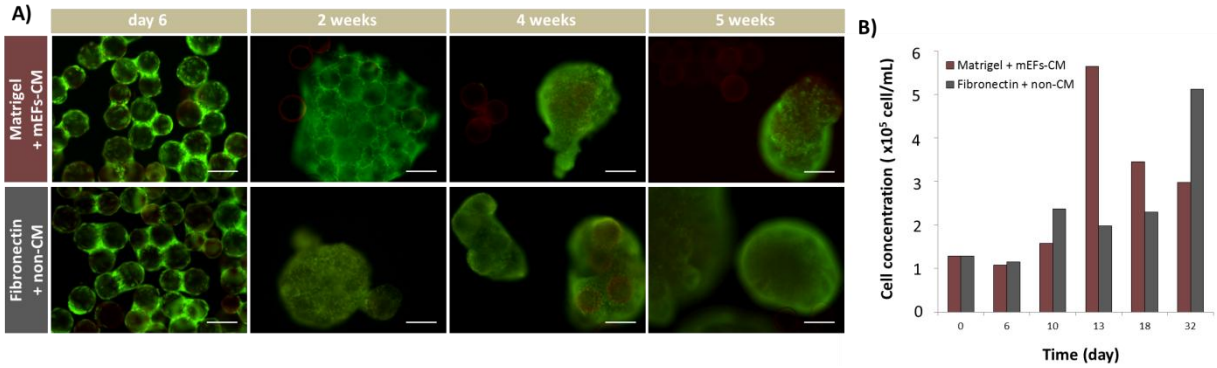

Fig. 4.8 - Effect of culture parameters in expansion of hESCs immobilized on microcarriers. Cells were cultured on microcarriers, in Erlenmeyer, using animal free (microcarriers coated with human plasma fibronectin and non-conditioned media, non-CM) and non-animal free (microcarriers coated with Matrigel and mEFs conditioned media, mEFs-CM) culture components **A)** Viability analysis during culture time, using FDA (live cells, green) and PI (dead cells, red). **B)** Concentration of hESCs during time in each system.

4.4.1.2. Effect of the encapsulation day in expansion of hESCs immobilized on microcarriers

Encapsulation was tested at different culture time points: 8 hours (day 0), 1, 3 and 6 days after cell immobilization to the supports. Cell viability of all cultures was assessed before and after encapsulation (**Fig. 4.9 A**) and it was observed that encapsulation at days 0, 1 and 3 was not efficient for expansion of hESCs. When the encapsulation was performed at day 0 or 1 the majority of the cells died within the following days of encapsulation and no cell growth was observed. At these culture time points, hESCs were still sensitive and weakly adherent on the supports therefore many cells detached during the encapsulation process. The few attached cells presented upon encapsulation probably were not in sufficient density to promote cell growth. In addition, when encapsulation was done at day 3, cell proliferation was only detected in some areas within the capsule (**Fig. 4.9 A**) and the percentage of populated microcapsules was low (not shown). Day 6 demonstrated to be the best time point for the encapsulation of hESCs immobilized on microcarriers. Almost all the microcapsules were populated, assuring more efficient microcarriers colonization than control cultures (non-encapsulated cells). At the end of culture (3rd week) expanded cells expressed alkaline phosphatase activity (**Fig. 4.9 B**). Therefore, the day 6 of culture was the time point selected to perform encapsulation in spinner culture experiments.

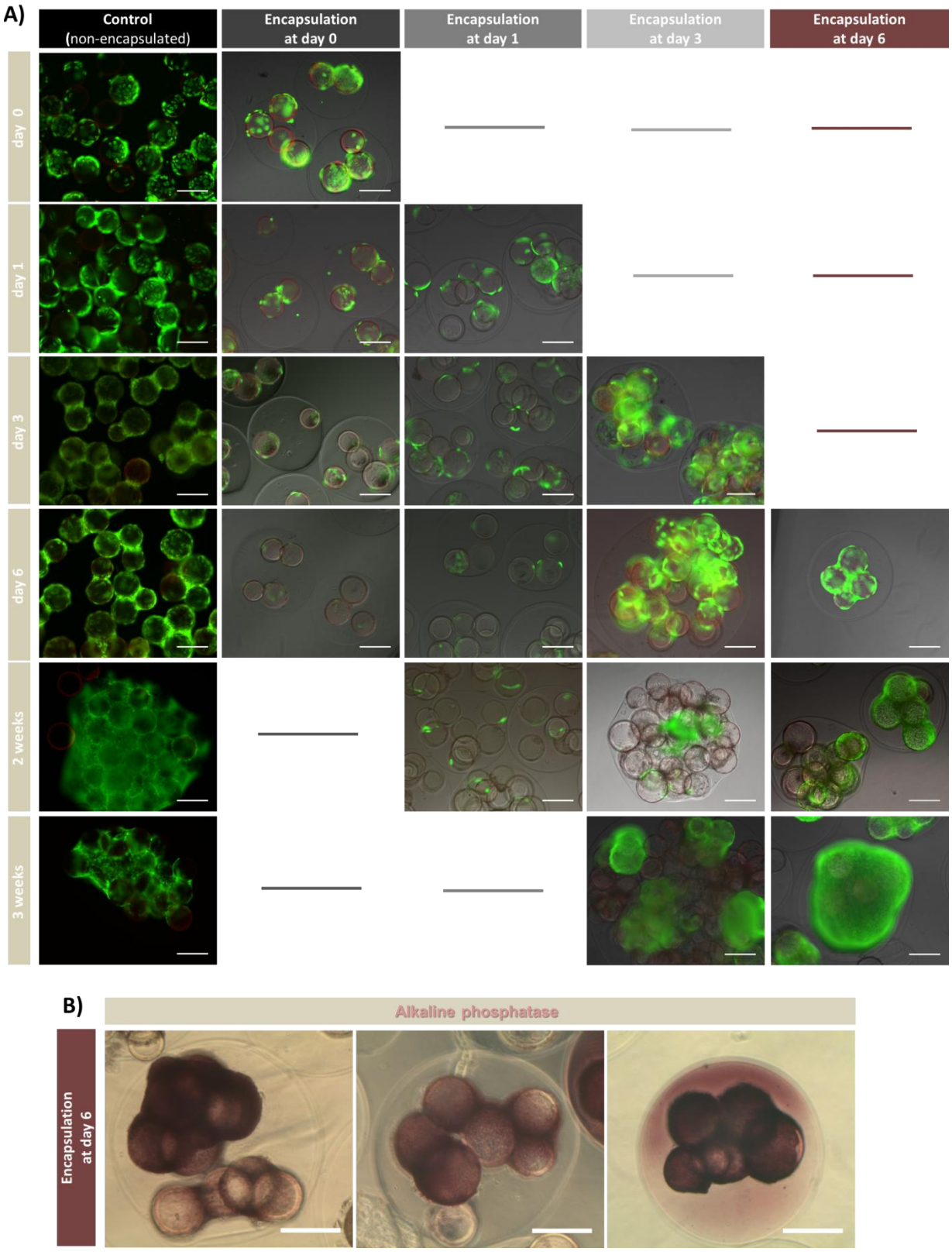

Fig. 4.9 - Effect of the encapsulation day in expansion of hESCs immobilized on microcarriers. Cells were encapsulated at day 0 (after 8 h), 1, 3 and 6 of culture on microcarriers, in Erlenmeyer. **A)** Viability analysis during culture time, using FDA (live cells, green) and PI (dead cells, red). **B)** Phenotypic assessment of alkaline phosphatase activity for hESCs encapsulated at day 6. Scale bars: 200 µm.

4.4.2. Expansion of encapsulated hESCs immobilized on microcarriers in spinner vessels

The culture parameters selected for efficient expansion of encapsulated hESCs-microcarriers aggregates in Erlenmeyer, were applied to spinner vessels. The viability analysis during culture (**Fig. 4.10**) indicated that cells proliferated in microcapsules. However, many microcapsules with empty microcarriers were also detected (**Fig. 4.10**), demonstrating that cell expansion process was not efficient. Due to the increase in the culture system scale, more encapsulations were performed and therefore cells remained longer time in the alginate solution. This step may have affected, cell viability and consequently cell density per support. Indeed, the release of cells from microcarriers was more pronounced than in Erlenmeyer culture, thus justifying the lack of cell growth observed in some microcapsules.

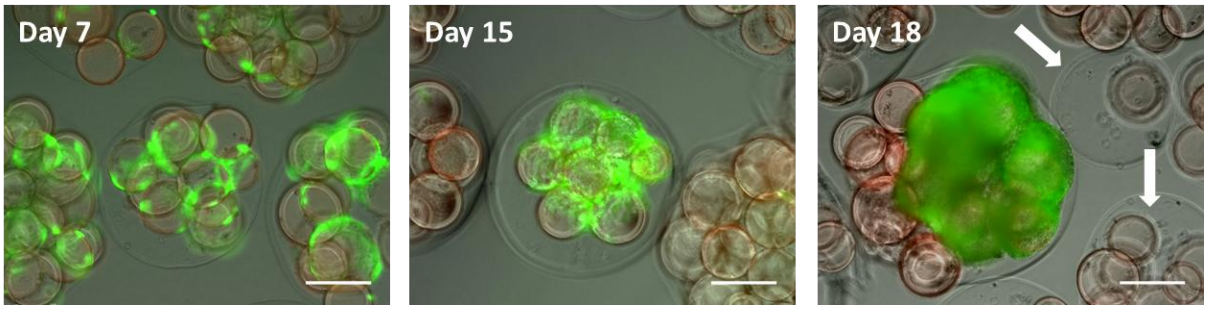

Fig. 4.10 – Expansion of hESCs-microcarriers aggregates in spinner vessels. Viability analysis of hESCs-microcarriers aggregates encapsulated at day 6 in UHV_{NT} alginate, using FDA (live cells, green) and PI (dead cells, red). Cell growth only occurred in microcapsules initially more populated. At the end of culture many non-populated microcapsules (indicated by the white arrows) were observed. Scale bars: 200µm.

To increase microcarriers colonization and cell density *per* support before encapsulation, the approach was to start the cultures with a lower concentration of microcarriers (2 g/L) and add new microcarriers immediately before encapsulation (1g/L thus yielding a final concentration of 3g/L). The addition of fresh coated supports could also promote cell migration and further proliferation inside the capsule, ultimately increasing the number of populated microcapsules and cell yields.

Two types of alginate (0.7% Ba^{2+}-UHV_{NT} and 1.1% Ca^{2+}-UP MVG) were evaluated. The results obtained in viability analysis (not shown) and in alamarBlue test demonstrated that cell proliferation and metabolic performance were similar in both alginate cultures; metabolic activity increased twice from immediately after encapsulation to the end of the culture (**Fig. 4.11 A**).

Since it was possible to dissolve 1.1% Ca^{2+}-UP MVG alginate and efficiently recover the cells, cell concentration was monitored during time. The results demonstrated that there was a small decrease in cell concentration after the encapsulation (**Fig. 4.11 B –**

encapsulation time point is indicated by the black arrow). This can be justified by the occurrence of some cell detachment from the supports during the encapsulation process, as mentioned above. For non-encapsulated culture a stationary phase was observed from day 6 to day 10. This profile could be related to culture adaptation after addition of fresh microcarriers.

Importantly, higher cell concentrations were obtained in encapsulated than in non-encapsulated cultures, yielding higher fold increase at the end of expansion process (10.7±0.8 and 7.7±0.2, respectively, **Fig. 4.11 B** and **Tab. 4.1**). The profile of cumulative LDH also proved that cell lysis was higher in non-encapsulated cultures (**Fig. 4.11 C**). Moreover, no differences were observed in specific cell growth rates in both cultures (**Tab. 4.1**). Taking together these results confirm that encapsulation did not compromise cell growth performance while, at the same time, protected the cells from the hydrodynamic shear stress. Also, the results demonstrated that when combining encapsulation with the two-step addition of microcarriers cell migration was enhanced. The number of populated microcarriers and microcapsules in culture increased significantly.

Aiming at improving cell expansion yields, the concentration of fresh microcarriers was increased: 2 g/L of supports were added before encapsulation, giving a final concentration of 4 g/L. In fact, the addition of more microcarriers to the cultures, i.e. more surface area available for cell growth, contributed to the higher cell yields obtained (2.9 × 10^6 cell/mL corresponding to a fold increase of 19.2±1.8, **Tab. 4.1**). Within the microcapsules cells migrated and colonized most of the microcarriers, presenting also high viabilities (**Fig 4.11 D**). It is important to highlight that, using this strategy cells did not stop their

Tab 4.1 – Growth kinetics characterization of hESCs expansion using different culture strategies. The main results achieved with encapsulation of hESCs immobilized on microcarriers in spinner vessels are listed. Cells were inoculated at 4.5x10^5 cell/mL in all stirred systems. The symbol (*) means calculated after day 6 of culture. Abbreviation: mc, microcarriers.

Culture strategy	**2D - monolayers[65]**	**3D - Non encapsulated 3 g/L mc**	**3D – Encapsulated 3 g/L mc**	**3D – Encapsulated 4 g/L mc**
Microcarrier concentration	-	2g/L+1g/L	2g/L+1g/L	2g/L+2g/L
Initial cell concentration (day 3) (x10^5 cell/mL)	0.2±0.1	1.7±0.3	1.8±0.1	1.5±0.6
Maximum cell concentration (x10^5 cell/mL)	1.9±0.9	12.7±0.5	19.0±2.4	28.8±3.8
Fold increase in cell concentration	10.8±2.0	7.7±0.2	10.7±0.8	19.2±1.8
Apparent growth rate, μ (day^{-1})	Not determined	0.14±0.03* (R^2=0.99)	0.15±0.07* (R^2=0.99)	0.16±0.15* (R^2=0.94)

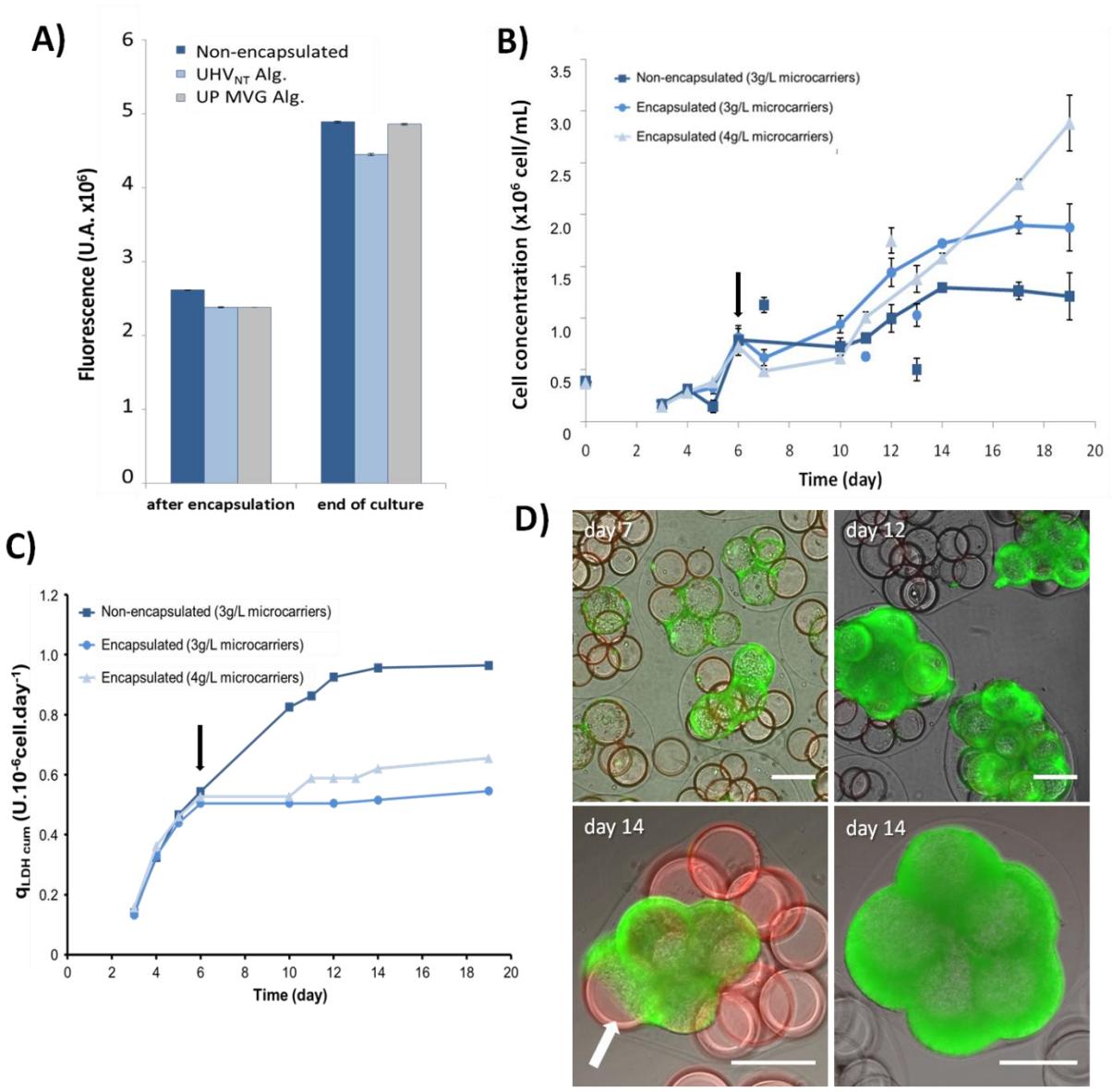

Fig. 4.11 – Expansion of encapsulated hESCs immobilized on microcarriers after addition of fresh microcarriers in spinner vessels. Before encapsulation fresh microcarriers were added, yielding a final concentration of 3 and 4 g/L. **A)** AlamarBlue metabolism test. Error bars denote SD of 3-4 measurements. **B)** Growth curves expressed in terms of cell number *per* volume of medium. The results presented are only for Ca^{2+}-UP MVG Alginate due to the difficulty in dissociating Ba^{2+}-UHV$_{NT}$ alginate without interfere with cell viability. Black arrow indicates the encapsulation time point. Error bars denote SD of 2-4 measurements. **C)** Cumulative values of specific rates of LDH release during culture time. Black arrow indicates the encapsulation time point **D)** Viability analysis of cells encapsulated in Ca^{2+}-UP MVG alginate with 4g/L of microcarriers. White arrow indicates cell migration inside the microcapsule.

exponential growth. However, culture was finished at day 19, since overgrowth was observed in some microcapsules (results not shown). On the other hand a stationary phase was detected, in the other two cultures (encapsulated and non-encapsulated cultures in which 1g/L of fresh microcarriers were added) from day 14 onwards. The reason why cell concentration was maintained until the end of culture should be related to the perfusion system used, since it allowed a constant renewal of the nutrients and prevented the

accumulation of toxic metabolites. Also, supplementation the medium with Rapamycin may have enhanced hESC viabilities[65, 134].

4.4.3. Characterization of expanded hESCs

After expansion in spinner vessels, hESCs were characterized for their undifferentiated and pluripotent status. The results obtained by both immunofluorescence microscopy and flow cytometry analysis (**Fig. 4.12 A, C**) demonstrated that high numbers of hESCs that stained positive for specific stem cell markers (OCT4, SSEA-4, hESCellect^{TM} and TRA-1-60) were observed at the end of the expansion process. When compared to non-encapsulated cultures, the flow cytometry analysis results for pluripotent stem cell markers were very similar, except for TRA-1-60 where higher percentage of positive cells were detected in encapsulated cultures (**Fig. 4.12 C**). This difference could be related to the experimental protocol itself that could have affected the analysis/result.

Regarding SSEA1 detection, higher levels of positive cells were obtained in non-encapsulated (13.0±0.4) than in encapsulated cultures (7.8±0.3) (**Fig. 4.12 D**), suggesting that, at the end of the expansion process, more cells in an early differentiated state were presented in the formers.

Encapsulated cells maintained the capacity to form undifferentiated colonies in 2D traditional monolayer systems **(Fig. 4.12 B)** and presented *in vitro* pluripotency. Cells were able to spontaneously differentiate into cells of the three embryonic germ layers **(Fig. 4.12 D)**.

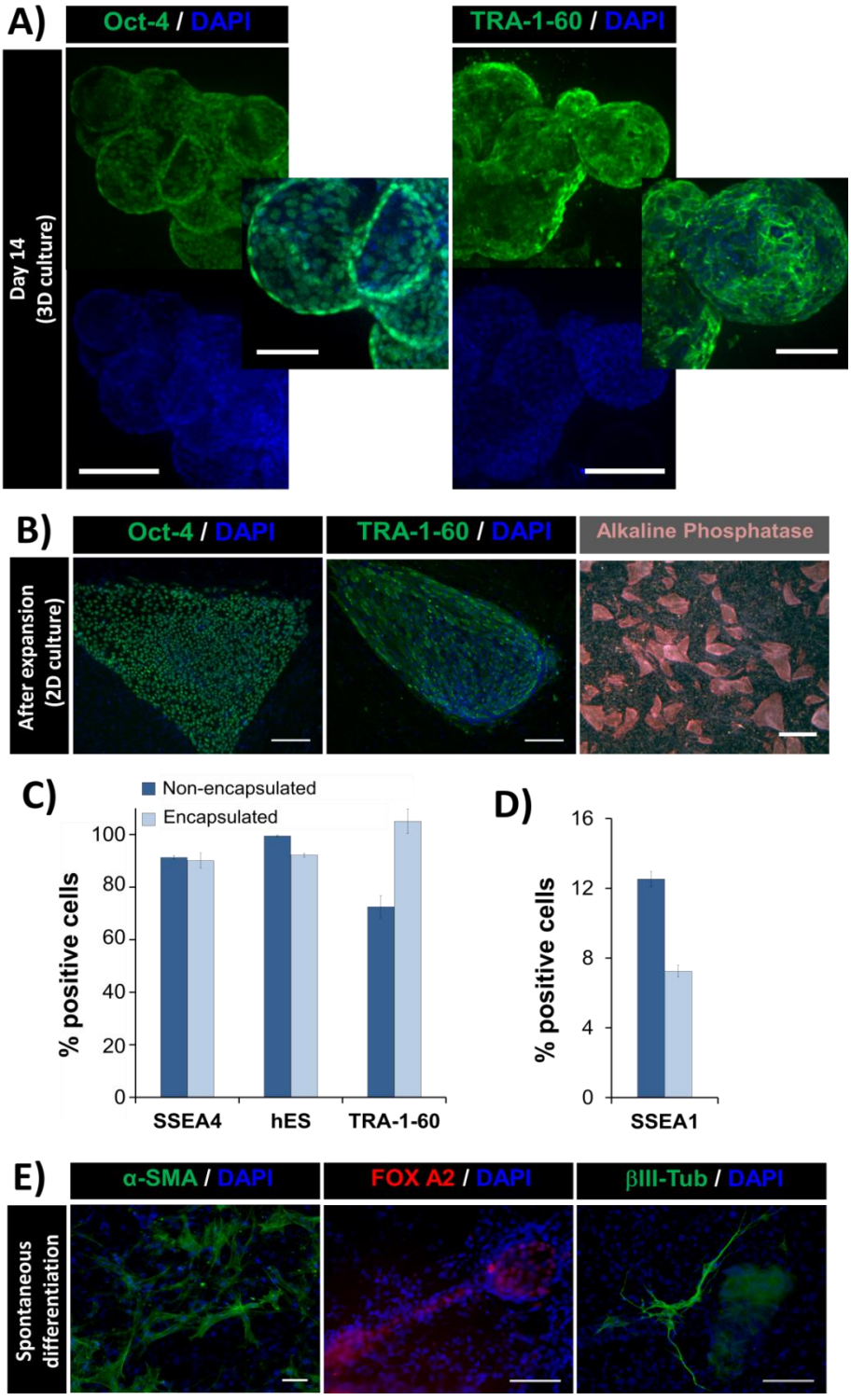

Fig. 4.12 - Characterization of hESCs after expansion in spinner vessels. **A)** Confocal images of Oct-4 and TRA-1-60 labeling at day 14 of encapsulated 3D culture in UP MVG alginate. Nuclei were labeled with DAPI (blue). Scale bar: 200 µm, merge images 100 µm. **B)** Immunofluorescence images of Oct-4 and TRA-1-60 labeling and phase contrast pictures of alkaline phosphatase activity after expansion (2D culture). Nuclei were labeled with DAPI (blue). Scale bars: 200 µm and 1 mm for immunofluorescence and phase contrast images, respectively. **C)** Flow cytometry analysis of the expanded population: percentage of SSEA-4, hESCellect^{TM} and TRA-1-60 positive cells in relation to the control population (cells growing in 2D – on feeders). **D)** Percentage of SSEA-1 positive cells after expansion determined by flow cytometry analysis. **C-D)** Error bars represent SD of 2 measurements **E)** *In vitro* pluripotency analysis. hESCs encapsulated in UP MVG alginate formed embryoid bodies (EBs) and spontaneously differentiated into cells of the three germ layers. Staining for α-SMA (mesoderm), FOX-A2 (endoderm) and βIII-Tub (ectoderm) are shown. Scale bars: 100 µm.

4.4.4. Cryopreservation of encapsulated hESCs immobilized on microcarriers

The development of an effective protocol to cryopreserve hESCs on microcarriers was investigated. Non encapsulated and encapsulated hESCs were harvested at day 13 of spinner cultures and cryopreserved using two different procedures: slow freezing and vitrification.

The vitrification method was not suitable for cryopreservation of hESCs in microcarriers. In both non-encapsulated and encapsulated cultures the profile was similar. High cell death was registered immediately after thawing (**Fig. 4.13**). In addition, some supports were damaged after the fast cooling/thawing process.

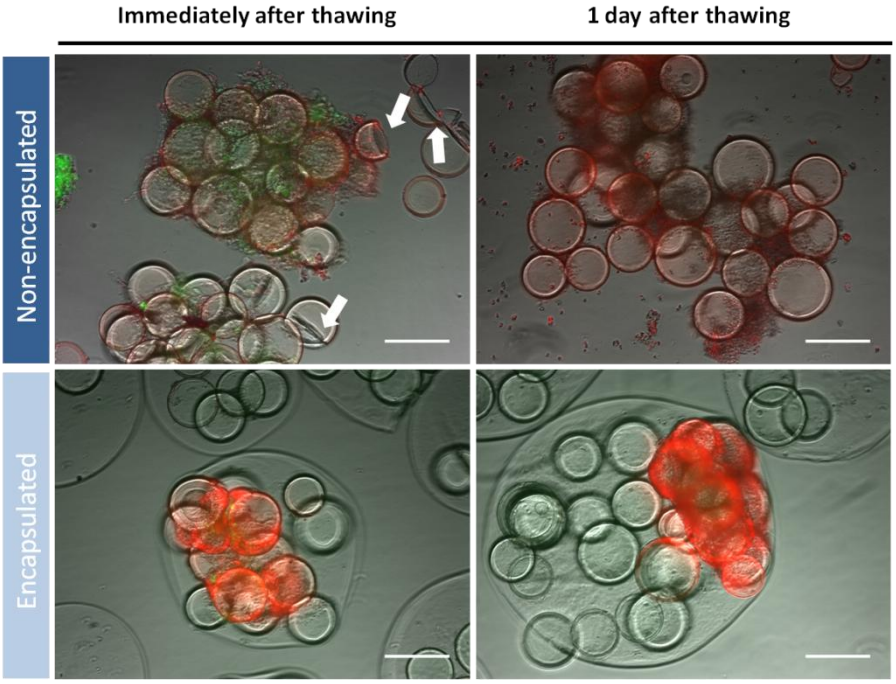

Fig. 4.13 – Vitrification of hESCs-microcarriers aggregates. Cell viability analysis, immediately and 1 day post-thawing, using FDA (live cells, green) and PI (dead cells, red), for encapsulated and non-encapsulated cells. White arrows indicate damaged microcarriers. Scale bars: 200 µm.

On the other hand, the slow freezing procedure demonstrated to be a promising method to cryopreserve encapsulated hESCs-microcarriers aggregates. Most of the cells remained viable after thawing, using both alginates (**Fig. 4.14 A**). Moreover, higher cell recovery rates were obtained in encapsulated culture (day 0= 103.7±8.8% and day 1= 71.0±5.0%) than in non-encapsulated culture (day0= 55.7±4.6% and day1= 24.9±2.8%) (**Fig. 4.14 B**). Although there has been some cell death in the first days post-thawing (probably due to post-thawing apoptosis), encapsulated hESCs recovered quickly their proliferative and metabolic activity, as confirmed by phase contrast/fluorescence microscopy and alamarBlue ssay (**Fig. 4.14 C**). In non-encapsulated cultures, cell death was more pronounced; it was detected loss of cell membrane integrity and a significant cell detachment from the

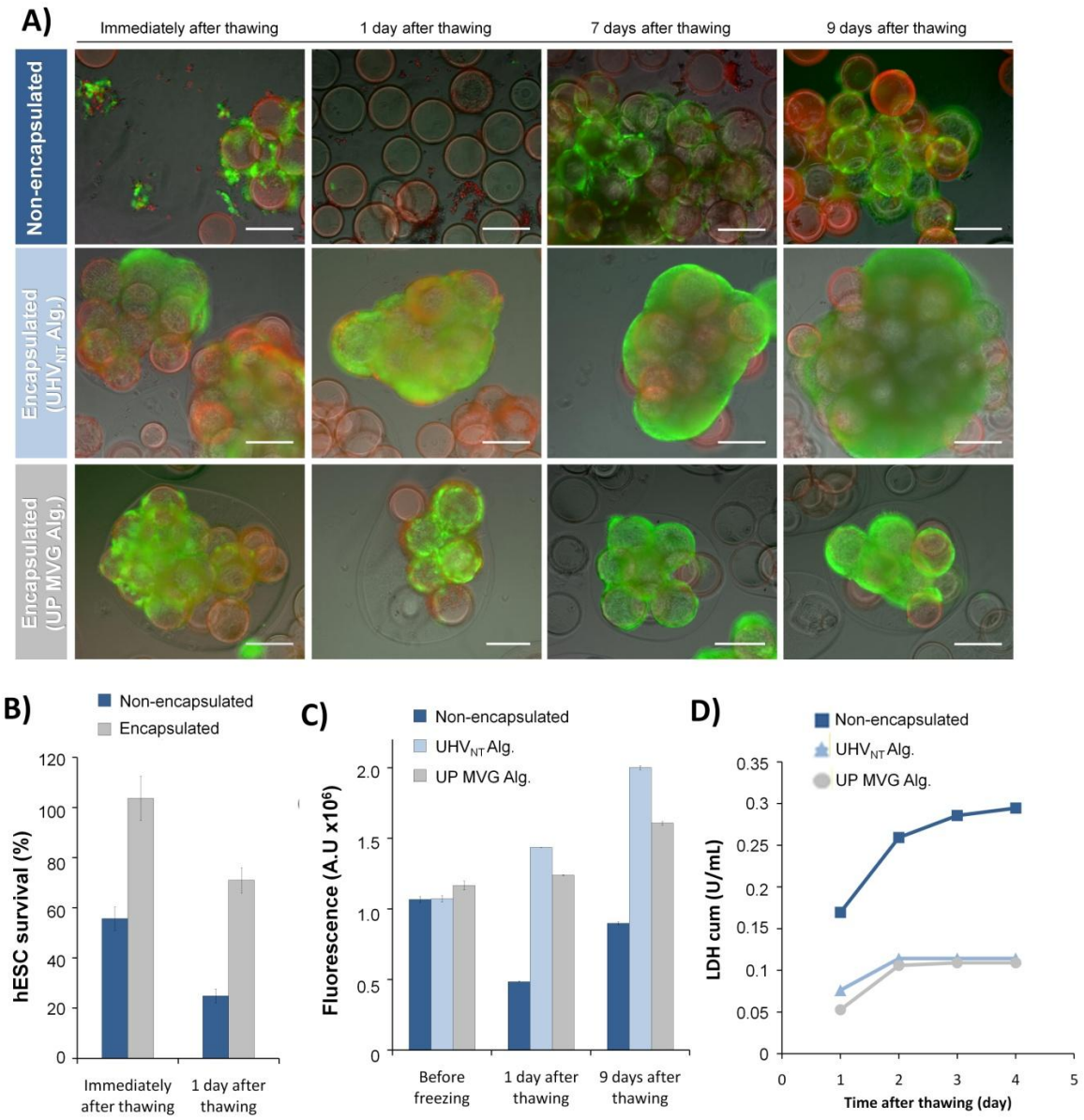

Fig. 4.14 - Cryopreservation of hESCs-microcarriers by slow freezing rate. After thawing, encapsulated and non-encapsulated hESCs-microcarriers aggregates were cultured in suspension in conditioned media. **A)** Phase contrast and fluorescence images of cryopreserved hESC immediately, 1, 4, 7 and 9 days after thawing. Viability analysis of hESCs stained with fluoresceine diacetate (FDA-live cells, green) and propidium iodide (PI- dead cells, red). Scale bars: 200 µm. **B)** Percentage of cells survival (estimated by the ratio between the number of live cells after thawing and the number of viable cells cryopreserved) immediately and one day after thawing. Error bars denote SD of 2 measurements. **C)** Metabolic activity of non-encapsulated and encapsulated cultures measured by alamarBlue test immediately, 1 and 9 days after thawing. Error bars denote SD of 4 measurements. **D)** Cumulative values of LDH release.

microcarriers after thawing (**Fig. 4.14 A**). Only highly populated hESCs-microcarriers aggregates remained viable. By alamarBlue assay (**Fig. 4.14 C**) it was verified that non-encapsulated cells did not reestablish the metabolic activity that they presented before cryopreservation and the levels of LDH release were always higher than in both encapsulated cultures (**Fig. 4.14 D**), where the profiles were similar.

Cells encapsulated in UP MVG alginate were released from the microcapsules/supports and plated onto ihFF monolayers immediately after thawing. These cells maintained their ability to form undifferentiated pluripotent colonies for at least 5 passages as confirmed by immunofluorescence microscopy, flow cytometry, alkaline phosphatase activity test and colonies morphology (**Fig. 4.15 B, C**). These cells also retained the ability to differentiate *in vitro* into cells from the three germ layers (**Fig. 4.15 D**).

Immunofluorescence microscopy for stem cells markers of encapsulated cells in UP MVG alginate at day 9 post-thawing, show that cells maintained the undifferentiated character also when expanded after thawing (**Fig. 4.15 A**).

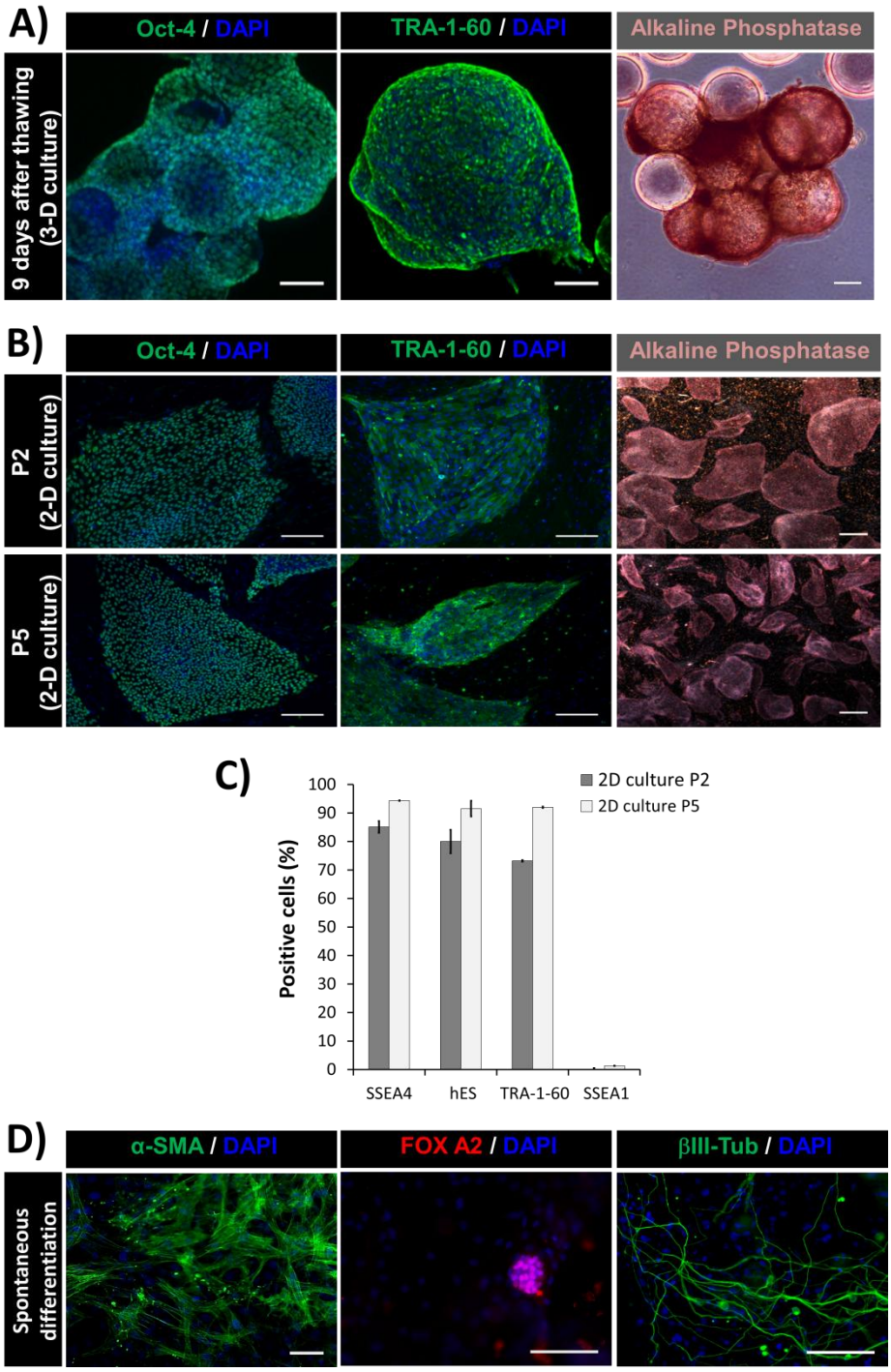

Fig. 4.15 - Characterization of hESCs after cryopreservation by slow freezing rate. Phenotype analysis of encapsulated hESC immobilized on microcarriers after **(A)** 9 days of culture post-thawing and **(B)** after 2 and 5 cell passages in 2-D culture systems (P2 and P5, respectively). **A, B)** Immunofluorescence images of Oct-4 and TRA-1-60 labeling, and phase contrast pictures of alkaline phosphatase activity. Nuclei were labeled with DAPI (blue). Scale bars: **(A)** 100 µm and **(B)** 200 µm for immmunofluorescence images; **(A, B)** 1mm for phase contrast images. **C)** Fow cytometry analysis; percentage of SSEA-4, hES-Cellect^{TM}(hES), TRA-1-60 and SSEA-1 positive cells. Error bars represent SD of 2 measurements. **D)** *In vitro* pluripotency analysis. Microcapsules were dissolved and hESCs were dissociated from the microcarriers and transferred to a monolayer of ihFF. At confluence, colonies were dissociated and hESCs were able to form embryoid bodies (EBs) in non-adherent conditions and differentiated into cells from all three germ layers. Phase contrast micrograph of human embryoid bodies and fluorescence images of differentiated cultures labeled for α–SMA (α smooth muscle actin, mesoderm), FOX-A2 (Forkheadbox A2, endoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bars: 100 µm.

5. Discussion

This work evaluates the use of alginate microencapsulation technology to support and integrate the expansion and cryopreservation of hESCs as a 3D culture system. The main results achieved were described in **Tab. 5.1**.

Tab. 5.1 - Main results obtained in this work for each strategy of hESCs culture. The symbols mean: + resulted efficiently; - did not result

Culture strategy	Expansion	Cryopreservation		Main results
		Slow freezing rate	Vitrification	
Encapsulation of single cells	-	-	-	High cell death after encapsulation
Encapsulation of hESC aggregates	+	-	-	Culture of undifferentiated spherical and compact aggregates for two weeks
Encapsulation of hESCs immobilized on microcarriers	+	+	-	High microcarriers colonization High cell yields High cell recovery after slow freezing rate

Firstly, it was shown that hESCs microencapsulation in an alginate matrix allow an efficient expansion of undifferentiated hESCs (**Tab. 5.1**). Either the encapsulation of hESC aggregates or the encapsulation of hESCs immobilized on microcarriers did not compromise cell proliferation.

In fact, one important aspect to have in consideration in cell microencapsulation is the matrix design. The matrix should be enough strength in order to maintain the structural integrity of microcapsules and also enough flexible to accommodate cell expansion within. One parameter that should be well define is the concentration of alginate gels, since it determines the matrix flexibility/porosity to permit cell growth within the bead[145] and the diffusion of molecules (nutrients, gases, metabolites, growth factors, cytokines, etc.). Matrix pore size decreases as the alginate concentration increases[146] and the diffusion rate, in turn, decreases with decreasing pore size[147].

In this study, two concentrations of UHV_{NT} alginate were tested: 0.4% (w/v) and 0.7% (w/v). The lower concentration did not allow the formation of microcapsules with sufficient mechanical strength to support a long-term culture. On the other hand 0.7% UHV_{NT} alginate matrices fulfill the requisites of permeability, stability and elasticity since it supported an efficient hESC culture.

Also, two distinct alginate matrices (UHV_{NT} and UP MVG alginate) were evaluated in this study. It was showed that cell growth performance and viability within both alginates

were similar. These observations can be explained by the fact that both polymers are high-G content (>60)[115, 117]. In general, alginates with high content of G monomers have shown to form stable gels with a high permeability[91] and therefore have the necessary properties to support an efficient cell growth.

The only difference observed between the two alginate matrices was related with alginate dissociation and consequent recovery of hESCs. The dissociation of UHV_{NT} alginate significantly affects cell viability, preventing an efficient recovery of the cells. On the other hand, UP MVG alginate dissociation does not compromise cell viability, allowing the recovery of viable cells and their further characterization after expansion. This difference should be related with the use of different crosslinkers. The gelification of UHV_{NT} alginate was performed using Ba^{2+} whereas UP MVG alginate microcapsules were formed in a Ca^{2+} bath. In fact, it is known that Ba^{2+} ions give better long-term mechanical stability than Ca^{2+} ions do, since Ba^{2+} forms stronger cross linkers with alginate than Ca^{2+} [148]. However, this effect is only observed for alginates with a high G content (more than 60% G)[149]. To dissociate the strong linkers between Ba^{2+} and the UHV_{NT} alginate, a solution of Na_2SO_4 was used. This procedure changes drastically the osmotic pressure, leading to cell shrink and ultimately cell death. The size of the cells recovered after decapsulation was so small that it was difficult to distinguish them from cellular debris. Cross linkers between Ca^{2+} and the UP MVG alginate were easily disrupted using an EDTA solution and cells maintained their viability.

Overall, these considerations support the assumption that 0.7% Ba^{2+}-UHV alginate is more appropriate to be used for long-term immunoprotection of differentiated cells during transplantation. It will be useful for the treatment of human disorders such as hormone or protein deficiencies. Ca^{2+}-UP MVG alginate is more suitable to be integrated in stem cell bioprocesses for efficient high production of cell-based products. Calcium forms weaker alginate gels and is also more sensitive to chelators such as citrate, phosphate, and lactate[89], therefore long-term survival of Ca^{2+}-alginate microcapsules *in vivo* should be limited. However this can also be advantageous if the aim is the transplantation of autologous cells for repair, remodeling or regeneration of tissues[107]. In this case, the capsule will only minimize the initial immune-rejection of the cells by preventing surgery induced activation of innate immunity and could enhance the graft delivery to a certain target. Indeed, easily biodegradable alginates have been studied as promising approaches for tissue engineering and regenerative medicine. It should be noted that Ca^{2+}-UP MVG alginate was not tested yet for routine clinical application. Although, this alginate is manufactured in compliance with

current Good Manufacturing Practice and presents an endotoxin concentration less than required for *in vivo* studies (≤100 EU/g)[91].

The encapsulation of hESCs as single cells resulted in a pronounced cell death even when different cell densities and alginate matrices were used. Thus this strategy is not useful for hESCs expansion. This result is in agreement with other published studies[139, 146], confirming that cell–cell interactions are an important environmental factor that influences hESCs survival and proliferation. In fact, the formation of hESCs aggregates within the microcapsules was only observed when higher cell densities were used ($3x10^6$ cell/mL alginate), since some small clusters were already encapsulated and single cells within the alginate matrix were closer to each other. It is important to highlight that hESCs are vulnerable to apoptosis after complete dissociation[51], therefore when the cells were handled as single cells (before and after both harvesting and encapsulation steps) they were treated with ROCKi. Previous reports shown that this compound prevent apoptosis and enhance the survival and cloning efficiency of dissociated hESCs without affecting their pluripotency[51]. However, in this case no significant improvements in cell viability/expansion were observed.

On the other hand, it was demonstrated that alginate microencapsulation of aggregates is an efficient strategy for the expansion of hESCs in spinner vessels. Cells were protected from the harmful effects of hydrodynamic shear stress, allowing a more efficient cell growth, control of aggregate size and maintenance of pluripotent status for two weeks of culture.

Compact, spherical and size uniform aggregates were formed in both alginate cultures. Furthermore, cells presented high viability within the aggregates during the two weeks of culture, confirming that limitations in the diffusion of nutrients and metabolites within alginate matrices did not occur. In fact, a high improvement in culture performance was achieved comparatively to non-encapsulated culture, where many dead cells were observed in suspension as well as aggregate clumping. The formation of these large aggregates compromised cell viability; necrotic centers were observed as a consequence of diffusion limitations within the 3D structure. These observations confirmed that shear stress (promoted by stirring) was harmful for the cells.

The expansion of undifferentiated hESCs as aggregates using spinner vessels has been reported recently. Krawetz *et al.* cultivated hESC aggregates in stirred spinner vessels (stirring rate - 100 rpm) using the repeated dissociation strategy (every 6 days the cultures

were dissociated into single cells and split at 1:5) in order to avoid the formation of large aggregates and achieve high levels of pluripotency[134]. In another study, Singh *et al.* successfully reported the culture of hESCs for 7 days as uniform spherical and viable aggregates using spinner vessels equipped with a low shear impeller (single stirring pendulum at 40 rpm)[131]. When compared to the conditions used herein for non-encapsulated culture, these studies used less time of culture and less abrasive stirring, which probably contributed to the efficient expansion of pluripotent hESCs as aggregates. Importantly, the results obtained in this study demonstrated that the culture of encapsulated aggregates seems to be a more efficient system for the expansion of hESCs.

Few reports of culturing hESCs on scaffolds or hydrogels have been published, demonstrating that the culture of hESCs in these 3D matrixes assured the maintenance of their undifferentiated phenotype for weeks without passaging. Gerecht *et al.* demonstrated that encapsulated hESCs in hyaluronic acid hydrogel maintained their undifferentiated status in the presence of conditioned medium from mEFs for more than 20 days in culture[150]. Siti-Ismail *et al*. showed that encapsulation supported the maintenance of hESCs in the undifferentiated state for 260 days, without the need for feeders or passaging. In this case the encapsulated hESCs were cultured in non-conditioned medium supplemented with bFGF. The larger capsule sizes used may allow such a prolonged culture of encapsulated hESCs[138].

Nevertheless, in this study, after two weeks of culture, signals of cell differentiation in encapsulated aggregates were observed. This can be explained by the increase in aggregate size that could led to the formation of gradients in the concentration of gases and regulatory molecules (e.g. bFGF) within the aggregate, thus promoting spontaneous differentiation of some cells. Alternatively, this profile can be considered advantageous if the aim is to integrate directed differentiation after a previous expansion step. Inducing directed differentiation at this culture time point (2nd week), by changing specific culture conditions (addition of inducible factors, medium replenishment, etc.), would bypass the step of EBs formation, which is time-consuming and uncontrolled, and ultimately increase differentiation efficiency and yields. Several studies have successful reported the differentiation of encapsulated embryonic stem cells, for instance into definitive endoderm[139], pancreatic insulin-producing cells[146], hepatocytes[151], cardiomyocytes[140], and bone cells[152].

In this study it was evaluated for the first time the encapsulation oh hESCs adherent on microcarriers. This 3D strategy demonstrated to be very efficient to the production of high cell numbers required for clinical or pharmacological applications. At the end, it was possible to achieve approximately $3x10^6$ cell/mL, corresponding to an improvement of 15.2-fold in cell yields when compared to standard 2D protocols[65].

It should be noted that the best result was obtained when 2g/L of empty and fresh coated microcarriers were added to the hESCs-microcarriers aggregates cultures immediately before encapsulation. The two-step addition of microcarriers allowed to improve microcarriers colonization, microcapsules population and consequently enhance cell yields in spinner vessels. Despite of being an effective system for the production of high numbers of pluripotent hESCs, this strategy cannot be conjugated with transplantation studies, due to the presence of supports within the capsules. Alternatively, cells could be released from the matrix, detached from the supports and then used clinically. Moreover, a biodegradable clinical approved support could eventually be used. For instance, Tatard *et al.* developed pharmacologically active biodegradable microcarriers made with poly(D,L-lactic-*co*-glycolic acid) (PLGA) and coated with adhesion molecules to serve as a support for cell culture[153]. Gelatin microcarriers are also under study to be used in 3D cartilage and bone like tissue engineering[154].

Although the culture of hESCs on microcarriers has been reported to be an efficient strategy to expand hESCs, future studies should be focus on the development of define xeno-free culture conditions. For instance, coating the microcarriers with Matrigel has proven effective in enhancing the poor adhesion of hESCs to the supports but its animal origin makes it unsuitable for use in cell therapy. The same is true about the use of mEFs-conditioned media. Preliminary studies performed in this work suggested that the use of non-conditioned media and microcarriers coated with human plasm fibronectin is able to support the proliferation of hESCs. However, the specific growth rate was reduced. This preliminary knowledge might be helpful for the future development of an efficient xeno-free culture system.

The feasibility of using microencapsulation technology for hESCs cryopreservation was also investigated.

The results shown that the encapsulation of cells adherent on microcarriers is a valuable strategy for the efficient cryopreservation of pluripotent hESCs, by slow freezing

rate. In fact, comparatively with non-encapsulated culture, higher cell survival was obtained immediately and one day after thawing (improvement of 2- and 3- fold, respectively) and hESCs recovered faster their proliferative and metabolic activity. These results indicate that the encapsulation process had an important role in cell recovery. Indeed, cell microencapsulation within alginate beads has previously been reported as a promising strategy to improve post-thaw viability of different cell types which were shown to poorly survive in the cryopreservation process, such as hepatocytes[155, 156], pancreatic islets[110, 157] and neural cells[130]. Although the underlying mechanisms are still unclear, cell entrapment may help protect cells from the adverse effects of cryopreservation by decreasing exposure to cryoprotectants[127], limiting extracellular ice growth near the cell's membrane and the initiation of intracellular ice formation[158, 159], providing cell immobilization thus preventing detachment of the cells from the supports and breakage of cell-cell contacts[114, 130].

On the other hand, the recoveries reported after conventional slow freezing of hESCs vary from 0% to only 48%[75, 77-79, 81, 160]. In addition Nie *et al*. verified that cryopreservation of hESCs on mEFs-microcarriers improved in 1.5-1.9 times the recovery of hESCs frozen as free colonies. However, when hESCs were cryopreserved adherent to Matrigel-microcarriers cell recovery was not improved[63]. Cells in these microcarriers were more prone to detach from the supports after thawing. This result is similar to that obtained in this study. These results reinforce the assumption that maintaining both cell-cell and cell-matrix contacts improve hESCs recovery following cryopreservation through slow-rate freezing[63, 73, 76].

The slightly decrease obtained for cell recovery at day 1 after thawing, in encapsulated cultures must be caused by post-thaw apoptotic and necrotic mechanisms[161, 162]. Therefore the addition of anti-apoptotic factors at this time should have a great importance. In this study, ROCKi was used before, during and after the cryopreservation process since it was reported that the addition of this inhibitor also increases the survival rate after thawing[163, 164].

Importantly, in the system developed herein, hESCs can be efficiently recovered after cryopreservation without compromising their stem cell characteristics. These cells maintained their pluripotency over at least five passages in standard culture conditions.

The slow freezing rate of both encapsulated and non-encapsulated hESC aggregates proved to be an inefficient strategy. The higher cell death observed in aggregates in comparison with cell-microcarrier cultures may be explained by the limitation in heat and mass (water and cryoprotectant) diffusion within the aggregates derived from their larger

size and more compact 3D structures. This results in different cryoprotection effect from the surface to the center of the aggregate[130, 166], possibly leading to cryodamage. Another explanation is the presence of components of the extracellular matrix (ECM) when hESCs were cryopreserved immobilized on Matrigel coated Cytodex3 microcarriers. This may have contributed to enhance cell survival during slow freezing rate and thawing[76, 80]. Indeed, it has been demonstrated that hESCs embedded in Matrigel exhibit an improvement in cell recovery[76, 82]; Heng *et al.* argued that Matrigel entrapment improves hESCs colony recovery by preserving gap junctions and other intercellular adhesion contacts during freezing and thawing[165].

It is important to highlight that cryopreservation of encapsulated hESCs using the vitrification protocol did not work in all strategies tested in this study. The reasons why cells lose their viability are not clear. However, it should be noted that only preliminary tests were performed and the optimization of this technique using, for example, straws and by improving specific steps during the fast cooling and/or thawing process(es) should be considered. The addition of more apoptotic inhibitors may be also important, since some encapsulated aggregates remained viable immediately after thawing process and only died in the days after-thawing. Nevertheless, it is important to note that slow freezing rate is a more appropriate procedure to cryopreserve hESCs than vitrification since the last technique is labor consuming and impractical on a large scale; it requires special devices, is extremely dependent on the expertise manipulation skills and is unsuitable for handling bulk quantities of hESCs[67, 73, 167]. The high risk of contamination with pathogenic agents[85] is another disadvantage of the vitrification protocol.

In future, more fundamental studies regarding the physical-chemical and bio-physical phenomena occurring during freezing/thawing of encapsulated hESCs should be performed in order to improve the cryopreservation of hESCs aggregates.

Summing up, this work has shown that alginate cell microencapsulation is a promising approach for the cultivation of hESCs because: it allows the culture of hESCs in a 3D conformation similarly to what occurs in vivo; it enables the culture of cells in fully controlled bioreactors, where scalability automation and tight control of the culture environment are combined to assure high cell yields; it protects cells from shear stress and also prevents excessive clumping, permitting a tighter control of the process and a prolonged cell culture; it allows the differentiation of the cells within; and finally, it allows

higher cell recovery of pluripotent hESCs after cryopreservation on microcarriers by a scalable slow freezing procedure.

In addition microencapsulation technology is useful to protect cells from the immune-response during transplants. However, to increase the efficiency of microencapsulation technique so that it could be used in the clinic and industrial fields some parameters have to be optimized. Bubbles as well as tails during the manufacture of the microcapsules should be minimized or eliminated for instances by applying sinusoidal pressure waves[114]. Ca^{2+}-alginate microcapsules should be coated with polycation, such as poly-L-lysine (PLL)[91], to increase its integrity and stability during long-term culture. Immune reactions should be avoided, for example, by covering microcapsules with a cell-free layer of high M alginate, that could also guarantee long-term stability to the microcapsules[114]. Prolonged incubation time periods with Ba^{2+} should be avoided since it inhibit the potassium channels in the membranes of encapsulated cells, leading to their death[115]. Cells should be symmetric positioned within the capsule, since thin layers of alginate do not immunologically protect the entrapped cells and could also lead to breakage of the microcapsules[114]. Knowing that cells tend to agglotinate during the encapsulation process and induce the production of empty beads, another challenge is to separate these empty microcapsules from the filled microcapsules. Finally, standardization and validation of the entire manufacturing process from the material production site to the clinics should be performed.

6. Conclusion

This thesis shows that alginate microencapsulation is a powerful tool to integrate expansion and cryopreservation of pluripotent human embryonic stem cells. When microencapsulation technology is combined with microcarriers to grow hESCs in stirred systems, higher cell yields can be achieved (approximately 3×10^6 hESC/mL) without compromising cell pluripotency. Moreover, cells can be cryopreserved immediately after expansion by using a simple and easy scalable technique, that assures high recoveries of undifferentiated and pluripotent hESCs after thawing. The integrated strategy developed herein would be amenable to scale-up (e.g. using large-scale stirred tank bioreactors), therefore it would ensure the production, banking and distribution of clinical relevant numbers of high-quality hESCs, in a scalable and straightforward manner.

Furthermore, this work also demonstrated that microencapsulation confers physical protection to hESC aggregates from damage caused by stirring, allows the control of aggregates size and the maintenance of pluripotency for two weeks. Also, this strategy is very promising to integrate both expansion and directed differentiation steps in a controlled bioprocess. In future, using a xeno-free, clinical-grade alginate (such as UHV_{NT} alginate) the differentiated cells derived from the process could be eventually used in (human) transplantation studies.

Hopefully, the 3D culture strategies developed here represent a relevant step forward in the promising transition of hESCs to regenerative medicine, tissue engineering and toxicology fields.

7. References

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