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Stem cell cultivation in bioreactors

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ABSTRACT

Cell-based therapies have generated great interest in the scientific and medical communities, and stem cells in particular are very appealing for regenerative medicine, drug screening and other biomedical applications. These unspecialized cells have unlimited self-renewal capacity and the remarkable ability to produce mature cells with specialized functions, such as blood cells, nerve cells or cardiac muscle. However, the actual number of cells that can be obtained from available donors is very low. One possible solution for the generation of relevant numbers of cells for several applications is to scale-up the culture of these cells in vitro. This review describes recent developments in the cultivation of stem cells in bioreactors, particularly considerations regarding critical culture parameters, possible bioreactor configurations, and integration of novel technologies in the bioprocess development stage. We expect that this review will provide updated and detailed information focusing on the systematic production of stem cell products in compliance with regulatory guidelines, while using robust and cost-effective approaches.

Contents

Abbreviations: 2D, Two-Dimensional; 3D, Three-Dimensional; BFU-E, Burst-Forming Units-Erythrocyte; CFCs, Colony-Forming Cells; CFU-F, Colony-Forming Units-Fibroblast; CFU-GEMM, Colony-Forming Units-Granulocyte-Erythrocyte-Macrophage-Megakaryocyte; CFU-GM, Colony-Forming Units-Granulocyte-Macrophage; EBs, Embryoid Body; EMEA, European Medicines Evaluation Agency; ESCs, Embryonic Stem Cells; FDA, Food and Drug Administration; GMP, Good Manufacturing Practices; HARV, High Aspect Ratio Vessel; HSCs, Hematopoietic Stem Cells; HSPCs, Hematopoietic Stem and Progenitor Cells; iPSCs, Induced Pluripotent Stem Cells; LIF, Leukemia Inhibitory Factor; LTC-ICs, Primitive Long-Term Culture Initiating Cells; MAPCs, Multipotent Adult Progenitor Cells; MEF, Mouse Embryonic Fibroblast; MESCs, Mammary Epithelium Stem Cells; MNCs, Mononuclear Cells; MSCs, Mesenchymal Stem Cells; NSCs, Neural Stem Cells; PB, Peripheral Blood; PET, Polyethylene Terepthalate; PSCs, Pluripotent Stem Cells; Ri, ROCK Inhibitor; SBs, Stirred-Suspension Bioreactors; STLV, Slow Turning Lateral Vessel; UCB, Umbilical Cord Blood.

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1. Introduction

Stem cells are undifferentiated cells with self-renewal capacity and the ability to differentiate into mature cells (Passier and Mummery, 2003). These properties are very appealing for therapeutic applications, but the actual number of cell therapy products that have reached the market is still very low (Parson, 2008). Most likely, the success of these approaches will be dependent on the development of novel technologies that allow the systematic production of cells in a robust and cost-effective manner (*e.g.* bioreactors) (Kirouac and Zandstra, 2008).

In fact, for some applications the number of cells needed to treat an adult patient greatly surpasses the number of cells available from donors (Laflamme and Murry, 2005; Sohn et al., 2003). Moreover, the need to develop fully controlled large-scale bioreactors arises not only from the limited number of cells that can be obtained from available donors, but also from the need to comply with strict regulatory guidelines (FDA, EMEA) (Cabral, 2001). Since the desired products are cells, further challenges related to good manufacturing practices (GMP) and product safety also need to be overcome (Unger et al., 2008) (Fig. 1a). Donor-to-donor variability, microbiological contamination, potential tumorigenicity of the transplanted cells, among others, are examples of such issues (Ahrlund-Richter et al., 2009).

Furthermore, stem cell engineering strategies can also contribute for studying the mechanisms controlling cellular events such as proliferation and differentiation (Vazin and Schaffer, 2010), and consequently greatly benefit process development (Amanullah et al., 2010). Additional empirical and mechanistic modeling, along with other rational approaches for process optimization (Lim et al., 2007), may also contribute to further comprehend the factors that affect a given system. Successful in vitro models will therefore enable the study of the mechanisms and dynamics of stem cell differentiation and organ development (Abranches et al., 2009). Moreover, meaningful pharmacological studies can also be carried out using such strategies (Lee et al., 2008).

Therefore, the propagation and differentiation of stem cell populations under controlled conditions remains a major technical challenge due to the complex kinetics of the heterogeneous starting culture population, the transient nature of the subpopulations of interest, the lack of invariant measures, and multiple interactions between culture parameters, such as growth factor concentration, dissolved oxygen tension, or cell–cell interactions. Advances in bioreactor culture have been reviewed for specific populations of stem cells, like mesenchymal stem cells (Godara et al., 2008), hematopoietic stem cells (Cabral, 2001; Cabrita et al., 2003; Safinia et al., 2005), neural stem cells (Kallos et al., 2003) or pluripotent stem cells (Azarin and Palecek, 2010a; Kehoe et al., 2010). Interesting points of view were also published regarding key issues related with this field, like stem cell bioprocessing (Placzek et al., 2009), challenges for the development of novel cellular therapies (Kirouac and Zandstra, 2008) and the application of engineering principles to understand and manipulate stem cell behavior (Ashton et al., 2011). In this review, we focus on the fundamental issues related to bioprocess and bioreactor development towards the in vitro expansion, maintenance and/or controlled differentiation of stem cells, while keeping their functional characteristics, including the ability to differentiate into appropriate tissues. In the following sections we give an overview of the progress already achieved with different stem cell populations, in different bioreactor systems, and describe recent developments and new technologies for stem cell cultivation. We expect to provide an updated and integrated perspective based on initial reports from the literature, and also on recent developments from this field.

1.1. Stem cell isolation and characterization

The isolation of stem cells from donor sources and their functional characterization represent the initial steps in the design of a new process (Kirouac and Zandstra, 2008). In fact, different stem cell populations can be used for the clinical production of cellular products. Cells isolated from embryonic, fetal or adult tissues and, more recently, pluripotent stem cells (PSCs) generated using cellular reprogramming (Takahashi and Yamanaka, 2006), represent available sources of cells for potential clinical use.

Embryonic stem cells (ESCs), for example, have the potential to generate all the cell types derived from the three embryonic germ layers, a property best known as pluripotency (Smith, 2001). However, their clinical usage is undermined by their innate tumorigenicity (*i.e.* ability to form teratomas upon implantation), lack of efficient culture systems to control their differentiation, and ethical constraints due to the destruction of the embryo. On the other hand, ethical concerns are alleviated with adult stem cells that can be directly obtained from available donors. Nonetheless, cell features are quite dependent on donor characteristics (*e.g.* age, sex, genetic background, etc.). In addition, they possess limited proliferative capacity in vitro and their differentiation potential is restricted to the original cell lineage. Nevertheless, hematopoietic stem cells have been widely used in the clinic since the 1960s (Thomas et al., 1957), and more recently mesenchymal stem cells have been already tested with success in cell therapy settings as well (Caplan and Bruder, 2001).

In the adult, bone marrow was originally the cell source of excellence for transplantation, but other tissues like peripheral blood after mobilization, adipose tissue, placenta and umbilical cord, are also promising alternatives. For instance, the isolation of hematopoietic stem cells (HSCs) from these sources can be performed by magnetic or fluorescence-activated cell sorting based on surface antigen expression ($CD34^+$, $Thy1^+$ and $CD38^-$) (Wognum et al., 2003). Likewise, human mesenchymal stem cells (MSCs) have been characterized based on cell adherence to tissue culture plastic and a specific pattern of surface antigen expression — more than 95% of expression of CD73, CD90 and CD105, without expressing hematopoietic markers (Dominici et al., 2006; Pittenger, 2008). In addition, cell surface antigen expression is not only useful for cell isolation from donor tissues, but also as a quality control measurement during *ex-vivo* cell culture.

On the other hand, human ESCs have been isolated and derived from blastocysts using feeder cell layers and serum-containing medium (Thomson et al., 1998). The maintenance of these cells in culture may also be assessed using the expression of key pluripotency markers, such as the cell surface markers SSEA3, SSEA4, TRA-1-60, and TRA-1-81, and the transcription factors Oct4, Nanog, Sox2 or Rex-1 (Carpenter et al., 2003). Importantly, ethical concerns related with the destruction of human embryos have led to the establishment of several protocols for derivation of pluripotent stem cell lines that do not require embryo destruction (McDevitt and Palecek, 2008). Among these, reprogramming adult cells to generate induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) is of great interest, not only due to the ethical concerns surrounding human ESCs, but also because it allows the derivation of patient-specific pluripotent stem cell lines

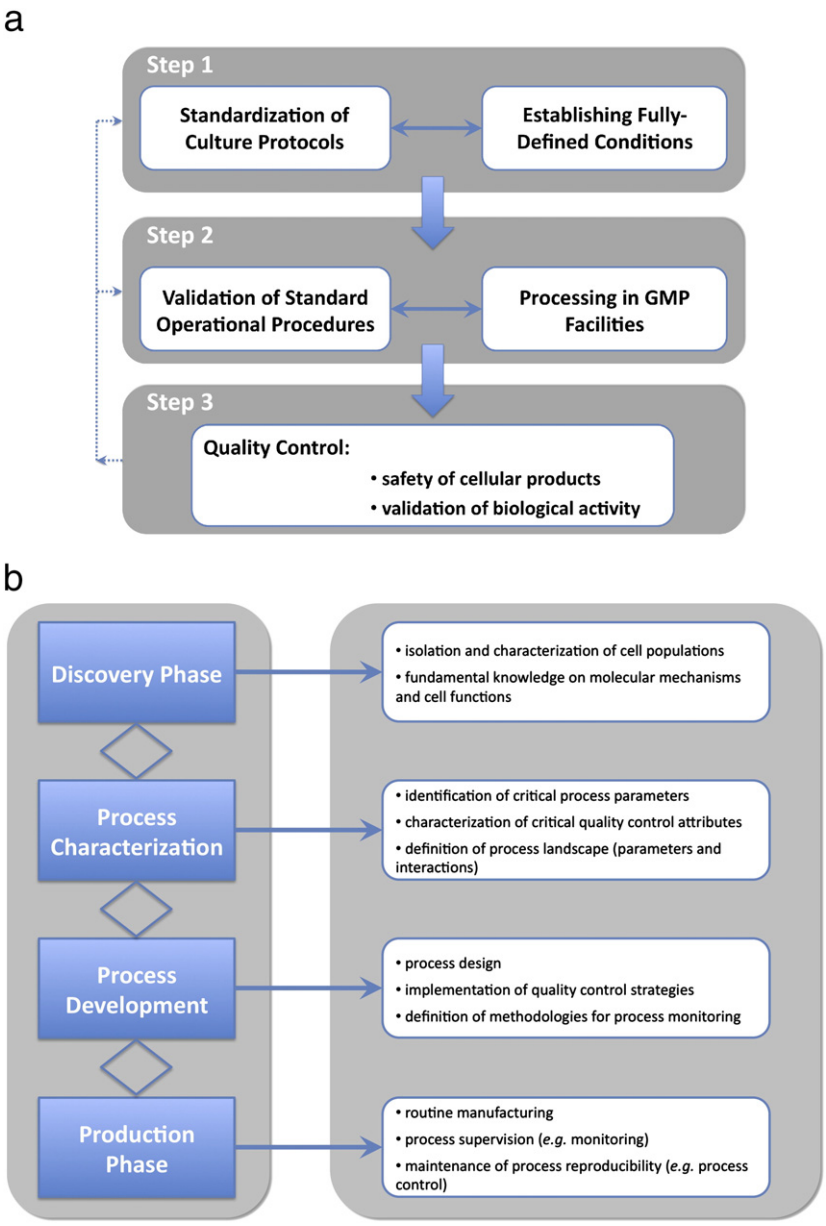

Fig. 1. Roadmap for the production of stem cell-derived products in compliance with GMP practices. (a) Schematic representation of a feedback, stepwise model for implementation of stem cell bioprocesses under GMP conditions. The first steps highlight a quality control scheme covering both design and standardization of production to ensure that end products meet critical specifications. This requires control of raw materials, and also that protocols follow validated standard operational procedures. The final step represents quality control of the final product. The dashed arrows stand for the feedback control mechanisms designed to monitor and maintain critical parameters within the required levels. (b) Flowchart depicting the critical attributes of each phase of bioprocess development for GMP production of stem cell-derived products.

(Ebert et al., 2009; Soldner et al., 2009), thus generating cells suitable for clinical and pharmacological applications.

In the future, patient-specific cells will become important models for studying human disease, for testing responses to potential drugs, and might also be used to develop patient-specific cell therapy, circumventing host immune responses and laying the foundations of personalized medicine (Nishikawa et al., 2008).

1.2. Bottlenecks and critical issues in stem cell bioprocess development

It is obvious that additional efforts are needed to tackle the existing challenges towards the routine production of stem cell products under good manufacturing practices (GMP) conditions. Many of these difficulties are related to a fundamental understanding of the cellular and molecular mechanisms involved in stem cell functions. Other important issues include the variability of the starting cell population, along with several technical bottlenecks, such as lack of sensing techniques that allow quantification of important culture parameters (*e.g.* cell numbers, differentiation stage, or metabolism). Limited means for predicting the culture outcome (*e.g.* bioprocess modeling), and inefficient bioprocess monitoring and control strategies also represent important limiting factors. In fact, to better control and standardize key product properties such as cell identity, purity and potency, all these points should be taken into consideration (Placzek et al., 2009).

Thus, understanding the scientific aspects of a given stem cell system will help to identify critical features, such as factors involved in stem cell expansion and differentiation, which can then be used for process control and assurance of product safety. With this fundamental knowledge, the next step would involve the identification of key process parameters for the biological system under study (*e.g.* growth factor supplementation, metabolite concentration, or dissolved oxygen,

among others). Finally, it is clear that monitoring and control capabilities, which are available in bioreactor systems, represent additional resources for bioprocess development towards the production of cellular products in compliance with GMP conditions (Rathore, 2009) (Fig. 1b and (Kirouac and Zandstra, 2008)).

2. Bioreactor design and operation

2.1. Critical parameters

For the successful implementation of stem cell culture in bioreactor systems several critical parameters need to be addressed. These include physicochemical variables, such as pH, dissolved oxygen, or temperature, and biochemical input, including the levels of key nutrients and metabolites, or growth factors (Table 1).

Oxygen tension is an important component of the stem cell microenvironment and appears to influence stem cell self-renewal/maintenance and differentiation (Ivanovic, 2009; Mohyeldin et al., 2010). Mesenchymal stem cells, for example, are obtained in relatively low numbers and need to be expanded in vitro, but prolonged cultivation at atmospheric oxygen levels may decrease cell viability due to oxidative stress. In order to optimize culture conditions while increasing the number of viable MSCs for successful engraftment, dos Santos and coworkers have studied the influence of low oxygen levels (2%) on human MSC proliferation kinetics and metabolism (dos Santos et al., 2010). Their results highlight a more efficient expansion at 2% O_2, when compared to normoxic conditions. Similarly, in the case of mouse ESC-derived neural stem cells, results have shown that 2–5% oxygen levels led to higher cell proliferation without affecting multipotency (Rodrigues et al., 2010). In addition, oxygen tension has been recognized as an important cell fate determinant in HSCs as well, namely by modulating the production of cytokines and transcription factors (reviewed in (Guitart et al., 2010)).

In mouse ESCs, oxygen tension has a considerable effect on differentiation. Culture under low oxygen levels induces spontaneous ESC differentiation (Kurosawa et al., 2006), with inhibition of cell self-renewal via negative regulation of STAT3 signaling even in the presence of leukemia inhibitory factor (LIF) (Jeong et al., 2007). Nevertheless, the work by Fernandes and coworkers have shown that this effect varies on culture conditions, depending on the specific signaling pathway that is being used for keeping the pluripotency threshold (Fernandes et al., 2010a). This may explain why hypoxic conditions actually favor maintenance of pluripotency in human ESCs (Ezashi et al., 2005), since these cells do not rely on LIF/STAT3 signaling for self-renewal. Nevertheless, during neural commitment, low oxygen tension exerted a positive effect on early differentiation of mouse ESCs (Fernandes et al., 2010a; Mondragon-Teran et al., 2009). Hypoxia also increased the yields of cardiomyocytes (Bauwens et al., 2005) and hematopoietic cells (Dang et al., 2004) in differentiation from ESCs. These examples demonstrate the complex signals that arise from manipulating oxygen tensions in culture, and the importance of this parameter in proliferation and cell-fate commitment.

Hydrodynamic shear stress may also influence the culture outcome, and have been shown to influence stem cell fate in vitro (King and Miller, 2007). In stirred bioreactors, agitation results in energy transfer from the impeller to the culture medium, causing the formation of areas of intense turbulence. This effect results in localized shear on particles in the bioreactor, including the surface of cell aggregates, cells attached to microcarriers, or single cells in suspension, which results in cell damage. In addition, damage may also occur due to sparging with gas bubbles, to achieve aeration of the bioreactor. Beyond agitation rate, other parameters have implications in the bioreactor shear stress, namely impeller diameter, geometry and position, and the presence of probes or other vessel internals (Baghbaderani et al., 2008). Impeller geometry may also be critical, especially for minimizing cell damage near the tips (Schroeder et al., 2005). Nevertheless, the optimal values for shear stress may vary among different stem cell subtypes (*e.g.* 0.21 Pa for mammary epithelial stem cells or 0.61 Pa for mouse ESC) and therefore need to be determined for each case (Cormier et al., 2006; King and Miller, 2007; Youn et al., 2005). Although shear stress may have a detrimental effect on cell culture, it can also be a stimulus for cell differentiation into particular cell types, like endothelial cells (Yamamoto et al., 2003) or osteogenic cells (Yourek et al., 2010).

Growth factors are also potent regulators of the stem cell behavior, namely by providing survival, proliferation and differentiation signals to the cells. Multi-factorial interactions between growth factors and other process parameters further contribute to the complex nature of the culture milieu. It is therefore critical to systematically quantify these interactions and optimize the culture process (van der Sanden et al., 2010). To address this question, systematic strategies for optimizing cytokine concentrations have been developed (Andrade et al., 2010). Using statistical tools, this study contributed for the rational delineation of the cytokine concentration range, while unraveling complex cytokine-to-cytokine interactions, for the efficient expansion of hematopoietic stem/progenitor cells towards transplantation.

Nutrient and metabolite concentrations also influence cell expansion, differentiation or death in culture. For example, the biochemical profile of mouse ESC expansion under serum-free conditions was evaluated, which allowed measuring the consumption of important substrates, such as

Table 1
Critical process parameters influencing stem cell expansion and/or differentiation in bioreactors.

Variable category	Examples of critical parameters
Physicochemical	• pH values above or below the physiological levels may be detrimental for cell maintenance. Since pH levels may vary during cell culture (for example, due to accumulation of lactate), a tight control of this parameter in bioreactors is needed. • Temperature is an important parameter, and it is typically maintain at 37 °C for stem cell cultivation. • Osmolarity is a measurement of the osmotic pressure of a given growth medium and was found to have a large effect on stem cell functioning (Kallos and Behie, 1999), specifically in cell numbers produced and cellular viabilities. • Dissolved oxygen tension is also an important parameter for stem cell cultivation. Hypoxia, or physiologic oxygen tensions, appears to influence many cellular processes such as stem cell maintenance and differentiation, and therefore the levels of dissolved oxygen in liquid medium should be precisely monitored and controlled. • Hydrodynamic shear stress can occur at the cell boundary due to fluid movement, thus causing cell physiology damage and affect cell functions. In stirred bioreactors, hydrodynamic shear stress is due to mechanical agitation of the liquid and to sparging with air bubbles. Stem cells are especially sensitive to this culture parameter, and therefore it is crucial to control hydrodynamic shear in bioreactors.
Biochemical	• Nutrients are important for efficient cell metabolism. Glucose and glutamine are the main sources of energy to the cells, providing carbon and nitrogen for cell functions, metabolism and biosynthesis. • Metabolic waste products, especially lactate and ammonia, may inhibit cell growth and should be tightly controlled. • Growth factors and Cytokines are signaling proteins that modulate a wide range of cell functions, including self-renewal, differentiation or survival. For example, LIF and BMP4 can be used in combination to sustain mouse embryonic stem cells in culture, while a cocktail of growth factors composed of SCF, Flt-3 L and TPO can be used for the *ex-vivo* expansion of HSCs.

glucose and glutamine, and the production of metabolic byproducts, like lactate (Fernandes et al., 2010b). This allowed confirming a more efficient cell metabolism under serum-free conditions and the identification of glutamine as an important energy source during cell expansion at low seeding densities. Similarly, metabolic studies of human ESCs have shown that high levels of metabolic waste products, particularly lactate, and low pH obtained at the late stages of cell propagation could cause cell growth arrest and reduce the population of cells expressing pluripotency markers (Chen et al., 2010). Therefore, a close monitoring and control is needed for the development of fully controlled bioprocesses to produce relevant numbers of cells for therapy or high-throughput drug screening.

These examples highlight that variations in the culture environment could be used to direct cell behavior in vitro. However, the mechanism of action of these parameters is not always clear and it is often related to other factors, which further contributes to the convolution of stimuli present in the culture milieu.

2.2. Microscale approaches for bioreactor development

Microscale high-throughput screening approaches enhance our ability to analyze multiple conditions in a fast and parallel fashion, contributing to foster our understanding of a given system. This can accelerate bioprocess development, leading to a rapid transition from biological observation to optimized, clinical-scale bioreactor systems. Thus, the development of in vitro high-throughput profiling methodologies for evaluating the effects of different culture conditions in cell models might assist in the establishment of novel bioprocesses, and also increase our knowledge on conditions that selectively control cell fate (Fernandes et al., 2009b).

The first microengineered systems used in stem cell research focused mainly on the multiplexed analysis of signaling environments that control stem cell fate (Flaim et al., 2005; Soen et al., 2006). In fact, signals emanating from the stem cell microenvironment, or niche, are crucial in regulating stem cell functions. Nevertheless, advances in microfabrication and microfluidics have also driven the generation of microscale platforms that allow bioprocess optimization (Gómez-Sjöberg et al., 2007). Microbioreactor arrays, containing independent micro-bioreactors perfused with culture medium, have been fabricated using soft lithography (Hung et al., 2005; Kim et al., 2006). These systems were used for adherent cell culture at variable levels of hydrodynamic shear, or alternatively for cell encapsulation in hydrogels. Automated image analysis was used to detect the expression of specific cell markers, and several configurations were validated for different cell types, including mouse myoblasts, primary rat cardiac myocytes and human ESCs (Figallo et al., 2007). In addition, these devices can also be used to study design parameters, mass transport phenomena and shear stress using numerical simulations (Korin et al., 2009), providing efficient means to analyze multiple parameters and parameter interactions.

However, one major limitation of these high-throughput cell culture platforms is the ability to quantify specific cellular responses in an accurate and straightforward manner. Several methods have been developed to address this question, including immunofluorescence-based assays for high-throughput analysis of target proteins on three-dimensional cellular microarray platforms (Fernandes et al., 2008), or cellular microarrays with integrated multifunctional sensing elements that allow immunodetection of secreted proteins (Jones et al., 2008).

In conclusion, high-throughput, high-content screening platforms have the potential to provide valuable information regarding the mechanisms controlling cell proliferation, differentiation or death (Fernandes et al., 2010c; Peerani et al., 2009). This can greatly benefit process development, as the underlying aspects of the stem cell biology are becoming further understood. Additionally, microscale strategies can also be directly employed for parameter measurement (*e.g.* specific cell phenotypes), ultimately leading to the development of an integrated process for clinical-scale production of stem cells and/or their progeny.

2.3. Bioreactor configurations

The usual choice for the culture of mammalian cells, and therefore stem cells, at laboratory scale are flat two-dimensional surfaces like tissue culture flasks (T-flasks), well plates or gas-permeable blood bags, which mostly consist of a single non-agitated compartment where nutrients diffuse to cells. However, despite the simplicity, ease of handling and low cost, these static culture systems present serious limitations. First, gas exchange (*e.g.* oxygen and carbon dioxide) occurs only at the medium/gas interface and the static nature of the culture leads to concentration gradients (pH, dissolved oxygen, nutrients, metabolites, etc.) in the culture medium. Although the on-line monitoring of culture parameters such as pH or pO_2 is currently possible, even in systems as compact as 96-well plates (Deshpande and Heinzle, 2004; Kensy et al., 2005), tight control of these variables is mostly impossible. Scale-up of these culture systems is also difficult. When a very high number of cells is required, the reduced surface area/volume ratio, for instance in T-Flasks, implies the use of multiple culture vessels or flasks with multiple trays, with the subsequent increase in incubator occupancy, handling time (for feeding, culture monitoring and harvesting of cells), risk of contamination and also an increased price.

Automation and robotics (Terstegge et al., 2007; Thomas et al., 2009) could minimize the impact of some of the issues described, but still culture would be done in static conditions. An increase in surface area for cell adhesion and growth can be achieved with three-dimensional (3D) constructs that more closely resemble the *in vivo* environment (Ott et al., 2008; Placzek et al., 2009), leading to higher cellular concentrations, but at the expense of an increase in mass transfer limitations.

More advanced bioreactor systems that can accommodate dynamic culture conditions may be the solution to minimize mass transfer limitations and other problems described above (Table 2) and are crucial when large numbers of cells are needed, accessory cells are used or very high cell densities are desired. Perfusion and stirring are strategies that have been used for this purpose, resulting in different bioreactor configurations, which can be used in stem cell culture (Cabrita et al., 2003; Godara et al., 2008; Ulloa-Montoya et al., 2005).

Mammalian cells can be easily cultured under dynamic conditions in devices known as roller bottles (Kunitake et al., 1997; Mitaka, 2002), where multiple cylindrical bottles are placed into a rotating apparatus that may accommodate up to hundreds of bottles. This system is limited in terms of control of culture parameters and, if large numbers of bottles are used, are expensive and require thorough handling. Widely used and characterized for the culture of both microbial and animal cells, mostly for production of recombinant proteins and monoclonal antibodies, stirred-suspension bioreactors (SBs) are more appealing for large-scale production of stem cells and/or their progeny. In conventional SBs, concentrations of 10^6–10^7 cells/mL can be attained (Kehoe et al., 2010) and thus for clinically relevant applications (where 1×10^9 to 1×10^{10} stem cells and stem cell-derived cells may be required) working volumes of a few hundred milliliters to a few liters may be necessary. In SBs, cells can be cultured as single cells, as aggregates or, in the case of anchorage-dependent cells, attached on microcarriers or other scaffolds (Kehoe et al., 2010). The vast experience acquired over the years with SBs, as well as the wide range of models and components commercially available, significantly facilitate their application to stem cells as well as the scaling up of the cultures.

Having in mind the minimization of shear and turbulence in cell culture, NASA's Biotechnology Group developed an alternative bioreactor design, the rotating wall vessel, with interesting and unique features for mammalian cell cultivation (Goodwin et al., 1993; Hammond and Hammond, 2001). This bioreactor, which simulates microgravity conditions, exists in two different configurations, the High Aspect Ratio Vessel (HARV) and the Slow Turning Lateral Vessel (STLV). However, scaling up these systems may be complicated.

Table 2
Summary of the main characteristics of different bioreactors used for stem cell culture.

Bioreactor configuration	Main characteristics	
Roller bottles	• Versatile system with simple operation and usage • Low-cost solution • Monitoring and control is possible, but not straightforward	• Only allows anchorage-dependent cell culture • Concentration gradients are minimized, but still persist
Stirred suspension bioreactor	• Simple design. Homogeneous conditions are achieved • Bioreactor operation and sampling are easily performed • Monitoring and control solutions are widely available	• In addition to suspension culture (as cell aggregates or single cells), also allows adherent growth when microcarriers are used • Hydrodynamic shear stress due to mechanical agitation can be harmful to cells • Microcarrier bridging and/or cell agglomeration may occur
Wave bioreactor	• Disposable system and easily scalable • Contamination issues are minimized and sterilization is not needed, rendering it suitable for GMP operations	• Sampling, monitoring and control are not as simple as with other systems • High-cost solution
Rotating wall vessel	• Low-shear stress environment and efficient gas transfer	• Complex system, not easily scalable
Parallel plates bioreactor	• High productivities can be achieved • Accumulation of toxic metabolic side-products is minimized, but continuous removal of secreted factors may be detrimental	• Medium-intensive culture system • Effects of hydrodynamic shear stress are unknown
Hollow-fiber bioreactor	• Low shear stress environment, provides better mimic of the cellular microenvironment • Monitoring and control is not straightforward	• Spatial concentration gradients are formed at the hollow-fibers interfaces • Scale-up is difficult to perform
Fixed and fluidized bed bioreactor	• Provides 3D scaffolding for cell attachment and growth • Cell–cell or cell–matrix interactions are possible, providing a better mimic of the *in vivo* intricate structure • Low volumes and difficulties in scaling-up, when compared with other systems	• Spatial concentration gradients (in the fixed bed configuration) • Possible shear stress effects (in the fluidized bed configuration)

When working in compliancy with GMP guidelines is required, a very attractive option to these configurations is the Wave bioreactor (Singh, 1999). This bioreactor consists of a disposable bag, partially filled with media and inoculated with cells, with the remainder inflated with air. The culture bag is placed on a special platform, which through a rocking motion generates waves at the liquid–air interface. Since the culture bags are pre-sterilized and disposable, reduced cleaning and validation are required, which makes the wave reactor suitable for clinical applications. Although culture parameters can be tightly controlled in the wave bioreactor and the scale-up is possible, the high cost of the disposable bags may limit the use of this reactor for research purposes.

Bioreactors can also operate with perfusion. In this case mass transfer is enhanced by continual exchange of media, as fresh or recycled medium is introduced and exhausted medium removed (King and Miller, 2007). Although most bioreactors, if not all, can operate with perfusion, some configurations were developed based in this concept. Examples include parallel plates, hollow-fibers, fixed bed and fluidized bed bioreactors.

Parallel plates bioreactors consist of an upper gas compartment separated by a membrane from the liquid-filled bottom compartment, which has a tissue culture plastic surface for attachment of anchorage-dependent cells (Godara et al., 2008; Koller et al., 1993b; Peng and Palsson, 1996). These reactors have the advantage of simple automation, providing continuous and automated feeding of the culture but present difficulties in the collection of representative samples from the system, except through total harvest (Nielsen, 1999).

Hollow-fiber bioreactors are two-compartment systems consisting of a hollow-fiber bundle encased in a cylindrical shell with ports for flow of medium in the intracapillary and/or extracapillary spaces (Godara et al., 2008). Hollow-fiber bioreactors provide an increased surface area for cell culture but present difficulties in culture monitoring and scale-up (Safinia et al., 2005; Sardonini and Wu, 1993).

Finally, fixed and fluidized bed bioreactors consist in an immobilized scaffold arranged in a column, the bioreactor bed, where cells are seeded. The column may consist of particles, either packed (fixed bed) or floating (fluidized bed). Although these reactors provide 3D scaffolding for cell attachment and growth, better mimicking *in vivo* conditions, spatial concentration gradients (in packed bed reactors) and shear stress effects (fluidized bed) may occur.

2.4. Bioprocess monitoring and control

Intelligent strategies can be developed in order to minimize process and product variability while maximizing productivity in compliance with regulatory guidelines. Such strategies include monitoring and control of bioreactor systems, and represent important tools for bioprocess development. Therefore, process standardization can potentially be achieved when physicochemical culture parameters (pH, pO_2, etc.) are monitored and controlled (Lim et al., 2007).

Process control thus have the potential to increase the robustness and stability of cellular products obtained in the manufacturing process. Also, models predicting population dynamics by incorporating kinetic data like growth rates or cell death, metabolite uptake and production rates, or cellular events such as differentiation or transition between quiescence and active cycling cell states, provide the means to improve process performance, as it was shown for the *ex-vivo* expansion and differentiation of hematopoietic stem cells (da Silva et al., 2003). In addition, *in silico* mathematical modeling of stem cell functions also allows the prediction of many cellular events, relating process parameters and environmental variables with measurable cellular outcomes (Kirouac et al., 2009). Therefore, models incorporating cell-level kinetics, physicochemical culture parameters, and microenvironmental variables are valuable tools for process development, while providing insights into biological questions important to understand stem cell dynamics.

In conclusion, the implementation of online monitoring together with automated control systems is essential for the large-scale culture of stem cells under controlled and reproducible conditions. Moreover, these strategies also provide a mean to unveil the effects of multiple parameters on the dynamics of specific cell populations. Thus, the kinetic analysis, along with hydrodynamic and mass transfer characterization of the bioreactor, can be incorporated in predictive models that might be useful for establishing optimal operational conditions. Nevertheless, most models deal with a limited set of inputs and are built from a small number of experimental runs, which means that they do not fully describe all aspects of a dynamic biological system.

3. Stem cell cultivation in bioreactors

The different bioreactor configurations described above have been adapted for stem cell expansion and differentiation (Table 3). Systems

Table 3
Stem cell cultivation in bioreactor systems.

Design of bioreactor	Stem cell cultivation strategy	References
Stirred suspension bioreactor	• Mouse ESC culture as cellular aggregates (including EBs)	• (Zandstra et al., 2003; Fok and Zandstra, 2005; Cormier et al., 2006; zur Nieden et al., 2007; Kehoe et al., 2008)
	• Mouse and human ESC encapsulation	• (Dang et al., 2004; Jing et al., 2010)
	• Mouse ESC cultivation in microcarriers	• (Abranches et al., 2007; Fernandes et al., 2007)
	• Human ESC culture as cellular aggregates (including EBs)	• (Krawetz et al., 2009; Niebruegge et al., 2009; Singh et al., 2010)
	• Human ESC cultivation in microcarriers	• (Fernandes et al., 2009a; Lock and Tzanakakis, 2009; Oh et al., 2009; Kehoe et al., 2010; Lecina et al., 2010; Storm et al., 2010)
	• NSC cultivation as cellular aggregates (neurospheres)	• (Kallos et al., 1999; Sen et al., 2001; Gilbertson et al., 2006; Baghbaderani et al., 2008)
	• HSC suspension culture	• (Sardonini and Wu, 1993; Zandstra et al., 1994; Collins et al. (1998a, 1998b))
	• MSC suspension culture	• (Baksh et al., 2003; Frith et al., 2010)
	• MSC cultivation in microcarriers	• (Frauenschuh et al., 2007; Yang et al., 2007; Schop et al., 2008; Sart et al., 2009; Yu et al., 2009; Eibes et al., 2010)
Rotating wall vessel	• Mouse and human ESC culture as cellular aggregates (including EBs)	• (Gerecht-Nir et al., 2004; Come et al., 2008; Hwang et al., 2009; Fridley et al., 2010)
	• NSC encapsulation	• (Low et al., 2001; Lin et al., 2004)
	• HSC suspension Culture	• (Liu et al., 2006)
	• MSC suspension culture	• (Chen et al., 2006)
	• MSC osteogenic and chondrogenic differentiation	• (Duke et al., 1996; Granet et al., 1998; Song et al., 2006)
Microbioreactors	• Human ESC cultivation in perfused micro-bioreactors	• (Figallo et al., 2007; Cimetta et al., 2009; Korin et al., 2009)
	• Human HSC cultivation in microliter-bioreactors	• (Luni et al., 2010)
Parallel plates bioreactor	• HSC culture in flat-bed single-step perfusion	• (Koller et al., 1993b; Palsson et al., 1993; Jaroscak et al., 2003)
	• HSC culture in flat-bed multi-step perfusion	• (Koller et al., 1993a)
	• MSC culture in flat-bed single-step perfusion	• (Dennis et al., 2007)
Hollow-fiber bioreactor	• Extra-capillary cell culture with intra-capillary perfusion for HSC cultivation	• (Sardonini and Wu, 1993)
Fixed and fluidized bed bioreactor	• HSC or MSC cultivation in packed bed bioreactor	• (Wang et al., 1995; Highfill et al., 1996; Mantalaris et al., 1998; Meissner et al., 1999; Jelinek et al., 2002; Weber et al., 2010)
	• HSC cultivation in fluidized bed bioreactor	• (Meissner et al., 1999)

for adult, fetal and pluripotent stem cell culture have been established and some examples are illustrated in the following sections.

3.1. Hematopoietic stem and progenitor cells

In its natural environment, hematopoiesis resides in a microenvironment characterized by local geometry (structure and vasculature), by accessory cells of mixed origin (stromal cells) and the extracellular matrix produced by them (Nielsen, 1999). Since the first in vitro reconstruction of the *in vivo* murine hematopoietic microenvironment to culture Hematopoietic Stem and Progenitor Cells (HSPCs) by Dexter et al.(1973), which was later adapted for human cells (Gartner and Kaplan, 1980), hematopoietic cell cultures have been typically performed in static conditions (Haylock et al., 1992; Lemoli et al., 1992).

The potential of stirred suspension cultures to support hematopoiesis *ex-vivo* has been investigated since the 1990s. HSPCs are relatively shear-sensitive cells, and agitation is thought to affect surface marker expression (McDowell and Papoutsakis, 1998), thus low agitation rates (30–60 rpm) are necessary in these systems in order to avoid cell damage (Collins et al., 1998a; Sardonini and Wu, 1993; Zandstra et al., 1994).

The short-term maintenance of both colony-forming cell (CFC) numbers and their precursors, detected as long-term culture initiating cells (LTC-IC), was initially demonstrated to be possible in stirred suspension (Zandstra et al., 1994). After 4 weeks the number of LTC-ICs and CFCs present in stirred cultures initiated with 1 million cells increased an average of 7- and 22-fold, respectively. Later on, the same authors studied the parameters that possibly limit the cytokine-mediated expansion of primitive hematopoietic cells in stirred suspension cultures (Zandstra et al., 1997). More primitive cells (LTC-ICs) were shown to deplete cytokines from the medium much more rapidly than their more mature progeny according to a mechanism that is strongly dependent on the concentration of cytokines to which the cells are exposed.

Cultures of umbilical cord blood (UCB) mononuclear cells (MNCs), peripheral blood (PB) MNCs, and PB $CD34^+$ cells were also performed in spinner flasks and in T-flasks, both in serum-containing and serum-free media (Collins et al., 1997). Glucose and lactate metabolic rates were determined and correlated with the percentage of CFC present in the culture for a broad range of culture conditions. The proliferation and differentiation characteristics of these populations in spinner flask cultures were also examined by the same authors (Collins et al., 1998a). Cell proliferation in spinner flasks was dependent on both agitator design and agitation rate, as well as on the establishment of critical inoculum densities. The expansion of UCB and PB MNCs in a stirred-tank bioreactor system with pH and dissolved oxygen control was also described, as well as oxygen uptake and lactate production in these cultures (Collins et al., 1998b). Expansion of total cells and CFCs was greatly enhanced by the use of a cell-dilution feeding protocol (as compared to a cell-retention feeding protocol). The different metabolic profile of CFCs and more mature cells may allow the prediction of the content of several cell types in culture by monitoring the uptake or production of oxygen, lactate and other metabolites.

A number of perfusion reactors have also been developed for HSPCs culture. The greatest success has been achieved with two flat-bed reactor systems: a multipass perfusion system (Koller et al., 1993a), and one single-pass perfusion reactor (Koller et al., 1993b; Palsson et al., 1993). Both systems support 10- to 20-fold total cell expansion and ~10-fold progenitor expansion, whereas expansion of primitive cells has only been reported for the second system. The multipass reactor was further extended for use with or without stroma by the introduction of multiple microgrooves at the chamber bottom, allowing rapid medium exchange with low shear stress (Horner et al., 1998; Sandstrom et al., 1995, 1996).

The single-pass system has been employed in several clinical trials. The cell production system consists in a disposable cassette where cells are injected on top of a layer of stromal cells grown on a tissue culture plastic surface. Nutrients are continuously perfused to the cassette,

while a chamber, located just above, is filled with oxygen that diffuses to the cassette through a gas-permeable, liquid-impermeable membrane (Palsson et al., 1993). The device has been integrated into a GMP fully automated, closed system with pre-sterilized, disposable reactor cassettes and automated perfusion and sterile cell recovery for clinical-scale culture (Goltry et al., 2009; Mandalam et al., 1999). The system has been used for expansion of small volumes of bone marrow aspirates (Palsson et al., 1993) and UCB cells (Jaroscak et al., 2003; Koller et al., 1993a). Mesenchymal components of the bone marrow can also be expanded in this platform, which is also being used in clinical trials for bone and vascular regeneration (Dennis et al., 2007; Goltry et al., 2009). More than 625 *ex-vivo* cell production lots and treatment of over 260 patients in phase I/II clinical trials have been achieved (Goltry et al., 2009). However, each cassette supports relatively low cell densities (Nielsen, 1999) and thus expansion for bone marrow transplants requires several cassettes.

Indeed, different systems were evaluated for the scale-up of bone marrow cultures, including airlift bioreactors and hollow fiber bioreactors (Sardonini and Wu, 1993). Cell culture in the airlift bioreactor led to MNCs expansion, but less extensive than in the static culture used as control. The experiment in the hollow fiber system demonstrated no observable expansion of HSPC when compared to control static cultures.

An alternative configuration that more closely mimics the *in vivo* bone marrow environment, providing the cell–cell and cell–matrix interactions absent in stirred bioreactors, is provided by packed bed reactors (Cabrita et al., 2003). In fact, in these 3D culture systems an attachment-dependent stromal cell culture is started on the bed particles, whereupon HSPCs can be co-cultivated (Highfill et al., 1996; Jelinek et al., 2002; Mantalaris et al., 1998; Meissner et al., 1999; Wang et al., 1995). By co-culturing HSPC with stromal cells immobilized in porous glass carriers in a fixed-bed reactor, early progenitor cells (CFU-GEMM) and later progenitor cells (CFU-GM and BFU-E) were expanded up to 4.2-fold, 7-fold, and 1.8-fold, respectively (Meissner et al., 1999). Fluidized bed bioreactors were also tested (Meissner et al., 1999), but the carrier movement inhibited adhesion of HSCs to stromal cells hampering the success of this approach.

Two more recent studies have shown that HSPC expansion is more extensive in stirred or rotating wall vessels (Li et al., 2006; Liu et al., 2006). At the end of 200 h of culture, over 400-fold increase in total cell number was observed (Liu et al., 2006) as well as a ~30-fold increase in $CD34^+$ cells, and ~20-fold in colony-forming units of granulocyte-macrophage (CFU-GM). Both rotating wall vessel and spinner flasks were also used to perform simultaneous serum-free expansion and harvest of HSPC and MSC derived from the UCB, with the support of microcarriers (Kedong et al., 2010).

In addition to high expansion capacities, cell production for clinical settings must be robust and guarantee the safety of the cellular products generated. For this purpose, a clinically relevant single-use, closed-system bioprocess capable of generating high numbers of UCB-derived HSPCs was developed (Madlambayan et al., 2006). The system consists of 2 gas-permeable cell culture bags and incorporates inline subpopulation selection and medium dilution/exchange capabilities. In addition to expanded numbers of CFCs and LTC-ICs, the bioprocess also generated more long-term repopulating cells than present at input.

Finally, genetic changes caused by different culture microenvironments were studied by comparing gene expression profiling of $CD34^+$ HSPCs in static and stirred cultures (Li et al., 2006). Genes involved in anti-oxidation, DNA repair, apoptosis and chemotactic activity were found to be differently expressed. This kind of data may provide new insights for culture optimization strategies in the future. A microliter-bioreactor array for HSPC culture was recently presented (Luni et al., 2010) and may be as well a powerful tool for high-throughput optimization of culture conditions, which may provide important data that can be translated for larger scale processes.

3.2. Mesenchymal stem cells

MSCs have an important role as accessory cells for *ex-vivo* HSPC culture (da Silva et al., 2010) but are clinically meaningful *per se*, for instance for the treatment of numerous clinical conditions, like graft-*versus*-host disease, renal failure, Crohn's disease or myocardial ischemia (Caplan and Bruder, 2001; Fang et al., 2006; Ringden et al., 2006), as well as a starting source for tissue engineering applications (*e.g.* bone, cartilage). However, the low frequency of MSCs in bone marrow ($1{:}10^4$ in young ages, decaying with age) makes expansion a prerequisite for MSC therapies (Ringden et al., 2006). The time-consuming and labor-intensive nature of conventional tissue-flask culture has limited target doses in clinical trials to about 10^8 cells per patient (Lazarus et al., 2005; Ringden et al., 2006), but to achieve higher therapeutic efficacy more cells will probably be required.

Although MSCs are anchorage-dependent cells, typically growing as a monolayer in conventional tissue culture flasks, these were successfully cultured as individual cells in a stirred suspension bioreactor, maintaining their ability to form functional differentiated bone, in a cytokine dependent manner (Baksh et al., 2003). The authors further demonstrated that MSCs grown under these conditions maintained the ability to differentiate along other multiple mesenchymal lineages (Baksh et al., 2005) and, using high-content screening approaches, soluble growth factor combinations that influence MSC growth in serum-free conditions were identified.

However, MSCs can be cultured on microcarriers, in stirred suspension. Cytodex 1 microcarriers were used for the attachment and growth of porcine MSCs (Frauenschuh et al., 2007), which retained their osteogenic and chondrogenic developmental potential over a cultivation time of 28 days. In another study, the growth and metabolism of goat MSCs in microcarrier spinner flask cultures was studied and the feeding regime was optimized (Schop et al., 2008). During cultivation, nutrient (glucose and glutamine) and metabolite (lactate and ammonia) concentrations in the medium were monitored allowing the determination of a correlation between nutrient consumption, metabolite production and cell growth. Rat bone marrow (Yang et al., 2007) and ear-derived MSCs (Sart et al., 2009) were also successfully cultured on gelatin macroporous microcarriers. Human placenta-derived MSCs were expanded in stirred bioreactors using microcarriers, in serum-containing medium, achieving higher fold expansions than in T-flasks and obtaining comparable antigenic phenotypes (Yu et al., 2009). More recently, a low-serum system was described for the culture of human MSCs on microcarriers (Eibes et al., 2010). An almost 10-fold increase in cell number was observed and cells retained their differentiation potential into adipogenic and osteogenic lineages, as well as their clonogenic ability.

MSCs isolated from bone marrow MNCs were also expanded in other bioreactor systems. The parallel plate perfusion device described above for HSC culture (Koller et al., 1993b), was used to significantly expand colony-forming efficiency-fibroblast (CFU-F) and progenitor cells with osteogenic potential from bone marrow MNCs (Dennis et al., 2007). A new tubular perfusion system allowed culture of MSCs in 3D scaffolds and supported early osteoblastic differentiation (Yeatts and Fisher, 2011). A perfusion system, where cells grow embedded in 3D polymeric matrices, maintaining multi-lineage differentiation potential after extensive expansion at high cell density, was also described (Zhao and Ma, 2005).

The model cell line MSC-TERT was used to demonstrate the use of a fixed bed bioreactor, based on non-porous borosilicate glass spheres, for the expansion of human MSCs, with automated inoculation, cultivation and harvesting of the cells (Weber et al., 2010). Bed volumes up to 300 mL were used and the simple design of the reactor may be suitable for the manufacture of a disposable system. Additionally, a model describing the process was developed, based in the collected data, in order to perform calculations for scaling up.

Bone marrow MNCs were also cultured in rotary bioreactor systems (Chen et al., 2006) and after 8 days of culture the numbers of Stro-1$^+$ CD34$^+$ CD44$^+$ MSCs, CD34$^+$ Stro-1$^-$ CD44$^+$ HSCs, and total cells increased by 29-, 8-, and 9-fold, respectively. The bioreactor-expanded MSCs expressed primitive mesenchymal cell markers, maintained a high level of CFU-F per day, and were capable of differentiating into chondrocytes, osteoblasts, and adipocytes upon appropriate induction protocols.

Bioreactors were also used for promoting MSC differentiation for tissue engineering applications (Zhang et al., 2010). Spinner flasks increase the efficiency of scaffold cell seeding and survival, in comparison to static culture (Godara et al., 2008), and have been used for cultivation of MSCs for osteogenic differentiation (Hofmann et al., 2007; Kim et al., 2007; Meinel et al., 2004; Mygind et al., 2007). The rotating-wall reactor has also been successfully used for osteogenic differentiation (Granet et al., 1998; Qiu et al., 1999; Song et al., 2006; Turhani et al., 2005) and cartilage engineering (Marolt et al., 2006). Perfusion bioreactors were as well used with success for chondrogenic differentiation of human ESC-derived MSCs using constructs prepared from porous silk fibroin scaffolds (Tigli et al., 2011).

A new approach for undifferentiated MSC culture was recently described where cells are cultured as 3D aggregates or spheroids (Bartosh et al., 2010; Frith et al., 2010). Since it is believed that this approach may lead to an increase of the MSC therapeutic potential (Bartosh et al., 2010), methods were developed for dynamic 3D in vitro MSC culture using spinner flasks and rotating wall vessel bioreactors (Frith et al., 2010). Altered cell size and surface antigen expression, together with enhanced osteogenic and adipogenic differentiation potential, were observed, as well as many differences in gene expression between 3D and monolayer cultured MSCs, including those related to cellular architecture and extracellular matrix. Together these results present 3D culture as a viable alternative to the usual monolayer methods.

3.3. Neural stem cells

Neural stem cells (NSCs) have the potential to differentiate into all cell phenotypes present in the central nervous system: neurons, astrocytes and oligodendrocytes. Primary fetal murine NSCs are typically grown in vitro as suspended spherical aggregates, known as neurospheres (Reynolds and Weiss, 1992). This approach was also the basis for the culture of mouse NSCs in stirred-tank bioreactors (Kallos et al., 2003). Indeed, the development and optimization of bioreactor protocols for mouse NSCs has been performed in detail and is one of the best-studied systems for stirred suspension bioreactor culture of stem cells. The development of a new culture medium for mouse NSCs, capable of high expansion rate and efficiency of neurosphere production, was the first step for the large-scale production of these cells (Sen and Behie, 1999) followed by the optimization of inoculation and culture conditions of mouse NSCs in bioreactors (Kallos and Behie, 1999). Optimal values for physico-chemical growth parameters, such as pH and osmolarity, as well as inoculation parameters, including initial cell density, were determined in this study. An important issue concerning neurosphere culture is the diffusion of adequate amounts of nutrients and oxygen to cells in the center of very large-diameter aggregates, which can be limited. In extreme cases, cell death can occur in the center of the spheres due to necrosis caused by nutrient/oxygen starvation. The diameter of mouse NSC aggregates in a bioreactor can be controlled below the limit at which necrosis would be expected to occur through manipulation of the agitation rate (Sen et al., 2001).

The authors subsequently developed protocols for the extended culture of mouse NSCs by successive passaging the cells over 35 days. An overall multiplication ratio greater than 10^7 was achieved with no evident loss in growth potential or stem cell attributes. These protocols were developed for 125–250 mL spinner flasks and the mass transfer, shear stress and hydrodynamic guidelines learned from these studies drove the scale-up to large-scale (500 mL) computer-controlled reactors (Gilbertson et al., 2006). Maximum viable cell densities of 1.2×10^6 cells/mL were achieved and the culture of mouse NSCs in these vessels was shown not to affect cell growth and cell characteristics in relation to the optimized small-scale systems.

The same group also presented protocols for serum-free generation of clinical quantities of human telencephalon-derived neural precursor cells (NPCs) in 500 mL computer-controlled suspension bioreactors (Baghbaderani et al., 2008). The bioreactor-derived human NPCs retained the expression of Nestin, a neural stem/progenitor cell marker, following expansion and were able to differentiate into glial and neuronal phenotypes under defined conditions.

Apart from these studies in stirred vessels, neural stem cell expansion and differentiation has also been performed in rotary bioreactors (Lin et al., 2004; Low et al., 2001). In this case, rat NSCs isolated from the embryonic brain and encapsulated in 3D collagen gels produced cell-collagen constructs containing, after 6 weeks in rotary culture, over 10-fold more Nestin-positive cells than those found in static cultures, as well as a greater number of GFAP (astroglial marker) and Tuj1 (neuronal marker) positive cells (Lin et al., 2004). In fact, the rate of proliferation of NSCs decreases with hydrogel stiffness, and a great enhancement in expression of neuronal markers can be achieved in soft hydrogels such as alginate, which have an elastic modulus comparable to that of brain tissues (Banerjee et al., 2009).

3.4. Pluripotent stem cells

3.4.1. Mouse embryonic stem cells

Mouse ESCs are a commonly used animal model in stem cell and developmental biology and thus, to fulfill the requirement of higher numbers of cells, their in vitro expansion has been considered an important biotechnological challenge, with significant breakthroughs already achieved.

Suspension aggregate systems have been successfully applied to mouse ESC culture (Cormier et al., 2006; Fok and Zandstra, 2005; Kehoe et al., 2008; zur Nieden et al., 2007). If LIF is present in the culture medium, mouse ESCs proliferate as aggregates without significant loss of viability and with doubling times and expression of pluripotency markers comparable to static culture when cell aggregate diameter is efficiently controlled. By efficiently adjusting shear forces the problem of excessive cell aggregation can be minimized.

Similar approaches have also been used for differentiating mouse ESCs, as these cells tend to form 3D aggregates, called embryoid bodies (EBs), when cultured under suspension conditions. EBs can be formed directly from enzymatically-dissociated mouse ESCs and cardiomyocyte differentiation has been shown possible either in rotary cell culture systems (E et al., 2006) or in spinner flasks with paddle-type impellers (Zandstra et al., 2003). A scaled-up version of the latter system, where cells were cultured in a fully automated 2-L bioreactor, was also shown to be able to generate in a single run, with a genetically engineered mouse ESC line, a total yield of 1.28×10^9 cells, consisting of essentially pure cardiomyocytes (Schroeder et al., 2005). Mouse ESCs expanded in suspension aggregates in spinner flasks could also be differentiated into cardiomyocytes, in a single process without an intermediate dissociation step (Fok and Zandstra, 2005). Differentiation of mouse ESCs into osteoblasts in spinner flasks has been described as well (Alfred et al., 2010).

In a recent study (Fridley et al., 2010), spinner flasks and rotary bioreactors were compared in terms of EB formation. Parameters like cell seeding densities or rotation speed were varied and their effects on hematopoietic differentiation efficacy and progenitor cell profile were examined. Optimal conditions for HSPC generation were determined for both systems and, additionally the authors described unique profiles of progenitors generated by the different bioreactor configurations. cDNA microarrays were used to monitor mouse ESC

gene expression profile during differentiation under dynamic conditions and it was observed that cells from all three germ layers were generated during bioreactor cultures, with distinct profiles in each bioreactor, in particular for hematopoietic differentiation.

Agglomeration of the EBs is a concern in some of these cultures and encapsulation of the cells in agarose beads has been proposed as a solution to minimize this problem (Bauwens et al., 2005; Dang et al., 2004). Less extensive mouse EB aggregation can also be obtained by forming EBs on tantalum scaffolds suspended in a spinner flask (Liu and Roy, 2005). Mouse ESCs encapsulated in alginate beads were also cultured in 50 mL HARV bioreactors, being differentiated toward osteogenic lineages (Hwang et al., 2009).

Alternatively to suspension aggregates, mouse ESCs can be cultured on microcarriers. Successful proliferation was described on microporous collagen-coated dextran beads (Cytodex 3), glass microcarriers, and macroporous gelatin-based beads (Cultispher S) in spinner flasks (Abranches et al., 2007; Fernandes et al., 2007; Fok and Zandstra, 2005) with an increase in cell number up to 70-fold (in 8 days). Microcarrier cultivation has the disadvantage of requiring dissociation of the cells from the carriers once the cells reach confluency. However, EB-like suspension cultures require periodic dissociation of the aggregates after a few days, which is more labor intensive and can potentially damage the cells, turning the scale-up of these cultures likely unfeasible.

The use of perfusion bioreactors, in which the medium is pumped through the culture vessel, has been also reported (Oh et al., 2005) for the expansion of mouse ESC lines on Petriperm (a *Petri* dish with a gas-permeable base). The cell densities obtained were 64-fold greater compared to *Petri* dish controls which only originate a 9-fold increase compared to the initial inoculum, over 6 days. However, these cultures are still at the laboratory-scale and present difficulties in terms of scale-up. Expansion and differentiation of mouse ESCs in a perfused 3D fibrous matrix has been reported (Li et al., 2003). A polyethylene terepthalate (PET) matrix was applied for construction of the scaffold, which provided a larger surface area for adhesion, growth, and reduced contact inhibition. A perfusion bioprocess for efficient ESC-derived cardiomyocyte production was also developed (Bauwens et al., 2005). This system harbored monitoring and control oxygen tension and pH in 500 mL vessels with continuous medium perfusion. Oxygen tension was shown to be a culture parameter that can be manipulated to improve cardiomyocyte yield.

An innovative system to grow mouse ESCs in manual fed-batch shake flask bioreactors, similar to those used for culturing bacteria, may open the door for novel developments in this field (Mohamet et al., 2010). Abrogation of the cell surface protein E-cadherin leads to loss of cell-cell contact by mouse ESCs and subsequently to reduced cellular aggregation as well as EB agglomeration. Cells were grown for 16 days, as a dispersed cell suspension in the presence of an E-cadherin blocking antibody, with a cumulative expansion of 2775-fold. The expression of pluripotency markers was retained, as well as a normal karyotype and high viability. Furthermore, the cells could be differentiated into the three germ layers. This system may provide an effective alternative to the "usual" bioreactor systems (aggregates or microcarriers) circumventing most of the limitations described for these methods. However, the use of a blocking antibody for E-cadherin is not cost-effective for large-scale cultures and the system could not be directly translated to human ESCs. In fact, the limitation of the system just described highlights the difficulties in translating the bioreactor systems developed for mouse ESCs to their human counterparts. The in vitro establishment of human ESC lines and the recent derivation of human iPSCs created high expectations around their potential to revolutionize tissue engineering and regenerative medicine but the culture of both these pluripotent human cells present significant differences from the mouse model. Although human PSCs can already be cultured in feeder cell-free conditions (like mouse ESCs), for instance LIF does not support, at least only by itself, the expansion of human ESCs and a poor survival is normally observed upon enzymatic colony dissociation (Watanabe et al., 2007). Nevertheless, the generation of high numbers of cells stands as an essential step for their numerous applications and thus some advances have already been made in terms of bioreactor cultivation.

3.4.2. Human pluripotent stem cells

As mentioned before, microscale devices may constitute useful tools for optimization of culture conditions, providing precise control over the cell microenvironment (Azarin and Palecek, 2010a). Human ESCs have been studied in arrays of microbioreactors (Cimetta et al., 2009; Figallo et al., 2007), as well as in microbioreactors with periodic "flow-stop" perfusion systems for co-culture of with human feeder cells (Korin et al., 2009).

Nevertheless, the first successful attempt of culturing human ESCs in bioreactors consisted in using STLV and HARV rotating bioreactors for human ESC differentiation as EBs (Gerecht-Nir et al., 2004). Although agglomeration and cell death within the EBs was observed in the HARV, a 70-fold expansion occurred after 28 days in the STLV and human ESCs could still originate cells of the three germ layers. This system was later improved with two additional features (Come et al., 2008). Perfusion was implemented to provide continuous delivery of medium to the cells and external control of medium parameters and, moreover, a dialysis chamber was included improving even more the control of the culture environment. Dialysis also leaded to a better process economy as less quantity of expensive molecules, like growth factors, were required. As a result, faster and more synchronized differentiation was observed in the optimized system, in relation to static cultures. Spinner flasks were also used for human EB culture (Cameron et al., 2006), with superior expansion of EB-derived cells in relation to static conditions as well as a more homogenous morphology and size, with comparable hematopoietic differentiation potential. Different bioreactor configurations were then evaluated for EB culture (Yirme et al., 2008) concluding that the highest fold increase in total cell number (6.7-fold in 10 days) could be obtained with glass bulb impeller-equipped spinner flasks. Spinner flasks equipped with the same bulb-shaped impellers were also used for the generation of cardiomyocytes, from cultures of human iPSCs, reprogrammed without the oncogene c-Myc (Zwi et al., 2011).

A possible strategy to control human ESC differentiation consists in the precise control of niche properties using micropatterning technology (Bauwens et al., 2008) and this approach was used to create size-controlled aggregates of human ESCs (Niebruegge et al., 2009). These aggregates were cultured in a controlled bioreactor system in a two-step process and it was shown that, together with low oxygen concentration (4% oxygen tension), this approach was beneficial for cell expansion and generation of mesoderm and cardiac cells.

The successful expansion of pluripotent human ESCs, as aggregates, in stirred suspension bioreactors has also been achieved (Krawetz et al., 2010). The small molecule Y-27632, an inhibitor of Rho Kinase known as ROCK inhibitor (Ri), increases the survival rate of dissociated single human ESCs (Watanabe et al., 2007) and was used along with continuous treatment with rapamycin. This system was able to maintain cells with high expression levels of pluripotency markers, a normal karyotype and the ability to form teratomas *in vivo*. As a result, different strategies have been already developed for culturing human PSCs as aggregates in suspension (Olmer et al., 2010; Singh et al., 2010). One of these studies describes a process for culturing both human ESCs and iPSCs in suspension, with an initial step of dissociation into single cells. An almost fully defined, serum-free, medium (mTeSR) was shown to have the best performance, when continuously supplemented with Ri, and allowed successful long-term expansion of human pluripotent stem cells. Contradictory observations were made in relation to the study by Krawetz et al., (2010). For instance, the continuous exposure to the Ri did not inhibit cell growth, as reported in the first study, and supplementation with rapamycin was not required to prevent differentiation or dissociation

of the aggregates. Although Olmer and collaborators worked mostly in the small scale, pilot studies were done in agitated Erlenmeyer flasks suggesting the scalability of the process (Olmer et al., 2010). Singh et al. (2010) developed also protocols for scalable suspension aggregate culture of human ESCs, relying on Ri (and without rapamycin), in addition to an optimized heat shock treatment. The cells were successfully cultured in 50 mL spinner flasks with retention of pluripotency marker expression and ability to form teratomas. Importantly, the authors alert to some variability in behavior among different cell lines, which can explain the discrepancies found in these studies (Singh et al., 2010).

A first study under static conditions indicated that human PSCs could be cultured on microcarriers as well (Phillips et al., 2008a) and cultivation in spinner flasks on dextran (Fernandes et al., 2009a) and cellulose-based (Oh et al., 2009) microcarriers, coated with denatured collagen and Matrigel respectively, was also demonstrated. Superior expansion was attained compared to static cultures and the pluripotency of the cells was maintained. These results were obtained with mouse embryonic fibroblasts (MEF)-conditioned medium (Fernandes et al., 2009a), as well as with two different types of defined media (Oh et al., 2009). The system developed by Oh and co-workers allowed the culture of two human ESC cell lines for more than 25 passages without pluripotency loss and was extended to differentiation into cardiomyocytes (Lecina et al., 2010) using GMP-compliant reagents. The same group also found cell line specific effects of agitation on cell growth (Leung et al., 2011). Although agitation did not affect the expression of pluripotency markers or doubling times of the HES-2 cell line, both the human ESC line HES-3 or the human iPSC line IMR90 were shown to have an increased differentiation in agitated conditions, even with the addition of different cell protective polymers.

Using mouse ESCs as an initial model, other authors (Storm et al., 2010) tested different microcarriers and applied the optimized conditions to human ESCs. Successful integration of human ESC expansion and differentiation into definitive endoderm was also achieved using microcarriers in stirred bioreactors. However, in this case microcarriers were coated with the animal-derived matrix Matrigel (Lock and Tzanakakis, 2009).

Additionally, perfusion has been shown to improve human ESC culture in culture dishes (Fong et al., 2005) in an analogous system to what was used for mouse ESCs (Oh et al., 2005), as well as in controlled stirred tank bioreactors with O_2 controlled to 30% air saturation (Serra et al., 2010). Encapsulation of human ESCs in alginate beads was also performed and after up to 260 days in culture, under static conditions, cells remained pluripotent (Siti-Ismail et al., 2008). Other materials have also been used for cell encapsulation (Dang et al., 2004; Gerecht et al., 2007) with positive results. Encapsulation of human ESCs in poly-L-lysine-coated alginate capsules led to the generation of heart cells, with serum-containing media, in stirred suspension bioreactors (Jing et al., 2010).

3.5. Other stem cell populations

In the previous sections, we described significant advances on the bioreactor culture of the most well documented stem cell populations. However, other important findings were made with different stem cell subtypes that are also noteworthy.

A good example is given by the limitations faced in breast cancer research caused by the scarcity of mammary epithelium stem cells (MESCs), which may be implied in the disease process (Youn et al., 2005). These cells can be obtained from primary tissue but expansion is required for application of analytical techniques. Murine MESC aggregates, known as mammospheres, were thus expanded in suspension bioreactors and scaled up to 1 L vessels. The protocols developed for MESCs were adapted for the expansion of murine breast cancer stem cells, also with the purpose of addressing the limited supply of cells for research (Youn et al., 2006). Cells were propagated as tissue aggregates, called tumorspheres, in spinner flasks and in 500 mL stirred suspension bioreactors. Experimental design was performed to assess the effects of inoculum density and hydrodynamic shear on cell yield and oxygen mass transfer was also studied in detail. More recently, bioreactor protocols were also developed for brain cancer stem cells (Panchalingam et al., 2010).

Apart from cancer stem cells, a population of cells isolated from the bone marrow, with extensive proliferation capability and differentiation potential into various cell types, like endothelial and smooth muscle cells or hepatocytes, known as multipotent adult progenitor cells (MAPCs) were cultured in stirred bioreactors as well (Subramanian et al., 2011). MAPCs derived from rat bone marrow were for the first time cultured as 3D aggregates, first in static conditions and then in a scalable dynamic suspension in 250 mL spinner flaks. A 70-fold expansion of MAPCs was achieved after culture in spinner flasks and the cells generated maintained the potential for differentiation into hepatocyte-like cells, which can be useful for drug bioavailability and hepatotoxicity studies or other applications.

4. Final remarks and future perspectives

As described in the previous sections, cultivation in bioreactors is a powerful method to generate large numbers of stem cells for their numerous applications. Importantly, beyond the question of scalability, bioreactor culture allows continuous monitoring and control of the physical and chemical environment of the culture (Ulloa-Montoya et al., 2005), which allows studying the effect of dynamic temporal nutrient, oxygen or pH profiles, culture parameters known to have crucial influence over stem cell fate.

The use of stem cell-based in vitro models in toxicology may constitute a very attractive alternative to costly and labor-intensive studies performed with primary cells or *in vivo* animal models (Davila et al., 2004). For the successful implementation of this technology large amounts of cells will be required and may be supplied by bioreactor cultures. For example, hepatocytes and cardiomyocytes derived from stem cells in vitro can be used for screening new chemicals for hepatotoxicity or cardiotoxicity, both important causes of failure in drug development, as well as reproductive toxicology (Wobus and Loser, 2011). Other opportunities in this field are the development of assays for neurotoxins and skin or renal toxicity. Successful bioreactor production of cardiomyocytes from hPSC has already been achieved (Lecina et al., 2010) and thus the development of reproducible and validated toxicological assays with these cells would constitute an invaluable resource for this field.

Concerning the use of stem cell bioreactors in clinical environments, several issues still need to be addressed (Kehoe et al., 2010; Ulloa-Montoya et al., 2005). In general, for this application culture media must be chemically defined, nonimmunogenic (without animal-derived products), and, obviously, pathogen free. Also important is a robust manufacturing process, to eliminate lot-to-lot variability. Although important progress has been done in this direction, the use of serum (Schop et al., 2010) or feeder layers of animal origin (Nie et al., 2009) is still often reported, especially when cells grow under adherent conditions. Regarding this issue, the availability of novel microcarrier particles, designed for use in serum-free conditions would be of great benefit for this area. A considerable number of available products include animal-derived components, like collagen or animal-derived matrices like Matrigel. Different defined and xeno-free substrates were recently described for successful culture of hESCs (Azarin and Palecek, 2010b) and could be the basis for the production of new microcarriers, assuming the manufacturing cost would be competitive with the currently available options.

Apart from the general questions, individual stem cell populations face specific challenges. In the case of human pluripotent stem cells some issues are clear. Seeding these cells as clumps on microcarriers/scaffolds is

not optimal and this may hinder the performance of the culture in bioreactors (Kehoe et al., 2010). Alternative approaches for single-cell dispersion of cell colonies prior to seeding may alleviate this issue. In addition, most methods described so far require coating with MEFs or Matrigel and, as stated above, these animal-derived products are unsuitable for therapeutic applications. Clinical-grade human fibroblasts (Phillips et al., 2008b) could provide a good alternative, but functionalization of beads with defined molecules to enhance the initial cell attachment and to allow maintenance of pluripotency capacities as well as to maximize cell proliferation would offer an optimal xeno-free system for large-scale bioreactor culture of pluripotent stem cells. Finally, the culture media used for cell expansion should not only be fully defined but also cost affordable, thereby making the bioprocess economically attractive and competitive. Hence, a systematic optimization to minimize the use of growth factors in the culture medium should be performed. With the advent of high-throughput screening technologies, small-molecule libraries can now be analyzed to identify molecular interactions leading to particular stem cell responses (Ding and Schultz, 2004). For example, identifying natural or synthetic small molecules capable of supporting stem cell self-renewal or differentiation can lead to economically attractive alternatives to current culture media.

Although bioprocesses for the production of clinical-grade stem cell derivatives should follow strict GMP guidelines (Kehoe et al., 2010), the majority of the available hESC lines have been exposed to animal cells or proteins, rendering them unsuitable for therapeutic applications. Alternative methods for deriving hESCs and the advent of iPSCs (Takahashi and Yamanaka, 2006; Yu et al., 2007) may constitute relevant solutions for these problems. Recently it was reported the generation of hPSC with similar biological and epigenetic characteristics to those of mESCs (Hanna et al., 2010). These cells, for instance, display high single-cell cloning efficiency in contrast to what happens with the "classical" hESC lines, even with the use of ROCK inhibitor. Although more studies are required to fully understand this cell population, it has the potential to open new horizons for bioreactor culture of hPSC. Importantly, methods for depletion of undifferentiated tumurogenic cells in order to provide purified stem cell derivatives in medically relevant quantities, and for fast probing chromosomal and other genetic abnormalities also need to be developed.

The same issues described for human PSCs have to be considered in other stem cell models as well. HSC and MSC culture in completely defined, xeno-free, conditions is not frequently described. Operation under GMP compliancy will be essential and for this purpose the wave bioreactor may bring additional advantages, at the expense of an increased cost. An interesting biotechnological challenge will be improving the expansion of HSCs through the co-culture with MSCs in bioreactors, in an attempt to reproduce the bone marrow microenvironment (da Silva et al., 2010).

Human NSCs have already been successfully expanded in bioreactors (Baghbaderani et al., 2008), but studies in mouse models of disease revealed that further understanding of this system is needed for successful clinical applications (Mukhida et al., 2008). Alternatively, integrated bioreactor expansion and differentiation into specific cell types could constitute a promising strategy. Methods for bioreactor expansion of NSCs from different origin, for instance derived from PSC (Conti and Cattaneo, 2010), could be developed and the expanded cells could provide better results in terms of integration in diseased hosts. A strategy, not only interesting for NSC but also for other stem cell populations, is the creation of synthetic microenvironments, which may encourage successful survival and incorporation of the stem cells into diseased or injured regions (Little et al., 2008). Biomaterials with different mechanical properties may be used to create scaffolds or to encapsulate the cells and the synergistic presentation of biological signals, by the inclusion of relevant molecules in the culture medium could also be a strategy to explore. If cells are cultured on surfaces or scaffolds, immobilization of those molecules or controlled release from a matrix could be also interesting possibilities (Kirouac and Zandstra, 2008).

We can thus conclude that the fast growing field of stem cell bioreactor cultivation is expected to contribute for the future development of systems suitable for clinical or pharmacological grade production of stem cell-derived cellular products. Invaluable tools for progress in this area will be high-throughput screening platforms that can elucidate the factors that modulate stem cell fate, computational models and engineered cell microenvironments (Ashton et al., 2011). This field will thus benefit from multidisciplinary collaborations between bioengineering, computer science and medicine.

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