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REVIEW

Stem cell bioprocessing: fundamentals and principles

Mark R. Placzek, I-Ming Chung, Hugo M. Macedo, Siti Ismail, Teresa Mortera Blanco, Mayasari Lim, Jae Min Cha, Iliana Fauzi, Yunyi Kang, David C. L. Yeo, Chi Yip Joan Ma, Julia M. Polak, Nicki Panoskaltsis† and Athanasios Mantalaris*

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In recent years, the potential of stem cell research for tissue engineering-based therapies and regenerative medicine clinical applications has become well established. In 2006, Chung pioneered the first entire organ transplant using adult stem cells and a scaffold for clinical evaluation. With this a new milestone was achieved, with seven patients with myelomeningocele receiving stem cell-derived bladder transplants resulting in substantial improvements in their quality of life. While a bladder is a relatively simple organ, the breakthrough highlights the incredible benefits that can be gained from the cross-disciplinary nature of tissue engineering and regenerative medicine (TERM) that encompasses stem cell research and stem cell bioprocessing. Unquestionably, the development of bioprocess technologies for the transfer of the current laboratory-based practice of stem cell tissue culture to the clinic as therapeutics necessitates the application of engineering principles and practices to achieve control, reproducibility, automation, validation and safety of the process and the product. The successful translation will require contributions from fundamental research (from developmental biology to the 'omics' technologies and advances in immunology) and from existing industrial practice (biologics), especially on automation, quality assurance and regulation. The timely development, integration and execution of various components will be critical—failures of the past (such as in the commercialization of skin equivalents) on marketing, pricing, production and advertising should not be repeated. This review aims to address the principles required for successful stem cell bioprocessing so that they can be applied deftly to clinical applications.

Keywords: stem cell; bioprocessing; tissue engineering

1. INTRODUCTION

The success of stem cell bioprocessing relies on robust and reproducible culture conditions and processes. For stem cell bioprocessing, this includes the scale-up of stem cells to a differentiated end product of sufficient quality and quantity for clinical and commercial goals. The considerable cost with respect to consumables, labour and time as well as the inherent variability in manual processes not only make this an unattractive option but also render it commercially unviable. Automation and the use of an efficient bioprocess paradigm are imperative for the creation of successful clinical products.

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2. DESIGN PRINCIPLES

The design principles (Lim *et al.* 2007) pertinent to stem cell bioprocessing can be categorized into three groups: (i) process components, (ii) process requirements, and (iii) process function, as summarized in figure 1. A combination of generic, 'off-the-shelf' and personalized manufacturing paradigms must be considered as no single technology satisfies all requirements. The *process components* consist of the cell source and type, and the elucidation of appropriate signals required for cellular development, in addition to the scaffold and bioreactor design and implementation. *Process requirements* address practical considerations of bioprocessing, satisfying good manufacturing practices (GMPs) such as quality assurance, bioprocess monitoring control and automation, in addition to

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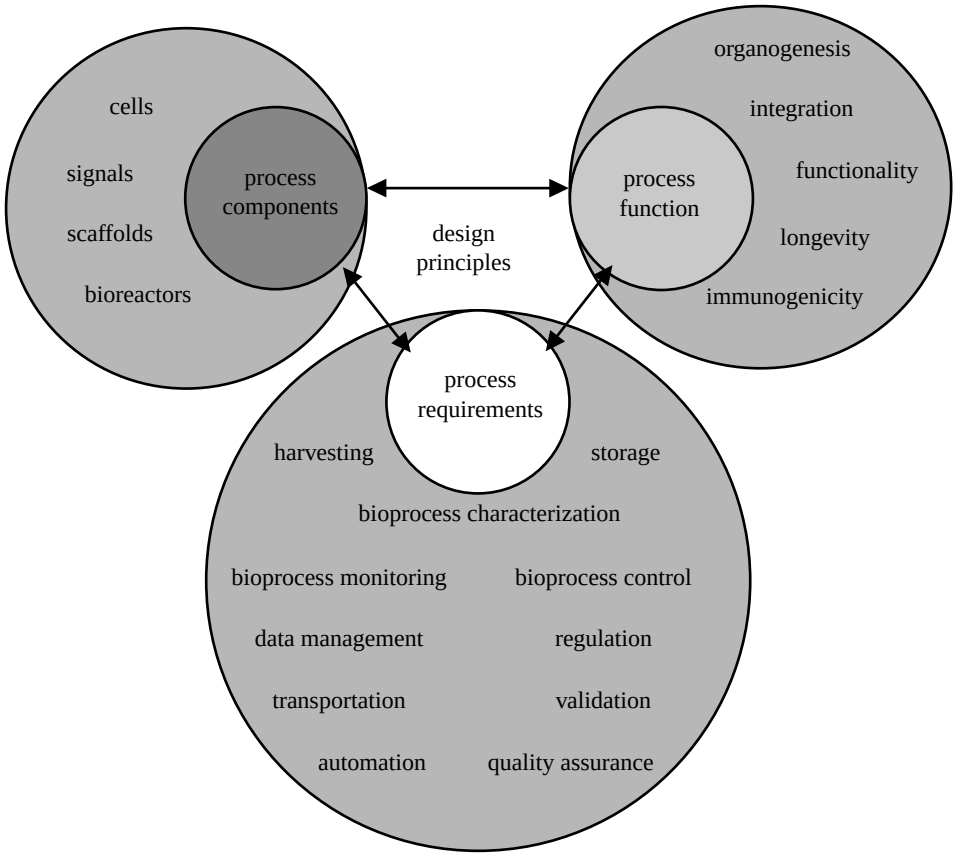

Figure 1. Design principles for stem cell bioprocesses.

product transportation. Finally, the *process components* and *process requirements* need to ensure the end product's functionality, integration and longevity, to name but a few vital factors included in the *process function*. This review will address only the *process components* and *requirements*.

2.1. Process components

Identification of the optimal cell source and important signals for cellular development, as well as delivering a suitable growth mimicry in terms of the scaffold used, are essential tailor-made requirements in stem cell bioprocessing and tissue engineering. In addition, provision of a controlled culture environment through the use of an appropriate bioreactor is critical. Clearly, application-specific approaches are required; however, certain common methodologies can be applied.

2.1.1. Stem cells. A stem cell is defined as a clonal precursor producing either identical stem cells (self-renew) or differentiating into other specialized cells. There are several classes of stem cells, each one presenting its own challenges and benefits for the manufacture of therapeutics for the clinic (figure 2). This review will focus only on embryonic and adult stem cells.

Embryonic stem cells (ESCs) are capable of indefinite expansion, and are pluripotent. Currently, attempts are being made to generate patient-tailored ESC lines that overcome immunological complications (Takahashi & Yamanaka 2006). However, they also are capable of teratoma formation, are difficult to control with respect to their differentiation fate, and elicit ethical considerations due to the destruction of the embryo, until recent advances (Petersen & Niklason 2007). By contrast, adult stem cells do not elicit ethical considerations and are obtained directly from the patient or the donor. For autogenetic grafts, adult stem cells do not present immunogenic complications on implantation and, depending on the source, could be obtained relatively easily. Adult stem cells, however, do present their own unique challenges. Specifically, they have been found to vary in quality with respect to the age and health of the donor/patient, and differentiation is often restricted to the original lineage of the cell source (multipotent). Some adult stem cell types have been shown to be able to transdifferentiate; however, most adult stem cell therapies require cells to be harvested from specific sources (Smith *et al.* 2007). An important consideration with respect to bioprocessing is the expansion potential of these cells. ESCs are believed to have an almost unlimited expansion capability while adult stem cells, depending on donor and source, may be capable of only tens of population doublings.

Adult stem cells Adult stem cells, or somatic stem cells, are undifferentiated cells found throughout the adult body. They reside within specific niches in the different organs where they produce cells that regenerate damaged tissue and replace dying cells. Adult stem cells have been used clinically since the 1950s when stem cell transplantation was pioneered using bone marrow-derived stem cells. Since then, other types of adult stem cells have been identified in the body, which

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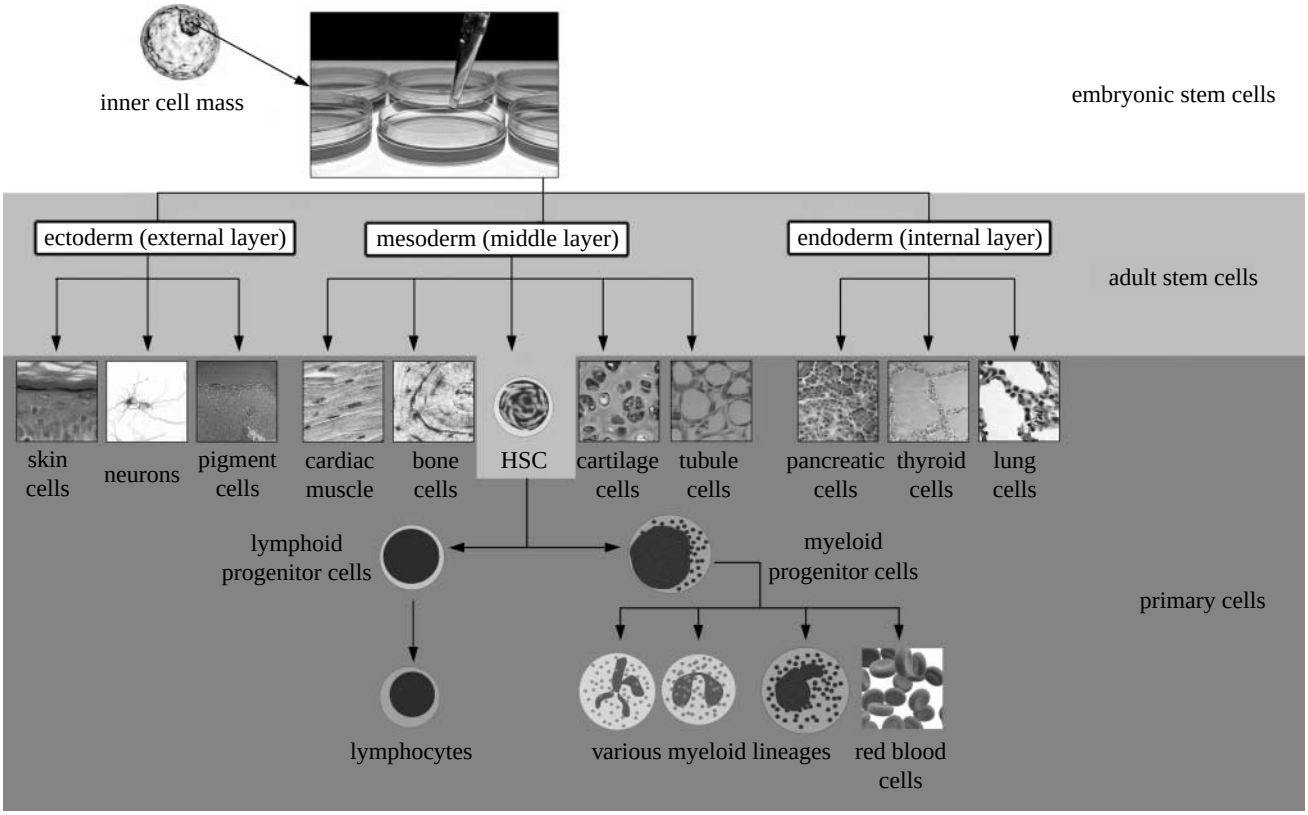

stem cell type	expansion potential	differentiation potential	cell availability	immunogenicity autologous	immunogenicity allogenic	teratogenicity	ease of regulation	ethical acceptance	bioprocess complexity	established clinical practice	regulatory approval
embryonic	+++	+++	+++	–	medium	yes	+	no	+++	no	no
adult											
– mesenchymal	+	++	+	low	high	no	++	yes	++	yes	yes
– haemopoietic	+	++	+	low	high	no	++	yes	++	yes	yes
– cord blood	+	++	++	low	low	no	++	yes	++	yes	yes
primary cells	–	–	+	low	high	no	+++	yes	+	yes	yes

Figure 2. Cell types available for regenerative medicine applications.

have offered alternative tissue repair possibilities and originate from easier to harvest cell sources, such as fat, skin, olfactory cells and peripheral blood (PB). Haemopoietic stem cells (HSCs) can be isolated from several sources besides the adult bone marrow. These include PB, umbilical cord blood (CB) and the placenta. Isolation is generally carried out by fluorescence-activated cell sorting (FACS) or magnetic cell sorting (MACS), an immunomagnetic selection technique where paramagnetic microbeads or adsorption columns coupled with specific monoclonal antibodies (e.g. anti-CD34) are employed to capture and enrich the target cells in the presence of a magnetic field (Sandstrom *et al.* 1995; Roots-Weiss *et al.* 1997; Kekarainen *et al.* 2006). Isolation strategies vary from the depletion of committed lineage cells and late multipotent progenitor cells using a variety of lineage-specific markers, or by positive selection using markers such as $CD34^+$, $CD59^+$, $CD90^+$ and $CD133^+$ (Baum *et al.* 1992). HSCs isolated from the different sources possess roughly similar properties; however, the volumes harvested vary considerably as do the specific populations of haemopoietic stem cells (McAdams *et al.* 1996; Urbano-Ispizua 2007). HSCs are capable of giving rise to all blood cell types, including the myeloid and lymphoid lineages. Some transdifferentiation capability has been shown by various research groups reporting differentiation of HSCs into skeletal muscle (Ferrari *et al.* 1998), cardiac muscle (Bittner *et al.* 1999), liver (Petersen *et al.* 1999; Theise *et al.* 2000*a*,*b*), endothelial cells (Shi *et al.* 1998) and neurons (Eglitis & Mezey 1997; Brazelton *et al.* 2000; Mezey *et al.* 2000). Interestingly, it appears that HSCs may be capable of sustained symmetrical division, explaining how a small number of HSCs are capable of repopulating the bone marrow rapidly after depletion (Sherley 2002). The rapid and prolonged expansion capability of HSCs renders them a suitable candidate cell type for bioprocessing.

Bone marrow was originally the only source of progenitor cells used for allogeneic transplantation. In the early 1990s, mobilized [mainly by granulocyte colony-stimulating factor (G-CSF)] PB was also introduced clinically, which presented certain advantages. For instance, a greater volume of cells were able to be harvested from PB, thus increasing the number of progenitor and accessory cells in the collection. For transplantation, this leads to faster myeloid and lymphoid recovery facilitating engraftment and full T-cell chimerism (Sheridan *et al.* 1992; Bensinger *et al.* 1993; Chao *et al.* 1993). Additionally, irrespective of the small inherent risks involved with mobilized PB collection and the use of G-CSF (Martinez *et al.* 1996, 1999; Majolino *et al.* 1997; Tabilio *et al.* 1997; de la Rubia *et al.* 1999), the risks and side effects involved in the BM donation are

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considerably greater in comparison (Buckner *et al.* 1984; Stroncek *et al.* 1993; Pulsipher *et al.* 2006). For bioprocessing, however, the greater volume of cells is offset by their lower expansion capability, making PB HSCs less amenable than bone marrow-derived stem cells. In the late 1990s, CB was used as a source of HSCs and, by 2004, 3 per cent of the total allogeneic transplants in Europe and 7 per cent of the unrelated allogeneic transplants used CB (Gratwohl *et al.* 2005). However, the use of CB is clinically hindered, primarily due to its low harvest volume. This causes complications resulting in graft failure or relapse. The therapeutic potential of CB, however, should not be underestimated; its low human leucocyte antigen (HLA) compatibility restrictions, low-risk collection from donors and off-the-shelf availability render CB a promising source of HSCs provided sufficient numbers can be obtained (Locatelli *et al.* 1999; Barker *et al.* 2001*a*). Consequently, this remains an active research area with a number of different approaches currently being tested, such as double CB transplants (Barker *et al.* 2001*b*, 2005) and co-infusion of CB with either haploidentical haemopoietic progenitor cells (Fernandez *et al.* 2003; Magro *et al.* 2006) or mesenchymal stroma cells from a third party (Noort *et al.* 2002). In terms of *ex vivo* expansion of haemopoietic stem and progenitor cells, Koller *et al.* (1993*a*,*b*) pioneered the concepts of stem cell bioprocessing by using scalable perfusion bioreactor systems for the expansion of haemopoietic stem and progenitor cells from bone marrow, PB or CB in the early 1990s as discussed below.

Mesenchymal stem cells (MSCs) are multipotent stem cells that are primarily isolated from the bone marrow. Other populations of MSCs have been found, including from PB (Zvaifler *et al.* 2000), adipose tissue (Zuk *et al.* 2001), skin tissue (Chunmeng & Tianmin 2004), trabecular bone (Sottile *et al.* 2002) and umbilical CB (Erices *et al.* 2000). MSCs have been shown to differentiate into osteoblasts, chondrocytes and adipocytes (Muraglia *et al.* 2000). A broader differentiation capability of human MSCs (hMSCs) has also been suggested with hMSCs being differentiated into muscle cells (Taylor & Jones 1982) and limited myogenic potential being observed when human MSCs were co-cultured with murine skeletal myocytes (Lee, J. H. *et al.* 2005). Cardiomyocyte differentiation has also been demonstrated (Lee, J. H. *et al.* 2005), resulting in cells with the proper morphology, physiological cardiomyogenic response and expression of appropriate adrenergic and muscarinic surface receptors. MSCs are obtained by either (i) direct plating, which relies on the MSC adherent properties (Luria *et al.* 1971), or (ii) through the use of cell surface markers. Both methodologies present certain limitations. Specifically, the homogeneity of the purified population, which is obtained through adherence to culture plastic, is not high with only 10–20 per cent of the cells capable of multipotent differentiation (Phinney *et al.* 1999). In addition, in the absence of a ubiquitous MSC-specific marker, marker selection varies between groups, with different protocols using a combination of CD146, CD271 (Buhring *et al.* 2007), CD105 (Aslan *et al.* 2006), CD29 (Jackson *et al.* 2007) and Stro-1 (Gronthos *et al.* 1994). Culture of MSCs has indicated that isolated cells are limited to 40 population doublings—25 for elderly donors (Stenderup *et al.* 2003)—which is the result of telomere shortening due to the loss of telomerase activity in tissue culture (Simonsen *et al.* 2002). Furthermore, of importance to clinical applications, cultured cells have demonstrated genetic instability after 250 population doublings when using this method of extended expansion (Serakinci *et al.* 2006), highlighting the need to develop efficient scale-up methods and effective differentiation protocols so that sufficient numbers of target cells can be delivered without prolonged *ex vivo* culture time.

In general, a firm correlation exists between the expansion/differentiation ability and availability of the stem cells and their clinical applicability in terms of process complexity, and ethical and regulatory restrictions. The implication of this correlation is that it directs, and potentially restricts, the manufacturing and scale-up approaches available and the associated costs. Clearly different stem cell types will require different operating conditions; however, this variability is also likely in any one stem cell type from initial expansion to differentiation. The process is transient in nature and operating conditions require tight control. Finally, stem cell standardization necessitates the identification of appropriate markers as well as the development of suitable evaluation assays.

ESCs The discovery of mouse ESCs over 25 years ago (1981) and the derivation and culture of human embryonic stem cells (hESCs) more recently (1998) represent major advances in biology with immense potential for tissue engineering and regenerative medicine (Hirai 2002; Prelle *et al.* 2002; Keller 2005). Culture of ESCs requires precise methodology in maintaining their undifferentiated state and directing their differentiation. The traditional ESC culture process is fragmented consisting of a maintenance/expansion phase, an embryoid body (EB) formation phase, followed by terminal differentiation to the desired cell lineage. Each phase presents obstacles that need to be overcome before widespread clinical application becomes standard practice. Specifically, during maintenance/expansion, the ESCs, especially the hESCs, are traditionally cultured on feeder cells, such as mouse embryonic fibroblast (MEF), in order to retain their undifferentiated state (Thomson & Marshall 1998; Reubinoff *et al.* 2000). Consequently, the quality of the feeder cells and the consistency of the culture conditions are critical elements in generating high-quality ESCs that retain their 'stemness'. Additionally, it has been suggested that prolonged culture of hESCs can ultimately result in culture adaptation where the hESCs lose their true undifferentiated state (Draper *et al.* 2004). More recently, culture protocols have been developed in order to move away from the traditional MEF-dependent methods to more clinically relevant feeder-free systems. The use of human feeder cells, such as foetal muscle, foetal skin, adult fallopian tube epithelial cells (Richards *et al.* 2002, 2003; Amit *et al.* 2003), foreskin fibroblasts (Hovatta *et al.* 2003), adult marrow cells (Cheng *et al.* 2003), adult endometrial cells

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(Lee, J. B. *et al.* 2005) and hESC-derived fibroblasts (Stojkovic *et al.* 2005) has proven useful for generating viable hESCs (Cheon *et al.* 2006). However, while these systems eliminate direct contact between hESCs and feeders, they are limited by low success rates in the initial transfer of hESCs from feeder to feeder-free conditions (Cheon *et al.* 2006) and can lead to the development of a mixed population of undifferentiated and differentiated hESCs (Amit *et al.* 2004; Carpenter *et al.* 2004; Rosler *et al.* 2004). More recently, it has been demonstrated that the encapsulation of hESCs within hydrogels can sustain their undifferentiated state for over six months in the absence of mechanical or enzymatic passaging and without co-culture with feeder cells, which represents a potentially crucial development (Siti-Ismail *et al.* 2008). In addition, efforts are being made towards moving away from animal-derived products, such as conditioned media and serum, towards defined media, serum replacement and the use of growth factors (Amit *et al.* 2004; Carpenter *et al.* 2004; Rosler *et al.* 2004). This would represent a significant step towards clinically relevant protocols. Additional considerations are the standardization and automation of ESC culture; however, the trade-off between expensive automation systems (Mason & Hoare 2006) and standardization remains an open challenge for the stem cell bioprocessing community.

Several methods of expansion and differentiation of adherent ESCs exist, in either static or bioreactor systems, including EB cultivation (Fok & Zandstra 2005; Schroeder *et al.* 2005; Cormier *et al.* 2006; zur Nieden *et al.* 2007), encapsulation (Dang *et al.* 2004; Bauwens *et al.* 2005; Randle *et al.* 2007), use of microcarriers as a substrate for cell attachment (Fok & Zandstra 2005; Abranches *et al.* 2007), automated tissue platforms (Narkilahti *et al.* 2007; Terstegge *et al.* 2007) and perfusion bioreactor systems (Fong *et al.* 2005; Oh *et al.* 2005). Currently, the most robust method for generating differentiated cells from ESCs is through the formation of embryoid bodies, where ESCs spontaneously differentiate and form tissue-like spheroids in suspension culture (Dang *et al.* 2004). EB differentiation has been shown to recapitulate aspects of early embryogenesis, including the formation of a complex three-dimensional arrangement where cell–cell and cell–matrix interactions are thought to support the development of the three embryonic germ layers and their derivatives (Keller 1995; Itskovitz-Eldor *et al.* 2000). The length of EB culture is dependent on the desired cell type, and EB differentiation appears to correlate temporally well with the post-implantation development of embryos (Keller 1995). Mesodermal and ectodermal precursors form within a few days, whereas some endodermal cell types may benefit from more extended culture times, up to 10 days, to a stage where most EBs have cavitated and become cystic (Abe *et al.* 1996; Leahy *et al.* 1999). The conventional methods of inducing EB formation, which include suspension culture in bacterial-grade dishes, hanging drops and methylcellulose, have been shown to produce small numbers of EBs, limiting the ability of the ESCs to differentiate into the desired cell type. Following formation of EBs, cells are returned to adherent culture conditions upon which specialized cells develop in the outgrowth area of differentiation (Kurosawa 2007). However, the conventional methods of EB formation are impractical for large-scale cell production (Fok & Zandstra 2005) and generate heterogeneous cell types not suitable for therapeutic applications. To address this, the use of bioreactors is essential to meet large-scale production needs, with the inherent benefit of amenability to process control strategies.

2.1.2. Operating conditions and signals. Successful stem cell bioprocessing, in terms of expansion and differentiation, depends on the control of key process variables: (i) the physiochemical environment, (ii) nutrients and metabolites, and (iii) growth factors (Lim *et al.* 2007). Physiochemical culture parameters include pH, dissolved oxygen and carbon dioxide tensions and temperature. Even though almost all expansion cultures are run at pH 7.4, 20 per cent oxygen, 5 per cent carbon dioxide and 37°C, the proliferation and differentiation of different stem cell cultures have been found to operate at different optima. Specifically, higher pH (pH 7.60) was found to enhance the differentiation and maturation of megakaryocytic progenitors and also favours erythroid differentiation (McAdams *et al.* 1998). By contrast, granulopoiesis is optimal at a lower pH (pH 7.21), which enhances granulocyte colony-stimulating factor receptor (G-CSFR) expression and granulocyte proliferation and differentiation (Hevehan *et al.* 2000*b*). More work has been done on the effects of pH on differentiation as opposed to optimization for the expansion of stem cells; interestingly, a pH of 7.1 has been found to increase the expansion of megakaryocyte progenitors (Yang, H. *et al.* 2002). Oxygen tension is a critical factor in haemopoiesis. In culture, oxygen tension can greatly affect the expansion of cells by modulating the production of cytokines, surface markers and transcription factors (Mostafa *et al.* 2001). Oxygen demand varies for different cell lineages and maturation stages—low oxygen concentration (5%) is better for progenitor cell expansion, while high oxygen (20%) promotes the growth of mature megakaryocytes and erythrocytes (McAdams *et al.* 1998). Low oxygen tension was also found to enhance granulocyte differentiation (Hevehan *et al.* 2000*a*). In murine embryonic stem cells (mESCs), oxygen tension has a considerable effect on differentiation. The mESCs cultured in 40 per cent oxygen retained alkaline phosphatase activity and Oct-4 gene expression for longer than at lower oxygen tensions, and normal oxygen tension (20%) allowed spontaneous differentiation of the mESCs (Kurosawa *et al.* 2006). These findings establish the presence of pH and oxygen gradients *in vivo*. Fewer investigations have been conducted on the effects of temperature, though in most haemopoietic cell cultures, the operating temperature is maintained at 37°C. Interestingly, a higher temperature of 39°C has recently been found to enhance megakaryopoiesis in CD34-enriched CB cell cultures (Proulx *et al.* 2004), and a lower temperature

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(32°C) has been shown to reduce oxidative damage while retaining viability and population doubling times in MSCs. MSCs are obtained in relatively low numbers and extended *in vitro* expansion can lead to reduced viability from oxidative damage. Therefore, optimized culture conditions are of great importance in producing suitable numbers of long-term viable MSCs for successful engraftment. This evidence highlights the fact that process variations in the culture environment could be strategically applied to direct and manipulate cellular behaviour *in vitro*. Nutrient and metabolite concentrations determine cell growth, differentiation and death in a culture, and therefore should be closely monitored and controlled in bioprocessing. Growth factors regulate stem cell behaviour by providing survival, proliferation and differentiation signals to the cells. They have specific functions, both positive and negative in nature, and can act on either a specific cell lineage or multiple lineages. Interactions between these growth factors and/or with other process parameters are in many cases not fully understood. It is therefore crucial to quantify and qualify these effects and their interactions with respect to one another in order to tailor the culture process for optimal production of a specific cell type population.

2.1.3. Cell adhesion and scaffolds. Organogenesis, which involves the finely regulated proliferation and differentiation of stem cells, depends, to a large extent, on cell adhesion and the provision of a three-dimensional growth environment. Cell adhesion to the extracellular matrix (ECM) is mediated by a class of heterodimeric transmembrane cell surface receptors called integrins. ECM proteins typically affect cell behaviour by binding to specific integrin cell surface receptors, thus activating intracellular signalling pathways that control gene expression, cytoskeletal organization and cell morphology (Giancotti & Ruoslahti 1999; Hynes 2002). For instance, fibroblastic, epithelial and endothelial cells must adhere to appropriate ECM components in order to survive, a phenomenon termed 'anchorage dependence' (Danen & Yamada 2001). Furthermore, the signal-transducing capacity of integrins has been indicated as a likely regulator of the *in vitro* differentiation of several types of stem cells and committed progenitor cells (Prosper & Verfaillie 2001; Schneider *et al.* 2001; El-Amin *et al.* 2002; Carvalho *et al.* 2003; Salasznyk *et al.* 2004; Klees *et al.* 2005; Chastain *et al.* 2006; Hayashi *et al.* 2007). Consequently, differentiation of stem and progenitor cells can be manipulated through the modification of cell culture surfaces. Indeed, numerous studies have shown that the modulation of cell adhesion properties via surface chemistry, surface microarchitecture or the types of ECM ligands present on the culturing surfaces could be applied to regulate stem cell differentiation (Prosper & Verfaillie 2001; Schneider *et al.* 2001; El-Amin *et al.* 2002; Boyan *et al.* 2003; Carvalho *et al.* 2003; Levenberg *et al.* 2003; Salasznyk *et al.* 2004; Klees *et al.* 2005; Chastain *et al.* 2006; Sun *et al.* 2006; Hayashi *et al.* 2007). Specifically, integrin activation has been implicated in the maintenance of mESCs' self-renewal and differentiation (Hayashi *et al.* 2007). When the mESCs were cultured on surfaces coated with type I and IV collagen, gelatine or poly-D-lysine, the cells remained undifferentiated; however, the mESCs differentiated into primitive ectoderm even in the presence of LIF when cultured on surfaces coated with ECM ligands, such as laminin or fibronectin. MSC adhesion to culture surfaces coated with various ECM proteins (fibronectin, collagen I, collagen IV, vitronectin and laminin-1) has also been examined as a function of their osteogenetic potential (Salasznyk *et al.* 2004). It was reported that MSCs adhered with varying affinity (fibronectin > collagen I ≥ collagen IV ≥ vitronectin > laminin-1), which suggested that appropriate ECM contact alone may be sufficient to induce differentiation in these cells.

The majority of research on ESC differentiation *in vitro* has so far focused on their differentiation in adherent culture following EB formation. However, as discussed above, precisely controlled surface chemistry and ECM ligand presentation can enable regulation of gene expression with increased differentiation efficiency towards the desired target cell. Additionally, for many tissue engineering applications, the incorporation of differentiated cells into higher order structures will be essential for implants to be functional (Rippon & Bishop 2004). To this end, the use of three-dimensional scaffolds has been explored and needs to be taken into consideration. Designing a tissue-engineered scaffold requires the consideration of a large number of variables: material; porosity; pore size; mechanical stability; degradability; biocompatibility; hydrophobicity; and bioactivity (table 1). Scaffolds are typically fabricated by either natural materials, which are inherently bioactive but lack mechanical strength, or synthetic materials, which lack inherent bioactivity but are mechanically strong and can be engineered with the desirable macro- (shape) and microarchitecture (pore size and porosity), as well as being modified to possess desired bioactive properties (Safinia *et al.* 2006) that will facilitate cellular growth and organogenesis in a biomimetic manner (Stevens & George 2005). Polymeric scaffolds should possess a network of channels and interconnected pores to facilitate mass transport of nutrients/metabolites and to guarantee the penetration of large numbers of cells allowing the formation of cellular associations (Elema *et al.* 1990). Pore size and high surface area to volume ratio are also key parameters for cell spreading and expansion (Kim *et al.* 2005). For *in vivo* applications, the impact of pore size and osteogenic differentiation was studied using polyHIPE polymer; the minimum recommended pore size is suggested to be 100 μm (Hulbert *et al.* 1970) and the maximum to be 500 μm (Karageorgiou & Kaplan 2005) with the larger pores registering formation of fibroblastic tissue (Wake *et al.* 1994). Pore size affects cellular adhesion, viability, distribution and formation of an ECM by specific cell types; it has been suggested that the pore size in scaffolds can be used to preferentially support or prevent the ingrowth of specific cells. A high surface area to volume ratio is necessary for *in vitro* cell attachment, proliferation and subsequent matrix deposition (Zeltinger *et al.* 2001).

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Table 1. Scaffolds used in tissue engineering.

scaffold material	cultured cell type	biodegradable	regulatory approval	scaffold properties	references
poly(L-lactic-co-glycolic acid) (PLGA)	human skin fibroblasts, porcine oesophageal smooth muscle cells (ESMCs), BM cells, chrondrocytes, ligament and synovial cells	yes	yes	porosity/pore size: vary depending on polymer ratio (80–95%)/ 125–500 μm	Yang, H. *et al.* (2002), Yang, J. *et al.* (2002), Wu & Ding (2004) and Uematsu *et al.* (2005)
polyglycolic acid (PGA)	chondrocytes, 3T3 fibroblasts, human foetal extensor tenocytes and BM MSCs	no	yes	surface area: 2 $m^2\ g^{-1}$; pore size: 100–300 μm	Drewa *et al.* (2006), Moutos (2006), Hannouche *et al.* (2007) and Wang *et al.* (2008)
poly L-lactic acid (PLLA)	3T3 fibroblasts, neural stem cells, MSCs and mESCs	yes	yes	porosity/pore size: 95%/180 μm	Yang H. *et al.* (2002), Yang J. *et al.* (2002), Taqvi & Roy (2006) and Hwang do *et al.* (2008)
macroporous collagen carriers	human fibroblasts and CB $CD34^+$, CHO-K1, BHK-21, bovine endothelial and murine BM cells	yes	yes	pore size: large rectangular pores 100–1000 μm	Wang *et al.* (1995), Itoh *et al.* (2001) and Panoskaltsis *et al.* (2005)
cellulose porous microspheres	stromal cells, osteoclasts, murine BM and human BM MNCs	yes	yes	surface area: 2.8 $m^2\ g^{-1}$; pore size: 100 μm	Tomimori *et al.* (2000) and Mantalaris *et al.* (2004*a*,*b*)
porous biomatrix (Cellfoam)	human BM $CD34^+$ cells, haemopoietic progenitor cells (HPCs), 5/9 Ma3-18	no	no	porosity/pore size: 80%/NR	Banu *et al.* (2001)
tantalum-coated porous biomaterial (TCPB)	human $CD34^+$ HSC and BM stromal cells	no	no	porosity/pore size: 90%/300 μm	Rosenzweig *et al.* (1997) and Bagley *et al.* (1999)
polyester non-woven fabric porous disc carriers (Fibracel)	murine BM and stroma cells	yes	FDA-validatable in their original form	surface area: 0.12 $m^2\ g^{-1}$; 6.5 cm^2 per disc	Takagi *et al.* (1999) and Tomimori *et al.* (2000)
nylon filtration screen	human BM mononuclear cells (MNCs)	yes	FDA-validatable in their original form	porosity/pore size: NR/210 μm	Naughton *et al.* (1991)
porous gelatin microspheres (CultiSpher-G)	human BM stroma and MNCs, human nasal chrondrocytes	yes	FDA-validatable in their original form	porosity/pore size: NR/5–15 μm	Xiong *et al.* (2002)
non-woven polyethylene terephthalate (PET)	human CB MNCs and $CD34^+$	yes	yes	porosity/pore size: 85%/10–60 μm	Li *et al.* (2001)
porous polyvinyl format (PVF)	murine BM cells and hepatocytes	no	no	porosity/pore size: 90%/130 μm	Tun *et al.* (2002)

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Biocompatibility is also an important factor in the design of synthetic scaffolds, especially for clinical applications. Furthermore, in the event of biodegradable scaffolds, the degradation rate can induce inflammation due to the localized acidity of hydrolysed products as in the case of poly(L-lactic-*co*-glycolic acid) and needs to be taken into consideration (Uebersax *et al.* 2006). Finally, the lack of cell recognition signals and the low surface energy or the hydrophobicity of many synthetic polymers impose many challenges and constrains (Yang J. *et al.* 2002). Whereas past wisdom required bioinert scaffolds, the requirement for engineered bioactivity is essential to achieve proper organogenesis (Shin *et al.* 2003). Specifically, synthetic polymers have been hybridized with fibres and proteins to combine the distinct advantages of each for cell growth and interaction (Dunn *et al.* 1997; Chen *et al.* 2004; Vance *et al.* 2004; Battista *et al.* 2005). The use of cell adhesion peptides (RGDS, KQAGDV and VAPG) has also been widely spread although some studies show that peptides initially increase cell attachment; however, they affect proliferation and matrix production (Mann & West 2002).

Diverse techniques are available to produce scaffolds with a network of channels and interconnected pores, with different pore sizes and surface area to volume ratio, biocompatible and hybridized with various molecules. A novel microscale three-dimensional technique was used in an attempt to recreate the mechanical properties of a native articular cartilage where polyglycolic acid (PGA) yarns were weaved into an orthotropic porous textile (Moutos 2006). The investigation showed that three-dimensional woven scaffolds can be designed to mimic the articular cartilage with anisotropic, nonlinear and viscoelastic properties. Additionally, numerous groups have also tried to mimic bone marrow using scaffolds derived from natural materials, such as collagen carriers (Wang *et al.* 1995), cellulose porous microspheres (Tomimori *et al.* 2000; Mantalaris *et al.* 2004*a*) or from polymers such as porous biomatrix (Cellfoam) (Banu *et al.* 2001), tantalum-coated porous biomaterial (TCPB) (Bagley *et al.* 1999), polyester non-woven fabric porous disc carriers (Fibra-cel) (Sasaki *et al.* 2003), nylon filtration screen (Naughton *et al.* 1991), porous gelatin microspheres (CultiSpher G) (Xiong *et al.* 2002), non-woven polyethylene terephthalate (PET) (Li *et al.* 2001) or porous polyvinyl format (PVF) (Tun *et al.* 2002). Other techniques used to create scaffolds include microfabrication and solid free form formation using micro-to-nanoscale features; examples include photopatterned hydrogels or microfabricated scaffolds with large rectangular pores (Liu & Bhatia 2002). Three-dimensional scaffolds that express structural and physiological features can also be designed to resemble native cardiac muscle using a variety of materials such as foetal or neonatal rat cardiac myocytes on collagen fibres (Akins *et al.* 1999) or porous collagen scaffolds (Freed *et al.* 2006).

Supplying nutrients to the seeded cells in large constructs needs to be considered when designing scaffolds; thin scaffolds (less than 100 µm) are feasible without an internal blood/nutrient supply, but thicker scaffolds (more than 100 µm) need to be engineered to contain a 'capillary network' as part of the structure. Angiogenesis can be induced by the addition of growth factors such as vascular epithelial growth factor (VEGF) that promotes vascularization (Soker *et al.* 2000). An alternative to overcome the problem is to design materials that induce vascularization even without the use of growth factors; some examples include the use of expanded PTFE (ePTFE; Boswell & Williams 1999), porous polyvinyl alcohol (PVA; Sharkawy *et al.* 1998) and polyacrylate containing traces of methacrylic acid (MMA; Gorbet *et al.* 2003).

The hESCs have been differentiated on polymeric scaffolds designed to support complex tissue structures resulting in the formation of structures with the characteristics of neural tissues, cartilage and liver, as well as a network of blood vessel-like tubules (Levenberg *et al.* 2003), suggesting that the ESCs might be able to generate histologically complete tissue constructs (Rippon & Bishop 2004). In addition, the use of hydrogels represents another useful method for *in vitro* organogenesis. Traditionally, alginate has been extensively used as it possesses good characteristics, such as low immunogenicity and controllable biodegradability, for tissue engineering applications. Furthermore, alginate hydrogels produce low mechanical or frictional irritation to the surrounding tissue, can be physically and chemically modified for specific surgical applications, and have high permeability for transport of nutrients and metabolites (Chia *et al.* 2000; Uludag *et al.* 2000; Bienaime *et al.* 2003). Magyar *et al.* (2001) encapsulated mESCs that were capable of forming embryoid bodies, followed by smooth muscle lineage differentiation. Randle *et al.* (2007) demonstrated a reproducible bioprocess for the production of osteogenic cells from mESCs, integrating several laborious culture steps within a bioreactor. Chondrogenic differentiation from MSCs encapsulated in alginate beads has also been reported (Ma *et al.* 2003). As with other biomaterials, the chemical and physical properties of the hydrogels may be altered to improve tissue development (Elisseeff *et al.* 2005). A combination of alginate with gelatin has been found to provide a biodegradable delivery vehicle for tissue engineering applications (Balakrishnan & Jayakrishnan 2005).

2.1.4. Mechanical stimulation. Mechanical stimuli on cells provide an additional dimension to the complexity of the microenvironment that is often overlooked in cell culture systems. Mechanical stress-induced changes in the ECM alters the cell surface directly by deforming integrin-binding sites resulting in mechanobiological responses that depend greatly on the type of mechanical loading, the type of cell and the location where the stimulus was applied. For instance, the application of mechanical stretching on myofibroblasts grown in three-dimensional porous scaffolds induced the differentiation into smooth muscle cells with a consistent cellular alignment after a certain time course of cyclic strain (Cha *et al.* 2006). Similarly, physiological deformational loading applied to hydrogels seeded with chondrocytes

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(Hung *et al.* 2004) or embryonic mesenchymal progenitor cells (Terraciano *et al.* 2007) demonstrated functional cartilage formation *in vitro,* which had mechanical properties corresponding to those of the native tissue as well as increased gene expression and ECM formation. Consequently, the ability to manipulate and control cell adhesion and function through specific ECM–integrin interactions and mechanical stimulation suggest an alternative and economical way of guiding stem cell differentiation *in vitro* with potentially minimal deployment of expensive growth factors (Hayashi *et al.* 2007).

2.1.5. Bioreactors. The successful transfer of stem cell technology and cellular products into widespread clinical applications needs to address issues of cost, automation, standardization and generation of clinically relevant cell numbers of high quality. Laboratories and industry alike have dealt with similar problems in the past through the use of bioreactors. Consequently, stem cell bioprocessing will involve the use of these specialized devices that aim to facilitate mass transport, high cell density, monitoring and feedback and tissue-specific functional specialization, thus mimicking the ultimate bioreactors, which are the tissues/organs within the human body. An optimal and universal system for stem cell culture does not exist; however, bioreactor development throughout the last 40 years has advanced the technology considerably (table 2).

As previously discussed, adult stem cells are found in relatively low numbers while large numbers are usually required for clinical applications. This highlights the need for the *in vitro* expansion of stem cells prior to their commitment into tissue-specific applications. The potential of bioreactors to address this is demonstrated by their capacity to support high cell densities in relatively small volumes, while the scaling up of the design, given mass transfer limitations, will depend on the type of bioreactor chosen. Traditionally, culture of stem cells is performed on flat two-dimensional surfaces that are used to support the growth of the cells (Dexter *et al.* 1977). Well-plates, tissue-culture flasks (T-flasks; Mellado-Damas *et al.* 1999; Liu *et al.* 2006) and gas-permeable blood bags (Collins *et al.* 1998) are widely used in stem cell bioprocessing due to their simplicity, ease of handling and low cost, making them the ideal choice for research screening purposes as well as for engineering simple tissues, such as skin, bone and cartilage (Bilodeau & Mantovani 2006). However, the lack of online monitoring, limitations in scaling-up due to the limited surface area per volume, as well as their inability to support complex cellular growth configurations render two-dimensional surface-limited systems inadequate for biomanufacturing and clinical applications. Three-dimensional culture systems that would closely resemble the *in vivo* conditions by accounting for the cell–cell, cell–matrix and cell–growth factor interactions (Mantalaris *et al.* 2004*b*) are required in many clinically relevant cases. Consequently, a variety of matrices, such as nylon screens (Naughton *et al.* 1991) as well as other natural or synthetic scaffolds, as described earlier, were used to provide support for cellular growth, proving to be more efficient than their two-dimensional counterparts (Mantalaris *et al.* 2004*b*) and allow the development of three-dimensional constructs (Ott *et al.* 2008). However, three-dimensional cultures with their increased available surface area for cellular attachment and growth, higher cell density and the ability for higher cell expansion face increased mass transport limitations.

Static bioreactors (Sardonini & Wu 1993), in which the 'ingredients'—cells, nutrients, metabolites, oxygen and other important molecules—experience mass transport that is exclusively through the process of diffusion, result in an inhomogeneous environment that can only support low cell densities and has a low total cell output (Panoskaltsis *et al.* 2005). To overcome the mass transport limitations of static cultures, bioreactors that can accommodate dynamic culture conditions are used. Primarily, perfusion and stirring have been the main means for enhancing mass transport. Stirred suspension bioreactors require careful impeller design to avoid high shear stress that can damage the cells (Zandstra *et al.* 1994), can be operated either in batch or continuous mode, and result in at least a 10-fold increase in cell density compared with the traditional methods. The scaling-up is usually straightforward due to the very good mass transport achieved by stirring. However, the flow environment created by the impeller renders them less suitable for the support of three-dimensional constructs (Nielsen 1999), although the use of porous microcarrier beads has been considered and studied (Zandstra *et al.* 1994). Several perfusion bioreactors have been designed to achieve a much lower shear stress environment as well as enhanced mass transport that facilitates the supply of nutrient and the removal of metabolites—perfusion rates having to be optimized based on cell type. However, different cell types have different sensitivities/necessities in terms of the shear stress, which is an important parameter in the design of bioreactors (King & Miller 2007). Shear stress is defined as the force exerted over the cells due to the flow of the media (Chen & Hu 2006), and a low rate has been described to result in cell clumping on aggregation supporting EB cultures (leading to lower mass transport to the cells; King & Miller 2007), while high rates could be deleterious for the cells. Thus, an optimal fluid velocity promoting the proper shear stress for the cell type being cultured is crucial. As an example, mammary epithelial stem cells aggregate cultures that have an optimal shear stress of $2\,\mathrm{dyn.\,cm^{-2}}$ (King & Miller 2007), while endothelial cells can support shear stresses of the order of 20–30 $\mathrm{dyn.\,cm^{-2}}$ (Sarkar *et al.* 2007). On the other hand, it has been described that the mechanical stimuli promoted this way can be beneficial for certain cell types: shear stresses of the order of $15\,\mathrm{dyn.\,cm^{-2}}$ magnitude have promoted differentiation of ESCs towards the lineage of endothelial cells, when compared with static controls (Ahsan & Nerem 2006). Several bioreactors have been designed for promoting this scenario *in vitro,* by promoting controlled shear stress levels: using dynamic tension for growing and developing cardiomyocytes, MSCs, skeletal muscle and macrophages; compression for chondrocytes; and

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Table 2. Cell culture systems. (n.a. not available)

bioreactor type	ease of harvesting	ease of monitoring	ease of scale-up	shear stress (dyn. cm^{-2})	mass transfer	culture surface area ($cm^2\,l^{-1}$)	cell output (cells $\times 10^6\,ml^{-1}$)	operation		standard culture environment		references
								batch	perf	three dimensional	two dimensional	
static culture (tissue culture flask)	high	low	low	0	low	290	0.1	yes	no	no	yes	Chaudhuri & Al-Rubeai (2005) and Lemoli *et al.* (2005)
permeable blood bag (wave bioreactor)	high	medium	high	0.1–0.5	medium	n.a.	10–20	yes	yes	no	yes	Singh (1999) and Ohashi *et al.* (2001)
stirred (or in suspension)	high	high	high	2–40	high	2800	1–10	yes	yes	yes	yes	Jelinek *et al.* (2002), Chaudhuri & Al-Rubeai (2005), Portner *et al.* (2005) and Goodwin *et al.* (2007)
airlift	medium	high	medium	10–30	high	2800	0.5	yes	yes	yes	yes	Merchuk & Berzin (1995) and Cabral (2001)
packed bed (Bellocell)	medium	low	high	1–5	medium	18 000	1.5–200	no	yes	yes	yes	Perry & Wang (1989), Jelinek *et al.* (2002), Chaudhuri & Al-Rubeai (2005), Portner *et al.* (2005) and Meuwly *et al.* (2007)
fluidized bed	medium	low	high	3–6	medium	25 000–75 000	5–100	no	yes	yes	yes	Reiter *et al.* (1991), Nakhla & Suidan (2002), Chaudhuri & Al-Rubeai (2005) and Portner *et al.* (2005)
perfusion chamber (Aastrom Replicell)	medium	medium	medium	1–5	medium	18 000	10–100	no	yes	no	yes	Palsson *et al.* (1997) and Chaudhuri & Al-Rubeai (2005)
grooved bioreactor	medium	medium	medium	0.1–0.5	medium	18 000–20 000	10–100	no	yes	no	yes	Horner *et al.* (1998) and Chaudhuri & Al-Rubeai (2005)

(*Continued.*)

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Table 2. (*Continued.*)

bioreactor type	ease of harvesting	ease of monitoring	ease of scale-up	shear stress (dyn. cm^{-2})	mass transfer	culture surface area (cm^2 l^{-1})	cell output (cells $\times 10^6$ ml^{-1})	operation		standard culture environment		references
								batch	perf	three dimensional	two dimensional	
perfusion hollow fibre (PluriX)	low	low	medium	0	medium	100 000–200 000	100–200	no	yes	yes	yes	Martin *et al.* (2004) and Chaudhuri & Al-Rubeai (2005)
rotating wall vessel (Synthecon)	medium	medium	low	0.5–2	medium	18 000–22 000	n.a.	no	yes	yes	yes	Martin *et al.* (2004), Portner *et al.* (2005), Liu *et al.* (2006) and Goodwin *et al.* (2007)

hydrodynamic pressure for bone cartilage (Korossis *et al.* 2005). Thus, optimal shear stress levels will depend largely on the cell type being grown/differentiated, as each type will have different sensitivities and/or stimulation needs (Palsson *et al.* 1998).

When low shear stress but high fluid velocities are required, a flat bed-grooved bioreactor can be used with cells growing within the grooves being protected from the flow. Such a system has been used for the expansion and maintenance of colony-forming units granulocyte–macrophage (CFU-GM), progenitor cells and long-term culture-initiating cells (LTC-IC) in the absence of stromal cells (Cabral 2001). Perfusion bioreactors also have the advantage of simple automation providing continuous and automated feeding of the cultures. Aastrom Biosciences, Inc., have developed a design whereby the cells are injected into a disposable cassette and grown on top of a previously established layer of stromal cells with nutrients being continually perfused to the cassette, while a chamber, located just above, is filled with oxygen that diffuses to the cassette through a liquid-impervious/gas-permeable membrane (Armstrong *et al.* 1996, 1999, 2000; Palsson *et al.* 1997). This system has been used to expand bone marrow mononuclear cells and umbilical CB cells for clinical applications. Several other bioreactor designs have been implemented with varying degrees of success. Hollow-fibre bioreactors can achieve a low shear stress environment with enhanced mass transport properties. They contain a number of hollow fibres, which are responsible for carrying nutrients and oxygen to the cells, by diffusing through the selective hollow-fibre membrane, thus avoiding the shear stresses caused by perfusion. At the same time, the inclusion of membrane technology in these designs greatly increases the surface area per volume available for cell growth (over 350 times that of a normal T-flask), allowing higher cell densities while still promoting efficient mass transfer of nutrients, oxygen and other important signalling molecules. Hollow-fibre bioreactors face certain limitations, such as decrease in mass transfer through the membranes due to cells growing in their periphery, especially in hollow-fibre bioreactors that support three-dimensional growth through the use of scaffolds (Yu *et al.* 2003). Other designs include: (i) packed or fluidized bed bioreactors (widely used for the expansion of hepatocytes, cardiocytes, osteoblasts and others; Portner *et al.* 2005), (ii) the rotating wall vessel (successful in the culture of HSCs, chondrocytes, cardiac cells, various tumour cells and others), which is a suspension culture adapted for lower shear stresses (Hammond & Hammond 2001; Liu *et al.* 2006), and (iii) the wave bioreactor, which uses wave agitation induced by a rocking motion to provide good nutrient distribution, off-bottom suspension and excellent oxygen transfer without damaging fluid shear or gas bubbles (Singh 1999; Ohashi *et al.* 2001). Unlike other cell culture systems, such as spinners, hollow-fibre bioreactors and roller bottles, scale-up is simple, and has been demonstrated up to 100 l of culture volume. The main differences in bioreactor designs can be associated with mass transport (addressed by diffusion, perfusion or bubbling), shear stresses (by developing

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ways to enhance mass transfer without increasing flow velocity or by which mechanical stimulation can be achieved), the ability to support three-dimensional constructs or even the end purpose (research versus large scale, cell type characteristics, etc.).

With respect to industry, automated cell culture allows faster and more accurate monitoring and analysis of cell cultures than comparable traditional methods. Events such as apoptosis, cell division, cellular movement and attachment can be continuously monitored and observed and recorded for up to several weeks (Narkilahti *et al.* 2007). Terstegge and colleagues have recently described a system for semiautomated cell plating, media change, growth factor addition and cell harvesting in both human and mouse ESCs (Terstegge *et al.* 2007). Furthermore, Narkilahti and colleagues, through monitoring dynamic growth of hESCs in both automated and conventional culture systems, reported that automated cell culture and analysis provides the optimal tool for the evaluation of hESC culture, allowing continuous monitoring of living cells that would be impossible to discover by conventional methods (Narkilahti *et al.* 2007). Furthermore, the expansion and/or differentiation of ESCs in a variety of bioreactors have been investigated. Stirred and perfusion bioreactors maintain significant advantages over static culture due to the homogeneous environment they provide and the control over crucial culture parameters, such as oxygen and nutrient supply, pH and metabolite removal (King & Miller 2007). In general, perfusion, or frequent feeding, enhances culture performance by replacing exhausted nutrients and removing inhibitory metabolic by-products (King & Miller 2007).

For the expansion of haemopoietic progenitor cells (CFU-GM), continuous perfusion bioreactors and automated pH and DO controllers were able to maintain culture conditions within desired ranges while minimizing nutrient step changes and physical disruptions. Compared with static cultures, these systems demonstrated faster cell growth. In addition, the expansion of primitive haemopoietic progenitors (LTC-IC) was obtained only with synergistic cytokine combinations (e.g. IL-3/IL-6 and SCF) and perfusion (Koller *et al.* 1993*a*). Culture parameters, such as gas-phase oxygen concentration, seeding density and time of cell harvest were also found to play important roles in the expansion of various types of haemopoietic progenitors in perfusion bioreactor systems (Palsson *et al.* 1993). To determine the feasibility of producing a large dose of haemopoietic cells through *ex vivo* expansion, Koller *et al.* scaled up their perfusion bioreactor 10-fold by employing 10 culture chambers in the system. Their results showed that more than three billion cells containing 12 million CFU-GM progenitors (the target number of CFU-GM routinely sought to support haemopoietic engraftment following autologous bone marrow transplantation is 15 million) were reproducibly generated from the equivalent of just 10–15 ml bone marrow aspirate and, more importantly, the number of primitive LTC-IC (stem cells) consistently increased with time in all cultures resulting in a 7.5-fold expansion. Consequently, this report represented the first published quantitative evidence of human stem cell expansion in an *ex vivo* bone marrow culture system, and such *ex vivo* expansion may have direct applications in clinical and experimental bone marrow transplantation (Koller *et al.* 1993*b*). As for the *ex vivo* expansion of PB mononuclear cells (PB MNCs), Sandstrom *et al.* (2005) investigated how $CD34^+$ selection and/or perfusion affect the performance of PB MNC cultures. Their results indicated that while perfusion supported higher LTC-IC numbers for both MNC and $CD34^+$ cell cultures, the selection of $CD34^+$ cells was not required to obtain extensive CFU-GM expansion from PB. In fact, unselected MNC cultures produced 1.5-, 2.6- and 2.1-fold more total cells, CFU-GM and LTC-IC, respectively, than the same sample selected and cultured as $CD34^+$ cells (Sandstrom *et al.* 1995). To date, *ex vivo* expansion of CB cells has demonstrated their promising expansion capability, far exceeding that of PB. After three weeks in culture, CB $CD34^+$ cell preparations continued to proliferate with the average cell dividing more than five times with a retained engraftment potential. By contrast, PB $CD34^+$ cell preparations did not show any continued growth after just a week in culture and had lost engraftment potential (Tanavde *et al.* 2002). Clinical trials have highlighted the potential improvements to engraftment through *ex vivo* expansion of CB stem cells (McNiece *et al.* 2000; Elizabeth *et al.* 2002). However, these studies relied on the expansion of cells in static culture systems; the more recent use of bioreactors, such as rotating wall vessels, has been trialled with considerably greater success with respect to the expansion of CB stem cells (Liu *et al.* 2006) and highlights the significant gains possible with the advancement of bioprocessing.

The complexity and fragmentation of ESC culture has directed several groups to demonstrate that mouse EBs can be formed directly from enzymatically dissociated mESCs in stirred bioreactors (Schroeder *et al.* 2005) or a rotary cell-culturing system (E *et al.* 2006). Mouse EBs can also be formed in stirred vessels by removing LIF from mESC aggregates produced in the same vessels (Fok & Zandstra 2005) or by encapsulating mESC aggregates in agarose beads (Bauwens *et al.* 2005). After formation, the EBs grew in size and retained the differentiation potential, such as cardiomyocyte differentiation (Bauwens *et al.* 2005; Schroeder *et al.* 2005). Tightly controlled mixing conditions have proven critical in these stirred suspension bioreactors in producing EBs of uniform size and quality. Furthermore, more recently, an integrated bioprocess for the culture, expansion and differentiation of mESCs has been demonstrated (Randle *et al.* 2007). In this system, encapsulated undifferentiated mESCs are cultured in rotary cell culture systems in a single-step process that does not require the formation of EBs or the disruption of cell aggregates, resulting in the formation of three-dimensional mineralized constructs (Randle *et al.* 2007). Differentiation of encapsulated ESCs has been used successfully for a wide range of tissue engineering applications and into several specific lineages. Dang *et al.* (2004) demonstrated the controllable and scalable culture of mouse and human ESCs

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Table 3. Estimated costs of cell culture systems. [The start-up costs producing 10^9 cells are determined based on the published cell outputs per volume (table 2) and their respective costs. n.a.: prices were not provided by the companies.]

bioreactor type	bioreactor price per volume ($ ml^{-1})	start-up costs ($ per 10^9cells)	automatable operation	company
T-flask	0.15	1500	no	Corning
permeable blood bag (Wave bioreactor)	0.25	5	yes	GE Healthcare
stirred bioreactor (Cytostir)	0.3	30	yes	Kimble/Kontes
airlift (Cytolift)	0.9	1800	yes	Kimble/Kontes
packed bed (Bellocell)	3.0	60	yes	Cesco Bioengineering Co., Ltd.
fluidized bed (System 10)	n.a.	n.a.	yes	
perfusion chamber (Aastrom Replicell)	43	430	yes	Aastrom Biosciences Inc.
grooved bioreactor	40–45	400–450	yes	Baxter International
perfusion hollow fibre (Fibercell)	3.3	16.5	yes	FiberCell Systems, Inc.
rotating wall vessel (Synthecon)	25	250	yes	Synthecon, Inc.

encapsulated in agarose hydrogel capsules. Encapsulation permitted the use of high cell-density culture and enabled EB formation and differentiation to haemopoietic cells in a suspension bioreactor (Dang *et al.* 2004).

As already mentioned, the diversity of stem cell sources and their respective culture conditions means that no one bioreactor system is suitable for all stem cells. Therefore, a comparison of bioreactor suitability for their expansion and associated cost is somewhat futile. However, with respect to industry and commercialization, cost is a consideration of great importance. Table 3 examines current stem cell output examples from literature and determines an estimated cost in achieving a target cell number following expansion. There are several important considerations that should be noted in view of the estimated costs. First, substantial variability with respect to cell output exists among the different culture systems, which is a result of differing starting materials and protocols between laboratories. For example, the stirred bioreactor can be used for either the expansion of cells that grow in suspension, or adherent cells (with the addition of microcarriers or other appropriate substrates). This changes the surface/volumetric area that can be occupied by cells and, therefore, the obtainable outputs. Additionally, for some bioreactor systems, little work has been done with stem cell cultures, and the published outputs may only represent a fraction of what may be achievable with further development.

The ultimate bioreactors, namely the organs/tissues within the body, share common operational characteristics. Specifically, mass transport in the form of circulation and diffusion to the cells is excellent—no cell is located more than 400 µm away from blood supply (size, shape and structure being determined by function). Subsequently, the cell density supported is high (can reach billions of cells ml^{-1}) and the spatial arrangement of cells is critical for proper organogenesis. Monitoring is performed real time, online and *in situ* with signal processing and feedback being executed coherently. Additionally, these *in vivo* bioreactors are specialized, performing different functions in a cohesive and integrated manner. Hence, the optimal bioreactors achieve production not by embracing traditional scale-up principles (larger bioreactors) but through process intensification, modularity, specialization and integration. The use of bioreactors is critical for TERM applications for control, scale-up, automation and regulatory reasons. The laboratory practices of cell/tissue culture in dishes/flasks would have to be transformed, even in the discovery phase so that bioprocesses are developed and can be directly applied to the clinic. The variability in the results and the lack in standardization represent a current obstacle. Modular, integrated and interconnected culture systems that are fabricated from standard components and are amenable to automation are also required. Specifically, modules that integrate inoculation, culture, separation and harvesting need to be developed. Furthermore, specialized culture modules that are able to provide the required mechanical stimulation and to produce the final cellular product with desired features, such as three-dimensional structure, need to be developed. Certainly, a universal bioreactor does not exist and manufacturing should be directed towards modularity and integration. The lessons learned from the biopharmaceutical industry are valuable but, in the view of the authors, do not apply to scale-up in the traditional manner (Lim *et al.* 2007).

2.2. Process requirements

Ultimately, the integration of the various *process components* will be required in order to achieve a clinically relevant product through a regulated and controlled bioprocess that is reproducible, standardized, automatable (when needed), integrated and certified. Furthermore, *process requirements* will also be, by the nature of the problem, application specific.

2.2.1. Bioprocess monitoring and quality control. Stem cell culture complexity, heterogeneity of cell types and the inherent variability in process performance over time and between batches render the control in bioprocess culture systems a tremendously challenging task (Lim *et al.* 2007). However, bioprocess control in the manufacturing of biopharmaceutical products is critical in maintaining high product quality and consistency. Efforts are continuously being made in

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this area to improve process monitoring and control techniques in complex bioprocesses. Specifically, the process analytical technology (PAT) initiative was established to promote better understanding and control of manufacturing bioprocesses through the use of process and end point monitoring tools, process control tools, multivariate data acquisition and analysis tools, process analytical chemistry tools and knowledge management tools (Junker & Wang 2006). The goal is not only to gain better insight into the bioprocesses of interest, but also to implement tight process control and reduce process variability. Integration of various engineering tools is therefore necessary to achieve these goals. With advances in sensor, optical and computer technology, stronger emphasis is being placed on the integration of *online, real time, in situ* monitoring systems. However, certain challenges in sensor technology still remain, such as the need for novel sensors for the detection of cytokines and cell density, overall sensitivity and stability of *in situ* biosensors, and sensor multiplexing capabilities (Clementschitsch & Bayer 2006). Other sophisticated measurement techniques, such as spectroscopy, though powerful and comprehensive, are often expensive and do not give direct measurements of process variables or provide insights into the cell's metabolite state (Clementschitsch & Bayer 2006). Advancements in computer technology and the availability of sophisticated data management systems capable of processing high volumes of information have made it easier for the implementation of sophisticated control systems (Junker & Wang 2006). Much of this work relies on the use of multivariate data analysis and principal component analysis (PCA) to generate models for the detection of process abnormalities and control of process variations (Gunther *et al.* 2007). This wide array of multivariate techniques has provided the necessary tools for enhanced process understanding through the identification of critical sources of process variation and the generation of predictive control models. Consequently, continued emphasis on improving and generating novel measurement technologies, along with the required product quality verification, will ultimately facilitate bioprocess control that generates precise, reliable and high-quality products for clinical applications.

2.2.2. Design of experiments. Design of experiments (DOE) is a powerful engineering tool mainly used for process characterization and optimization that has been widely applied in the materials, chemical, pharmaceutical and semiconductor industries. It is a systematic methodology for investigating a process using minimum effort to obtain the maximum information (Montgomery 2001). Process investigations performed via DOE are, therefore, highly efficient and informative. Specifically, one of the distinct advantages in using DOE is the ability of the design to reveal both individualistic and interactive effects for a process using the minimum number of experimental runs, reducing time and cost, and, more importantly, providing an accurate and quantitative characterization of the process of interest. Traditional experimentation methods, such as dose–response studies, often do not paint a 'true' picture of the story as they only examine changes due to one factor at a time, while other corresponding process factors remain unaltered, thus masking interactions. When studying a complex system, a systematic approach is required to obtain useful information and determine optimal operating conditions (Lim *et al.* 2007). Typically, this would involve the following three steps: screening; characterization; and optimization, using the appropriate design(s) in each stage. The objective *process screening* is to rapidly identify factors that have a significant influence on the process and correctly discriminate against factors that have little or no influence at all. Some of the commonly used designs for such investigations are the fractional factorial design and Plackett–Burman design (Montgomery 2001; Myers & Montgomery 2002), which enable rapid screening of a large number of factors using a very small number of experiments: in the Plackett–Burman design, for example, a 12-run experiment can screen up to 11 factors. In *process characterization*, the goal is to obtain a more detailed and quantitative description about the process by way of three-dimensional surface response plots. Common designs include the central composite design (CCD) and Box–Behnken design (Montgomery 2001; Myers & Montgomery 2002), which reveal process relationships that can be described using a quadratic or cubic model. In *process optimization*, the generation of three-dimensional plots generates optimal operating regimes and yields the best conditions for process optimization. The outcome of this systematic framework is a reproducible and statistically valid elucidation of bioprocesses that do not require *a priori* assumptions for the process of interest. To date, the use of DOE in stem cell bioprocessing has been somewhat limited; much of the research still uses traditional dose–response methods. However, a handful of investigators have been quite successful in using DOE for stem cell culture studies. These include the use of fractional factorial designs to perform screening experiments (Yao *et al.* 2003, 2004), and the use of central-composite or full factorial designs to perform process characterization (Zandstra *et al.* 1997*a*,*b*; Cortin *et al.* 2005). However, full process characterization, which considers all the process factors including physiochemical parameters, nutrient, metabolite and growth factor concentrations, has not yet been established. Owing to the complexity of these interactions and lack of sensor technology to monitor them simultaneously in culture, the investigation of these factors in combination is not straightforward. The successful application of DOE methodologies and monitoring techniques to resolve the complexity in stem cell bioprocessing will not only yield invaluable information for stem cell cultures but also provide a novel approach for other cell culture studies.

2.2.3. Bioprocess modelling. As elaborated earlier, stem cell bioprocessing will require the design and integration of different processes and systems that would be

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application specific. Consequently, designing such application-specific systems or scaling up of a system to produce the necessary cell numbers requires knowledge of the momentum and mass transport characteristics of the system(s) under various operating conditions, which will also be useful for the subsequent successful operation of the system (Williams *et al.* 2002). Such modelling can be implemented by employing computational fluid dynamic (CFD) software packages and will facilitate the optimization of culture system specifications and operating conditions, such as porosity and perfusion flow rate, so as to achieve the best possible conditions for cell growth (Begley & Kleis 2000). A number of mathematical models have been developed to study the fluid dynamics and nutrient distribution in perfusion bioreactors (Horner *et al.* 1998; Begley & Kleis 2000, 2002; Williams *et al.* 2002; Pathi *et al.* 2005; Coletti *et al.* 2006), as well as haemopoietic cell growth dynamics (Ching-An Peng 1996; Hevehan *et al.* 2000*c*; McNiece & Briddell 2001; da Silva *et al.* 2003). For instance, oxygen supply in bioreactors was addressed by the comparison of convective and diffusive nutrient supply (Pathi *et al.* 2005). Bioreactor design operating parameters, such as the perfusion rate, liquid depth and length of culture, have been investigated to determine their effect on long-term cell culture, thus enabling optimal bioreactor design. More recently, the simulation of a realistic multilineage *in vitro* HSC expansion culture has also been presented (Ma *et al.* 2007). A culture period of 14 days yielded an increase in progenitor cells (CFU-GMs) similar to experimental data, matching the spatial variation of oxygen concentrations representative of the multiple cell lineages in bone marrow *in vivo* to the oxygen distribution within the bioreactor sufficient to support the 14-day expansion of HSCs (Mukhopadhyay *et al.* 2004). These 'realistic' stem cell culture simulations provide relevant insights into understanding the stem cell expansion while also reducing experimental cost. Ultimately, modelling can be used to provide the guidelines for 'designer tissues' engineering *in vitro*.

2.2.4. Product/process characterization—proteomic and genomic analyses. Genomic technologies, such as microarray chromatin immunoprecipitation (ChIP–chip) and ChIP sequencing, allow for the total characterization of transcription factors and other DNA-bound proteins in a high-throughput and cost-effective fashion. Comparative genomic hybridization (CGH) can determine total genomic chromosomal losses or gain with respect to a control and test genome. Such technologies are powerful tools to help elucidate the molecular mechanisms that regulate the formation, self-renewal and differentiation of stem cells. The influence of genomics in stem cell research has been eloquently demonstrated with the induced expression of four important transcription factors. OCT4, SOX2, NANOG and LIN28 were shown to be sufficient to reprogramme mouse fibroblasts to undifferentiated, pluripotent stem cells (Takahashi & Yamanaka 2006; Maherali *et al.* 2007; Okita *et al.* 2007; Wernig *et al.* 2007). Subsequently, the same factors have been shown to have a comparable effect on human somatic cells, creating cells that meet all the criteria of ESCs with the exception of not being derived from embryos (Yu *et al.* 2007). Furthermore, comparative genomics not only helps elucidate important transcription factors during the growth and differentiation of stem cells, by observing changes in the transcriptome of the cell, but can also be used to determine the effect of drugs and growth factors on cells they are exposed to.

Similarly, proteomics is a tremendously important tool allowing proteome mapping, differential analyses and elucidation of signals and mechanisms, thus enabling the understanding of the complex biological processes and protein-regulated signalling pathways that constitute basic embryonic development and stem cell differentiation (Elliott *et al.* 2004; Nagano *et al.* 2005; Salasznyk *et al.* 2005; Baharvand *et al.* 2006; Hoffrogge *et al.* 2006; Van Hoof *et al.* 2006). Specifically, the proteome dataset of the mESC line, E14-1, has identified 1790 proteins, including nuclear proteins (Nagano *et al.* 2005). Similarly, the proteome profiles of three hESC lines are also being been analysed (Baharvand *et al.* 2006), which will result in the identification of proteins involved in protein synthesis, processing and destination, probably reflecting the ability of hESCs to remain undifferentiated or to rapidly differentiate. Proteome maps for the other stem cell lines have also been established, including hippocampal neural stem cells (Maurer *et al.* 2003), human adipose-derived stem cells (DeLany *et al.* 2005) and human umbilical CB MSCs (Feldmann *et al.* 2005). Proteomics has facilitated the elucidation of the complex environment provided by feeder cells (Lim & Bodnar 2002; Prowse *et al.* 2005) leading to the development of feeder-free, defined culture conditions, which are essential for the clinical applications of cellular products. It has identified key proteins involved in the maintenance of hESC pluripotency including Wnt, BMP/TGF-β1, activin/inhibin and insulin-like growth factor-1. Furthermore, proteomics is a powerful differential analysis technique. Several studies have compared differentiated and undifferentiated stem cells identifying candidate regulators of differentiation (Guo *et al.* 2001; DeLany *et al.* 2005; Wang & Gao 2005; Puente *et al.* 2006). Some of the proteins identified were exclusively expressed in undifferentiated ESCs suggesting possible use as ESC biomarkers, which will further facilitate standardization. Finally, stem cell regulation is also greatly influenced by culture parameters and signals, as discussed earlier. Proteomics can be used to characterize an environment that supports maintenance of undifferentiated stem cells and to help identify factors critical for their differentiation (Baharvand *et al.* 2007; Sze *et al.* 2007). In addition, more recently developed proteomic equipment enables us to optimize and control the quality of the manufacturing process for biopharmaceutical products. This can be usefully employed to monitor the bioprocess of stem cells for drug discovery and therapeutic applications in the future.

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2.2.5. Regulation. The clinical application of human stem cell products will require the whole process of cell separation, manipulation, culture, characterization, storage and delivery to be tightly controlled and satisfy strict regulatory requirements. The Food and Drug Administration (FDA) in the USA has divided cell therapies into two groups, commonly referred to as '361 products' and '351 products'. Traditional blood and bone marrow progenitor cell products as well as some tissues for transplantation are usually considered 361 products. Cells that are more than minimally manipulated or intended as a drug are considered 351 products. The 361 products typically have short processing times and require closed systems under good tissue practices (GTPs), whereas 351 products must be manufactured according to current GMP in addition to the GTP requirements. As most cell therapy protocols fall within the 351 products category, GMP facilities need to be taken into consideration when developing a cell therapy programme. Dietz *et al.* (2007) recently gave a very detailed practical guide on GMP facility development, including what is GMP, purpose of the GMP facility, general layout of GMP and some design considerations.

There are challenges relating to every aspect of the manufacture of cell therapy products. The raw material, which comes from the patient in case of autologous cell therapy, must be shipped in and processed in finite time. The shelf life of cellular products is commonly less than a few days. Transport devices must be developed both for biopsy material and the final product to ensure that they will not lose their functionality due to temperature or time. Final product packaging and design must be carefully developed and reviewed to meet all International Air Transport Association (IATA) requirements and regulatory requirements, apart from protecting the final product from contamination and ensuring its intended potency. In-process and final product testing must be carefully considered and designed. Development of rapid detection and quantitative assays are essential or the product may expire prior to release.

3. CONCLUSIONS

Bioprocessing and commercialization of stem cell/tissue-engineered products in regenerative medicine can translate breakthroughs from the research bench to the bedside of the patient. Though many of these tasks cannot be readily addressed and may require long-term commitment, some of the current challenges must remain the primary focus of our research and development. Process characterization and optimization is the key for any bioprocess start-up operation. Standardized operating procedures and know-how must be made possible for a process to be translated into a manufacturing operation. Improvements in the currently available process monitoring systems for bioreactors should be made for nutrients and metabolites, so that these key culture parameters can be monitored continuously and in real time for good process control. Future challenges in bioprocessing and manufacturing will include advanced and sophisticated monitoring platforms that allow monitoring at the cellular level. Completely integrated, modular, automated and controlled systems in a fully enclosed bioprocess operation from harvest to delivery will need to be considered. Ultimately, scale-up of stem cell/tissue-engineered bioprocesses, in the view of the authors, can be achieved by small-scale modular systems operating in an 'in-series and in-parallel' mode where overcapacity is considered and the whole process is addressed as a supply chain model. Scale-up can be delivered by lower infrastructure cost systems where the integration, modularity and parallel operation are the keys to the problem.

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