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REVIEW

Stem cell bioprocess engineering towards cGMP production and clinical applications

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Abstract Stem cells, including mesenchymal stem cells and pluripotent stem cells, are becoming an indispensable tool for various biomedical applications including drug discovery, disease modeling, and tissue engineering. Bioprocess engineering, targeting large scale production, provides a platform to generate a controlled microenvironment that could potentially recreate the stem cell niche to promote stem cell proliferation or lineage-specific differentiation. This survey aims at defining the characteristics of stem cell populations currently in use and the present-day limits in their applications for therapeutic purposes. Furthermore, a bioprocess engineering strategy based on bioreactors and 3-D cultures is discussed in order to achieve the improved stem cell yield, function, and safety required for production under current good manufacturing practices.

Keywords Stem cells · Bioprocess engineering · Bioreactor · Differentiation · Safety

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Introduction

In recent years, stem cells have generated a lot of excitement for their potential biomedical applications. The discovery of these unique cell populations has overturned certain scientific dogmas. For instance, the existence of mesenchymal stem cells (MSCs) in vivo has considerably changed the perception of the stromal cells (Pittenger et al. 1999), which are now tested in more than 300 clinical trials for treating various diseases (www.clinicaltrials.org). Moreover, the isolation of embryonic stem cells (ESCs) has enabled the development of new tools to study embryonic tissue morphogenesis (Thomson et al. 1998). The derivation of induced pluripotent stem cells (iPSCs) opens the era of disease modeling and drug screening, as in the example of cardiac toxicity testing (Takahashi et al. 2007; Mandenius et al. 2011). Furthermore, the capacities of both ESCs and iPSCs, known as pluripotent stem cells (PSCs), have been demonstrated in the restoration of most cell types.

Especially, the use of human ESC (hESC)-derived cells has been explored for treating patients with spinal cord injury and macular degeneration in Phase I clinical trials (Alper 2009; Schwartz et al. 2012). The possible use of PSCs in cell therapy has generated high hope for combatting incurable and degenerative diseases.

Consequently, there is an urgent need for large amounts of stem cells (e.g. 10^{10}–10^{12} per batch of production for PSC-derived cardiomyocytes) in a functional and safe state for their cost-effective use in therapy, drug discovery, and disease modeling (Serra et al. 2012). Traditional practices of cell culture in 2-D plastic dishes are clearly inadequate to supply large numbers of cells. In addition, 2-D cultures are not able to recreate a physiological environment similar to that of the original niche or to promote sufficient signaling for stem cell differentiation with high efficiency. Additionally there are increasing concerns regarding the tumorigenicity as well as the potential immunogenicity of stem cell populations. Bioprocess engineering approaches resulting from well-established practice in recombinant protein production or environmental science (e.g. bioremediation) could offer rational tools to alleviate the above limitations of ex vivo stem cell cultivation.

Focusing on the potential applicability of stem cells for clinical uses, this survey summarizes the key characteristics of stem cell populations, as well as the current hurdles in their use in a clinical setting. To overcome these hurdles, the operational objective and the tools of a bioprocess engineering strategy are discussed for large scale production of stem cell-derived progeny under current good manufacturing practices (cGMP) and for controlling stem cell population behavior to enable safe transplantation.

Stem cells and stem cell niches

Stem cells are defined as a cell population sharing self-renewal and differentiation potential along various distinct lineages (Watt and Hogan 2000). For instance, ESCs display unlimited self-renewal and differentiation potential along the three embryonic germ layers: ectoderm, mesoderm, and endoderm (Thomson et al. 1998). From somatic cells or progenitor cells, iPSCs can be generated by reprogramming the cells with pluripotent genes or even small molecules and these reprogrammed cells display similar characteristics to ESCs (Hou et al. 2013; Stadtfeld and Hochedlinger 2010). MSCs are adult stem cell populations that at least have the in vitro differentiation potential into adipocytes, chondrocytes, and osteoblasts (Pittenger et al. 1999; Augello et al. 2010). MSCs are also able to secrete trophic factors which can stimulate the endogenous progenitors after in vivo MSC transplantation, including immune-regulatory proteins, growth factors, pro-angiogenic factors and anti-scarring factors (Caplan and Dennis 2006; Wagner et al. 2009). Stem cells may also exist in cancers. Some cancer stem cells (CSCs) were shown to be able to differentiate towards a phenotype resembling their tissue of origin, in reverting their tumorigenicity (Massard et al. 2006; Takehara et al. 2011).

The stem cell identity is closely linked to a proper dynamic "niche", which is composed of a specific extracellular matrix (ECM) network, soluble endogenous and exogenous growth factors, and appropriate levels of metabolism-associated molecules such as oxygen to regulate cell proliferation or differentiation (Scadden 2006). Manipulating stem cell niches controls self-renewal versus lineage commitment as well as maintaining the differentiated cell functions (Lutolf and Blau 2009; Scadden 2006). For instance, modulation of mechanical forces (i.e. 1.5–15 dynes/cm^2) through ECM and hydrodynamic environment was found to affect the degree of ESC self-renewal and lineage commitment into three-germ layers (Przybyla and Voldman 2012; Wolfe et al. 2012). Hence, recreating the stem cell niche in vitro has been the focus of recent investigations in developing new culture systems.

Current limitations in the use of stem cells for therapeutic applications

Capability of large scale production of stem cells in a functional state

Stem cells are supposed to constitute a scarce cell population, and the readily obtainable number of a stem cell population for therapeutic use is limited. For example, hematopoietic stem cells (HSCs) represent a small percentage (0.1–0.3 %) of the bone marrow (BM) cells (Spangrude et al. 1988). Similarly, BM-MSCs were reported to represent a minor fraction of a

bone marrow aspirate (i.e. 0.001–0.01 %) (Pittenger et al. 1999). These primary stem cells have to be expanded in vitro to reach the clinical demand in cell number (e.g. 10^8–10^9 cells per patient). The initial derivation of ESCs and iPSCs was achieved from a small cell population as well. ESCs were derived from a minimal number of cells at the blastocyst stage, i.e. 14 inner cell masses were initially isolated to generate the first five ESC lines (Thomson et al. 1998). For iPSCs, the reprogramming efficiency is generally low, ranging from 0.001 to 1 % of the starting somatic cells (Stadtfeld and Hochedlinger 2010). After ESC and iPSC lines are established, they can be expanded indefinitely in theory. However, to fulfill the potential of the proliferation capacity of PSCs, large scale systems have to be developed to produce clinical-relevant quantity and quality of cells. Even for a low dose study (e.g. 10^6 cells per injection) using hESC-derived oligodendrocyte progenitor cells (OPCs), the cells required for multiple patients, safety testing, quality control, and the retained samples (e.g. samples used for the testing of sterility, endotoxin, identity/purity, impurity, stability, potency, etc.) would exceed a total of 10^9 cells for each batch of production. To use PSC-derived cardiomyocytes in drug screening or cell therapy (needs 10^9 cells per patient), large numbers of differentiated cells up to the order of 10^{10}–10^{12} per production batch are clearly required (Sharma et al. 2011).

In addition to meeting the quantity demand, stem cells or their derivatives need to maintain the desired purity and function. Especially for PSCs, the degree and heterogeneity of the differentiation affect the efficiency of stem cell implantation and these cells' in vivo therapeutic effects (Hedlund et al. 2008; Hwang et al. 2010). The difficulty of in vitro maturation of PSC-derived cells indicates that the currently used culture environment has not been able to recreate the in vivo-like stem cell niches, at least not fully predictably and consistently until now. Similarly, MSCs need to be primed in vitro to maximize their therapeutic effects, such as the secretion of trophic factors and pro-angiogenic factors (Wagner et al. 2009). Indeed, the limited performance of MSCs in clinical trials, e.g. for the treatment of chronic obstructive pulmonary disease (Weiss et al. 2013), indicates that the functions of MSCs have to be enhanced ex vivo. For instance, the MSC priming by hypoxia was found to enhance the pro-angiogenic properties when treating cardiovascular diseases (Wagner et al. 2009). All these considerations trigger the development of novel systems for large scale stem cell expansion and differentiation in a controlled manner.

The risks of immunogenicity and tumor formation

Stem cells have the risks of immunogenicity in transplantation. ESCs and iPSCs are not immune-privileged and are prone to immune rejection (Taylor et al. 2011; Dressel 2011; Zhao et al. 2011). Allogeneic MSCs have also been reported to retain some degree of immunogenicity by modifying the functions of innate and adaptive immunity (Griffin et al. 2010). Neural progenitors derived from teratocarcinoma have been shown to require strong immunosuppression (Hara et al. 2008). All these observations indicate that reducing the immune rejection is critical for the clinical applications of stem cells. The use of appropriate raw reagents (e.g. xeno-free) in culture systems, the characterization of stem cell populations prior to and after expansion, and the creation of stem cell banks based on human leukocyte antigens (HLA) should reduce HLA mismatching and immunogenicity (Unger et al. 2008).

ESCs and iPSCs, once injected in vivo without a complete in vitro differentiation, will form teratomas, a hallmark of pluripotency (Fong et al. 2010). Despite their high telomerase activity to allow indefinite growth, long-term culture-expanded PSCs are prone to chromosomal and genetic aberrations, leading to abnormal growth (Shay and Wright 2010; Blum and Benvenisty 2009; Knoepfler 2009). MSCs could be transformed by natural oncogenes (Lazennec and Jorgensen 2008). Recent studies indicate that mouse MSCs readily undergo chromosomal instability while human MSCs are more genetically stable (Rodriguez et al. 2012). However the trophic functions of human MSCs may stimulate tumor cell invasion, proliferation motility, and metastasis (Cuiffo and Karnoub 2012). Finally, HSC expansion was found to be associated with the increased expression of genes implicated in oncogenic transformation as well (Okamoto et al. 2007).

Consequently, an optimized bioprocess approach for large scale stem cell expansion and differentiation is required not only to reach the amount of primed cells necessary for therapeutic purposes, but also to

control the cell population with reduced therapeutic risks. For example, process intensification to minimize the time in culture, and thus to limit the risk of cell genetic aberrations is necessary. Using xeno-free culture reagents, such as growth medium and substrates, is preferable to reduce the risk of pathogen transmission. Moreover, implementing the cell separation steps compatible with the upstream culture process is also important. The selection, purification, and quantification of suitable populations of stem cell-derived tissue-specific cells constitute an obligatory step to ensure their safe therapeutic use.

Regulations and guidelines for stem cell-derived products

In the United States, treatments with adult stem cells which have been minimally manipulated and are intended for autologous use need to comply with the PHS (Public Health Service) act 361 and the 21 code of federal regulations (CFR) 1271 regulated by the Food and Drug Administration (FDA) (FDA 2008; Sensebe et al. 2011). Part C of Title 21 CFR 1271 defines good tissue practices (GTPs), which includes the guidelines for the procurement, the processing storage, and the distribution of stem cells. Adult stem cells and PSC-derived products that are manipulated (e.g. by gradient selection, activation, large scale expansion, or genetic manipulation) or intended for allogeneic use must comply with the PHS act section 351, which includes the regulation of drugs, devices, and/or biological products. The safety studies of PSC-derived products include the evaluation of toxicity, tumorigenicity, and bio-distribution. The guidelines of cGMP as described in Title 21 CFR 210, 211, 312, and 600 provide regulations for screening, testing, processing, labeling, and packaging of the products (Carpenter et al. 2009).

In Europe, stem cell therapy is regulated by the directive on Advanced Therapy Medicinal Products (ATMPs) following the European Commission directive No 1394/2007. The directive 2004/23/EC delineates the unmodified or modified stem cells. Stem cell modification includes large-scale expansion, activation, genetic manipulation, and use for allogeneic purpose. Preclinical studies on stem cells must be performed according to the good laboratory practices (GLPs) following the directive 2004/10/EC, while the clinical trials must comply with the directive 2001/20/EC following the GMP guidelines defined in the 2003/94/EC and 91/356/EEC directives (Martin et al. 2014). GMPs require the documentation for all raw materials and equipment, the controlled production process, and the controlled product storage and release according to pre-established standards and specifications approved by the quality assurance system.

A bioprocess engineering approach for the controlled production of stem cells

Bioprocess engineering aims at rationally designing the well-controlled equipment (e.g. bioreactors) and processes (e.g. efficient cell seeding and harvesting, microcarriers, serum-free medium, etc.) for mass scale production of stem cells and/or their derivatives (Godara et al. 2008). An analogy could be drawn from the well-established bioprocess engineering platforms applied to recombinant protein production from microbial and animal cells, although in the applications of tissue engineering and regenerative medicine the cell population itself instead of the secreted protein is the product. Thus the requirements for the control of process parameters during manufacturing are more stringent for stem cell bioprocessing (Fig. 1).

Automation devices for stem cell production

The immediate solution in stem cell bioprocessing to produce a large number of cells is the development of multi-layer culture vessels and flasks (e.g. CellFactory from Nunc, USA; CellSTACK from Corning, USA). However, the use of a large number of 2-D culture vessels (such as hyperstacks and hyperflasks) is labor intensive. Automated platforms have been developed to remove the human operator dependency and reduce the process variation for both MSC and human PSC cultures, such as the CompacT SelecT system (Thomas and Ratcliffe 2012; Ratcliffe et al. 2013) and the Cell^{host} system (Terstegge et al. 2007). The automation of partial processing can also be performed using devices such as the Pipeline Dispenser peristaltic pump for feeding (Essen Bioscience). However, this scale out approach is still limited in cell number and only suitable for a small number of patients and low dose studies. In particular, these

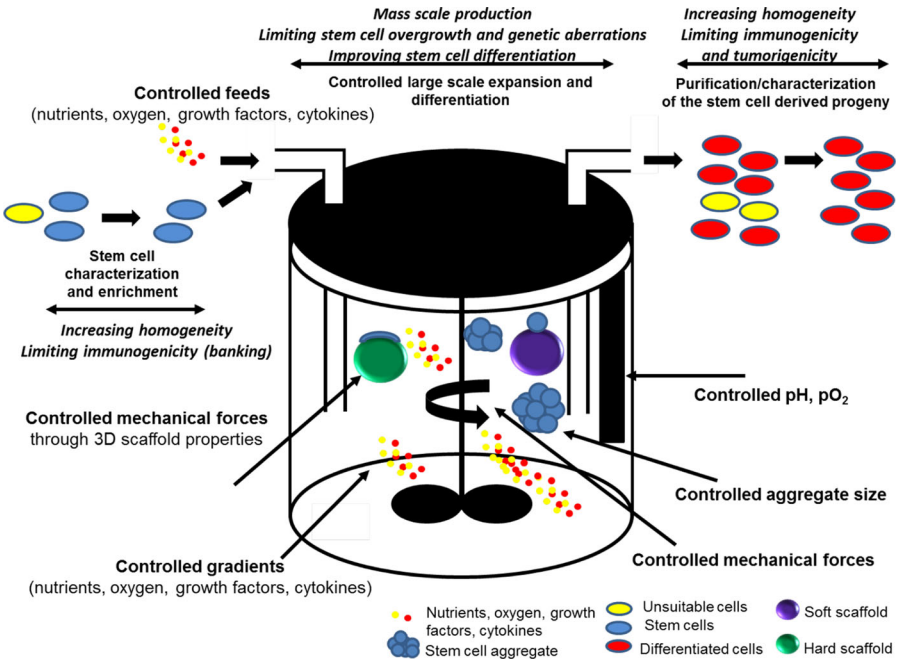

Fig. 1 Illustration of a bioprocess engineering approach with bioreactors to alleviate current limitations in the use of stem cells for clinical application. Bioreactors enable the large scale stem cell expansion and differentiation in a controlled environment. Bioreactors provide the controlled mechanical forces, feeds, the gradients of nutrients and growth factors, pH/oxygen, as well as aggregate size. With the detailed characterizations of input cells and output cells, the homogeneity of the produced cell population will be increased

systems are not amenable for 3-D stem cell cultivation.

Stem cell engineering for enhanced homogeneity, safety, and biological functions

To ensure the homogeneity of stem cell populations and avoid their potential tumorigenicity, stem cells should be characterized by the expression of cytogenetic, genetic, and surface/intracellular markers (e.g. by flow cytometry or proteomic analysis) (Bongso et al. 2008; Baker et al. 2007; Taylor et al. 2011). For example, hESC-derived OPCs are characterized by a set of surrogate purity markers NG2, Nestin, and PDGFRα, as well as the impurity markers Tra-1-60 and Oct-4 for residual undifferentiated cells (Li et al. 2013). Although the direct correlations of the surrogate markers with stem cell potency is yet to be established, these stem cell characterizations provide the filtering step for the follow up pre-clinical and clinical studies. The selective elimination of undifferentiated PSC populations could be performed via specific induction of apoptosis (e.g. mab 84 or ceramides) or cell depletion with magnetic-activated cell sorting (Bieberich et al. 2003; Tan et al. 2009; Diogo et al. 2012). The detection of histocompatibility complexes enables the creation of stem cell banks to preserve patient-specific cell lines (e.g. HLA-matched cell lines) (Fig. 1) (Heng et al. 2009; Bongso et al. 2008).

The development of defined serum-free culture systems constitutes a key tool to ensure the reproducibility of stem cell performance and to limit the putative introduction of pathogens. For instance, it has been reported that ESCs, cultured with animal-derived ingredients, might take up and express foreign non-human immunogenic sialoprotein, increasing the risk of immune rejection (Grinnemo et al. 2008). A number of serum-free media (e.g. E8 medium for human PSCs) have been developed for the expansion of PSCs and MSCs (Chen et al. 2011; Lennon et al. 1995), but relatively few exist for lineage-specific differentiation of stem cells (Outten et al. 2011).

Alternatively, a transgenic approach involving the modification of PSCs to enhance their specific properties such as promoting the expression of protective proteins (e.g. A20, Bcl-xL, HO-1, FasL, or IDO) could be a way to minimize rejection (Taylor et al. 2011). Similarly, the genetic modification of MSCs could enhance the therapeutic effect (e.g. in overexpressing insulin-like growth factor-1) as reported in the treatment of myocardial infarctions (Wagner et al. 2009) or in cancer therapy (e.g. overexpressing interferon-β, interferon-γ) (Lazennec and Jorgensen 2008). The

Table 1 Summary of bioreactors and their effects on stem cell expansion or differentiation

Stem cell type	Bioreactor type	Effect on stem cells	Reference
MSCs	Perfusion	Enhanced osteogenesis, proliferation and the secretion of vascular endothelial growth factor	Kreke et al. (2008)
MSCs	Perfusion	Enhanced chondrogenesis	Gonçalves et al. (2011)
MSCs	Biaxial spinner flask	Enhanced osteogenesis	Zhang et al. (2010)
MSCs	Rotating wall vessel	Enhanced adipogenesis	Meyers et al. (2005)
MSCs	Compression bioreactor	Enhanced chondrogenesis	Huang et al. (2004)
mESCs	Perfusion microfluidic bioreactor	Induced three-germ layer commitment	Przybyla and Voldman (2012); Wolfe et al. (2012)
mESCs	Spinner flask	Enhanced self-renewal	Gareau et al. (2012)
mESCs	Rotating wall vessel	Enhanced hematopoiesis	Fridley et al. (2010)
mESCs	Rotating wall vessel	Enhanced hepatic differentiation	Wang et al. (2012)
mESCs	Perfusion	Endothelial and hematopoietic differentiation	Wolfe and Ahsan (2013)
mESCs	Rotary orbital shaker	Enhanced cardiomyocyte differentiation	Sargent et al. (2009)
hESCs	Spinner flask	Induced three-germ layer commitment	Leung et al. (2010)
hESCs	Spinner flask; Rotating wall vessel	Induced three-germ layer commitment	Yirme et al. (2008)
iPSCs	Spinner flask	Integrated iPSC derivation, expansion, and cardiomyocyte differentiation	Fluri et al. (2012)

MSCs mesenchymal stem cells, *mESCs* mouse embryonic stem cells, *hESCs* human embryonic stem cells, *iPSCs* induced pluripotent stem cells

GTP and GMP (i.e. Title 21 CFR 1271 and the European directive 1394/2007) compliances require the characterization of the stem cell population as well as any materials required to manipulate stem cells, such as the relevant plasmids (Horwitz et al. 2002). Engineering the input cells, the process of expansion and differentiation, and the output cells enhances the homogeneity, safety, and biological functions of stem cells for therapeutic uses.

Bioreactors to modulate the mechanical and biochemical niches of stem cells

Bioreactors enable the control of the physiological microenvironment of stem cells, and thus are well-placed to improve culture performance towards mass scale production (King and Miller 2007; Liu et al. 2013). Moreover, 3-D stem cell aggregates have been recently shown to increase the therapeutic potential and differentiation efficiency of stem cells through the sustainment of endogenous signaling (Sart et al. 2013c, d; Fridley et al. 2010). In addition, to meet the requirement for high dose cell therapy (e.g. 10^{12} cells per batch of production), multi-stack devices would require 1×10^4–1×10^5 layers, which is not a feasible process (Simaria et al. 2014). Bioreactors (e.g. spinner flasks) support the expansion of 3-D cellular organization and are well-placed for cost-effective systems towards potential commercial processes (Abbasalizadeh and Baharvand 2013; Sart et al. 2013d).

Various bioreactor systems can be used for stem cell expansion and differentiation (Table 1). For instance, perfusion reactors can continuously deliver fresh nutrients and at the same time expose cells to

Table 2 Culture mode affecting stem cell proliferation or differentiation in bioreactors

Stem cell type	Bioreactor type	Culture mode	Parameter controlled	Effect	Reference
MSCs	Spinner flask	Fed batch	Growth factor concentration	Enhanced stem cell yield	Sart et al. (2010)
MSCs	Perfusion bioreactor	Hypoxia (5 % oxygen) versus normoxia (20 % oxygen)	Oxygen tension	Adipogenesis (normoxia) Chondogenesis (hypoxia)	Lovett et al. (2010)
mESCs	Microfluidic bioreactor	Gradient perfusion	Wnt3a, activin A, BMP-4 concentration	Mesodermal differentiation	Cimetta et al. (2013)
hESCs	Spinner flask	Hypoxia (4 % oxygen)	Oxygen tension	Cardiomyocyte differentiation	Niebruegge et al. (2009)
hESCs	Spinner flask	Fed batch	Glucose concentration	Enhanced stem cell yield	Chen et al. (2010)
hESCs	Spinner flask	Perfusion	Oxygen tension	Improved stem cell expansion	Serra et al. (2010)

MSCs mesenchymal stem cells, *mESCs* mouse embryonic stem cells, *hESCs* human embryonic stem cells

controlled fluid mechanical forces (Li et al. 2009). Stirred-tank bioreactors eliminate the concentration gradients of nutrients and create a homogeneous physicochemical environment (Kaiser et al. 2013). Rotating wall vessels display similar properties as the stirred culture vessel, while allowing the low shear stress and the control of gravity to mimic the in vivo tissue environment (Sheyn et al. 2010).

Stem cells are mechano-sensitive cell populations and bioreactors can provide controlled mechanical signaling to the cells (Table 1) (Sun et al. 2012). In addition to the commonly observed cell damage due to shear stress in bioreactors, shear stress could modulate stem cell fate decision for self-renewal or lineage commitment. For instance, perfusion-based bioreactors enhance MSC proliferation and osteogenic differentiation due to the presence of flow shear (Kreke et al. 2008; Liu et al. 2010). Shear stress (1.5–15 dynes/cm^2) has also been shown as a potent inducer of mesodermal commitment of ESCs through the modulation of FLK1 membrane protein (Wolfe and Ahsan 2013). Conversely, lowering shear stress in rotating wall vessels was found to favor MSC adipogenesis (Meyers et al. 2005) as well as hepatogenic differentiation of ESCs (Fig. 1) (Wang et al. 2012).

The rational design of culture mode in bioreactors is also essential in order to meet the physiological requirements of a stem cell population for optimized proliferation and differentiation (Table 2) (Lo et al. 2011). While simple batch mode does not support efficient MSC and ESC expansion, adapted feeding of nutrients (e.g. glucose or glutamine) or growth factors (e.g. fibroblast growth factor) with a fed-batch mode significantly increases the stem cell yields in bioreactors (Fig. 1) (Sart et al. 2010; Chen et al. 2010; Schop et al. 2010). The regulation of oxygen tension (i.e. hypoxia vs. normoxia) in bioreactors also modulates the proliferation and differentiation of MSCs and PSCs (Table 2) (Dos Santos et al. 2010; Lovett et al. 2010; Niebruegge et al. 2009). Moreover, bioreactors enable the tight control of cytokine and growth factor gradients, leading to the enhanced stem cell differentiation (Fig. 1) (Cimetta et al. 2013).

Modulation of stem cell behavior through 3-D culture configuration in bioreactors

Bioreactors enable the cultivation of stem cells as functional 3-D constructs. The culture on 3-D scaffolds is amenable to scale up of stem cell production while recreating the stem cell niches to maintain the cellular functions (Li et al. 2003; Abranches et al. 2007; Singh et al. 2010; Stenberg et al. 2011). Scaffold or substrate biomaterials provide additional mechanical signaling to stem cells. The pattern and the stiffness of scaffolds/substrates regulate stem cell shape, proliferation, and differentiation potential. For instance, large ECM islands promoted proliferation and osteogenic differentiation of MSCs as well as

Table 3 Three-dimensional culture configurations enhancing stem cell properties

Stem cell type	Bioreactor type	3-D culture configuration	Effect on stem cells	Reference
MSCs	Spinner flask	Aggregates	Enhanced cell survival, VEGF secretion	Bhang et al. (2011)
MSCs	Spinner flask	Aggregates	Enhanced IL-24 secretion (anti-tumor factor)	Frith et al. (2010)
MSCs	Spinner flask	Aggregates	Enhanced osteogenesis	Frith et al. (2010)
MSCs	Rotating wall vessel	Aggregates	Enhanced adipogenesis	Frith et al. (2010)
MSCs	Spinner flask	Cytopore-2 microcarriers	Enhanced chondrogenesis compared to 2-D	Sart et al. (2013a)
MSCs	Spinner flask	Cytodex-3 microcarriers	Enhanced osteogenesis compared to 2-D	Goh et al. (2013)
mESCs	Static	Aggregates	Enhanced cell survival	Sart et al. (2013b)
mESCs	Spinner flask	Aggregates	Enhanced osteoblast differentiation	Alfred et al. (2010)
mESCs	Spinner flask	CultiSpher-S	Supported stem cell expansion	Storm et al. (2010)
hESCs	Static	Aggregates	Enhanced self-renewal	Singh et al. (2010)
hESCs	Spinner flask	Tosoh 10 microcarriers	Enhanced cardiomyogenesis compared to EBs	Lecina et al. (2010)
hESCs hiPSCs	Spinner flask	DE-53 microcarriers	Enhanced neurogenesis compared to EBs	Bardy et al. (2013)

MSCs mesenchymal stem cells, *mESCs* mouse embryonic stem cells, *hESCs* human embryonic stem cells, *hiPSCs* human induced pluripotent stem cells

sustained ESC self-renewal (McBeath et al. 2004; Peerani et al. 2009). Conversely, small ECM islands induced MSC chondrogenic differentiation and limiting ESC self-renewal ability (Gao et al. 2010; Peerani et al. 2009). The biomechanical property of the scaffold, e.g. modulus, regulated ESC self-renewal versus commitment (Zoldan et al. 2011), and MSC proliferation versus osteogenic differentiation (Shih et al. 2011). To grow the adherent stem cells in suspension, various types of microcarriers have been investigated to support cell expansion and differentiation. In bioreactors, microcarriers enhanced ESC differentiation potential into neural lineage compared to EB-based protocols (Bardy et al. 2013). Importantly, the physical and biochemical properties of microcarriers modulate MSC proliferation and differentiation through the control of cell shape and cytoskeleton re-organization (Table 3) (Sart et al. 2013a).

Both MSCs and PSCs can also self-assemble as 3-D aggregates and be expanded in suspension bioreactors. The scaffold-free stem cell aggregates were found to reinforce cell–cell contacts (Singh et al. 2010) and autocrine/paracrine signaling (Kabiri et al. 2012), leading to the desired stem cell survival and biological function (Table 3). MSC aggregates grown in bioreactors displayed higher differentiation efficiency and enhanced trophic factor secretion (e.g. IL-24) compared to static and 2-D cultures (Frith et al. 2010; Bhang et al. 2011). Using bioreactors, the collision of aggregates can be controlled through hydrodynamic mixing, which leads to homogeneous aggregate size distribution and improved mass transfer and/or diffusion in the aggregates.

To overcome the diffusion barriers in the multicellular aggregates, growth factor delivery using nanoscale or microscale bioactive particles has been explored. The effects of nano-/micro- scale particles have been observed for embryoid bodies (EBs), the aggregate-like structure mimicking embryonic development. EB formation has been extensively used for lineage-specific differentiation because of intimate cell–cell contacts and cell-ECM interactions (Bratt-Leal et al. 2009). However, EBs usually form a shell-like structure which limits the diffusion length within 100 μm. Engineering EBs through the incorporation of microparticles containing retinoic acid has been shown to replicate embryonic tissue development (Bratt-Leal et al. 2011; Ferreira et al. 2008). These novel approaches using 3-D culture configurations provide the appropriate biomimetic microenvironment in the corresponding bioreactors and

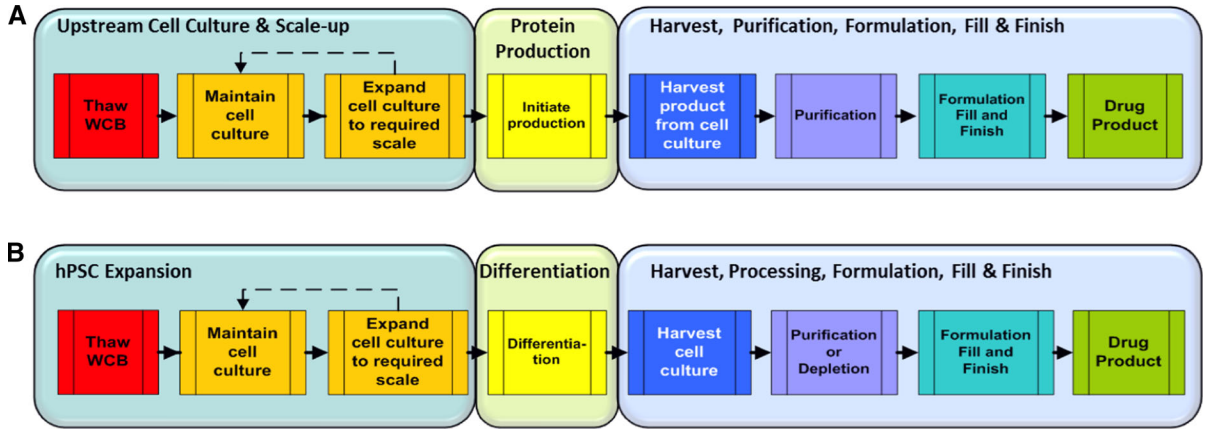

Fig. 2 cGMP production process flow for pluripotent stem cell-derived products and recombinant protein production. **a** Bioprocess flow for recombinant protein production, **b** Bioprocess flow for the production of pluripotent stem cell-derived products. For both types of products, the bioprocess consists of three stages: upstream cell culture/expansion, protein production/differentiation, and downstream purification and formulation. *WCB* working cell bank, *hPSC* human pluripotent stem cell

bioprocesses, thus ensuring precise control of stem cell fate decisions.

Production of stem cell products under cGMP

Stem cell-based cellular products have been produced in cGMP facilities for large scale clinical applications, such as hESC-derived OPCs for Geron's phase I clinical trial on spinal cord injury treatment (Geron, unpublished data). For MSCs, a cGMP process usually starts from the cell isolation, which requires the determination, screening, and testing of donor eligibility. For PSC-derived products, the process can start from a working cell bank (WCB). However, the source, history, and the generation of a WCB need to be well documented and the banks need to be cleared from adventitious agent testing. Similarly to recombinant protein production, the bioprocess of manufacturing pluripotent stem cell-derived products contains an upstream expansion stage, a differentiation process to produce the cell product (such as cardiomyocytes or neural progenitors), and a set of downstream purification and formulation processes (Fig. 2). To produce these novel cellular products, the process control aimed at reducing variability and the development of stringent bioreactor-based stem cell differentiation protocols to increase the production scale are becoming very critical. The process control includes the use of consistent and safe raw materials (such as replacing mouse tumor-derived Matrigel coated surface by synthetic peptide surface), the development of serum-free media for cell expansion and differentiation, and the implementation of in-process monitoring of parameters such as the metabolic activity (e.g. glucose consumption and lactate production) (Li et al. 2013). In bioreactors, as stem cells are exposed to shear stress, the impact of the hydrodynamic environment on stem cell differentiation needs to be better understood (Gareau et al. 2012; Leung et al. 2010). Stirred-tank bioreactors are more applicable for cGMP production compared to other types of bioreactors due to their simplicity and scale up feasibility. As some differentiation processes, such as the production of hESC-derived OPCs, involve multiple stages (i.e. suspension and adherent cultivation) over long-term periods (i.e. 6–8 weeks), novel strategies of process integration at the bioreactor level become critical (Liu et al. 2013, Oh and Choo 2006). To make the production successful, the peripheral processes such as reagent acquisition and preparation, personnel training, equipment specification and validation, and documentation system such as the history of batch production record (BPR) are needed to be in place to support the core production processes (Ratcliffe et al. 2011).

Compared to recombinant protein production, the challenges of bioprocess engineering for stem cell-derived products include:

1. Reducing the variations from cell source and during the differentiation procedure. MSCs are primary cells typically isolated from various tissue types of the patients. For human PSCs, various culture methods have been developed by different groups. Hence, tight quality control for the starting cell population and the culture procedures has to be implemented to ensure consistent cell products.
2. Increasing the yield and purity of the differentiated cells. The differentiation protocols have been significantly improved in recent years, while most protocols still result in low purity (<30 %) of the lineage-specific cells. In addition, most protocols have not yet been evaluated in bioreactors. A good baseline protocol will ease the process development efforts to translate a laboratory procedure into a bioprocess in a cGMP facility.
3. Detailed characterization of the produced cell populations. Developing the clinically relevant product specifications is not trivial. New bioprocess and biomarker development to predict and/or eliminate the impure cells is critical for safe clinical use of stem cell products. The link of the stem cell products with the potential clinical outcome needs to be established before cGMP manufacturing.
4. Increasing the scale in bioreactors. Not every protocol in the laboratory can be translated into a bioreactor-based process. Understanding the change in culture environment is important and appropriate modifications of differentiation protocols are required.
5. Reducing the cost. Most current materials used in stem cell expansion and differentiation are still costly, such as serum-free media, growth factors, and the culture substrates. Understanding the signals to regulate stem cell self-renewal and lineage commitment using simplified reagents (e.g. E8 medium from Life Technologies) will be quite helpful to reduce the cost.

Conclusion

As learned from traditional bioprocesses in recombinant protein production, bioprocess engineering applied to stem cells and their products requires a horizontal approach: the selection of stem cell populations, bioreactor optimization, media development, and the detailed characterization of stem cell-derived progeny to improve safety. Such a strategy for a well-controlled process could rationally address the current issues and overcome the hurdles in stem cell-based clinical applications.

As analyzed above, the application of rational bioprocess engineering strategies to stem cell cultivation constitutes the best way to monitor their microenvironment. This should lead to the improvement in stem cell expansion and the priming ex vivo that should ultimately meet clinical demands with the proper safety considerations. Since various stem cells display different potential or physiological requirements, stem cell bioprocessing should be closely coupled to the specific physiological needs in both expansion and differentiation steps.

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