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Large Scale Production of Stem Cells and Their Derivatives

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Abstract Stem cells have been envisioned to become an unlimited cell source for regenerative medicine. Notably, the interest in stem cells lies beyond direct therapeutic applications. They might also provide a previously unavailable source of valuable human cell types for screening platforms, which might facilitate the development of more efficient and safer drugs. The heterogeneity of stem cell types as well as the numerous areas of application suggests that differential processes are mandatory for their in vitro culture. Many of the envisioned applications would require the production of a high number of stem cells and their derivatives in scalable, well-defined and potentially clinical compliant manner under current good manufacturing practice (cGMP). In this review we provide an overview on recent strategies to develop bioprocesses for the expansion, differentiation and enrichment of stem cells and their progenies, presenting examples for adult and embryonic stem cells alike.

Keywords Bioreactor, Cell therapy, Differentiation, Process development, Stem cells, Teratoma

Contents

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Abbreviations

(NOD/SCID) mice	Nonobese diabetic/severe combined immunodeficient
(RWV) bioreactor	Rotating wall vessel
bFGF, FGF-2	Basic fibroblast growth factor
BM	Bone marrow
BMP	Bone morphogenetic protein
BMP	Morphogenetic protein
cGMP	Current good manufacturing practice
CHO	Hamster ovary cells
EBs	Embryoid bodies
ESC	Embryonic stem cells
GM-CSF	Granulocyte macrophage colony stimulating factor
hESC	Human embryonic stem cell
hNPC	Neural precursor cells
HSC	Hematopoietic stem and progenitor cells
LIF	Leukemia inhibitory factor bone
MASC	Magnetic activated cell sorting
MI	Myocardial infarction
MSC	Mesenchymal stem cells
NSC	Neural stem cells
PB	(Mobilized) Peripheral blood
SCF	Stem cell factor
SNM`	Spherical neural masses
TGF-beta	Transforming growth factor beta
UCB	Umbilical cord blood

1 Introduction

The title of this review is a bold claim. It implies that large scale production of stem cells is, to some extent, an established practice. Process scale-up of common mammalian cell lines such as Chinese hamster ovary cells (CHO), human tumor cells lines (such as HEK 293 and HeLa), and myelomas, which have been extensively used to produce large quantities of biopharmaceutical products (e.g., antibodies and cytokines), has indeed resulted in fermentation volumes of >1,000 or even >10,000 L in recent years [1–4]. In contrast, stem cell production and differentiation in vitro is in its infant stage. Process optimization experiments are often performed in 0.1–10 mL medium in tissue culture dishes. Spinner flask and other bioreactor volumes of 50–250 mL are considered a substantial up-scaling and lab-scale processes exceeding 1 L reactor volume are an exception.

One major underlying reason is the still limited knowledge of stem cell biology hampering the development of efficient and commercially viable processes. Not surprisingly, a recent leading edge analysis by Ann B. Parson [5] underscores that ramping up the process for stem cells products is currently one of the key success hurdles for biotech companies in the field.

1.1 Cells for Therapies: Estimating Cell Number Requirements

How many cells are actually necessary for future therapies? Obviously, this will depend on the respective application but some of the presently utilized cell therapy applications serve to highlight the dimensions. In the field of heart repair, for example, one can assume that the left ventricle of a human heart contains about 4–6 billion cardiomyocytes [6–8]. Individuals can survive myocardial infarction (MI) that affects about one-third of the left ventricle. Cardiac regeneration would thus require the replacement of as many as 1–2 billion cardiomyocytes that are irreversibly lost through hypoxia-reperfusion injury.

Similar numbers apply to beta-cell replacement in type 1 diabetic patients. The Edmonton protocol, a pancreatic islet transplantation procedure, typically utilizes a transplant of approximately 600,000 islet equivalents comprising abut 1,000 beta cells each [9] derived from cadaveric donor pancreata. This would mean that about 1 billion stem cell-derived functional beta-cell equivalents would be required per patient [10].

Another example documents the dimension of donor cell requirement to reconstitute stably blood formation in patients after chemotherapy or irradiation treatment. Using umbilical cord blood (UCB) as a cell source, cell doses of 15 million mononucleated cells containing about 1% CD34+ hematopoietic stem and progenitor cells per kg patient weight appears to be the threshold for safe transplants [11]. An adult of 80 kg receiving an unrelated UCB transplantation will thus need about 1.2 billion (1.2×10^9) nucleated cells including 12 million CD34+ cells. Supposing

that UCB samples can contain about 1×10^8 mononucleated cells comprising 1% CD34+ cells, in vitro expansion would require a 12-fold increase of the cell population, thereby keeping the proportion of CD34+ cells intact, which is a key factor for successful transplant products as discussed in more detail below.

These examples suggest that 1–2 billion stem and/or differentiated progenitor cells per patient is a useful ballpark number to estimate production requirements in bioprocess development.

1.2 Cell Sources for Therapies: Adult vs Embryonic Stem Cells

Stem cells are defined as being self-renewing, pluri- or multipotent, and clonogenic. Clonogenic cells are single stem cells that are able to generate a line of genetically identical cells thereby maintaining their self-renewal and differentiation potential. Stem cells exist at different hierarchical levels throughout the development of an organism and persist in adult tissues. At one end of the spectrum, pluripotent embryonic stem cells (ESC) can give rise to all cell types in the body whereas tissue specific, multipotent stem cells only retain the ability to differentiate into a restricted subset of cell types.

With the exception of hematopoietic stem and progenitor cells (HSC), which have been used in the clinic for more than 50 years [12], the routine therapeutic application of stem cells is limited to date. Ten years after the first derivation of stable human embryonic stem cell (hESC) lines by Thomson and coworkers [13], no clinical trial based on this cell source has yet been initiated. Although trials have been announced for spinal cord repair and ophthalmic disorders by biotech companies', initiation was repeatedly delayed due to profound safety and ethical concerns [5].

Present experimental trials aimed at cell-based tissue repair have thus focused on cells isolated from patients own tissue. Autologous approaches avoid donor cell rejection and the risk of teratoma formation (benign tumors containing cells from various differentiated tissues) imposed by ESC. These personalized cell treatments require no or limited small-scale expansion of harvested cells. Examples are (1) calf biopsy-derived in vitro expanded skeletal myoblasts and (2) nonexpanded, bone marrow-derived mononucleated cells. Both of these cell types are currently being tested for heart repair in patients post MI [14]. However, poorly defined mixtures of autologous cells are often used in experimental trials simply because the (stem-) cell type(s) with a supposed therapeutic potential is not known [14]. Crude bone marrow biopsies or fractions thereof are being tested for heart repair whilst the discussion on the adequate cell type, the optimal modus of application, and the expected clinical outcome is in full swing [14–16]. Considering the controversial observations from animal models, the distrust of numerous investigators towards ongoing clinical trials is not surprising [16–18]. Results observed in rodent hearts range from efficient cardiomyogenic differentiation of bone marrow derived cells [19] and mesenchymal stem cells (MSC [20, 21]) to negligible heart muscle cell differentiation of these cell types [22, 23] and even deleterious effects like the calcification of MSC injected into heart muscle [24].

This debate not only concerns the question of which cell type is most suitable to repair a particular organ. It also relates to the underlying question of whether primitive, undifferentiated stem or progenitor cells could be delivered to regenerate damaged tissue (where the differentiation will be guided in vivo by signals in recipients damaged organ) or whether stem cells must be directed to differentiate into mature, tissue specific progenies in vitro and then transplanted. Apparently, these considerations define the goals and strategies for bioprocess development.

Notably, the interest in stem cells lies beyond direct therapeutic applications. Stem cells, or differentiated progenitors thereof, provide a promising source of valuable human cell types that have not been available for in vitro assays before. This will allow the development of novel, scalable screening platforms for compound discovery and toxicity testing which might help to develop more efficient and safer drugs [25]. Another area of stem cell research is the study of developmental and differentiation processes as well as stem cell malignancy and genetic disorders in vitro.

The heterogeneity of stem cell types as well as the numerous areas of application suggests that differential processes are mandatory for their in vitro culture. Many of the envisioned applications would require the production of a high number of stem cells and their derivatives in scalable, well-defined and potentially clinical compliant manner under current good manufacturing practice (cGMP). In this review we will provide an overview on recent strategies to develop scalable bioprocess for the expansion and differentiation of stem cells, providing examples for adult and embryonic stem cells alike.

2 Strategies in Stem Cell Scale-Up

Development of clinical/industrial scale process for cell production requires a focus on key questions of process efficiency and eventually commercial viability of an envisioned strategy. This includes estimating the process dimension defined by the (1) number of cells to be transplanted per treatment, (2) bioreactor dimensions needed to generate multiple cell doses, (3) required total medium throughput, and (4) process duration; subsequently process costs can be calculated.

Using cardiomyocytes and pancreatic cells as examples, we have calculated above that 1–2 billion cells per patient will theoretically be needed to replace the loss of functional tissue. Notably, true cell numbers for successful organ repair might be extensively higher. Recent animal models suggest that only a single-digit percentage of transplanted donor cardiomyocytes eventually survive and integrate in the heart [26, 27]. Also, the physiological potency of surrogate cells generated in vitro might require higher donor cell doses. For example, the insulin release in response to a defined glucose challenge, a potency assay used to assess beta-cell functionality in vitro, is much lower in ESC-derived beta-like cells compared to cadaveric donor-derived beta cells embedded in functional islets. The latter comprise the gold standard in the field [10, 28].

In addition, differentiation of stem cell in vitro usually results in a mixed culture with the desired cell type being a minority even if protocols for directed differentiation are applied. Let's assume that a target cell type such as beta-cells or cardiomyocytes can be generated from ESC with a relative high efficiency of 20%. Subsequently a bioprocess must generate the total amount of 5 billion differentiated progenies to produce 1 billion target cells, which would thus impact on process dimension. The resulting cell mixture might be subjected to subsequent purification steps to achieve lineage purity.

Equipped with such estimations, process development is concerned with experimental-scale approaches to provide initial real-world figures on process efficiencies, dimensions, and costs, which are subsequently subjected to up-scaling and optimization.

2.1 Culture Media and Cell Attachment Matrices: Critical, Expensive, and yet Poorly Defined

One of the most essential and costly components in stem cell production is the culture medium. Development of media that either support stem cell self renewal and proliferation or, in contrast, direct differentiation into desired lineages is at the heart of current research. Experimental reports often utilize media comprising relative high amounts of serum. Unfortunately, serum is subjected to batch-to-batch variations and represents a xenogeneic component that might conflict with the generation of clinically-compliant stem cell products. In mouse and human ESC research, the need for defined media has resulted in broad usage of commercially available serum replacement (e.g., Invitrogen, Carlsbad, CA, USA) but the formulations still generally contain bovine serum albumin.

However, studies have begun to unravel signal transduction pathways controlling self renewal and differentiation in more detail resulting in chemically-defined, xeno-free media as outlined below. In this context, synthetically manufactured compounds that can control signaling pathways and subsequently stem cell behavior are progressively tested in the field [29]. Ultimately, this strategy will not only facilitate generation of chemically defined media. Applying small molecules might also support commercial viability of bioprocesses by replacing recombinant, costly growth factors and cytokines that are currently obligatory components of many media formulations. Prominent examples are fibroblast growth factor-2 (FGF-2) supplemented to culture media for hESC expansion or numerous hematopoietic growth factors including interleukins, granulocyte macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF) and others that are currently indispensable for the in vitro cultivation of HSC [12]. Other examples include the transforming growth factor beta (TGF-beta) family members activin and bone morphogenetic protein (BMP)-4, arguably among the most expensive molecules on the planet, which have recently been suggested in a sequential protocol to direct cardiomyocyte differentiation from hESC [26].

In conjunction with the culture medium, another key component controlling stem cell characteristics in vitro is the matrix provided for cell attachment. With the

exception of HSC, which have historically been grown on stromal feeder cells but are now generally expanded in suspension culture, most other stem cell types have been isolated under conditions depending on surface adherence. Mouse, primate, and human ESC were all derived on a layer of embryonic fibroblast. Much effort is currently being applied to replace this coculture system, which strongly interferes with up-scaling strategies, by defined matrices. MSC, per definition, are tissue culture plastic adherent cells. Thus, it's easier to comply with their demands regarding the surface matrix used for expansion. However, culture surface enlargement to ensure efficient and reasonable mass expansion of anchorage-dependent cells is a central challenge in bioreactor design.

2.2 Bioreactors and Microcarriers: Providing Stem Cells with a Home and a Bed

A bioreactor may be defined as a system that simulates physiological environments for the creation, physical conditioning, and testing of cells, tissues, precursors, support structures, and organs in vitro. It thus provides for a regulated and controlled environment. At first glance, bioreactors look like highly complicated and sophisticated equipment, and indeed, very heterogeneous designs and setups exist. However, exempting some exotic models, they can be divided into a few simple categories.

The simplest and among the most extensively used reactor types in mammalian cell culture are stirred tank reactors (usually a cylinder-shaped vessel). Spinner flasks represent a simple lab-scale format of this reactor type (typical working volume of 50–250 mL), and are placed in tissue culture incubators to provide the basic growth environment, which is controlled temperature and aeration gas mixture. Spinner flask aeration is usually limited to the gas exchange at the headspace. Homogeneous mixing of the culture solution is ensured via impeller(s), turbines, or bulb-shaped stirring devices. Design of these impellers and vessel geometry as well as the stirring speed define the medium flow (direction, velocity) and thus homogeneity of culture mixing, efficiency of gas exchange, and, importantly, shear forces acting on the cells.

Compared to spinner flasks, instrumented stirred tanks allow online measurement and adaptation of parameters like the pH and oxygen tension (pO_2). Installed ports enable the simple and regular collection of culture samples. This facilitates offline (or even online) measurement of additional parameters such as cell density, cell vitality, glucose consumption, accumulation of potentially toxic metabolites such as ammonia, medium osmolarity and others. Instrumented tanks also enable additional culture aeration through a so-called sparger, a device that generates gas bubbles at the bottom of a vessel thereby adding to the gas diffusion from the headspace, to keep the pO_2 constant even in dense cultures demanding high oxygen supply. This is particularly important for stem cell cultures, as the pO_2 has been shown to impact on stem cell differentiation into specific lineages as outlined below. Another feature is the possibility for continuous feeding. Fresh medium is

constantly added at a defined speed and an equivalent medium amount is constantly removed from the culture, usually without cell removal (cell retention techniques). Continuous medium perfusion, in contrast to batch feeding which is the standard feeding technique in tissue culture, results in more homogeneous culture conditions which can have profound consequences in stem cell bioprocessing. It is well established that stem cells and their differentiated progenies release inhibiting and stimulating factors that can strongly feedback on cell pluripotentiality, proliferation and differentiation. Perfusion feeding of hESC, for example, enabled growth to much higher cell densities without inducing differentiation compared to batch fed controls ([30]; perfused stationary culture). Other examples of this topic are presented for HSC expansion and cardiomyogenic differentiation of ESC below.

Stirred tanks are favored in process scale-up because established culture conditions in lab-scale can often be transferred to much higher volumes with relative ease by keeping both physical (vessel and stirrer geometry, medium flow features/shear forces, medium throughput, feeding strategy, etc.) and physiological (pO_2, pH, glucose conc., metabolic waste conc., etc.) parameters constant [1–4]. However, cells often do not immediately 'take' to culture in stirred suspension systems. Consequently, in the biopharmaceutical industry, a critical scale-up step is the adaptation of initially anchorage-dependent production cell lines to (usually serum-free) suspension culture growth without interfering with the quality and quantity of the desired, cell-derived product [1]. Such adaptation steps, however, might strongly interfere with stem cell characteristics limiting translation of this strategy to stem cell research.

Another technique to enable the growth of attachment-dependent cells in suspension is the use of microcarriers. In 1976 Van Wezel describes the use of small particles (0.2 mm), microcarriers, for the growth of anchorage-dependent cells [31]. These spherical particles are kept in suspension by stirring or other mixing techniques and provide a massively enlarged attachment surface in a relative small reactor volume due to their high surface-area-to-volume ratio. Carriers have been previously used in conventional cell culture, e.g., for vaccine production.

As with bioreactors, a "plethora" of microcarriers exists; they come in all shapes and sizes. Aiming to provide optimal cell attachment properties for diverse cell type microcarriers made from numerous materials are available. One main category of microcarriers comprises solid, spherical or disc-shaped particles made of cross-linked dextran, cellulose or polystyrene [32]. The other category is termed micro- or macroporous carrier [33]. Macroporous carriers have a sponge-like structure. They are typically made of soft materials such as gelatin or collagen and allow cells to grow in their internal pores. Due to their rough external surface, macroporous carriers generate more microeddies, resulting in higher fluid shear that acts on surface attached cells compared to solid, spherical carriers [34]. However, solid carriers also impose high mechanical stress on cells in stirred culture whereas cells grown in the interior of porous particles might be well protected. Also, the microenvironment that might develop in the vicinity of cells grown in micropores might be different from the bulk of the culture vessel and either support stem cell maintenance or differentiation. Macroporous scaffolds have therefore been used for heterogeneous hematopoietic cell cultures which entail a mixed population of adherent and suspension cells [35, 36].

More recently, porous as well as solid type microcarriers have also been tested for mouse ESC cultivation and differentiation in spinner flasks [37, 38].

To prevent the potentially detrimental shear stress on cells in stirred microcarriers culture surface enlargement for anchorage-dependent cells can also be achieved in fixed/packed bed reactors. These are fully controlled bioreactors in which macroporous microcarriers or other substrates (e.g., glass or plastic beads of various sizes, discs made of porous material etc.) are embedded in a column-shaped vessel (cell compartment). To supply cells that have been seeded into the substrate, aerated culture medium is continuously circulated through the cell compartment; in most configurations bubble-free medium aeration is established through a semipermeable membrane. Fresh medium is added according the metabolic needs of the cells and metabolic waste products are removed. Configurations of this reactor type have been applied to engineer murine and human bone marrow models to mimic ex vivo hematopoiesis [36, 39]. Hollow fiber reactors, which have also been used for HSC culturing [40], utilizes a capillary-like fiber structure for surface-enlargement; again, oxygenated medium is circulated through these fibers for cell supply.

Finally, an even lower mechanical and hydrodynamic shear but still efficient mixing and agitation of cells in suspension is enabled by rotating wall vessel (RWV) bioreactors; in contrast to the reactor types described above the incubator vessel itself is rotated to mimic gravity-free culture conditions [41]. Improved RWV systems enabling parallel bi-axial vessel rotation were recently developed and applied for efficient three-dimensional tissue engineering [42, 43]. Examples applying RWV reactors for HSC expansion and hESC differentiation are further presented below.

In the following sections we will review the status of bioprocessing with respect to several stem cell types that have an established or an envisioned role in regenerative medicine.

3 Hematopoietic Stem and Progenitor Cells: Long Medical History but Limited Ex-Vivo Expansion of a Complex Cell Mixture

Hematopoietic stem cells reside as rare cells in the bone marrow in adult mammals and sit atop a hierarchy of progenitors that become progressively differentiated to mature blood cells, including erythrocytes, megakaryocytes, myeloid cells, and lymphocytes [44]. Hematopoietic stem and progenitor cell (HSC) transplants are used as part of the treatment of a variety of genetic disorders, blood cancers, some solid tumors and when the bone marrow is damaged or diseased. Since the main forms of cancer treatments, chemotherapy and radiotherapy, are nonspecific, healthy cells including bone marrow cells are also damaged. If the intensity of the therapy destroys the bone marrow function for blood regeneration, a transplant is necessary to prevent live-threatening complications such as infections and bleeding. Full long term reconstitution of blood formation in patients receiving HSC transplants is a paradigm for successful stem cell therapies.

3.1 Stem Cell Sources and Clinical Application

Bone marrow (BM) was the first source of HSC used for transplantation but in the meantime other sources including (mobilized) peripheral blood (PB; isolated via apheresis) and umbilical cord blood (UCB) have also been utilized [45–47]. A major limitation to the clinical application of HSC has been the absolute number of stem- and progenitor- as well as mature hematopoietic cells available in stem cell products. On the other hand it has been proposed that a single stem cell is capable of more than 50 cell divisions and has the principal capacity to generate up to 10^{15} progenies, or sufficient cells for up to 60 years blood formation in an adult human [48, 49]. This potential level of expansion, if realizable ex-vivo, may have an impact on the cellular genetic stability and the differentiation potential of HSC due to the loss of telomere length [50] and oxidative stress [51]. However, even a modest in vitro expansion would have a significant effect on HSC availability and investigators have evaluated this possibility to achieve the following clinical needs:

- Generating a sufficient number of stem cells from a single bone marrow aspirate or apheresis procedure to reduce the need for large marrow harvests or multiple leukaphereses
- Generating sufficient cells from a single umbilical cord blood harvest to reconstitute an adult following high-dose chemotherapy
- Supplementing stem cell grafts with more mature precursors to limit pancytopenia (shortage of all types of blood cells)
- Increasing the number of primitive progenitors in stem cell grafts to ensure hematopoietic support for multiple cycles of high-dose chemotherapy

Adapted from Ian McNiece [12].

3.2 In Vitro Expansion and Scale-Up

Challenges associated with in vitro HSC propagation are generally applicable to most other adult stem cells as well. Particular considerations include (1) the heterogeneity of the cell source(s) available for process inoculation, (2) absence of definitive stem cell surface markers, and (3) absence of fast and reliable assays to test stem cell function. However, within the population of donor-harvested mononucleated cells expression of the CD34 antigen (CD34+), a cell surface glycoprotein, paralleled with the absence of lineage markers and CD38 expression (lin-, CD38-) has become the distinguishing feature used for the enumeration and isolation of HSC. CD34 is down regulated as cells differentiate towards hematopoietic lineages [52, 53]. Transplantation studies in several species have also shown that long-term marrow repopulation can be provided by CD34+ cells. Therefore, relevant clinical and experimental protocols aimed at the in vitro expansion of HSC are often quantifying the rare fraction of CD34+ cells pre- and postexpansion to determine

success of the bioprocess. Additionally, in vitro colony forming (e.g., methylcellulose assay) and differentiation assays are combined with the in vivo ability to reconstitute multilineage hematopoieses in a xeno-transplantation model using nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice to ensure the quality of the expanded cell population [54].

Aiming to reconstitute the so-called HSC niche [55], many in vitro cultures have been designed to regulate the HSC microenvironment by coculture systems utilizing supportive feeder cell lines [56–59]. However, cocultures are not only challenging in process scale-up. Most of the systems have only demonstrated maintenance of HSC numbers without achieving the desired expansion, potentially because they model steady-state hematopoietic homeostasis in vivo to some extend. Finally, feeder-based cultures may not require direct cell–cell contact but rather the secretion of HSC-supporting factors by feeder cells. This observation has driven the development of serum- and feeder-free suspension culture protocols and extensive research has been devoted in identifying optimal cocktails of hematopoietic growth factors that simultaneously inhibit apoptosis, induce mitosis, and prevent differentiation. For reviews on this topic, please see Heike and Nakahata [60], Noll et al. [61] and McNiece [12], with the later being focused on clinical studies of in vitro expanded HSC.

Besides the medium composition, HSC cultures are influenced by many other factors. Considering the general donor-to-donor variability on the expansion potential of HSC [62] it has been shown that cell production is improved by using lower seeding densities, preenrichment of stem and progenitor cells for process inoculation, increased medium exchange via culture perfusion, and applying high concentrations of early-acting growth factors [63, 64]. Adding to the complexity, it was observed that human CD34+ cells as well as differentiated hematopoietic cell types secrete numerous growth factors acting as autocrine and paracrine factors in normal hematopoiesis [65]. Exploiting this observation recent studies have improved HSC expansion by removal of lineage marker expressing differentiated progenies from the culture to avoid feedback inhibition [66, 67]. Modifying this approach in future by selectively removing cell types that secrete inhibitory factors but leaving other progenies that produce stimulatory cytokines behind might create a self-stimulating environment, thereby limiting the need for adding costly cytokines [67].

Using bone marrow, peripheral blood or umbilical cord blood for culture inoculation, permeable blood bags and conventional T-flasks are still most widely used for the expansion of human HSC in the clinic. Although they are simple to handle, these systems have the typical limitations of static cultures such as the development of gradients (e.g., dissolved oxygen, pH, cytokines and metabolites), lack of online control for environmental conditions, and a limited surface area. Due to their long medical history, HSC were among the first stem cell types to be cultured in bioreactors; numerous types of reactors have been applied including hollow fiber-, perfusion chamber-, fixed bed-, and stirred vessel bioreactors reviewed elsewhere [68, 69]. Recent studies, however, are progressing towards the long term HSC expansion in increased culture volumes. For example, UCB- and PB-derived mononuclear cells were expanded in a stirred bioreactor equipped with dissolved oxygen and pH control, whereby the process efficiency was greatly enhanced by using a cell-dilution

feeding protocol [70]. Another stirred suspension approach in 250-mL scale was published by Kim and coworkers [71] documenting the expansion of human BM; supplementation with factors secreted by stromal feeder cells combined with growth promoting and growth inhibiting cytokines enabled the prolonged expansion of hematopoietic progenitors. Long term culture (several weeks) and expansion of UCB and PB was also achieved in a cocultivation setting utilizing a perfused fixed bed bioreactor seeded with immobilized stromal cells on porous glass carriers [72]. As mentioned above, perfusion has been suggested to facilitate HSC expansion by increasing the medium exchange rate [73]. However, it is also known that hematopoietic cells are extremely sensitive to shear forces which can limit their viability in stirred and perfused systems or at least affect gene expression including cytokine receptors [68, 69]. This aspect has prompted Liu and coworkers [74] to apply a rotating wall vessel (RWV) bioreactor (33 mL working volume) which ensured laminar flow, resulting in minimal shear stress and well-mixed culture conditions, and also avoided the formation of gradients. Culturing UCB in the RWV reactor, on average, enabled a 435.5±87.6-fold expansion of all mononucleated cells paralleled by a 33.7±15.6-fold increase of CD34+ cells within ~ 8 days. Although this result is encouraging the authors have calculated that a process scale-up to four 500-mL RWV reactors running in parallel would be mandatory to generate a clinically relevant transplant for an 80-kg patient. This calculation assumes that the process is inoculated with a single, typical UCB sample and the expansion kinetic observed in the current 33-mL reactor scale is translatable to the envisioned 2-L dimension process and prolonged cultivation time.

Given the complexity of this multiparameter system it's not surprising that, despite the large number of studies HSC growth in serum-free, cytokine-supplemented liquid suspension culture has been still modest to date. For the next generation of HSC bioprocess design, it was therefore proposed to perform dynamic system perturbations comprising extensive control of the cell-population (lineage selective removal/maintenance), media control (exchange/dilution), and selective growth factor supplementation to efficiently increase particularly the stem and progenitor population in the culture [75].

4 Embryonic Stem Cells

4.1 ESC Expansion: Providing the Raw Material for Future Therapies

Compared to tissue-derived adult stem cells, embryonic stem cells that were successfully derived from blastocyst stage embryos of several species including mice [76], primates [77], and importantly humans [13], offer the particular advantage of prolonged proliferative capacity and great versatility in the lineages that can be formed in culture. Translating these advantages into clinical benefits faces many

challenges, including the efficient differentiation into a desired cell type, maintaining genetic stability during long term culture, ensuring the absence of tumorigenic ESC persisting in a therapeutic product, and scalability of existing protocols for mass generation of donor cells. By focusing on recent approaches of both, mouse and human ESC expansion and differentiation in bioreactors we will discuss the impact of above challenges on process scale-up.

To exploit the growth capability of ESC in their pluripotent state, a vital strategy for process development would depend on the expansion of a large starting population, which can be used to inoculate differentiation processes. Since ESC are anchorage-dependent and grow in typical colonies, current methods to scale-up their numbers have focused on flat surfaces or matrices [78]. For mass expansion, the simplest surface enlargement could be achieved by utilizing multilayered tissue culture flasks, so-called cell factories (produced by several manufacturers). They provide a relative large growth surface in limited space under standard tissue culture conditions facilitating adaptation of established tissue culture protocols. Their disposable nature would also facilitate GMP and clinical compliance. However, homogeneous cell distribution for the inoculation of multilayered flasks would require single cell dissociation of ESC combined with a medium formulation that ensures robust self-renewal. Mouse ESC (mESC) fulfill these requirements. They can be passaged by single cell dissociation and differentiation is largely avoided when the cells are grown on a simple gelatin matrix in the presence of leukemia inhibitory factor (LIF), an interleukin-6 family member that activates the Jak/Stat pathway. Unfortunately, this pathway fails to maintain self-renewal in human ESC [79].

Research to unravel the apparently multifactorial network of growth factors and downstream signaling that controls hESC pluripotency is in full swing. Members of the FGF family, particularly FGF-2, have been shown to support hESC self-renewal whereas the blockage of BMP-signaling by noggin or activin is required to retain their phenotype. For details on this topic please see recent reports and reviews [80, 81].

At present, serum replacement-based media (to avoid fetal calf serum; a product form Invitrogen, Carlsbad, USA) supplemented with FGF-2 are still broadly used for hESC culture. In addition, hESC have been mostly grown on a variety of feeder cell lines or on extra cellular matrices such as matrigel, fibronectin, laminin, or heparan sulfate and supplemented with conditioned media derived from the feeder cells [78, 82, 83]. To enable up-scaling of these culture platforms a clinical grade-human feeder cell line grown on microcarriers in spinner flasks was recently established [84]. These extensively characterized feeders have also been used to derive clinical-grade hESC cell lines [85], an important step toward the generation of fully controlled products for clinical trials. Large scale production of clinical- and cGMP-grade feeder conditioned medium might be a commercially vital strategy for hESC mass culture thereby limiting the need for costly growth factor supplementation even if a definitive cocktail will finally be available.

However, a notable discovery identified that hESC are capable of taking up substantial amounts of the potentially immunogenic nonhuman sialic acid Neu5Gc [86] and acquire bovine apolipoprotein B-100 [87] from feeder layers and the serum replacement medium, which contains animal compounds such as bovine

serum albumin. Extensive research is therefore ongoing to replace bovine components either by recombinant human serum albumin and/or to simplify culture condition with just the essential serum components, such as sphingosine-1-phosphate and platelet-derived growth factor [78].

Notably, and in contrast to the still elusive definitive markers of hematopoietic stem cells, availability of numerous well established markers known to be expressed in pluripotent hESC strongly facilitates the mandatory development of completely defined culture media. Pluripotentiality markers that are downregulated upon hESC differentiation include surface antigens such as stage specific embryonic antigen (SSEA)-3 and SSEA-4, Trafalgar (Tra)-1-60 and Tra-1-81, and GCTM-2 as well as transcription factors including Oct4, Nanog, and Sox2 [88]. Immune cytology specific to these and other markers unraveled the heterogeneity of hESC cultures grown under most established culture condition suggesting progressive differentiation to some degree [88, 89]. The grade of culture heterogeneity, which also varies between independently derived hESC lines [90], apparently adds another level of complexity to the system, thereby imposing challenges to the sensitive issue of reproducibility in process development. Furthermore, the epigenetic stability of hESC is intensively discussed which may impact on the differentiation characteristics of, for example, genetically modified clonal sublines [91, 92]. Additional assays for quality control of hESC culture optimization comprise measuring the telomere length and, particularly, the regular analysis of the karyotypic integrity.

Processing of adherent cells strongly depends on single cell dissociation. It has implications for controlled scale-up and automation, where it is important to seed bioreactors or scaffolds with reproducible numbers of evenly distributed cells. This issue is particularly apparent in hESC culture where the majority of cells do not survive dissociation into a single cell suspension [93, 94]. Thus, hESC are still propagated as aggregates in standard tissue culture scale and colony dissociation is usually performed via manual scoring methods using plastic tips (with or without enzymatic pretreatment), scoring with more facilitated cutting machines developed by inventive colleagues [95], or commercially available “cake cutters” [96].

In addition to decrease survivability, single cell dissociation for passaging seems to interfere with the chromosomal integrity of hESC, particularly resulting in trisomias, probably reflecting the progressive adaptation of self-renewing cells to their culture conditions [94, 97]. Other authors have suggested that single cell adaptation and long term expansion are achievable in the absence of, at least macroscopic, chromosomal aberrations [98, 99]. If these findings are robust, reproducible, and cell-line independent, the approach might facilitate scalable hESC expansion, efficient generation of transgenic hESC lines (which has now been achieved in Christine Mummery’s group [100]) and the induction of differentiation from single cells via embryoid body formation in bioreactors. However, a recent study revealed that even conditions that prevent macroscopic aneuploidy of single cell-expanded hESC might result in subkaryotypic deletions and amplifications (identified by competitive genomic hybridization) over only 10 passages, reinforcing that present culture regimes

remain suboptimal [101]. Notably, chromosomal abnormalities occurring after prolonged culturing are not limited to hESC; a recent report on mesenchymal stem cells shows that abnormal karyotypes can be detected if the cells are extensively passaged [102].

As mentioned in the introduction, the increased, systematic screening and application of small molecule inhibitors might provide new ideas and viable solutions to the field. In a recent report, the transient addition of the p160-Rho associated coiled-coil kinase (ROCK)-inhibitor Y-27632 to non single cell adapted cultures promoted survival of single cell dissociated hESC without affecting pluripotency [103]. Efficient single cell rescue and high plating efficiency might slow down the selection pressure that currently results in karyotypically abnormal cells upon culture adaptation. Thus, the compound might facilitate the single-cell-based expansion of normal hESC, generation of transgenic lines, and also the controlled inoculation of bioreactors with a single cell suspension for differentiation processes.

The single cell issue might also be resolved by alternative, potentially scalable culture strategies. Several groups have established suspension culture expansion of mouse ESC in stirred vessels by forming cell aggregates where differentiation is prevented by medium conditions, serial passaging and mechanical shearing [104, 105]. Further optimization of culture media that can efficiently avoid differentiation might allow translating this and other more automated approaches to hESC [106]. Modifying culture conditions in such systems, for example, by decreasing shear stress to allow larger aggregate formation and replenishing expansion medium by a respective differentiation medium, might allow switching from growth to differentiation in a one-step process.

Seeding cells onto microcarriers is another strategy to translate adherent, matrix dependent cells into easy-to-scale, fully instrumented and controlled stirred tank reactors. Taking advantage of the robust mouse ESC system, the groups of Zandstra and Cabral have provided initial evidence that microcarriers can be adapted to provide surface enlargement for murine ESC culture in suspension [37, 38, 104]. A high degree of carrier and cell agglomeration resulting in heterogeneous clumps was observed in these studies which substantially limits the degree of surface enlargement provided by the carrier and might also induce cell differentiation in the core of these clumps. However, expression of the tested ESC surface markers was largely retained and the ability to form embryoid bodies was also shown by Fok and coworkers [104]. Cabral's groups presented some degree of mESC expansion in an 8-day process utilizing stirred spinner flask with a working volume of up to 80 mL; unfortunately only a single passage was documented in these studies limiting conclusions about an extended applicability. A more general issue concerns the need for efficient removal of microcarriers from the final stem cell product before clinical application. However, this obstacle might be resolved if other hurdles such as the increased shear stress in stirred, microcarriers containing cultures is compatible with hESC expansion, a platform that has not yet been published but is currently developed in several labs (Blaine Phillips, Institute of Medical Biology, Singapore; Andre Choo, Steve Oh, Bioprocessing Technology Institute, Singapore; personal communication).

4.2 Scaling up ESC Differentiation: A Focus on Cardiomyocytes

Given the challenges in hESC culture, published studies on ESC expansion in scalable bioreactors have so far been limited to mouse ESC. In tissue culture, however, substantial progress has been made towards the directed lineage differentiation of human and primate ESC [107] as well. Improved differentiation regimens towards clinical relevant cell types include insulin producing beta-like cells [10, 28], dopaminergic neurons [108], hepatocytes [109] and other lineages [110].

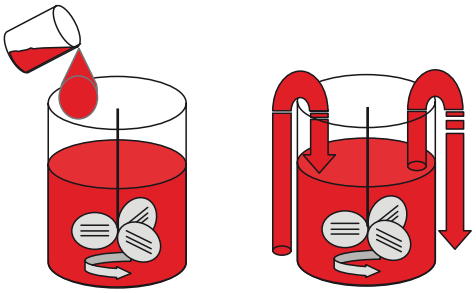
Nevertheless, mESC have a threefold shorter population doubling time ([PDT; ~12–16 h [93]) compared to the ~36 h observed in hESC [85]. Raw material can therefore be generated much faster and attempts towards scalable differentiation have mostly utilized murine ESC as well. Many studies have focused on the generation of ESC-derived cardiomyocytes. This might be driven by the high demand of this cell type for pharmacological screening purposes [111], tissue engineering approaches and cell-based heart repair. In the next step, extensive numbers of well-characterized cardiomyocytes from mouse, primate and human ESC will be mandatory for the functional testing of these cells in physiologically relevant large animal models of human heart failure such as pigs and primates [15, 112].

Besides media formulations, efficiency and robustness of differentiation processes strongly relies on, first, the homogeneity of ESC cultures used for process inoculation and, second, the consistent production of homogeneous embryoid bodies (EBs). These are spherical structures which are induced to initiate spontaneous differentiation of ESC in suspension; they are key to process reproducibility [7]. The heterogeneity of pluripotent hESC cultures has been discussed extensively elsewhere [88, 89], so we will focus our discussion on the formation of homogeneous EBs.

Controlling cell aggregation and agglomeration during EB formation has a profound effect on the extent of ESC proliferation and differentiation; EB size was found to be critical for cardiomyocyte formation and other lineages in the mouse and human system [113–116]. Spatiotemporal formation of these spherical structures was extensively studied in mESC utilizing numerous different formats all aimed at controlled sphere formation. This included the nicely controlled but non-scalable hanging-drop technique [117], cell-encapsulation in alginate beads [114], rotating-suspension culture in a 10 mL volume [118], stirred spinner flask cultures, and controlled reactors with up to 250 mL culture volume [119–121].

Recently, we have shown stirring-controlled EB formation and mESC differentiation in a 2-L instrumented and controlled bioreactor scale, thereby enabling the production of more than 1.2 billion cardiomyocytes in a single run [7]. This cell expansion approaches the 1–2 billion functional cardiomyocytes which are irretrievably lost in a patient's heart upon infarction, a number that could readily be provided by the bioreactor approach if translatable to hESC. A coefficient of 6.4 cardiomyocytes being generated per input ESC (CM/ESC) was found in our bioreactor approach utilizing a genetically engineered mouse ESC line that facilitates enrichment of pure cardiomyocytes.

In a follow-up study, applying multiple steps of process modification particularly applying lower medium throughput and continues perfusion feeding (in contrast to batch-feeding performed in our previous work [7]), this value was even improved to



Reactor working volume	2 l	2 l
Feeding strategy	Batch: 1 l medium/day	Perfusion: 0.5 l/day
Process duration	18 days	16 days
Total medium throughput	16 l	9 l
Inoculation (transgenic mouse ESC)	0.2 billion	0.2 billion
Total cardiomyocyte yield	0.86 billion	4.6 billion
Cardiomyocytes/ESC	4.3 CMs/ESC	23 CMs/ESC
Cardiomyocytes/l medium throughput	54 million/l	510 million/l

Fig. 1 Process optimization potential. Multiple steps of process modification, particularly perfusion feeding and reduced medium throughput, resulted in a fivefold increase in cardiomyocyte yield from a transgenic mouse ESC-line in a fully controlled 2-L stirred reactor. The efficiency in cardiomyocyte generation per liter total medium throughput even increase by almost 10-fold [7, 122]

23 CM/ESC [122] thereby underscoring the enormous process optimization potential (Fig. 1). More homogeneous culture conditions achieved via continuous feeding might support a better control of ESC differentiation. In vitro differentiation of ESC is notoriously variable due to the ongoing changes in cell density paralleled by the occurrence of differentiating cell lineages and thus changes in cell physiology, cell–cell interactions, growth factor secretion, etc. Inhibition of ESC differentiation following a noncontinuous, daily medium exchange was described by Viswanathan et al. [123] as cell-secreted factors were diluted. As outlined above, it has also been reported for hematopoietic cell cultures that the consumption and release of a variety of growth factors can affect the cell type(s) generated in a process [75]. Continuous feeding strategies ensure optimal process uniformity with respect to pH, pO_2, and concentration of metabolites while manual medium exchange, at least transiently, encounters alternating pH and gassing conditions. For example, high oxygen tension has been suggested to inhibit cardiac differentiation. In a study by Bauwens and coworkers [121] a controlled, perfusion fed system at a 250-mL scale was employed. Notably, the same cell line and similar differentiation and selection conditions as in the study by Niebruegge [122] were used, but EBs were formed from encapsulated ESC. Highest cardiomyocyte yield was archived under hypoxic conditions (4% oxygen tension) resulting in a CM/ESC-coefficient of 3.77 and a drastically lower value of 2.56 CM/ESC was found at normoxia. However, the significantly higher

CM/ESC coefficient of 23 described by us was achieved at an oxygen tension of 40%; whether hypoxic conditions would further increase this value in the controlled 2-L bioreactor setting applied by us requires further experimental evidence.

Another benchmark value which is key to the commercial viability of future cell replacement therapies, is the number of cardiomyocytes that can be generated per liter (of a potentially expensive) culture medium. Under optimized conditions >500 million cardiomyocytes per liter medium were generated in our optimized 18-day differentiation and enrichment process [122].

A first demonstration of the translation efficiency of hESC into cardiomyocytes was recently provided by a monolayer differentiation protocol (sequential addition of activinA followed by BMP4) yielding three CM/hESC [26]. However, scalability and economic feasibility for the mass-production of cardiomyocytes by this growth factor-dependent, two-dimensional monolayer approach needs to be determined.

Aiming at efficient cardiomygenic differentiation of hESC in suspension, we have recently converted a coculture based protocol for directed cardiomyocyte generation into a scalable suspension process (Fig. 2), using a serum-free medium conditioned

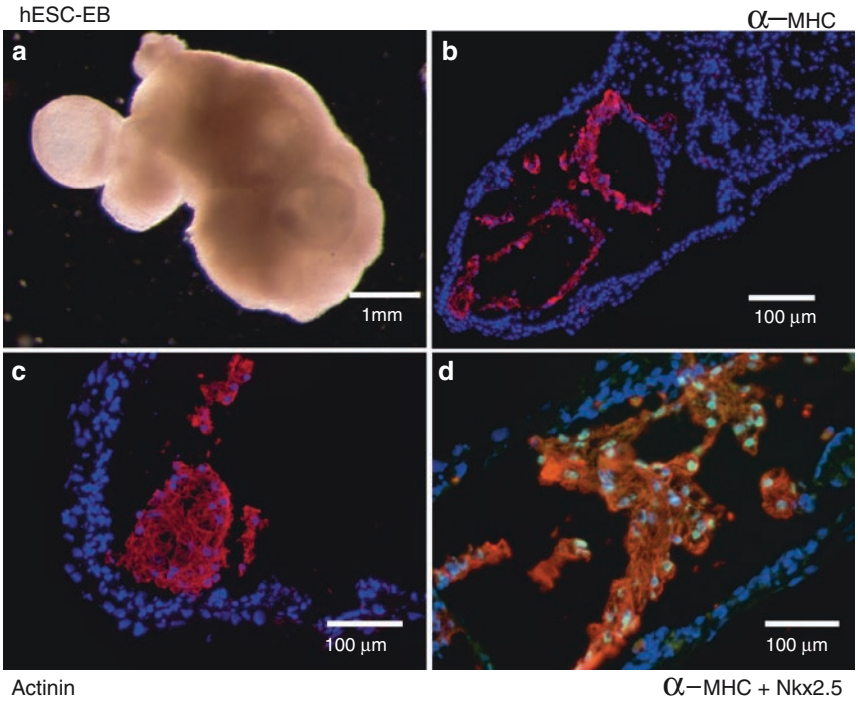


Fig. 2a–d Cardiomyocyte formation from hESC in scalable suspension culture. **a** A typical cystic embryoid body after about 12 days of differentiation in a serum free medium supporting cardiomyogenesis. **b** Immune histology of EB-sections specific to cardiac markers (*in red*) alpha Myosin Heavy Chain (alpha-MHC). **c,d** Actinin (**c**) and double-staining to alpha-MHC and Nkx2.5 (transcription factor, nuclear stain *in green* (**d**)) show the formation of cardiomyocyte-clusters in cysts [85, 124, 125]

by and endoderm-like cell line END2 (END2-CM; [124]). By screening small molecule inhibitors in this system, we have identified SB203580, a specific p38 MAP kinase inhibitor, as a potent, dose dependent promoter of cardiomyogenesis. SB203580 at an optimized concentration, induced >20% of hESC to become cardiomyocytes. A parallel increase in total cell number yield approximately 2.5-fold more cardiomyocytes compared to differentiation in END2-CM alone. Besides ascorbic acid, SB203580 is one of the first molecules to act as an efficient enhancer of hESC cardiac differentiation; other factors such as DMSO and retinoic acid, known inducers of mESC cardiomyogenesis, caused no significant improvement [15].

By systematically deconstructing the cardiomyocyte inducing activity of the "xenogenic" END2-CM we have found that the common media supplement insulin can have a dramatic inhibitory effect on the formation of cardiomyocytes [125]. The insulin effect, which was also triggered by the growth factor IGF1, was mediated through activation of the PI3/Akt pathway downstream of the insulin/IGF1 receptors during early steps of differentiation. Notably, this observation might also explain the varying compliance of serum batches for cardiac differentiation. The study further identified a small molecule, the prostaglandin member PGI2, as accumulating in END2-CM and enhancing cardiomyogenesis when added into a novel, insulin-free synthetic medium at optimized concentrations. Finally, combining SB203580 with the synthetic medium yielded a fully defined, cGMP-compliant medium, which enabled efficient hESC differentiation in suspension. In a second study we found that insulin redirects differentiation of hESC from mesendoderm to neuroectoderm [126].

One major difference between mouse and human ESC that is still hampering the systematic up-scaling of differentiation is the inability of the latter to reaggregate and form EBs once dissociated to single cells [127]. High expression levels of the cell adhesion molecule E-cadherin [113] seem to underlie the aggregation of mESC, and EB formation is focused on controlling the excessive fusion tendency interfering with differentiation. In contrast, although the majority of undifferentiated hESC also express E-cadherin [128], essentially all cells die when seeded in single cell suspension. This phenotype is seemingly independent of the cell line, the dissociation method, the culture medium and the seeding density [127, 129]. Consequently, most of the present differentiation studies rely on either enzymatic whole colony lifting (thereby separating hESC from the feeder layer) or other enzymatic and/or mechanical scoring techniques aimed at providing preformed hESC-aggregates of various size for EB formation in suspension [127, 129].

These hurdles might explain the limited number of studies on hESC differentiation scale-up. Gerecht-Nir and coworkers have used small cell clumps to inoculate RWV termed slow turning lateral vessels, or high aspect rotating vessels to control floating EB formation [115]. However, scalability of these specialized reactors might be limited. A first step towards hESC differentiation in impeller-stirred systems was published by Cameron and coworkers [130] employing a 250-mL spinner flask system, while another study translated the encapsulation approach of EBs in agarose from mouse to human ESC [114]. However, all of these studies depend on

the cumbersome, difficult-to-control, and hardly scalable preformation of hESC clumps before process inoculation. The only published strategies that seem to enable hESC-derived EB formation directly from single cell suspensions is seeding on three-dimensional porous alginate scaffolds [116] or the forced aggregation by centrifugation in round-bottom or V-shaped 96-well dishes [127, 129] which has recently been scaled to a 384-well format by custom-made silicon wafer-based microfabrication [131]. These studies indicate that the dissociation procedure, per se, is not irrevocably inducing hESC death but suggest that constraining physical cell–cell or cell–matrix interaction combined with chemical cues (from the substrate surface and/or the medium) are necessary to rescue single hESC. While both methods (porous alginate scaffolds and multi well dishes) are not straightforward for large-scale inoculation of stirred bioreactors, the underlying mechanism might be exploitable in future.

In summary, in vitro differentiation of ESC is a complex, continuously changing, and thus highly variable process. However, recent findings by us and others in controlled bioreactors indicate that reproducible and efficient production of differentiated lineages such as cardiomycytes is achievable. Translating highly controlled single cell inoculation and EB formation to hESC cultures and utilizing the recently developed fully synthetic differentiation media is another step towards this goal.

4.3 Enrichment of Differentiated Cell Types: The Need for Purity and Safety

Many of the envisioned hESC therapeutic as well as in vitro screening applications will require pure populations of a desired cell type such as cardiomyocytes that are devoid of any other lineage, in particular, residual, undifferentiated hESC [132]. A purification strategy is therefore essential and has proven to be effective for enrichment of hematopoietic stem cells from bone marrow and differentiated hESC populations. In the case of cardiomyocytes, however, there is no unique cardiac-specific surface marker that can be used for cellular isolation. Recently we have demonstrated that the surface marker CD166/Alcam which is specific to a transient population of heart-tube stage embryonic cardiomyocytes [133], is useful for isolating cells homologous to human embryonic cardiomyocytes from differentiated hESC populations (MASC; [134]). Using a sterile, magnet-assisted cell sorting system, we took advantage of this marker to produce cardiomyocyte populations that are greater than 60% pure from wild-type hESC.

Furthermore, Choo et al. [135] have demonstrated the ability to kill undifferentiated hESC using a cytotoxicity monoclonal antibody thereby eliminating teratoma formation in vivo in a SCID mouse model. The combination of positive and negative selection strategies will greatly facilitate in the enrichment of cardiomyocytes, which, until recently, was limited to improved differentiation strategies and hardly reproducible, selective dissociation protocols combined with Percoll gradient centrifugation [26, 136].

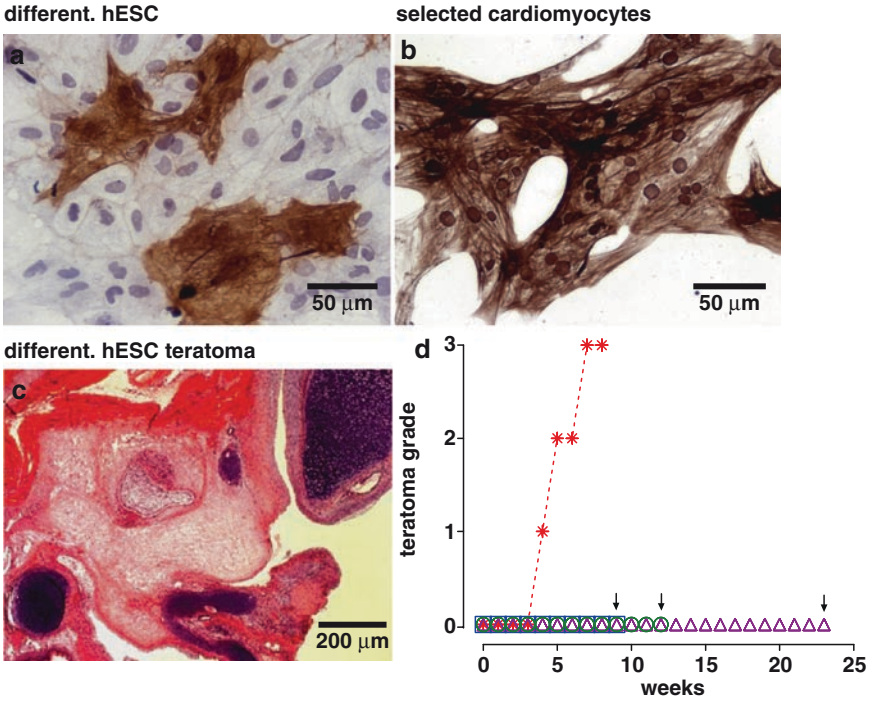


Fig. 3a–d Enrichment and safety of suspension-derived hESC-cardiomyocytes. Dissociated, differentiated embroid bodies (EBs) were seeded to generate a cell layer that contains a proportion of cardomyocytes presented *in brown* (**a**) (DAB stain specific to the cardiac marker alpha Myosin Heavy Chain). Antibiotic enrichment of cardiomyocytes (derived from a transgenic hESC-line in suspension) followed by cell seeding resulted in an essentially pure cardiomyocyte population (**b**). Injection of differentiated but not antibiotic-treated EBs as non-dissociated clumps resulted in teratoma formation is a SCID-hindlimb model (HE-stain of teratoma section in (**c**) within about 3–7 weeks (*red, dotted line* in (**d**)). In contrast, no teratoma formation was observed from an equivalent number of antibiotic-enriched cardiomyocyte-clumps injected in the same model when mice were analyzed after 9, 12, and 23 weeks (less time point tested) [142]

Previously, Field and his colleagues conceived a simple but ingenious genetic selection strategy for mouse ESC-derived cardiomyocytes. Introducing a transgene comprising the murine α-MHC promoter driving cardiomyocyte-specific expression of an antibiotic resistance gene enabled the enrichment of >99% pure cardiomyocyte populations [137]. This selection scheme was consequently applied to enrich for other cell lineages, including neural precursors and insulin-producing cells [138, 139], and adapted to mass production of cardiomyocytes in suspension culture [7, 118, 120, 122]. Aiming at the derivation of cardiomyocyte-subtypes, alternative constructs, such as the myosin light chain 2v (MLC2v) promoter in combination with a GFP-expression cassette followed by fluorescence based cell sorting (FACS), have also been utilized [140]. By generating stable transgenic lines using lentiviral vectors this strategy has been translated to hESC.

GFP expression under the transcriptional control of the human MLC2v promoter appeared to be cardiomyocyte-specific [141]. After FACS sorting, >93% of the isolated cells stained positive for cardiac-specific proteins and formed stable myocardial cell grafts for up to 4 weeks (the latest time point tested) following in vivo cell transplantation into immune suppressed Sprague–Dawley rats. The study provides the first proof-of-concept for the genetic lineage selection strategy to work in hESC. Although no teratoma formation was observed in this study, the animal model as well as the short follow-up time might not be useful to appraise this risk.

By applying the antibiotic-based lineage enrichment strategy introduced by Fields group to hESC, we have recently generated multiple transgenic hESC lines (via electroporation) and achieved >99% cardiomyocytes purity from differentiated hESC cultures [142] (Fig. 3) More importantly, applying a sensitive biosafety model for teratoma formation in SCID mice [143, 144] no teratomas were found for up to 23 weeks after the injection of antibiotic-selected cardiomyocytes clumps. In contrast, the injection of long term differentiated but not antibiotic treated EBs resulted in teratoma formation with high incidence [142]. These findings strongly underscore the necessity of efficient selection techniques and comprehensive long term safety studies in appropriate animal models. The therapeutic application of transgenic hESC lines might comprise yet another regulatory hurdle to clinical trials. However, where the genomic integration site of the transgene is well defined this technology clearly provides another level of safety in hESC-derived grafts. Whether other selection techniques will achieve the same level of scalability, purity, cell vitality, and safety remains to be demonstrated.

5 Bioprocessing of ESC- and Tissue-Derived Mesenchymal and Neural Stem and Progenitor Cells

Batch differentiation of an expanded ESC population which might be combined with a consecutive enrichment procedure is one possibility for the generation of specific progenies. The strategy is particularly useful if the differentiated cell type has no or only a limited proliferation potential such as cardiomyocytes [145, 146].

An alternative scenario is to generate intermediate cell types from ESC that are still capable of extended proliferation but are lineage-committed progenitors. Such intermediate stem- or progenitor type cells can also be derived from some adult tissues. However, the reproducible derivation of intermediate type stem cells from clinical-grade hESC might provide an invariable source of consistently uniform cells for therapeutic applications, thereby overcoming serious limitations imposed by the heterogeneity of donor tissue-derived cells.

Multipotent stem cells provide an expandable cell source that can either be used to produce more differentiated progenies or might serve directly for therapeutic or screening approaches. Recent studies on hESC- or adult tissue-derived mesenchymal stem cells and neural stem cells provide examples for this approach.

5.1 Neural Stem and Progenitor Cells

Cho and coworkers [108] have generated relative homogeneous spherical neural masses (SNM) from hESC colonies. SNM have a neural precursor phenotype and can be passaged long term in suspension culture without losing their differentiation capability. Finally, SNM have been directed into differentiated cultures consisting of 77% neurons. The vast majority, 86%, of these neurons comprise dopaminergic neurons, indicating a relative high purity of this desired cell type for Parkinson's treatment. At present SNM passaging requires mechanical handling and has not yet been scaled to bioreactors.

Neural stem cells (NSC) may also be isolated from both embryonic and adult tissue from the central nervous system (CNS). They are defined as tissue specific progenitor cells which undergo self-renewal in vitro and can be differentiated into all major cell types of the nervous system including oligodendrocytes, neurons and astrocytes [147]. NSC were thought to be particularly useful for the generation of dopaminergic neurons in vitro but the efficient differentiation towards this phenotype has been proven to be difficult. Hypoxic culture conditions appear to induce this process for human-derived tissue which forms dopamine neurons even less efficiently than NSC derived from mice [148]. A detailed description of multipotent neural stem and progenitor cell characteristics, their isolation from various sources and their envisioned therapeutic application is outside the scope of this publication. The interested reader is referred to a recent review by Hall, Li and Brundin [149]. However, the propagation of NSC in aggregates termed neurospheres is a paradigm for the expansion of pluripotent stem cells in bioreactors as outlined below.

Following the discovery of NSC in 1992 [150, 151] the group of Kallos and Behie has established and optimized scale-up of NSC cultures by controlling neurosphere size via hydrodynamic shear in stirred suspension culture [152, 153]. The process was scaled up to 500 mL culture volume in an instrumented bioreactor (temperature, pH, pO_2 control) enabling the generation of up to 1.2×10^6 cells/mL mouse NSC without interfering with the cells multipotentiality [154].

Notably, human neural precursor cells (hNPC) isolated from multiple fetal brain regions have recently also been expanded in stirred bioreactors aiming to provide tissue for neurodegenerative disorder treatments. In an initial study, reactor-expanded cells differentiated primarily into astrocytes after transplantation into the striatum or substantia nigra regions, and no behavioral improvement in a parkinsonian rat model was observed [155]. In a second study, telencephalic hNPC have been differentiated in highly enriched GABAergic cells following expansion in spinner flasks in 125 mL volume. Functional assessment in a rodent model of Huntington's disease revealed a significant behavioral improvement in motor and memory deficits following transplantation with differentiated GABAergic cells, whereas expanded but undifferentiated hNPC did not [156]. These recent studies on hNPC apparently suggest that stem cell differentiation into a desired cell type in vitro is mandatory for specific organ repair rather than to reliance on tissue specific differentiation of pluri- or multipotent stem cells following transplantation into a damaged organ. Next, it will be interesting to see at which scale primary hNPC can

be expanded under optimized condition in vitro without transformation and loss of differentiation properties. Finally, functional testing of GABAergic cells in primate models will be mandatory before entering clinical trials.

5.2 Mesenchymal Stem and Progenitor Cells

Mesenchymal stem cells (MSC; also known as multipotent mesenchymal stromal cells) comprise another cell type that has originally been derived from mammalian tissue but in vitro expandable MSC-like cells were recently also generated from hESC [157].

Following the pioneering work by Owen and Friendenstein on bone marrow stromal cells 20 years ago [158], MSC have also been isolated as plastic adherent, fibroblast-like cells from multiple other sources including placenta, adipose tissue, cord blood and liver (see recent review by Brooke et al. [159]).

The original stem cell term has been thought to be inadequate by many investigators as it has not been possible to grow human MSC indefinitely in culture while maintaining their multipotent properties. Currently, there is also no in vivo assay that can be used to define the repopulation ability of these cells analogous to existing assays for hematopoietic stem cells. The anatomical location and phenotype of MSC has also not yet been well defined in vivo. However, when isolated by plastic adherence and expanded, ex vivo human MSC have been shown to differentiate into mesodermal lineages including chondrocytes, adipocytes and osteocytes [160]. In addition to the in vitro differentiation potential the International Society for Cellular Therapy (ISCT) has recently proposed that MSC should be defined based on a panel of antibodies specific to CD105, CD73, and CD90 (>95% of the population should be positive) and CD45, CD34, CD14 and CD19 (<2% of the population should be positive), but notably none of these antigens are unique to MSC [161].

Nevertheless, MSC comprise an attractive cell type for therapeutic applications given their potential for organ repair, ease and reproducibility of isolation, some level of in vitro expandability, and immunosuppressive and/or immunoprivileged properties [162, 163], which particularly favor this cell type for the generation of allogeneic “off the shelf” stem cell products. In preclinical studies of tissue repair MSC have been shown to improve the function of the heart, brain, liver, and joint and they are currently tested for the regeneration of these and other organs in clinical trials as well as for immunological disorders and solid organ transplantation, the later being recently reviewed elsewhere [159]. However, it may become apparent that MSC exert many if not all effects via paracrine mechanisms, that is secreting factors and supplying the necessary environment for host tissue to repair itself recently noted by Brooke et al. [159].

Consequently, Timmers and coworker have infused a medium conditioned by hESC-derived MSC into the coronary vasculature of pig hearts in a myocardial infarct model [164]. This was associated with a 60% reduction of infarct size and marked improvement of systolic and diastolic cardiac performance. Development of large scale cGMP-compliant processes is currently underway to establish the

production of MSC-conditioned medium in sufficient quantities for clinical trials (Andre Choo and Steve Oh Bioprocessing Technology Institute, Singapore, personal communication).

Aiming at scalable MSC expansion, recent bioreactor studies have applied perfusion of human MSC embedded in three-dimensional scaffolds [165, 166]. These studies have shown that shear stress is an important biomechanical parameter in regulating MSC growth, and increased cell expansion was observed at lower perfusion rates [165]. Other culture systems, including static cultures, stirred reactors and rotated vessel reactors, which all impose highly differential shear conditions, consequently resulted in differential growth and differentiation properties of adult human bone marrow-derived MSC when cell proliferation and multilineage differentiation towards osteoblasts, chondrocytes, and adipocytes was analyzed [167, 168]. However, as with hematopoietic and other stem cell types, donor cell variability, variations in MSC isolation procedures, and a large number of cell culture variables makes direct comparison of results presented in independent studies problematic. Using hESC-derived MSC isolated under reproducible conditions and applying a meaningful side-by-side comparison of reactor systems might increase the knowledge on favorable culture conditions in future studies.

6 Conclusion and Outlook

In a recent assay on the future of stem cell biotechnology, Ann Parson [5] stated that only time will tell if "RegenMed 2.0" (Stem Cell based Therapy) will prevail or whether it will go the way of "RegenMed 1.0" (Gene Therapy based Regeneration). Unlimited availability of stem cells, the building stones of RegenMed2 in reproducible quality and at commercially viable conditions will be of fundamental importance to success.

Engineering has already provided bioreactors that can accommodate all major needs for large scale mammalian cell production. Sophisticated techniques to meet special demands posed by stem cells are continuously under development. Miniaturization has allowed scaling down (!) of bioreactor systems to a ~30 mL working volume which still allows full instrumentation and thus measurement and computational control and adaptation of key culture conditions (pH, pO_2, continuous medium supply, etc.) in multiple parallel bioreactors, thereby speeding up process development under conditions which in principle apply to 10- to 100-fold larger systems (for example from Dasgip, Juelich, Germany).

Another trend is the development of disposable bioreactors such as simple or more sophisticated spinner flasks some of which are readily equipped with active culture aeration modules (to enable increased cell densities) and ports for simplified sample collection. Establishing initial cGMP-compliant small scale processes based on disposable reactors to feed cells into phase1 clinical trials will benefit the field. Such step-by-step strategy providing stem cell products as a personalized treatment seems to be a more feasible approach, at present. Shooting for the ultimate goal, a

"one-fits-all of-the-shelf" (organ but not recipient specific) stem cell product that is generated in multi-liter tanks and stocked frozen until usage is apparently not yet enabled.

Basic research still needs to define complex, interwoven networks of molecular mechanisms controlling stem cell maintenance, genomic stability, and differentiation. Systematic high-throughput technologies like "omics" approaches (gen-, transcript-, prote-, metabol-omics etc.) as well as continuous progress in developmental biology and tumor cell biology will help to understand these fundamental questions; stem cell research will vice versa feed back into these research disciplines. These findings combined with systematic screens for small molecular effectors to control identified signaling pathways will finally lead towards commercially viable process and progressive increase in production scales.

Definitions

- Bioreactor: a system that simulates physiological environments for the creation, physical conditioning, and testing of cells, tissues, precursors, support structures, and organs in vitro
- Teratoma: benign tumors containing cells from various differentiated tissues
- Stem cells are defined as being self-renewing, pluri- or multipotent, and clonogenic cells
- Clonogenic cells are single stem cells that are able to generate a line of genetically identical cells, thereby maintaining their self-renewal and differentiation potential
- Pancytopenia: shortage of all types of blood cells
- Embryoid bodies: spherical structures which are induced to initiate spontaneous differentiation of ESC in suspension

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