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Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: Current technologies and challenges

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ABSTRACT

The long-term outlook for regenerative medicine predicts an increased need for scalable cell expansion technologies that utilize non-animal derived materials and are compatible with the limited number of downstream processing steps required for cell-based therapies. As more stem cell therapeutics progress through clinical testing, current *in vitro* culture methods using planar vessels are proving cumbersome to scale. Therefore, alternative processes are under investigation. Many human mesenchymal stem/stromal cell (hMSC) bioreactor-based manufacturing processes, in particular, are complicated by the requirement to separate cells from microcarriers with high cell yield and viability whilst maintaining target phenotypic and functional characteristics. Here we review currently available technologies and ongoing development for the expansion of cellular therapeutics, with focus on allogeneic hMSCs and microcarrier-based processes. Upstream challenges include the interplay between the cell culture substrate and media formulation, sourcing of high quality animal-free reagents, and considerations for the use of microcarriers in stirred-tank systems. Complications in downstream processes include harvest approaches for separation of cells from microcarriers and volume reduction.

1. Introduction

Stem cell-based therapies are distinct from traditional biopharmaceuticals in that the cell itself is the final treatment product rather than simply the means by which to produce a drug substance. Stem cells injected into a patient may engraft and/or secrete molecules that elicit an endogenous response. The injection is a complex therapeutic that must be administered in a certain functional state and can be influenced by the microenvironment or niche [1,2].

There are two categories of cellular therapeutics: autologous and allogeneic which can also be described as patient-specific and universal donor, respectively. For an autologous therapy, the individual donor is also the recipient of the treatment termed a "one-to-one" therapy. In the allogeneic approach, a single donor provides primary cells used to produce the therapeutic cells that will be administered to many recipients; thus considered a "one-to-many" therapy. Autologous and allogeneic therapies each require distinct criteria in order to meet manufacturers' and patients' needs. Autologous applications may require expansion of cells prior to administration; thus the scaling of these technologies is considered "scale-out" where many donors' cells may be processed in parallel. Key needs for autologous expanded cells are automated and closed manufacturing, as well as faster testing of the drug substance. Cell expansion is required for allogeneic therapies in order to generate ready-to-use doses for multiple patients; these are considered "scale-up" applications, calling for larger volumes to meet lot size needs. Key needs for allogeneic expanded cells are scalable expansion vessels up to 1–2kL with similar downstream processing capabilities [3,4]. There are examples of cell therapies that cross these commonly used descriptors, such as a cord blood transplants that are one-to-one, but in fact can be used in non-matched recipients. In all instances, cGMP grade raw materials, including serum replacement and ideally chemically-defined media, are important for ensuring that the highest quality product is manufactured.

There are a growing number of clinical and commercial activities in the cell therapy space including hundreds of global trials and

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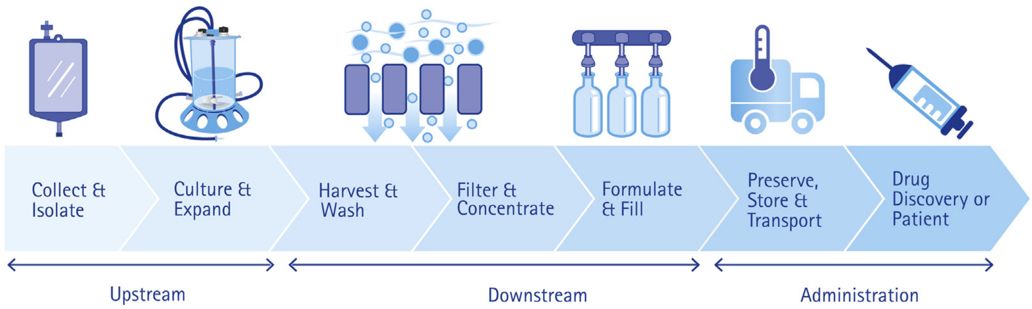

Fig. 1. A process for the manufacturing of a cellular therapeutic. Major challenges remain in all stages; in particular harvest, formulation and transportation.

marketed products for "stem cells", excluding gene modified therapies [5]. The vast majority of these employ adult, multipotent cells, such as human mesenchymal stem/stromal cells (hMSCs). Whereas many of the first cell therapy applications treated musculoskeletal and skin diseases, some of the most promising applications include those for acute diseases, such as cardiovascular or stroke events, and immunological dysfunction. Celyad (formerly Cardio3 Biosciences) is using hMSCs differentiated to a cardiac progenitor lineage in a phase 3 clinical trial to treat congestive heart failure [6]. Osiris Therapeutics recently divested their culture-expanded hMSCs to Mesoblast, and continues to investigate autologous applications in cell therapy [7], while Mesoblast has a phase 3 trial application for graft versus host disease using the expanded hMSCs [8]. Although a joint Athersys-Pfizer phase 2 trial did not find a significant effect of undifferentiated bone marrow-derived hMSCs on ulcerative colitis at midpoint outcomes, the safety profile of first round dosing was favorable and the trial continued to second round dosing and later time points [9,10]. Athersys is also evaluating these cells as possible treatment for other indications including ischemic stroke [11].

Challenges in generating cells for therapeutics include the lack of scalable, cost-effective systems utilizing processing equipment in which the product is not exposed to the immediate room environment (*i.e.,* closed systems). In order to affect this sort of system, solutions that ensure patient safety, operator ease of use and lot-to-lot reproducibility in the final product must be implemented. There has been intense focus on increasing scale and yield in order to drive down costs [3]; however, another avenue that can be investigated is to increase the potency of the cells produced. With a true understanding of the mechanism of action, the production of cells with specific quality attributes can be directed, thus requiring fewer cells for the same treatment. The result is a lower cost of goods and smaller batch size requirement. Both development of scalable manufacturing solutions and improvement of potency of the cells should be pursued in order to ensure the successful implementation cell therapy treatments.

The large-scale industrialization of stem cell production for their use as therapeutics (Fig. 1) presents several opportunities for manufacturers to develop regulatory-compliant, cost-effective processes. As discussed herein, it is clear that movement away from planar culture and towards stirred tank bioreactors where suspension culture using microcarriers is enabled, will be a requirement [4]. The bulk of current work to transition hMSCs to stirred tank bioreactors leverages microcarriers, however the growth of hMSCs as aggregates has also been described [12,13]. More recently, Alimperti et al.demonstrated the culture of hMSC spheroids in small-scale suspension culture [14]. Although this approach will not be discussed in detail, many of the concepts herein concerning reagent and process optimization may also be relevant to hMSC expansion in stirred systems without microcarriers. A complete single-use, end-to-end, manufacturing process is attractive due to its lower overall start-up cost versus traditional stainless steel processes, its inherent flexibility and adaptability to the emerging requirements of cell-based therapeutics, and the scale required. Some of the difficulties to this approach include ensuring that all components interacting with the process stream are low in extractables, do not contain materials derived from animal sources or at least comply with established regulatory guidance on minimizing the risk of transmitting animal spongiform encephalopathy as described in EMEA/410/01, while at the same time meeting the requirements of cell yield and performance. It should be noted that these are of critical importance as the cells undergo minimal processing before introduction into patients.

2. Scale-up approaches

2.1. Transition from planar to microcarrier-based systems

Adherent cell expansion has traditionally been performed on planar surfaces such as well-plates and tissue culture flasks for simplicity and easy handling when large numbers of cells are not required. For larger scale expansion, multi-layered flasks, spinner flasks and bioreactors offer higher capacity (available technologies summarized in Table 1); however, optimal culture conditions for stem cells are still under investigation [15] and will certainly depend on each particular cell type. In addition, there is concern regarding the population doublings required for expansion of cells to sufficient quantities to meet target dose requirements [16]. There are challenges with growing stem cells in multi-layered flasks as these vessels are cumbersome and time-consuming to handle, have limited scalability and typically limit the user's ability to monitor cell health or marker status during cultivation. Transitioning from planar-based culture to microcarrier-based systems not only allows for higher density culture, and thereby cost of goods reduction, but also for more stringent culture control and monitoring.

The transition to bioreactors allows for greater process control since samples can be collected during and following the expansion process and characterized by off-line analytics such as flow cytometry which aids in optimizing the process. In order to retain the functional characteristics of hMSCs, the passage numbers and cell doublings are monitored and minimized [17]. MSCs expanded both in planar and suspension formats are commonly evaluated for cell identity and purity by criteria outlined by the International Society for Cellular Therapy [18] as well as the International Federation of Adipose Therapeutics and Sciences [19], yet these criteria may not always be indicative of function [20]. Apart from these recommended criteria, there are additional markers which are used by several researchers in the stem cell field, often with the goal of isolating or defining a more homogeneous population of cells [21–23]. Moreover, a broad range of functional assays are employed to determine whether expanded hMSCs have maintained differentiation potential, cytokine release, immune modulation, migration capacity and/or angiogenic potency, as examples [23].

Table 1
Summary of available single-use cellular expansion technologies. Approximate surface areas for suspension systems are based on 15 g/L of a 360 cm^2/g microcarrier.

Technology	Volume	Surface area (approx. cm^2)	Example technologies	Features
T-flasks (single layer/≤5 multilayers)	30 mL/200 mL	150/1000	Vented flasks (Corning)/Millicell® HY multilayer culture flasks (EMD Millipore)	- Small scale - High volume:area ratio - No inline control or monitoring
Multilayer stacks (10/40 stack layers)	1.4 L/5.5 L	6,360/25,440	CellSTACK® cell culture chambers (Corning), Nunc Cell Factory™ systems (Thermo Scientific)	- Single-batch expansion - Small scale - High volume:area ratio - No inline control or monitoring - Cumbersome at larger scale, may require robotic handling
Closed system, multilayer stacks (12/36/120 layers)	1.3 L/3.9 L/13 L	6,000/18,000/60,000	HYPERFlask® Cell Culture Vessels (Corning)	- Single-batch expansion - Condensed, gas-permeable culture layers - Closed venting/gassing - Lower volume:area ratio than traditional stacks - No inline control or monitoring - Cumbersome at larger scale, may require robotic handling
Spinner flasks	125 mL– 3 L	675/2700	Corning® Disposable Spinner Flasks (Corning)	- Suspension systems offer lower volume:area ratio than planar culture - Suitable for small-scale process development and expansion - Surface aeration only - No inline monitoring
Mini-reactor systems	3–250 mL	16–1,350	DASbox® Mini Bioreactor System (Eppendorf), BioLevitator™ 3D Cell Culture System (Hamilton), TAP ambr™ microbioreactor (Sartorius), Micro-24 MicroReactor System (Pall Corporation)	- Suitable for small-scale process development with up to 24 parallel reactors - Small footprint - Controlled gassing - Inline monitoring - BioLevitator™ exclusively used with GEM™ magnetic microcarriers
Benchtop stirred reactors	1 L–5 L	5,400–27,000	Mobius® CellReady (EMD Millipore), CelliGen® BLU (Eppendorf), UniVessel® SU (Sartorius)	- Suitable for small-scale process development and expansion - Controlled gassing - Inline monitoring
Pilot scale, stirred reactors	50 L–300 L	2.7×10^5–1.62×10^6	Mobius® CellReady (EMD Millipore), CelliGen® BLU (Eppendorf), BIOSTAT® STR (Sartorius), Xcellerex™ XDR (GE Healthcare), HyPerforma™ Single-use Bioreactor (Thermo Scientific), Nucleo™ Single-use Bioreactor (Pall Corporation), Allegro™ STR 200 (Pall Corporation)	- Suitable for pilot scale and clinical scale manufacturing - Many systems scalable from lower volume offerings - Controlled gassing - Inline monitoring
Production scale, stirred reactors	500 L–2000 L	2.7×10^6–1.08×10^7	BIOSTAT® STR (Sartorius), Xcellerex™ XDR (GE Healthcare), HyPerforma™ Single-use Bioreactor (Thermo Scientific), Nucleo™ Single-use Bioreactor (Pall Corporation)	- Suitable for clinical scale manufacturing - Many systems scalable from lower volume offerings - Installation of large scale single-use bags is cumbersome - Controlled gassing - Inline monitoring
Oscillating motion reactors, surface aeration only	300–500L	1,620–$2.7 \times = 10^6$	WAVE bioreactor™ system (GE Healthcare), BIOSTAT® RM (Sartorius), SmartBag™ containers (Finesse), Appliflex™ systems (Applicon), CELL-tainer® (CELLution Biotech/Lonza), XRS-20 Bioreactor System (Pall Life Sciences)	- Suitable for development to clinical scale manufacturing - Many systems scalable from lower volume offerings - No sparging - Inline monitoring
Oscillating motion reactors, sparging	30–1000 L	162,000–5.4×10^6	BaySHAKE® (Bayer)	- Suitable for development to clinical scale manufacturing - Many systems scalable from lower volume offerings - Gas transfer via sparging - Inline monitoring
Vertical wheel/bubble column	50–500 L	270–2.7×10^6	Vertical-Wheel™ reactor (PBS), CellMaker PLUS™ system (Cellexus)	- Suitable for development to clinical scale manufacturing - Gas transfer via sparging - Mixing by air- or magnetic-driven wheel - Inline monitoring
Pilot scale, static	19.8 L	1.15×10^5	Integrity™ Xpansion™ Multiplate (Pall Corporation)	- Suitable for development to small scale manufacturing - Growth on planar surface - Gentle media circulation and gas transfer - Requires large incubator for temperature control - Inline monitoring - Closed system from seeding to harvest

2.2. Attachment surfaces for microcarrier-based systems

Microcarriers enable culture of adherent cells in suspension systems by providing suitable attachment surfaces while suspended in the culture medium *via* agitation. Microcarriers offer the advantage of a large surface to volume ratio, facilitating higher density cultures. They help alleviate some of the problems associated with static cultures including the large volumes of media required, inefficient gas transfer, the presence of concentration gradients, as well as inadequate monitoring and control [24]. The majority of currently available microcarriers are spherical in shape, but cylindrical (DE53, Whatman) and disc-shaped (Fibra-Cel®, Eppendorf) microcarriers are also available. Microcarriers can be manufactured from a variety of base materials including glass, polystyrene, alginate, cellulose, dextran or gelatin, and can be macroporous or microporous, the latter of which will be the focus of this review. Derivatizing microcarriers to carry charges, peptides or extracellular matrix proteins such as collagen, fibronectin and laminin can enhance cell attachment and promote growth. Collagen, a major component of extracellular matrix (ECM), is also commonly used as a surface coating for hMSCs grown on microcarriers. Although animal-free collagens are becoming more readily available, many collagen coatings are still derived from animal sources. Additional animal-free options include laminin, poly-D-lysine, fibronectin [25] and derivatives thereof, such as superfibronectin [26]. The surface characteristics of the microcarrier are paramount in determining its performance with respect to cell attachment, proliferation and to some extent differentiation. The GEM™ magnetic microcarrier is used specifically with the small-scale BioLevitator™ system by Hamilton, and potentially provides low shear suspension and additional options for cell—microcarrier separation during culture harvest, though available surface coatings and chemistries are limited in this format.

Components from serum used to supplement cell culture media, such as ECM proteins, can nonspecifically bind to the microcarrier surface and thereby aid in cell attachment. The surface properties of the microcarrier will determine which ECM proteins will bind, and thus the selection of an appropriate microcarrier/coating combination is essential for optimal performance. For example, positively charged microcarriers will selectively bind albumin whereas gelatin-coated microcarriers will preferentially bind fibronectin [27]. Both low-serum and ECM protein coating were shown to be essential for cell attachment in serum-free media [28,29]. Ideally, cell culture media should be optimized for use with standard tissue culture-treated surfaces, though this is certainly cell type-dependent and can result in a decline in differentiation potential in comparison to collagen-coated surface [30]. Synthetic surfaces have also been developed that support cultivation of hMSCs in serum-free media [31].

Current investigations concerning the use of engineered surfaces suggest that the planar structure, including stiffness, nanotopography and local curvature of the material, can impact proliferation, maintenance of phenotype and differentiation [32]. For example, it has been demonstrated that small changes in nanoscale features can either promote maintenance of stemness or osteogenesis in hMSCs [33]. Soft gels were demonstrated to support hMSC differentiation to neuronal cells, whereas stiffer gels supported an osteoblast-like phenotype [34]. Although the effect of the biomechanical properties has thus far primarily been evaluated on flat culture surfaces, it is likely they also play a role in microcarrier-based cell culture. The biochemical and biomechanical properties of surfaces could be potentially leveraged to improve the efficacy of stem cell culture.

A key consideration in microcarrier-based culture is whether the culture conditions support the transfer of cells from one microcarrier to another [35]. This migration of cells from "bead-to-bead" is likely influenced by an interplay between the cell type, media composition and microcarrier surface. Whether a given system supports cell transfer between microcarriers can be determined by adding new microcarriers to an existing culture and observing the transfer of cells. Routine monitoring of the culture helps determine the need for additional surface area in the form of fresh microcarriers for continued growth. In systems that do not support bead-to-bead migration of cells, enzymatic detachment can be performed within the vessel prior to the addition of new microcarriers and continuation of the culture.

2.3. Suspension culture vessels

A major challenge to stem cell-based therapy is the ability to generate the number of cells required to make its application practical and cost-effective. A variety of bioreactor vessels have been used with microcarriers to increase stem cell production for cell therapy (Table 1), including stirred-systems such as spinner flasks and stirred-tank bioreactors, and non-stirred systems such as oscillating and multiplate bioreactors. The Mobius® 3 L Bioreactor was the first single-use stirred-tank bioreactor reported to successfully expand hMSCs on microcarriers [36]. It was demonstrated that a yield of approximately 600 million hMSCs was obtained in a 2.4 L working volume using collagen-coated polystyrene microcarriers; these cells retained their basic defining characteristics of cell surface marker expression and differentiation potential. hMSCs have been successfully expanded in other stirred systems, such as the 1.3 L Bioflo® reactor in which approximately 1.4×10^5 and 6.0×10^4 cells/mL of bone marrow-derived and adipose-derived hMSCs were achieved, respectively [37]. Additionally, hMSCs have been expanded in the Biostat® B 1 L and 5 L reactors, and also retained phenotypic features after post-harvest from the microcarriers [38,39].

Using a non-stirred system Timmins et al. reported the expansion of placental hMSCs in a Wave bioreactor™ system using macroporous gelatin microcarriers [40]. Wave-mixed bioreactors rely on rocking motion to create a wave for mixing and bubble-free aeration. The rocking angle and rate must be carefully tuned to meet the changing gas transfer needs of a growing culture (reviewed in Ref. [41]). Conversely, in a parallel plate bioreactor such as the Integrity™ Xpansion™ Multiplate Bioreactor (Pall Corporation), cells remain stationary on the culture surface while the medium is circulated throughout the vessel. The Xpansion™ bioreactor is made of stacked circular plates, reminiscent of multitray 10-stack planar flasks. The system additionally enables control of pH and dissolved oxygen (DO) with installed sensors and inlets/outlets for gases. This type of hybrid system may be particularly well-suited for cells that cannot tolerate the hydrodynamic forces when attached to a microcarrier [42], though does not enable sampling of cells during the bioreactor run.

Single-use technologies are particularly amenable to cell-based therapeutics due to their flexibility and low start-up costs as compared to traditional stainless steel systems, and potential alleviation of sterility concerns [4]. Several scalable, single-use bioreactors from various manufacturers are currently available which are capable of processing up to 2000 L of cell culture (Table 1). Since single-use technology, by its very nature, lends itself to facile customization, the application of single-use bioreactors to microcarrier-based stem cell manufacturing offers the possibility of customization designed to meet the unique (*e.g.*, low hydrodynamic stress) requirements of a particular manufacturing process. Other single-use technologies, which have been established for many traditional cell-based recombinant protein manufacturing processes can be readily applied to regenerative medicine applications such as containers for preparation of media, buffer and microcarriers, customizable storage and transport bags, containers for collection

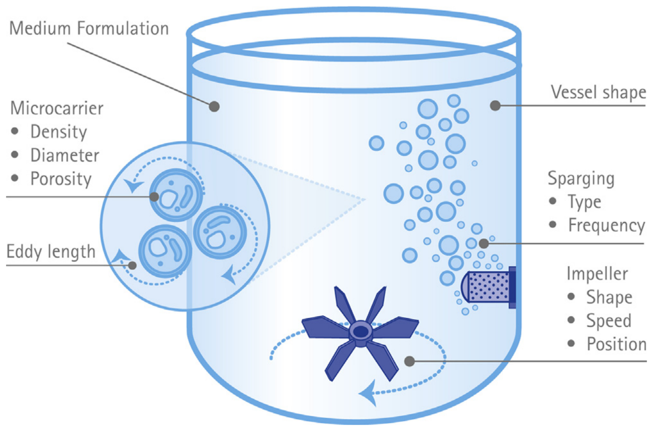

Fig. 2. Factors influencing hydrodynamic forces exerted on cells in a stirred-tank reactor. For a given bioreactor design, users need to balance several factors to ensure growth and viability of the cultures stem cells. The choice of microcarrier (*e.g.*, size, density, porosity *etc.*), impeller speed and gassing strategies must be considered together in order to ensure success in any particular stirred-tank bioreactor.

and storage of cells and validated sterile-to-sterile connectors. The availability of pre-sterilized single-use components is particularly attractive in autologous stem cell applications, where the culture volumes used for production are relatively small. Many of the current challenges that exist in implementing stirred-tank bioreactors for microcarrier-based manufacturing processes can be addressed by partnering with manufacturers of single-use technologies.

2.4. Culture of adherent cells in stirred systems—effects of fluid dynamics

Identification of the proper impeller speed is critical for optimization of fluid dynamic conditions for microcarrier processes in stirred-tank bioreactors. For microcarrier processes, it is desirable to maintain a homogeneous state of suspension to ensure a uniform growth environment throughout the entire vessel. However, since the sensitivity of cells to hydrodynamic shear forces that can cause damage resulting in cell lysis or decreased biopharmaceutical protein productivity [42] can be exacerbated when cells are grown on microcarriers [43,44], microcarrier users develop their processes with agitation rates that are as low as practically possible. Balancing these factors against the need to keep the microcarriers adequately suspended, while also maintaining sufficient bulk liquid mixing for appropriate aeration of the culture, can be challenging. Factors that may influence the hydrodynamic forces exerted on cells cultured on microcarriers in a stirred-tank are summarized in Fig. 2. These factors are highly dynamic and interconnected, and the relative importance of each factor can vary greatly depending on process specifics.

Identification of the minimum agitation requirement for microcarrier suspension is of utmost importance to ensure successful cell culture. If the impeller speed is too low, the microcarriers do not circulate in the medium and cell growth is poor [45]. While mixing and fluid dynamics in bioreactors have been studied extensively, the focus has been on liquid–liquid and gas–liquid interactions. The suspension of solid particles in liquid media has received considerably less attention [46]. To date, the empirical correlation derived by Zwietering in 1958 [47] is still the most widely-used method to characterize microcarrier suspension [46,48]. This method allows for the determination of N_{js}, or the minimum impeller speed to maintain complete suspension, ensuring that no microcarriers remain on the bottom of the vessel for more than 1 to 2 s [47]. This N_{js} is typically used as the minimum agitation requirement for microcarrier processes. At N_{js}, the microcarrier concentration will normally decrease with height and could fall to zero near the surface. As the impeller speed is increased to above N_{js}, the microcarrier distribution approaches homogeneity. At speeds far below N_{js}, microcarriers fall out of suspension, with microcarriers settling in areas of low flow at the bottom of the vessel. Such conditions result in poor cell growth due to the decreased microcarrier surface area in contact with the medium. It has also been observed that inadequate agitation can result in overgrowth of cells (forming bridges between microcarriers) [45]. This phenomenon is particularly prevalent towards the end of the culture cycle, due to the increase in particle density as cells begin to populate the surface of microcarriers. While it has not been extensively studied, Clark and Hirtenstein suggest that the best results are generally obtained by changing from lower to higher agitation rates as the culture progresses [45].

Identifying the upper agitation limit is as critical to a successful microcarrier process as identifying the lower agitation limit. High levels of shear stress can inhibit culturing of cells in stirred-tank bioreactors, since the cells adhering to the microcarrier surface are more sensitive to fluid-mechanical forces than freely-suspended single cells [49,50]. Damage incurred on microcarrier cultures have been investigated based on the Kolmogorov turbulence model described in 1941. In microcarrier culture, turbulent eddies are often intermediate in size between the cells and the microcarriers, and the high rate of local energy dissipation due to these eddies interacting with the surface of microcarriers can cause shear rates that are sufficiently large to damage or even remove cells from the microcarrier surface [51]. Little work has been done to apply the Kolmogorov model directly to hMSCs grown on microcarriers in stirred-tank systems, though Hewitt et al. have leveraged the theory to interpret a study examining the impacts of impeller design and microcarrier concentration on hMSC growth in spinner flasks [52].

Estimates of shear stress that can promote aggregate and/or embryoid body formation for a variety of stem cell types has been described [50] and support the need to develop systems and processes imparting minimal hydrodynamic shear. Studies of the impact of hydrodynamic forces (*i.e.*, energy dissipation rate) using a flow contraction device on suspension cells [53] suggest cells to be more resistant to the lethal effects of shear than anticipated. However, direct characterization of hMSCs in this manner, which are thought to be substantially more sensitive to shear than CHO cells, has not as yet been reported. Therefore, the determination of an optimal impeller speed range for hMSC growth on microcarriers in any stirred-tank system requires an empirical analysis to balance performance versus hydrodynamic forces. Using too low a speed will result in microcarrier settling, aggregation and poor process control. In contrast, too high a speed may result in hydrodynamic shear forces that have been shown to influence phenotypic characteristics of hMSCs including cell signaling and differentiation [54,55]. The choice of microcarrier is critical and users should consider their size in relation to the Kolmogorov eddy length, density in relation to settling, as well as their composition and surface area in order to optimize stem cell production in stirred-tank bioreactors. It will be critical to choose impeller speeds that not only support cell growth but also enable the cells to maintain stem cell identity and potency.

2.5. Controlling dissolved oxygen

Hypoxic culture conditions may provide solutions to overcome problems observed in regenerative medicine for hMSCs; for example, poor growth kinetics, genetic instability and poor engraftment after transplantation. Traditional cell culture is performed under normoxic oxygen concentration (21% O_2 in ambient air). However, this concentration of oxygen might not be required for the cultivation of hMSCs as they are derived from tissues that vary in oxygen tension (*e.g.*, 1–7% in bone marrow and 10–15% in adipose tissue) [56–58]. Using umbilical cord-derived hMSCs, Lavrentieva et al. found that self-renewal capacity of different hypoxic hMSC populations was increased compared to the normoxic culture, and hMSCs cultured under hypoxic conditions still maintained their differentiation properties [59]. The same group then further analyzed the expression of oxygen-dependent cytokines from hMSCs to link the proliferation and differentiation capacities [60]. It was found that growth factors and growth factor receptors were both over-expressed under hypoxic conditions, whereas differentiation factors were absent under both hypoxic and normoxic conditions. It was concluded that the enhanced growth potential and maintained undifferentiated status can be attributed to the oxygen-dependent expression of a particular set of cytokines. The effects of hypoxic culture for MSCs were well-reviewed by Haque et al. [61], and also by Sart et al.,describing how oxygen tension interacts with glucose levels to influence proliferation and differentiation [62].

Considering the numerous studies in planar culture that suggest some effect of oxygen tension of MSC characteristics, more extensive studies will be required to fully understand the oxygen requirements of hMSCs expanded in suspension-based systems. In a stirred-tank bioreactor, effective expansion of hMSCs was obtained under hypoxic conditions at 20% and 9% air saturation (4% and 2% oxygen, respectively) controlled *via* sparging, with no significant differences in growth, glucose or lactate levels [37]. Moreover, when controlled gassing is not applied, dissolved oxygen levels can drift down as the culture progresses. As much as a 50% drop in oxygen has been measured during hMSCs expansion in a stirred-tank bioreactor in which oxygen levels were monitored but not controlled [39]. In most single-use stirred tank bioreactors there are different options to control DO such as overlay, open pipe sparging and microsparging. Of them, an overlay is the least intrusive method and generally used for low cell density culture or low oxygen uptake rate cells. Open pipe is generally used to strip carbon dioxide in the system, whereas microsparging is more effective for oxygen transfer, especially during high cell density cultivation. Sparging paradigms in order to meet gas transfers demands must always be balanced with the possible generation of foam that can adversely affect the culture health. Although there is a body of work regarding oxygen levels and hMSCs in planar culture or for example 3D scaffolds [63], bioreactors introduce the opportunity not only to further characterize optimal oxygen levels, but also to determine the best method for that control.

3. Cell culture media and supplements

3.1. Fetal bovine serum—nutrient content and material sourcing

Fetal bovine serum (FBS) is commonly used as a supplement to cell culture media. It provides high concentrations of growth stimulatory factors, macromolecules, carrier proteins for lipids, trace elements, attachment and spreading factors, low molecular weight nutrients, hormones, and low concentrations of immunoglobins [64]. However, there are issues with the use of serum such as cost, availability, non-desirable/undefined components, lot-to-lot variability and the risk of transmission of Bovine Spongiform Encephalopathy (BSE) and/or viral pathogens. In addition, there are ethical concerns around the harvest and collection of fetal bovine serum [65]. Traceability and origin are important for FBS used in commercial processes. “Origin” is the country in which the raw material is collected. “Traceability” refers to the documentation roadmap that connects the serum's origin to final manufacturing and distribution. The United States Department of Agriculture permits the importation of bovine products only from those countries that are considered to be at low risk for BSE, such as Australia and New Zealand that together with the United States, supply 90% of the serum used for commercial therapeutics [66].

As described by Brindley et al., the current global FBS supply may not be able to support the manufacturing of multiple successful cell therapies [66]. There are limited suppliers with international standards organization (ISO)-grade environments for collection of serum suitable for cell therapy production. The authors go on to describe the declining global serum supply, following reduced demand from the vaccine industry that is transitioning to expression systems using serum-free mammalian or microbial platforms. They estimate that current production is at approximately 600,000 liters per year of which only 200,000 liters may be suitable for cell therapy production. Cell therapy manufacturing demand on the other hand, is on the rise; thus, the serum infrastructure may not be able to meet the demands of the projected cellular therapy market. A serum supply of 200,000 L is predicted to support production of 400,000 therapeutic doses per year. After that xeno-free or serum-free manufacturing strategies will be necessary to sustain the growth of the cellular therapy market.

3.2. Platelet lysate as a serum alternative

Various blood products derived from mature organisms have been investigated as suitable substitutes for FBS to support the hMSC cultivation, including antagonist-activated platelet-rich plasma and platelet lysate; these materials contain elements essential for cell expansion [64,67]. Platelets isolated from whole blood donations or by apheresis originating from single or multiple donors are used to produce human platelet lysate (hPL) *via* multiple freeze-thaw cycles to release large quantities of growth factors that support hMSC expansion [68]. Less processed forms of hPL require the use of animal-derived heparin to prevent coagulation, whereas other hPL forms go through refining steps to inhibit the effect of clotting factors. Moreover, an inactivation process involving psoralen and UVA light can be applied to the platelet lysate to help prevent donor-derived pathogen transmission [68]. hPL has low levels of insulin-like growth factor 1 (IGF-1) and protein content compared to human serum, likely due to removal of immunoglobulins and albumin [64], yet retains high levels of key growth factors including basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1) [69]. It supports the expansion of hMSCs without additional supplementation of recombinant growth factors as is typical of FBS-containing media [70]. Obviating this need for exogenous growth factor supplementation may help reduce costs.

Reported advantages of using hPL in place of FBS in media include a reduction of cell culture duration as a result of shorter doubling times [70,71] and a potentiation of the inhibitory effects of adipose-derived hMSCs on lymphocyte proliferation when the hMSCs were expanded in hPL-containing media [72]. Whereas hPL may offer advantages from a safety perspective since the source material is animal origin-free and approved for human use, the use of human-derived component presents other potential risks [15,73] as routine screening of blood may be insufficient to provide the required level of safety. Moreover, hPL often requires the use of animal-derived heparin to prevent coagulation, though there are heparin-free preparations available. Additional concerns include security of supply and batch-to-batch variation which could impact the adoption of hPL in clinical-scale production. The concerns raised from use of FBS or human-sourced supplements like hPL, suggests the need for the development of chemically defined media for cultivation of cell for therapeutic use.

3.3. Formulating chemically-defined media

Widely varied media formulations have been tested with mixed results on hMSC proliferation and maintenance of functional attributes (reviewed in [74]), the vast majority of which contain blood-derived components. One of the major complications in formulating cell culture media without the use of animal or human-derived products is replacement of serum albumin. This pleiotrophic blood protein carries a multitude of molecules that support and/or stimulate stem cell growth. In addition, albumin binds and tempers the potentially toxic effects of common culture additives such as antibiotics, and provides a degree of buffering capacity that helps regulate culture conditions. Although recombinant forms of the protein retain many of these beneficial properties, they lack critical albumin-associated molecules such as lipids, hormones and trace elements. Lipids, mainly fatty acids and sterols, are present in published serum-free formulations such as TeSR1 [75], and have long been recognized as essential for the cultivation of mammalian cells under serum-free conditions. Primary cells such as hMSCs pose a great challenge when transitioning away from FBS-containing medium as they are not immortalized, have limited replication capacity and may not adapt well to minimalistic culture conditions.

Cholesterol represents the major lipid in FBS, much of which is bound to albumin and to both low- and high-density lipoproteins, and is important for maintaining cell integrity and growth in serum-free culture [76]. Cholesterol has been well-studied for its contribution to membrane fluidity, and is a major component of lipid rafts that have recently been described as regulating structures of embryonic stem cell self-renewal [77]. It has also been shown to influence osteogenic differentiation in MSCs [78]. The difficulty with replacing cholesterol in chemically-defined media is twofold. It has limited solubility in aqueous solutions, an issue resolved when bound to a carrier protein such as albumin, and it is primarily sourced from animal-derived materials such as sheep's wool. Therefore, this raw material must be synthetically derived if included in an animal-free medium, and formulated in such a way to ensure solubility and stability. Approaches include cyclodextrin encapsulation, liposome formation, emulsions [79–81] and synthetic cholesterols such as SyntheChol™ supplement (Sigma–Aldrich).

There is strong interplay between the trace elements present in cell culture media; many trace elements are typically bound to proteins found in FBS and may thereby be lacking in serum-free formulations. Iron, copper, zinc and selenium are the main trace elements in culture media and participate in a variety of cellular functions, often as enzyme cofactors. A recent study by Bryan et al. not only established variations in donor-to-donor trace element composition of FBS, but also linked this variation to differences in protein expression of primary umbilical vein endothelial cells [82], in particular to that of CD54, a non-standard hMSC marker [83]. Iron is present as a salt in many classical media formulations that can be delivered and regulated by the transferrin present in FBS. However, in the absence of transferrin, iron salts are unable to enter the cell and thus must be substituted with a bioavailable source of chelated iron such as ferric ammonium citrate [84]. The use of chelated iron in the absence of transferrin must be carefully optimized to avoid iron-mediated oxidative stress in the manufacturing of an hMSC therapeutic, as excessive iron has been shown to alter their biological properties [85]. Alternatively, inclusion of recombinant transferrin in serum-free formulation can provide iron delivery and regulation. Likewise, copper and zinc are typically bound to proteins in FBS including insulin, recombinant forms of which may lack these essential elements.

3.4. Growth factors and feed strategy

Perhaps the most critical components to balance properly in cell culture media for regenerative medicine applications are the growth factors. Not only do they provide necessary mitogenic input to non-immortalized cells, but their correct balance is required to maintain the specific cellular phenotype desired. bFGF in particular is often used to enhance cell growth and maintain phenotype, though it may not be absolutely necessary to achieve expansion in the presence of FBS. The exact factors and concentrations thereof vary among cell types and applications; however, factors such as bFGF, PDGF-BB and TGFβ1 are commonly used in serum-free media for the expansion of hMSCs [86]. One of the most comprehensive serum-free media optimization studies published to date, by Jung et al. [87] explored these and other growth factors including EGF, HGF and FGF4. A mix of growth factors, hormones, as well as insulin, transferrin and notably human serum albumin was found necessary for hMSC serial passaging and expression of cell surface markers. Reliable sourcing of these materials may pose a challenge as the manufacturing of growth factors using a completely animal-free process is not trivial. For example, TGFb1 must be made in a more complex mammalian system such as Chinese hamster ovary (CHO) cells due to required post-translations modifications or in human cell expression systems such as the HumanKine® line of cytokines (HumanZyme) made in proprietary HEK 293 cells.

Development of an optimized growth factor supplementation strategy for expansion and retention of desired stem cell phenotype and potential is critical for keeping formulations simple and cost-effective. Considering the short half-life of many of these factors, feed strategies for scale up in suspension systems need to be optimized as media exchange becomes more complicated versus planar culture where 100% of the medium is typically changed every 2–4 days. In smaller scale vessels such as spinner flasks, batch culture will typically maintain hMSCs for approximately 4–6 days. The inclusion of a 50% media exchange to the process, at day 4 for example, can extend the culture to approximately 6–8 days; longer cultures are possible with additional media exchanges [38]. Not only does this replenish critical components such as the growth factors, but also dilutes accumulated metabolites including ammonia and lactate, and may allow for greater cell densities.

In larger scale reactors in which upwards of a 40-fold increase in hMSC cell number can be achieved [36], cultures are typically started either at the minimum working volume or half the maximum volume of the reactor. Fresh media and microcarriers are then added at designated points in the culture, either based on culture time or as the result of a predefined metabolic trigger (*e.g.*, glucose level) [88], until the maximum volume is reached. At this point, small additions of concentrated nutrients such as growth factors, glucose and/or glutamine can continue to be added as needed. This scale up and feed approach serves to dilute metabolites, maintain nutrients and growth factor concentrations, and can extend culture duration up to 10–14 days for hMSCs. Perfusion is another approach that can be used to extend the growth phase of microcarrier-based suspension cultures and has been demonstrated for hMSCs utilizing a proprietary xeno-free media formulation [37].

4. Downstream processing

Downstream processing in a cell therapy manufacturing process is fairly simple consisting of removal and separation of the cells from the growth surface, concentration of the cells and reformulation, usually into a cryopreservative. These steps become increasingly complex as a process transitions from planar culture to

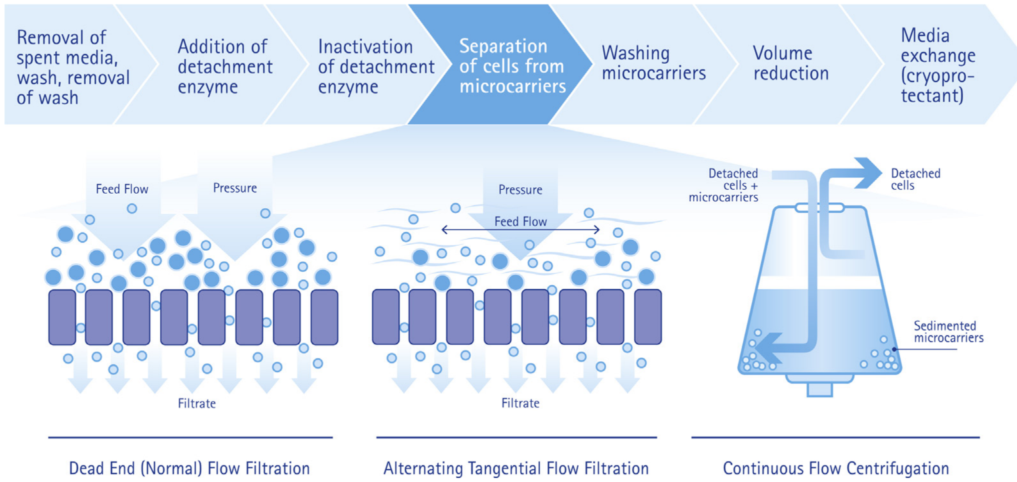

Fig. 3. Process for harvesting cells from microcarrier-based suspension cultures. Various approaches are under evaluation for the separation of cells from microcarriers, including both normal flow and alternating tangential flow filtration, as well as continuous centrifugation.

microcarrier based bioreactor culture and as the process is scaled up to large volumes. At each processing step care must be taken to preserve the viability of the cells while maximizing yield and ensuring that no contaminants enter the cell product. More detail regarding each of the steps and current technologies in use for downstream processing is described in this section.

4.1. *Detachment of cells from microcarriers*

Just as important as the culture medium in which cells for therapeutic use are cultivated, is the surface upon which they are grown. Cultivation of anchorage-dependent cells such as MDCK, VERO or certain CHO cell lines on microcarriers has been used for vaccine and antibody production. However, because the final biotherapeutic in these instances is secreted by cells into the culture medium, detachment and separation of cells from microcarriers is not required. The culture surface properties can greatly impact the ability to achieve this detachment. An extracellular matrix (ECM) component coating or surface charge aids in attachment of the cells, which in turn deposit their own ECM as they expand on the culture surface. Some cultureware such as CellBind® surfaces (Corning) are modified to improve cellular attachment, in this case by increasing oxygen-containing functional groups rendering the surface more hydrophilic. In cellular migration models, exogenous factors including TGFb1 and PDGF-BB have been shown to alter adhesion and migration capacity of MSCs [89,90], highlighting the complex interaction between media formulation and surface composition. Typical detachment approaches include proteases such as trypsin, cationic chelating agents such as EDTA, increased pH [91], application of mild hydrodynamic forces or some combination thereof. For example, Nienow et al.recently reported a harvest method for hMSCs grown on plastic microcarriers that includes agitation in the cell detachment step in order to minimize the duration of enzymatic exposure [92]. Detachment reagent concentration and detachment time should be carefully optimized to enable the maximum recovery of cells while preserving the quality of hMSCs for therapeutic use with respect to viability and cell surface characteristics [93], as well as other functional attributes. Stepping toward an enzymatic-free process, a thermoresponsive hydrogel surface material was described that sustained enzymatic-free passaging of adipose-derived hMSCs with retention of key cell characteristics [94]. Application of such technology to a microcarrier format could greatly ease the current challenges in cell-microcarrier detachment and separation [95] for a more streamlined process.

4.2. *Separation of cells from microcarriers*

Several technologies are available for the separation of cells from their expansion surface. Whereas enzymatic detachment alone is sufficient to separate cells from a planar culture system, cells grown in suspension require the development of methodologies to ensure efficient separation of cells from microcarriers. Furthermore, it is essential that the final cell therapy product meet the regulatory and safety standards for particulates (*e.g.*, USP <788>) for injectable and parenterally infused drugs. Cell therapy products have a unique set of challenges with regard to particulates, as they are more extensively processed than blood transfusion products, yet they lack the downstream filtration steps of protein biologic production that can remove particulates. Development and application of appropriate methods, standards and regulations with respect to particulate removal is an ongoing topic for the cell therapy field [96].

Microcarriers are larger than MSCs (generally ~150–200 μm compared to 15–20 μm, respectively) and can be filtered from cells *via* size exclusion after the cells are detached. For small scale processes, devices are available from BD or EMD Millipore with nylon filters and mesh sizes of 40–100 μm that are often employed to separate hMSCs from microcarriers [38,97,98], but as larger scale processes are envisioned a more scalable solution is required. Dead end or normal flow (NF) filtration with larger nylon mesh filters (Harvestainer, HyClone) or membrane filters (Opticap® Polygard®-CR 100, EMD Millipore) are feasible options for moderate scales (3–200 L), and are simple and cost-effective single-use options. However, to avoid fouling at scales greater than 200 L, more sophisticated harvest systems may need to be employed. Alternating tangential flow (ATF) filtration offers a possible way to keep microcarriers from fouling the mesh screen (Fig. 3). Repligen offers a variety of devices from the ATF™ 2 to the ATF™ 10 which can harvest microcarrier process scales from 1 L to 5000 L. Many of these separation tools, including the ATF™ device, currently need to be autoclaved or steamed in place, adding additional process and validation work to be performed by the end-user.

Continuous flow centrifugation is another option for microcarrier separation. KBI Biopharma's kSep® centrifuge systems offer closed, single-use contact surfaces and can sediment up to 6 L of microcarriers per cycle, corresponding to approximately 250 L of culture with 15 g/L of microcarriers. While the current system is well-suited for use with cell suspensions, modifications may be needed for use with higher concentrations of microcarriers. Thus, normal flow filtration with a mesh between 40 and 100 μm may work well for scales less than 200 L but for very large scales more development may be needed. In all cases it must be demonstrated that the method used to separate the cells from the microcarriers is capable of safe and effective clearance of microcarriers from the final drug product.

4.3. Cell concentration

It is estimated that for some indications up to 250 million cells per dose will be required [3]. In a 5 mL injection dose example, a cell concentration of 50 million cells/mL may be needed. Available technologies for the concentration or volume reduction of cells following microcarrier separation depend greatly on the scale of the process and the required volumetric reduction. Currently, small-scale centrifugation is effective for volumes up to approximately ten liters, but becomes cumbersome thereafter. The kSep® products previously mentioned enable process volumes up to 6000 L and can sediment up to 1.2×10^9 cells. Without microcarriers in the flow path, the chance of clogging is greatly reduced. The volume reduction (fold concentration) would depend greatly on the bioreactor feed, but for the kSep®400 system volume reductions of 100-fold may be possible from a 200 L bioreactor. Another possibility is the ATF™ device with hollow fiber membrane options than can concentrate the cells after they have been separated from the microcarriers. These ATF™ membranes are designed for perfusion, so they may need to be oversized to accommodate the flow rates necessary to perform the volume reduction in less than 8 h in order to maintain high viability. Tangential flow filtration offers a third type of technology that can be used to concentrate cells for therapeutic use. Open channel TFF products like the Pellicon® or Prostack™ cassettes from EMD Millipore utilize large flow paths for the cells to flow through while permeate is removed. Like the ATF™ offerings, pre-sterilization is a hurdle that needs to be overcome by vendors for these devices to best serve the cell therapy industry.

4.4. Cryopreservation

The drive towards clinical applications of allogeneic hMSCs has led to the reappraisal of conditions under which cells are preserved to ensure their safety and retain their potency. Cells for immediate use can be formulated differently than those that need to be cryopreserved. For hMSCs that need cryopreservation, one must select a medium and cryopreservative, develop a freezing protocol and secure stable storage in liquid nitrogen facilities [99]. Typical cryopreservation solutions are a mixture of cell growth medium and DMSO. However, there is mounting desire to remove both animal-derived materials present in some growth medium formulations and the DMSO used as the cryopreservative for clinical-grade stem cell banking, as there is evidence that DMSO is toxic to humans at high concentrations [100]. Thus, initial efforts have focused on reducing the concentration of DMSO required to achieve cryopreservation effectiveness. Replacing DMSO with alternatives such as glycerol, sucrose, and/or high molecular weight polymers such as polyvinylpyrollidone for cryopreservation of hMSCs is an area of current research [101]. An additional factor that is critical for successful cryopreservation and subsequent recovery of cells, is the ability to control the rate at which cells are frozen. Controlled-rate freezers are suitable for controlling temperature [102], however some units are lacking and thus it is difficult to ensure that all vials have been processed identically during large-scale banking of MSCs [101]. Hence, there is a need to develop controlled-rate freezers that can effectively accommodate large scale banking as well. Together these variables contribute to the stability of cells, and thereby their successful final use in patients and as such their continued refinement is critical.

5. Conclusions

Although hurdles remain for bringing safe and effective stem cell therapies for the benefit of patients, great strides has been made in the development of materials and processes to achieve this goal. hMSCs may be particularly well-suited as an allogeneic therapy due to their intrinsic immune-regulatory properties, and serve as a primary model for manufacturing platform development. Ultimately, cost-effective, closed-systems using high quality reagents with enough flexibility to address the needs of multiple cell types are desirable. Fundamental investigations into the influences of culture surface chemistry, hydrodynamic forces and media components on stem cell biology are helping to guide this development. More than ever, close collaboration between hardware, consumables and reagent manufacturers, and academic and clinical investigators will be required to facilitate this process if successful cell-based therapies are to be realized. Moreover, although the various phases of therapeutic production are often developed in isolation, it will be important to consider downstream challenges while establishing upstream parameters. This is especially true concerning the development of relevant analytics and lot release testing for which a deeper understanding of the mechanism of action of cells used as therapeutics may be required. Ongoing and future trials with hMSCs and other cell types will be an important clinical setting for advancement of this basic knowledge around the efficacy of stem cell therapy.

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