We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,300

Open access books available

172,000

International authors and editors

190M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

The Challenge of Human Mesenchymal Stromal Cell Expansion: Current and Prospective Answers

Christiane Elseberg, Jasmin Leber,
Tobias Weidner and Peter Czermak

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/66901>

Abstract

In the field of cell therapy, allogenic human mesenchymal stromal cells (hMSCs) are often used in clinical trials, creating a demand for cell mass production using efficient dynamic bioreactor systems. As an advanced therapy medicinal product (ATMP), such cells should meet certain special requirements, including product specifications requiring a production process compatible with good manufacturing practice (GMP). The development of processes in which the cells are the product therefore remains a significant challenge. This chapter describes the requirements at different steps in the upstream and downstream phases of such dynamic processes. Potential solutions are presented and future prospects are discussed, including the selection of media and carriers for the strictly adherent growing cells, allowing efficient cell adhesion and detachment. Strategies for dynamic cultivation in bioreactors are described in detail for fixed-bed and stirred-tank reactors based on GMP requirements and the integration of process analytical technology (PAT). Following cell harvest, separation and purification, the formulation and storage of the product are also described. Finally, the chapter covers important cell quality characteristics necessary for the approval of ATMPs.

Keywords: hMSC, cell expansion, stirred-tank reactor, microcarrier, fixed-bed reactor, PAT, GMP, ATMP, cell harvest, formulation, storage, quality approval

1. Introduction

Intensive research in the field of regenerative medicine has resulted in a large number of clinical trials over the last few years. The unique characteristics and differentiation pathways of human mesenchymal stem/stromal cells (hMSCs) make them promising candidates for future therapeutic strategies. There is a great interest in these cells because they can migrate

to injured tissues following implantation or intravenous injection, and they have anti-inflammatory and regenerative capacity due to the release of cytokines, specific growth factors and other bioactive molecules. In addition to non-differentiated cells, the differentiation of hMSCs into osteocytes, chondrocytes, adipocytes, tenocytes and muscle cells [1, 2] may allow the treatment of patients with bone and cartilage diseases, gastrointestinal diseases, diabetes, graft-versus-host diseases and limbal stem-cell deficiency [3–6]. The application of immunomodulatory hMSCs may involve autologous cells (isolated from the patient and used in personalized therapy) or allogenic cells (isolated from another individual, then expanded and used in the patient). Allogenic cells allow off-the-shelf treatment for indications affecting a large number of patients. In addition to primary hMSCs, which can be isolated from bone marrow, adipose tissue and the umbilical cord, the genetically modified and immortalized cell line hMSC-TERT achieves higher passaging numbers while retaining the differentiation potential of primary cells [7, 8]. ClinicalTrials.gov currently lists 51 clinical trials specifically involving autologous hMSCs, 32 of which are ongoing, whereas 67 studies are listed for allogenic hMSCs, 56 of which are ongoing (accessed May 9, 2016). Since 2005, the number of clinical trials has increased continuously, the majority using allogenic cells [9]. These trials have shown that cell-dose-dependent efficacy requires a minimum of $1.5\text{--}6 \times 10^7$ cells per dose, depending on the indication [10, 11]. Human cells are classed as advanced therapy medicinal products (ATMPs). Only four ATMPs are currently authorized, which may reflect the lack of suitable production processes [12]. This reveals the need for robust and efficient biomass expansion processes for adherent growing hMSCs that yield a high-quality product. The development of dynamic processes for sensitive, adherent growing cells is challenging because several requirements discussed in this chapter must be fulfilled to mimic *in vivo* conditions. This article focuses on dynamic processes for primary bone marrow-derived hMSCs and the hMSC-TERT cell line for allogenic applications of non-differentiated cells.

2. Requirements for stem cell expansion

2.1. Cells as a product

In contrast to biopharmaceutical production processes in which the cell synthesizes the product, in the field of cell therapy, the cells are the product. Like any other medical product, cells must be approved by the competent authorities, i.e., the European Medicines Agency (EMA) in Europe or the Food and Drug Administration (FDA) in the USA. Medicines based on genes, cells or tissues are usually defined as ATMPs, a category that includes both hMSCs and the hMSC-TERT cell line. ATMPs are subject to special guidelines in addition to the standard good manufacturing practice (GMP) requirements. In the EU, such products must be compliant with Regulation 1394/2007, guideline EMEA/CHMP/410869/2006, Directives 2004/23/EC, 2006/17/EC, 2006/86/EC and the revised guideline EMA/CAT/600280/2010. In the USA, such products must be compliant with the FDA Code of Federal Regulations (CFRs) covering investigational new drug (IND) applications (21 CFR 312), biological regulations (21 CFR 600) and GMP (21 CFR 211) [13–15]. The International Society for Stem Cell Research (ISSCR) published its updated “Guidelines for Stem Cell Research and Clinical Translation” in May 2016 [16]

with commentaries on manufacturing, safety and efficacy to provide standard guidelines and recommendations.

In addition to the regulatory framework, different societies and institutes have listed product characteristics that must be fulfilled [17]. One definition of hMSCs is provided by the International Society for Cell Therapy (ISCT) in their “minimal criteria for defining multipotent mesenchymal stromal cells” [18]. These criteria include the identity of cells, which is characterized by the following cell surface markers: positive expression (>95%) of CD105, CD73 and CD90, and no expression (<2%) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II. Also included in the ISCT definition is the adherence to plastic surfaces and the differentiation capacity in vitro into osteoblasts, adipocytes and chondroblasts. These characteristics must be defined and extended specifically for each cell line depending on its source [19].

To meet these regulatory demands and retain the defined cell characteristics, extreme care must be taken during process development in a dynamic system to achieve the highest final product quality. Only a few aspects can be covered in this chapter, showing the complexity of the issue. **Figure 1** shows the steps in a general production process including the various aspects that must be addressed.

Figure 1. General overview of a dynamic production process for hMSCs including various aspects that influence the final product quality.

Different aspects during each step determine the final product quality and process efficiency by affecting cell viability, potency, identity and safety. Each aspect summarized in **Figure 1** must also be regarded in the context of GMP, which provides general guidance rather than

detailed instruction. Briefly, the processes must ensure the quality of the product at each step and part of the system, as well as the final product. The basic materials should be chemically defined and proven to be within specifications. The quality and purity of all substances must be ensured. Robustness, reproducibility and efficiency must be shown using closed control systems to confirm quality, safety and efficacy throughout the process. The documentation required for each quality-relevant step is usually completed using standard operating procedures [20]. Process analytical technology (PAT) is one tool that can be used to ensure process quality based on the online and offline monitoring of different parameters [21]. Further examples of GMP include the requirement of animal-free materials, closed production systems and system validation that confirms the separation of different production processes, favoring disposable equipment. The biological source is one of the major factors that determine process success. The age and health of the human donor as well as the tissue of origin affect cell quality attributes. For both primary hMSCs and hMSC-TERT cells, the number of passages has a major influence on the final product quality and must be as low as possible [22].

2.2. Process requirements – upstream

2.2.1. Stem cell media

The maintenance of proliferating hMSCs requires special growth media, typically a basal medium comprising salts, glucose, amino acids and a buffer system supplemented with serum. The latter is routinely used because it contains proteins, adhesion factors, vitamins, growth factors, hormones, fatty acids and lipids that promote cell adhesion and attachment (e.g., to collagen, fibronectin, laminin and vitronectin), which is required for in vitro cell proliferation [23]. Important variables include the concentration of each ingredient, and the osmolality, buffering capacity, conductivity, substrate availability and stability (especially thermostability) of the medium. Serum constitutes a risk when hMSCs are produced for clinical applications under GMP and also increases the complexity of downstream processing. Serum should be avoided because it is derived from animals, thus increasing the risk of contamination (e.g., with viruses) and immunogenicity, and its complexity introduces unknown variables that make process standardization difficult to achieve.

To eliminate serum from stem cell production processes, several defined serum-free formulations have been developed. According to one classification system [24], a “defined medium” can be further divided into the following categories:

- Serum-free medium (SFM) containing a broad range of supplementary hormones, growth factors, proteins and polyamines, derived from bovine or human sources
- Protein-free medium (PFM) containing peptide fragments from enzymatic or acid hydrolysis of animal or plant source proteins
 - PFM variant A containing human serum albumin, human transferrin, human insulin and animal-derived lipids
 - PFM variant B, also known as xeno-free medium (XFM) containing human serum albumin, human transferrin, human insulin and chemically defined lipids

- Recombinant xeno-free medium (recXFM) containing either recombinant proteins, hormones and compounds or chemically defined lipids
- Chemically defined medium (CDM)—a protein-free basal medium containing low-molecular-weight components, synthetic peptides or hormones, and a few recombinant or synthetic versions of proteins.

In-house serum-free media for hMSCs have been reported in several studies, often containing additional factors such as bovine/human serum albumin, insulin, transferrin, hormones (e.g., progesterone, hydrocortisone and estradiol), growth factors (e.g., bFGF, TGF β , EGF or PDGF) or heparin [25–28]. The list of commercially available chemically defined, protein-free, xeno-free and serum-free is constantly increasing [29] as discussed in recent reviews [30, 31].

2.2.2. Growth surfaces

The characteristics of the growth surface have a major impact on the cultivation of hMSCs, especially in the context of production processes. The attachment, spreading and proliferation of these adherent cells are strongly influenced by the growth surface and the corresponding cell-surface interactions. These interactions can induce signaling pathways that are involved in regulating important cellular processes including cell migration, gene expression, cell survival, tissue organization and differentiation [32–34].

In dynamic bioreactor systems for adherent cells, microcarriers provide the growth surface in suspension or as a bed. The choice of an appropriate microcarrier, which not only supports attachment and growth but also allows efficient detachment without losing viability, is one of the key design aspects of an hMSC production process. For large-scale processes, bead-to-bead transfer strategies are necessary and should be promoted by the microcarrier. Several microcarrier types are commercially available, and a suitable microcarrier must be selected for each application by considering a combination of factors based on the following list of properties:

- geometry, size and size distribution, porosity, physical density
- GMP compliance
- mechanical stability, autoclavable or sterile delivery
- core and surface material (glass, polystyrene, gelatin, dextran, protein or synthetic protein fragments), source, surface characteristics (charge, stability, transparency, attachment and harvest, thermosensitivity)
- compatibility with downstream processing

Some commercially available microcarriers have been developed to improve cell attachment using new synthetic or natural materials, whereas others are optimized for the specific harvest requirements of hMSCs [35]. Nonporous microcarriers are most suitable for hMSC expansion and harvest because successful detachment with high viability is difficult to achieve using porous microcarriers [36].

2.2.3. Cell expansion in bioreactors

The reproducible, cost-efficient, scalable and automated production of hMSCs in a dynamic, three-dimensional system is best achieved using microcarriers because they allow controlled inoculation, expansion and cell harvest with the system remaining closed [37]. According to PAT requirements, control loops should include for example pH, temperature, stirrer speed or flow rate, aeration and feeding rate. Online process monitoring is necessary to ensure quality throughout the process, e.g., cell density, media properties (pH, temperature, pO_2 and pCO_2) and the concentration of metabolites such as glucose, lactate, ammonia and glutamine. The number of parallel systems should be minimized, aiming to achieve a high surface-volume ratio, which can be realized using systems compatible with microcarrier cultivation. Sterility must be guaranteed by minimizing the contamination risk, and single-use equipment is an option to achieve this. The system must allow low-shear oxygenation by internal or external aeration, and a homogeneous nutrient supply, which are also required for PAT. Well-characterized systems are favorable because they facilitate process validation. Furthermore, the systems should be as simple as possible to avoid errors during system handling.

Several bioreactor types have been described for the expansion of hMSC and hMSC-TERT cells. The most common are spinner flasks, stirred tank reactors (STRs), wave systems [38], fixed-bed reactors (FBRs), fluidized bed and wall-rotating systems [22, 39–41] as well as the application of Vertical-Wheel™ technology [42]. Most published studies describe cell expansion in spinner flasks, but these are difficult to scale up and automate, and the only process control options are pH and oxygen concentration monitoring. Similar drawbacks are associated with wave reactors. The most promising systems are STRs and FBRs. STRs are well-characterized, scalable and controllable systems that are suitable for automation, whereas FBRs avoid the need for media replacement and also offer the opportunity to combine inoculation, expansion and harvest in one system [43–45].

Once the bioreactor system is chosen, the cultivation procedure and process parameters must be defined. The choice of the process mode strongly influences the process parameters, which must be adapted to achieve the defined product specifications [39]. Batch processes are often successful, but fed-batch processes in STRs are more efficient and can be achieved by the partial addition of media and microcarrier [40, 46]. The microcarrier concentration or bed volume must be chosen in combination with the seeding density, followed by the appropriate inoculation strategy. The seeding density affects cell proliferation, and lower densities (100 cells/cm²) are more suitable than higher densities (5000 cells/cm²) [47], suggesting that a ratio of five cells per bead is optimal [48]. The dispersal of cells on the microcarrier follows a Poisson distribution [49]. Initial seeding densities of $1\text{--}3 \times 10^4$ cells/cm² are nearly independent from the available growth surface area. The inoculation strategy is often based on intermittent agitation or flow depending on the system [50, 51].

The rotation speed of a STR is a critical process parameter because the culture should be homogeneous but a low shear stress is necessary to avoid changes to the cell characteristics while at the same time avoiding the formation of bridges between microcarriers by overgrowth [40]. This topic has been reviewed in detail [22, 52]. One solution is to increase the agitation rate during cultivation [53]. The application of a suspension criterion [54] may also

facilitate process development and scale-up, using theoretical process analysis based on power input, microcarrier type and cell growth. Three-phase systems, such as aerated microcarrier-based stirred-tank cultures, show the complex effects of the working volume, carrier concentration and aeration on the power input. Understanding the mixing characteristics of such a system is required to optimize hMSC cultivation [55]. Similarly, for hollow fiber or FBR systems, the flow rate must be defined according to the cell line, microcarrier and system volume [43]. Media exchange is often necessary to avoid nutrient limitation and critical metabolite concentrations [49], but only partial replacement is possible [56] and this increases the risk of contamination.

2.2.4. Cell harvest

Because the cells are the product, adherent hMSCs must be detached and separated from their growth surface. Unlike standard processes, where the cells do not need to be detached, harvest is a very sensitive process step but must still remain cost-effective and GMP compliant. In static cultures, cell detachment is often achieved using peptidases, usually trypsin (EC 3.4.21.4), which cleaves peptide chains after most basic amino acids [57]. Cell detachment in dynamic systems is more complex, especially in terms of shear forces. Therefore, the enzyme used to detach cells grown on carriers in dynamic systems must be both gentle and efficient. The molecular basis of cell attachment depends on the microcarrier, so the choice of enzyme for detachment must take this into account. Furthermore, animal-derived enzymes such as pancreatic trypsin should be avoided [58, 59]. Further aspects that must be addressed include the commercial availability of the enzyme, the final cell density, the incubation time and the temperature. The aim is to maximize the harvest yield and cell viability while maintaining the characteristics of the cells after harvest, processing and formulation.

2.3. Process requirements—downstream

2.3.1. Cell separation, clarification and concentration

After cell detachment, the carrier must be separated from the cells, followed by cell clarification, washing and cell concentration (volume reduction) [60, 61]. For this part of the process, large-scale applications are still in their infancy. Appropriate methods, such as filtration, must ensure minimal processing time and must be based on inert, single-use materials. Cell recovery must achieve clarification and volume reduction while preserving cell viability and retaining cell characteristics. Thus, low shear processes are necessary. Impurities must be reduced to levels below 1 ppm. The system should be closed, automated and scalable [62].

For standard biopharmaceutical processes, tangential flow filtration (TFF) [63] is often used for the initial clarification of therapeutic proteins produced in mammalian, bacterial or yeast cell cultures [64]. For the production of ATMPs, TFF processes must be redesigned to meet product quality demands. The filter material, pore size and initial cell concentration, as well as operating parameters such as shear rate and permeate flux, may influence the recovery of viable, high-quality cells [63]. The impact of shear rate and shear stress on the viability and differentiation potential of hMSCs is well-characterized [65, 66]. High shear rates are known

to reduce membrane fouling during microfiltration [67], so a trade-off between fouling and cell damage is necessary to achieve a satisfactory hMSC concentration.

2.3.2. Formulation and storage

Allogenic stromal cells should be off-the-shelf products available on demand, and appropriate formulation strategies are therefore required allowing the cells to be stored for a specific period of time under defined conditions. Often, allogenic cells are formulated as cell suspensions. Alternative formulations, which prevent immune responses in recipient patients, are cell encapsulation. Inert and biocompatible materials, capsule size and a gentle encapsulation process must ensure that cells retain their viability, identity, purity and differentiation capacity.

The same requirements are relevant to the storage method, including the physical storage form, long-term product stability/availability and the eventual application of the stored cells to the patient, ideally without prior treatments. The storage form must also fulfill GMP and especially ATMP requirements. One common way to store cell culture and ensure long-term availability is cryopreservation in liquid nitrogen. Post-thaw cell survival is sensitive to the freezing and thawing processes, which require cryoprotective agents (CPAs) to prevent the formation of intracellular and extracellular ice crystals that would otherwise expand and destroy cell and organelle membranes [68, 69]. CPAs also minimize osmotic effects that would promote the denaturation of proteins [70]. The nature of the freezing and thawing cycle and the most appropriate CPA must be determined on a case-by-case basis for each cell line [71]. A detailed description of the development of cell line-dependent cryopreservation protocols has been published [72] and the topic has been recently reviewed [73].

A storage system in a GMP environment features a constant temperature in a controlled and monitored environment with a barcode-based vial-labelling system. Frequent thawing of the frozen vials and testing according the above-mentioned aspects are essential to ensure long-term stability.

3. Stem cell production in dynamic bioreactors

3.1. Media choice

As stated above, animal-derived raw materials should be avoided and SFM, XFM or preferably CDM should be used for hMSC expansion. Appropriate media are available from several commercial sources (**Table 1**) and studies published between 2011 and 2015 using these different media for the cultivation of hMSCs have recently been reviewed [74].

Human platelet lysate has been used as an alternative for bovine serum by several groups, e.g., for umbilical cord-derived hMSCs [75, 76]. XFM containing a mixture of proteins including human serum albumin and recombinant growth factors was used to expand hMSCs derived from bone marrow (bm-hMSCs) and adipose tissue on microcarriers [77] and a similar approach with a XFM containing components from human plasma has also been described [78].

Product name	Company	Classification and comments (if information available by manufacturer)	The literature and website
CellGro® MSC Medium	CellGenix	SFM: albumin (human-plasma-derived) insulin (human recombinant, yeast-derived), synthetic lipid contains chicken egg-derived lecithin (licensed medicinal product for human use)	Not cited Web: [136]
CTST™ StemPro® MSC SFM	Gibco by Thermo Scientific	SFM: coating with CELLstart™ CTST™ necessary	[77, 137, 138] Web: [139]
hMSC High Performance Media Kit XF	RoosterBio	XFM: standardized, “enriched” basal medium	Not cited Web: [140]
Human MSC medium, chemically-defined	AmCell Biosciences	recXFM: components are either chemically synthesized or recombinant produced and purified, none of its ingredients are directly derived from nonhuman animals	Not cited Web: [141]
Mesenchymal Stem Cell Growth Medium DXF (Defined Xeno Free)	PromoCell	XFM fibronectin-coated plates are necessary	[142] Web: [143]
Mesenchymal Stem Cell Medium—animal component free (MSCM-acf)	ScienCell Research Laboratories	XFM: no animal- or human-origin materials. Contains (quantitatively and qualitatively formulated) growth factors, hormones and proteins	Not cited Web: [144]
MesenCult™-XF Medium	STEMCELL Technologies	SFM used in conjunction with the MesenCult™-SF Attachment Substrate	Not cited Web: [145]
MesenGro® Human MSC Medium	System Biosciences/ StemRD	recXFM: does not contain animal-derived components, components are either chemically synthesized or recombinantly produced and purified	Not cited Web: [146]
MSC NutriStem® XF Medium	Biological Industries	XFM, defined formulation. Use of fibronectin coating is recommended. Drug Master File available	[11, 147] Web: [148]
MSC-GRO™ Serum-Free/ Xeno-Free, Complete Media	Vitro Biopharma	XFM	Not cited Web: [149]
PowerStem MSC1	PAN-Biotech	XFM: free of animal or human serum, without animal derived components, no undefined peptones or hydrolysates, contains hormones, growth factors and enriched human proteins and lipids	Not cited Web: [150]
PRIME-XV® MSC Expansion SFM	Irvine Scientific	SFM: pre-coated with PRIME-XV MatrIS F necessary	[100, 151] Web: [152]
SPE-IV Media	ABCell-Bio	SFM: animal protein-free, contains human albumin, synthetic iron carrier, rh-insulin, nucleosides, α - monothioglycerol, synthetic lipids, rh-IGF-1 and rh-b- FGF. Requires the addition of pre-adhesion molecules such as fibronectin and collagen	Not cited Web: [153]
Stem Cell 1	Cell Culture Technologies	CDM: chemically defined, protein/peptide-free	[35] Web: [154]
StemMACS MSC Expansion Media Kit XF	Miltenyi Biotec	XFM	Not cited Web: [155]
StemXVivo Xeno-Free Human MSC Expansion Media	R&D Systems	XFM coating with recombinant human fibronectin necessary	[138] Web: [156]

Product name	Company	Classification and comments (if information available by manufacturer)	The literature and website
TheraPEAK™ MSCGM-CD™ Mesenchymal Stem Cell Medium, Chemically Defined	Lonza	XFM: contains only constituents of known molecular structure, contains human albumin, recombinant human insulin, pasteurized human transferrin	[25, 157, 158] Web: [159]

Table 1. Overview of commercially available, SFM and XFM for the cultivation of hMSCs (as of July 2016).

A new CDM for hMSC expansion has been described in which each component has a Chemical Abstracts Service (CAS) registration number and none of the components frequently used in XFM formulations are present, such as serum albumin, insulin, transferrin, progesterone, hydrocortisone or estradiol [35]. The absence of attachment-promoting factors limits the attachment behavior of the hMSCs and the growth surface must therefore be coated with attachment-promoting substances. To our knowledge, this stem cell 1 medium (Cell Culture Technologies, Switzerland) is the only protein/peptide-free CDM for hMSC expansion, mainly comprising defined concentrations of low-molecular weight compounds (50–250 Da, with only one component larger than 1000 Da). The addition of recombinant growth factors in combination with surface coatings makes this medium suitable for the attachment, spreading, growth and detachment of hMSCs derived from different tissues [35].

3.2. Microcarrier choice

Several commercial microcarrier products are available that are suitable for hMSC expansion, as recently reviewed [34]. Microcarriers with surfaces comparable to static tissue culture plastic simplify the transfer of cells from static to dynamic cultivation environments. They have been used successfully for bm-hMSCs in a 5 L STR [79]. Microcarriers 2–5 mm in diameter are often used for FBR processes, whereas those used in fluidized bed reactors are typically 1 mm in diameter and those used in STRs are generally 100–300 µm in diameter [80]. Glass has been used as a cell culture growth surface for decades [81], and low-density microcarriers with a copolymer plastic core and a high-silica glass coating were used for the expansion of hMSC-TERT in serum-containing medium [51, 82]. These results are based on a microcarrier choice for the hMSC-TERT cell line resulting in strong proliferation and a good yield of detached cells for glass and polystyrene microcarriers [36].

Microcarriers should be selected on a case-by-case basis. For example, glass-coated and plastic microcarriers both achieved comparable maximum cell densities (0.91×10^4 and 1.08×10^4 cells/cm², respectively) for umbilical cord-derived MSCs expanded in XFM [75] but only the plastic microcarrier achieved an even distribution of cells (75% of carriers occupied after 72 h), which is essential for successful process scale-up.

Microcarriers coated with extracellular matrix proteins (ECMs) such as collagen can also be used for hMSC expansion [83]. The ECM components encourage cell attachment and growth by providing adhesion ligands on the surface. As discussed above, animal-derived proteins

are discouraged because they increase the risk of contamination and the composition is unpredictable, causing a lack of reproducibility. Microcarriers are therefore coated with synthetic protein fragments. For example, Synthemax microcarriers (Corning) achieved yields and metabolite profiles in bm-hMSC cultures that were comparable to collagen-coated microcarriers [84]. They are suitable for XFM applications [78], as are microcarriers with plasma-treated plastic surfaces that show improved hydrophilicity and wettability to encourage cell attachment [85].

3.3. Stem cell expansion

The following sections focus on the expansion of hMSCs and hMSC-TERT cells using STR and FBR systems due to the limitations of the other systems described above. We consider PAT applied in STR and FBR systems and the use of disposable bioreactors.

3.3.1. PAT applications

In basic processes, the pH, temperature and oxygen partial pressure are monitored to ensure quality throughout the process. In more advanced processes, the concentrations of cells, substrates and metabolites can also be measured. Online monitoring tools are ideal, but offline data analysis is needed for correlation. The cell density on a microcarrier is often determined offline by cell lysis followed by counting the released nuclei, but this is rather imprecise [86]. Alternatively, an intercalating fluorescent dye such as SybrGreen I can be used to estimate DNA levels, which are linearly related to the cell density, and this can be achieved without cell detachment [86]. Dielectric spectroscopy is a promising tool for the online measurement of cell density [21, 86, 87]. At a frequency of 300 kHz, there is a linear correlation between the permittivity and the cell density up to 5×10^4 cells/cm² (~80% confluence). This is sufficient because cell confluence should not be reached. The cell adhesion process can be monitored analyzing the critical frequency. As the cell volume changes during adhesion, the critical frequency declines and remains almost constant throughout the exponential growth phase. When monitoring a process limitation such as oxygen depletion, the permittivity of the cells changes and this is clearly shown in the signal [39, 86]. If the introduction of a probe is not possible (e.g., in FBR processes), the cell density can be determined indirectly, e.g., by calculation from the oxygen or substrate consumption. Mid-infrared spectroscopy combined with multivariate data analysis is another promising online tool to optimize process monitoring for spinner cultures, particularly in the context of process prediction, contamination risks, speed and economic modeling. An optimized partial least squares regression model has been used to estimate glucose, lactate and ammonia concentrations [88].

3.3.2. Fixed-bed reactor

FBR systems can be automated, but it is not possible to take samples, so the cell density is determined indirectly by measuring glucose or oxygen consumption. The homogeneity and scalability of FBR systems remain challenging despite intensive development work by Weber and colleagues [43, 44, 89, 90] and by Elseberg [39]. In bed volumes of 14–300 mL,

2 mm diameter solid glass carriers [91] were successfully used to cultivate hMSC-TERT cells in Eagle's minimal essential medium (EMEM) with serum. The inoculation strategy was 4×30 min for each 7 min with 2×10^4 cells/cm² at a superficial velocity of 0.48 cm/min, achieving an inoculation rate of 50%. During expansion, the superficial velocity was 1.6 cm/min. Process monitoring involved online oxygen measurement (>60%) as well as pH (7.4) and temperature (37°C) control in the conditioning vessel. The system was integrated into a process control system (**Figure 2**).

Figure 2. Schematic drawing of the expansion and harvest process for hMSC-TERT cells in a 300 mL bed. Peristaltic pumps and hose-crushing valves ensured process flow and switching. Single-use oxygen sensors were introduced before and after the packed bed. To avoid carrier settling below the bed, a sieve with a mesh size of 100 μ m was inserted at the bottom socket. D1: heating blanket, G1: harvest vessel, G2: buffer vessel, G3: waste vessel, G4: enzyme vessel, G5: reservoir, I1: pH-probe, I2: pO₂-probe, I3: temperature probe, I4-I5: pO₂-probe, I6: temperature probe, I7: dielectric spectroscopy, I8: mass determination, K1: measurement chamber, M: motor, P1-P2: pump, R1: packed-bed reactor, R2: conditioning vessel, T: water bath for constant temperature, V.1-V.8: valves, W: balance [39].

Offline measurements of glucose and lactate concentrations were used to determine the cell density. Partial media replacement was carried out at glucose concentrations below 0.4 g/L. Expansion was defined as complete at a cell density of $5\text{--}5.5 \times 10^4$ cells/cm². Analysis of residence time in the 300 mL reactor during cultivation clearly showed an inhomogeneous cell distribution, which caused an altered flow profile [39]. Shear stress (1.74×10^{-6} N/cm²) was below the critical value of 1.5×10^{-4} N/cm² [66]. In summary (**Figure 3**), cultivation for 8 days, starting with 1.2×10^4 cells/cm², achieved a final cell density of $6.3 \pm 0.86 \times 10^4$ cells/cm² (total per batch of approximately 3.16×10^8 cells for 5500 cm²) at an average growth rate of 0.27 d⁻¹ [39]. Complex models and scale-up considerations have been published [44]. Monod-based models showed that the glucose uptake rate, and therefore the cell density calculation,

depends on the number of passages and the scale. The system could potentially be improved to achieve a more homogeneous cell distribution by optimizing the inoculation strategy.

Figure 3. Cell and substrate concentration during hMSC-TERT expansion in a 300 mL fixed-bed bioreactor [39]. Model data are based on an initial cell concentration of 1.45×10^7 cell/mL and 0.9 g/L initial glucose concentration using the Euler-method for first order kinetics.

Placenta-derived hMSCs have been cultivated in a scalable packed-bed reactor in which the 13 mL bed was encased within a gas-permeable shell for indirect aeration and nutrient supply [92]. This achieved a low shear of 9.5×10^{-5} Pa at a flow rate of 5 mL/d. The growth surface (160 cm²) was provided by air plasma-treated polystyrene pellets endowed with a surface chemistry similar to tissue culture plastic. A 10-fold expansion of initially 1×10^4 cells/cm² in Dulbecco's modified Eagle's medium (DMEM) with serum was achieved after 1 week in culture, and the cells retained their differentiation capacity. Even so, cell growth in the packed bed culture was slower than growth in static two-dimensional cultures. Inoculation was performed by rolling the column at 5 rpm for 5 min then resting for a period of 3 h. The cell density was determined by AlamarBlue staining, which caused cell disruption. The system was shown to be scalable and suitable for the automated cultivation of murine MSCs to a volume of 235 mL and a growth surface of 2800 cm² and therefore appears suitable for hMSC expansion [92].

3.3.3. Stirred tank reactor

Intensive work on the development of a hMSC-TERT batch process in a 3 L glass STR with a working volume of 1.65 L was described by Elseberg and colleagues, and the cell characteristics were retained [39, 51]. The sterilized bioreactor (**Figure 4**) was filled with the sterile glass-surface microcarrier RapidCell [36] at 25 g/L suspended in high-glucose DMEM supplemented with 10% serum [51]. Cell suspensions were introduced by a syringe at a density of 7×10^3 cells/cm² in four cycles, comprising 45 min without stirring and 2 min

with stirring at 120 rpm [51]. The process parameters pH (7.4), temperature (37°C) and pO_2 (<60%) were monitored online, the cell density determined by dielectric spectroscopy. The fluorescence-based assay described above was used offline to determine cell density, combined with microscopy. During expansion, the rotation speed was increased stepwise from 120 to 160 rpm at 10-rpm intervals for every 1.2×10^4 cells/cm² [51]. The cultivation data confirmed that the cell density, substrate consumption and metabolite production were reproducible, and critical values of lactate and ammonia concentrations were not observed. In summary (**Figure 5**), after 6 days of cultivation, starting at 7×10^3 cells/cm², a final cell density of 4.5×10^4 cells/cm² was achieved (equivalent to $\sim 6.84 \times 10^8$ cells for a surface of $\sim 13,600$ cm²) with an average growth rate of 0.32 d^{-1} in three replicate cultivations [39]. The detailed protocol has been published [93]. Optimization studies should consider bead-to-bead transfer as a stepwise fed-batch process to reduce costs. Furthermore, the online analysis of glucose and lactate would be beneficial. The detailed analysis of each step may also allow the process to be adapted for primary cells.

Figure 4. Schematic drawing of the expansion process for hMSC-TERT cells in a 3 L glass STR including a picture of SybrGreen-stained hMSC-TERT cells on RapidCell microcarriers; D1: heating blanket, I1: pH probe, I2: pO₂ probe, I3: temperature probe, I4: dielectric spectroscopy, M: motor, R: glass stirred tank reactor, V1: valve [39].

One of the larger-scale processes for hMSC production (2.5 L in a 5 L STR) [79] required a cultivation time of 12 days to achieve a cell density of 1.7×10^5 cells/mL. The culture conditions were pH 7.2–6.7 at 37°C and pO_2 >45%. This allowed a sixfold expansion while retaining cell characteristics. Oxygenation only occurred during 50% media exchange every second day after day 3. The inoculation density was 6×10^3 cells/cm² on plastic-surface microcarriers (P102L by Solohill), which is equivalent to ~ 5 cells/bead. The static culture with a down-pumping impeller was run in static mode for 18 h at 75 rpm to ensure that all microcarriers were distributed

evenly throughout the reactor, resulting in better cell growth compared to spinner cultures. The authors found that homogeneity could be achieved with less power input compared to the spinner cultures. Optimization was required in terms of pH and oxygen control, as well as monitoring the cell density to determine the point of confluence during the process.

Figure 5. Cell density and substrate concentration during hMSC-TERT expansion in a 3 L glass STR [39]. Model data are based on an initial cell density of 4.24×10^7 cell/mL, and 3.5 g/L initial glucose concentration using the Euler method for first order kinetics.

A further process has been described for hMSCs in a 1.3 L working volume STR [94]. The expansion processes differed according to the source of the hMSCs, such as umbilical cord matrix-derived hMSCs (UCM-hMSCs), highlighting the need for tailored process development. The cultivation of UCM-hMSCs was recently demonstrated using gelatin-based microcarriers and XFM in a controlled STR culture with a working volume of 800 mL [41]. To increase process efficiency, an automated continuous process (e.g., without intervention for media replacement) was developed based on continuous perfusion in a STR and the cells retained their differentiation capacity [95]. Starting with 0.25×10^5 cells/mL in 400 mL at 40–60 rpm in Mesencult™-XF media, continuous perfusion was carried out including a cell retention device, such as the ATF-System for microcarriers by Repligen Corporation, with dilution rates of 0.2 d^{-1} starting on day 5, or a dip-tube adapter attached to the bioreactor cap. Inoculation was achieved by intermittent stirring on Synthemax II microcarriers at 16 g/L. This resulted in higher cell densities of 3.7×10^5 cell/mL (expansion ratio = 14.6) and growth rates of 0.016 h^{-1} . No media depletion was observed, and inhibitory substances such as lactate and ammonia remained below critical values of (<6 and <1 mM, respectively [50]). Bead-to-bead transfer was further shown to increase process efficiency by the addition of empty microcarriers on day 6 [78].

Single-use technology is appropriate for economic reasons and to meet GMP requirements because this eliminates the risk of cross-contamination and facilitates the validation and

qualification of the system. The first disposable STR used for the expansion of hMSCs on microcarriers was the Mobius® 3 L bioreactor by Merck-Millipore with 2.4 L working volume, which yielded ~600 million hMSCs on collagen-coated polystyrene microcarriers [83, 96]. The cells retained their basic defining characteristics, i.e., cell surface marker expression and differentiation potential. The cultivation of hMSCs over 12 days in this system was compatible with bead-to-bead transfer. This increased the expansion factor to 62-fold, six times higher than a normal batch process, achieving a yield of 49,750 cells/cm². Starting with a growth surface of 5400 cm², the addition of media and microcarrier after 7 days increased this to 10,800 cm² (+1 L) and 12,960 cm² on day 11 (+0.4 L) until the end of the process. This process involved non-cycling inoculation at 35 rpm. During cultivation, the stirrer speed was increased from 55 to 75 rpm and minimal aggregate formation was observed [46]. The mixing characteristics of this three-phase system showed a certain degree of inhomogeneity, but this was beneficial because the cells were allowed rest phases of low shear stress [55].

The transfer of a process for hMSC-TERT cells from a geometrically similar glass STR to the single-use Mobius® 3 L bioreactor has also been described [51]. All parameters were kept constant except the stirrer speed, which was reduced to 60–90 rpm, achieving a 6.9-fold expansion and comparable growth rates to the glass STR. Even so, carrier aggregation was observed, indicating nonoptimal culture conditions [46]. Other systems include the 1.3 L Bioflo® by Eppendorf for the production of 1.4×10^5 cells/mL, which is also available as a single-use device [77]. The UniVessel® by Sartorius for hMSC cultivation can produce up to 1.8×10^5 cells/mL in a maximum volume of 2 L [22]. The cell density can be increased by up to three-fold by reducing shear forces, which can be achieved by changing the impeller blade angle from 30° to 45°, reducing the off-bottom clearance from 0.411 to 0.26 and increasing the microcarrier density. To our knowledge, the largest volume for hMSC expansion used the BIOSTAT® CultiBag STR 50 L with 5% serum-supplemented stem cell media by Lonza [22, 54]. Inoculation was implemented during a 4-h cell attachment phase in 20 L of medium-containing equilibrated gelatin microcarriers, and the bag was then transferred to the CultiBag STR 50 L starting with a 35 L working volume. The process conditions were 37°C, pO₂ >20%, maximum air flow rate of 0.03 vvm, pH 7.2–7.3 and the impeller speed was set to 50–66 rpm. The peak viable cell density was 7.2×10^5 cells/mL, with an expansion factor of 51.5 ± 4.9 .

3.4. Cell detachment

As described in Section 2.2, enzymatic cell detachment is often a crucial step for the successful production of hMSCs. Researchers often use recombinant trypsin for process development, but there is an intensive ongoing search for alternatives, mostly studied using static cultures [82]. Here, we discuss the harvesting procedure for the dynamic systems described above and then consider alternatives based on studies using static cultures.

Studies reported by Weber [89] and Salzig and colleagues [82] have shown how cells can be harvested from a FBR system. Success depends on the combination of enzyme choice, incubation time and temperature, and the effects of downstream processing and formulation [82]. After flushing the reactor twice with phosphate buffered saline (PBS), harvesting was carried out by incubating the cells with TrypZean for 15 min at 21°C and cells were flushed out of

the reactor with media (37°C) at a superficial velocity of 19 cm/s. The yields were not reproducible due to the inhomogeneous cell and enzyme distribution, and the highest yield was $82.1 \pm 2.3\%$. The final cell density had a strong impact the cell harvest yield. Based on transport limitations, higher cell densities reduced the viability of the cells from 80–90% to 50% [39].

In a glass STR process [39, 46, 51, 93], a partial cell harvest of 300 mL was achieved using a sieve with a mesh size of 100 μm to separate the carriers with the cells from the media. The carriers were then rinsed with PBS and the entire sieve was transferred into a dish containing trypsin solution for incubation at room temperature for 10 min. Detached cells were rinsed off with serum-containing media. This method achieved a consistent high harvest yield with >95% cell viability. The viability, metabolic activity (determined with water-soluble tetrazolium salt) and adipogenic differentiation capacity of the cells were comparable to cells from static cultures in T-flasks, whereas cell growth of re-cultured cells was slightly slower.

A potentially scalable harvesting method has been developed to recover hMSCs from a 5 L STR with a working volume of 2.5 L, based on studies in spinner flasks with plastic-surface microcarriers [97]. The duration of the incubation step in trypsin-EDTA was limited to 7 min and combined with agitation (150 rpm) so that Eddy sizes exceeded the cell size. The harvest efficiency was >95% with a viability of 98%, and cells retained their characteristics [18]. Cell harvest from a 5 L STR would normally require 8–9 min incubation with the enzyme and agitation at 120 rpm. Further studies are required to optimize the incubation time, taking into account carrier-carrier effects, carrier density and the cell density prior to the harvest [97]. Other groups use the nonmammalian trypsin TrypLE for the detachment of hMSCs [94, 97–100] on cationic polystyrene charged microcarriers, which achieves high cell quality even in cultures based on SFM [63, 100].

A promising and safer alternative to trypsin is the prolyl-specific peptidase (PsP) expressed natively and isolated from the fungus *Wolfiporia cocos* [58]. Allogenic hMSC-TERT cells were detached more rapidly with 1.6 U/mL PsP than trypsin, and the new enzyme also showed less severe effects on the growth and metabolism of re-cultivated cells. The optimal harvest yield was achieved by incubating the cells in PsP for 20 min.

Studies using static cultures have shown that the choice of the enzyme must be considered in combination with the growth surface coating and the type of medium for each cell type on a case-by-case basis [35]. The authors compared the detachment of hMSCs and hMSC-TERT cells with trypsin, Accutase, collagenase and PsP when the cells were grown on untreated surfaces, or surfaces coated with collagen or fibronectin, combined with CDM or serum-containing media (SCM). These different conditions had a significant impact on the detachment of hMSC-TERT cells but little impact on primary hMSCs, although the detachment of hMSCs was slightly less in the absence of coatings (**Figure 6**). All detached cells remained highly viable [35].

For gelatin-based microcarriers, the total digestion of the microcarrier is possible using trypsin, yielding a single-cell suspension. Although this removes the need for cell-carrier separation, a longer incubation time is necessary which can change the immunophenotype of the cells, but this can be reverted during re-cultivation in static cultures [41].

A promising alternative is the use of thermosensitive microcarriers to avoid the need for enzymatic cell detachment. Various materials have been tested, i.e., poly-N-isopropylacrylamide, and recently, a successful application has been reported in dynamic systems [101–103]. Although proteolytic enzyme treatment is not necessary, mechanical forces are still required to achieve a single cell suspension, as cell-cell-contact is not dissociated due to the temperature shift [104]. Future research in this area could offer a replacement for the gold standard of cell detachment with trypsin or its derivatives, particularly if combinations of enzymes and functional surfaces are considered.

Figure 6. Detachment of hMSC-TERT cells using different enzymes for n = 3 measurements each. The cells were grown to confluence in coated or uncoated wells and were detached enzymatically. Cell detachment was analyzed by counting the cells in suspension [35].

3.5. Separation, clarification and concentration

Until recently, the scalable harvesting of hMSCs has received little attention [63, 97]. The separation of single cells from the carrier in STR systems can be achieved by dead-end filtration [63, 97, 100]. The pore size of the polystyrene filters must be $>75\ \mu\text{m}$ to maintain cell viability and quality because hMSCs fall within the size range $15\text{--}20\ \mu\text{m}$, whereas the microcarriers are $125\text{--}212\ \mu\text{m}$ [63]. Cells could also be harvested directly from a disposable STR by inserting a sieve [46]. Commercially available systems for cell-carrier separation in general have been reviewed, and the authors also suggest the option of continuous flow centrifugation, which is available as a closed, single-use device that can handle approximately 250 L of culture with 15 g/L of microcarrier, but will require modifications for compatibility with ATMPs [40].

For FBRs and the glass STRs described above [39, 51, 89], cell-carrier separation is achieved by flushing the cells from the bioreactor when enzymatic detachment is complete. The shear forces during this process transiently exceed the critical value of $1.5 \times 10^{-4}\ \text{N/cm}^2$, which may explain the loss of cell viability. The integration of a measurement chamber including a dielectric spectroscopy probe allowed the definition of parameters that maximized the harvest yield while minimizing the volume. Concentration was achieved by centrifugation and resuspension in a smaller volume [39]. For cells harvested from the FBR process described above, cells retained their capacity for adipogenic differentiation and their metabolic activity was only slightly lower than cells cultured in parallel using T-flasks. Cells harvested from the STR process after centrifugation consistently showed $>90\%$ viability, comparable to cells in static cultures in the same medium [39].

Clarification and volume reduction can be achieved not only by centrifugation [100] or dead-end filtration [105] but also by TFF to achieve better product purity [63, 106]. Many fully automated, disposable and integrated (concentration and washing) TFF systems are available that are compatible with ATMP processes. TFF systems have linear scalability and operate with low shear forces and pressures. A TFF system with hollow-fiber modules and polysulfone membranes ($24\ \text{cm}^2$ surface, pore size $>45\ \mu\text{m}$, sterilized with NaOH) was recently used for the downstream processing of hMSC suspension cultures [63]. The authors showed that 10-fold concentration in a 0.25 L volume is possible, removing 98% protein and maintaining $>95\%$ viability as well as cell identity and potency, at a shear rate of $3000\ \text{s}^{-1}$. The permeate flux was controlled at $250\ \text{L m}^{-2}\ \text{h}^{-1}$ and cell recovery was more than 80% at densities $>2 \times 10^5\ \text{cells/mL}$. The cell density, shear rate and permeate flux were shown to affect yield, viability and quality of the cells [63]. The incorporation of an expanded bed chromatography step using a multimodal prototype resin based on core-shell bead technology achieved a further 10-fold increase in efficiency, with a process recovery of 70% in negative mode. The best trade-off between cell recovery (89%) and protein clearance (67%) was achieved using an intermediate expansion bed rate (1.4) which also retained the cell characteristics. A further diafiltration step can be introduced using a CPA solution for formulation, fill and finish [95]. This reduces the overall diafiltration volumes and achieves a product purity sufficient for clinical applications. TFF combined with negative mode chromatography may therefore represent the beginning of a new generation of downstream processes for hMSCs that can be improved further by the investigation of novel adsorbents [106]. The cost of large-scale separation, clarification and concentration could be

reduced fluidized bed centrifugation [62], whereas TFF would be more appropriate for smaller lots. The raw materials and detailed process parameters must always be chosen according to the cell line, media, microcarrier and expansion process.

3.6. Formulation and storage of stem cells

As discussed above, cryopreservation is the standard storage mode for single-cell suspensions, and this is also the case for allogenic hMSCs [107]. The standard is a slow freezing rate of 1°C/min down to -80°C and a quick thawing rate (2 min at 37°C for 2 mL vials) using 5–20% dimethylsulfoxide (DMSO) in serum as a CPA [72]. Neither DMSO nor serum are suitable for ATMPs [69, 108]. For other stem cells, successful cryopreservation has been achieved using 5% DMSO in 5% human albumin [109]. A recently published study discussing the long-term cell banking (up to 8 years) of allogenic hMSC used a cryopreservation method with 20% DMSO, and the cells were suitable for clinical studies within 1 h after thawing and dilution in PBS [110]. Sodium pentaborate pentahydrate combined with low concentrations DMSO is beneficial for tooth germ stem cells [111]. SFM and XFM combined with 5–10% DMSO is suitable for different human progenitor cells [112]. Research is underway to find appropriate alternatives CPAs to avoid the need to remove DMSO (e.g., by diafiltration) before their use in the clinic.

Based on promising preliminary studies [69], ectoin and proline have been investigated as alternative CPAs for hMSC-TERT cells [108]. They were compared to commercially available Biofreeze SFM lacking DMSO (Biochrom, Germany [113]) combined with methylcellulose supplemented PBS. The cells were stored at -150°C and thawed quickly at 37°C. The highest cell survival rates after 48 h re-cultivation ($89 \pm 2\%$) without DMSO and serum were achieved by supplementing the medium with 1%/10% (w/v) proline/ectoin for 60 min before freezing, and then reducing the temperature by 1°C/min which was shown to be beneficial for other stem cells [108]. The best results ($\sim 99\%$ survival rate) were achieved with Biofreeze SFM in all approaches. Regardless of whether Biofreeze or 1%/10% proline/ectoin was used, the cells retained their adipogenic differentiation capacity. The impact of the duration of pre-freeze incubation and the cooling rate depended on the CPA combination. To improve outcome of cryopreservation, the authors suggested nucleation temperature control during the freezing process [108]. Another recent report described the formulation of hMSCs using the FDA-approved commercial serum-free and xeno-free CPA known as STEM-CELLBANKER™ (Amsbio, UK) [29]. The cell viability was $>90\%$ (better than DMSO) and no morphological differences were observed, but cell growth was slower. The mesodermal differentiation capacity was not regardless of which CPA was used [29]. The cryopreservation of hMSCs has also been tested using Prime-XV MSC FreezIS DMSO-Free (Irvine Scientific, USA) with 30 min incubation at room temperature before cooling to 4°C for 5 min followed by further cooling at 1°C/min down to -80°C for storage in liquid nitrogen vapor. The cells were thawed quickly at 37°C [100]. Other CPAs such as sucrose and high-molecular-weight polymers like polyvinylpyrrolidone [114] should be investigated as well as studies in larger volumes or geometries (such as syringes) that might be more appropriate for therapeutic approaches [108].

In addition to single-cell formulations, the encapsulation of cells may be beneficial to prevent allogenic cells triggering an immune response in the patient [115–117]. A semi-permeable membrane allows the diffusion of molecules (e.g., nutrients and therapeutic proteins)

but protects the cells from the host immune system and mechanical forces, thus potentially enhancing the therapeutic benefits of hMSCs [118]. Various production methods have been tested to generate small beads (200–400 μm) with a narrow size distribution [119]. The formation of core capsules is often suggested [120] and this can be used to induce line-specific differentiation [22] even when cells are cultivated in a FBR [90]. Biopolymers such as agarose, Pluronic F-127 [121] and clinical-grade alginate are used for this purpose, the latter forming three-dimensional structures in the presence of multivalent cations.

Cells harvested from FBRs or glass STRs can be encapsulated by suspending 5×10^6 centrifuged cells in 500 μL sodium histamine solution then adding 4.5 mL sterile 1.5% alginate solution. After incubation for 2.5 h, the suspension was dropped into a BaCl_2 solution and incubated for 2 h. Finally, the capsules were washed at least three times with PBS, and twice with EMEM and cultured in six-well plates for further analysis. Encapsulated cells from the FBR showed adipogenic differentiation capacity and high viability (80–90% decreasing after 48 h). Cells encapsulated from T-flasks consistently showed vitalities >95% [39, 91].

The cryopreservation of encapsulated cells is useful for the long-term storage and off-the-shelf availability of many cell types, including hMSCs [122]. A three-step slow cooling process [123] with induced ice nucleation using 10% DMSO (also suggested elsewhere [124]) has been shown to maximize cell viability, and the cells retain their metabolic and differentiation capacity. Cell encapsulation may also be beneficial for short-term storage, such as for transport [125]. The hypothermic (4–23°C) preservation of human adipose-derived cells encapsulated in 1.2% alginate in XFM/SFM has been discussed [126].

4. Quality approval

Prior to the product release, intensive quality control is required to confirm cell identity and safety according to validated protocols that comply with GMP, the International Conference on Harmonization (ICH) Guidelines and/or the European Pharmacopoeia [17].

4.1. Identity

Cell identity must be approved, including viability, differentiation capacity and the surface marker profile demanded by the ISCT [18]. Cell viability, metabolic activity and growth rates can be monitored during the production process after every step (after isolation, after expansion, after harvest and clarification, after final formulation). Viability testing and cell counting are achieved using methods such as flow cytometry, including dye exclusion. Different dyes make it possible to detect viable and dead cells even in encapsulated formulations [82].

Immunophenotyping by flow cytometry is used to detect surface markers. Antibodies that bind to specific antigens expressed by the cell are coupled to fluorophores. A repertoire of cell markers can be identified and quantified simultaneously using different dyes. Many of the antigens used to distinguish human cell populations are cluster of differentiation (CD) molecules (www.hcdm.org). Immunophenotyping by flow cytometry has become the method of choice to identify and sort cells, e.g., in bone marrow aspirates.

The differentiation of hMSCs into osteoblasts, adipocytes or chondroblasts can be induced using established methods [127–129]. Several differentiation media are commercially available, although the ingredients are often not fully disclosed (e.g., StemPro® Differentiation Kits for hMSCs by Gibco). The multi-step process of adipocyte development involves a cascade of transcription factors and cell-cycle proteins that regulate gene expression. Adipogenesis is induced by insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and indomethacin [130]. Differentiation towards the adipogenic lineage can be confirmed by Oil Red O staining of lipid vacuoles in the adipocytes. MSCs undergoing chondrogenic differentiation produce large amounts of ECM when cultured in pellet form, primarily composed of cartilage-specific molecules such as collagen type II and aggrecan [129]. The latter can be used as evidence for the chondrogenic differentiation of hMSCs and can be stained with Alcian blue, or by the immunostaining of collagen type II. Osteogenic cells show changes in cell morphology, from spindle shaped to cuboidal, and the cells accumulate extracellular calcium deposits (mineralization). Osteoblast mineralization is therefore indicative of the formation of bone mass and can be detected using the dye Alizarin Red S [127].

The successful differentiation of hMSCs and the expression of corresponding CD markers can also be verified by quantitative reverse transcription PCR (qRT-PCR), which requires the extraction of RNA. Different protocols for RNA extraction from tissues are available, and commercial kits can be used to capture RNA on silica membranes in spin columns or isolate the RNA by phenol/chloroform extraction, followed by precipitation. A recently published study compares different single-step RNA extraction methods focusing on embedded stem cells [131].

Lineage differentiation capacity needs to be approved only once for a validated process, if no further changes occur. A single-cell preparation does not need to be re-evaluated because the differentiation of cells *in vitro* takes 10–30 days, resulting in ethical discussions for on-demand product applications. Generally, the benefit-risk ratio of clinical applications must also be considered [16].

4.2. Safety

The sterility of therapeutic hMSC products must be guaranteed because any contamination (bacteria, bacterial endotoxins, mycoplasma and viruses) present a high risk to the patient. Protocols for tests are provided in the European Pharmacopoeia (EuPh) chapters 2.6.27, 2.6.14 and 2.6.2. The chapter on microbiological examination was revised in September 2016 [132]. This covers a selection of alternative tests because classical microbiological methods are often not suitable for products with a shelf-life of only a few hours or a few days.

The potential tumorigenicity of therapeutic hMSC products is also relevant [133] but chromosomal abnormalities are rarely observed in freshly-isolated primary hMSCs [134]. However, primary hMSC populations are heterogeneous, comprising cells of different ages that have undergone different numbers of divisions. The presence of abnormalities is dependent on both the donor and the *in vitro* culture method. In 2014, an investigation of 92 clinical-grade bm-hMSCs showed clonal mutations in only 3 of 86 cases, and none of these showed evidence of a malignant transformation or a change in phenotype [135]. To exclude products

containing cells with cytogenetic abnormalities, G-band karyotyping and fluorescence in situ hybridization (FISH) is necessary, as proposed by the EMA Cell Products Working Party (CPWP) and the Committee for Advanced Therapies (CAT) [133]. The cytogenetic testing of each batch as a release criterion is unnecessary if chromosomal abnormalities are not observed. The cryopreservation of batch samples during manufacturing is useful for later testing if needed.

5. Conclusion

The production of allogenic hMSCs is challenging, and there is intensive research focusing on the different steps in the process. Research institutes and industry have only recently published various reviews on this topic, and to our knowledge, there are still no large-scale processes with detailed protocols for every process development step, from cell line and media selection through to the final formulation and storage, focusing on the special demands of ATMP production. This chapter has summarized the major issues affecting such a process and has discussed potential future options. Further research is required to develop closed, continuous and efficient processes that meet regulatory demands for high product quality.

Acknowledgements

This research was financially supported by the Hessen State Ministry of Higher Education, Research and the Arts, within the Hessen initiative for scientific and economic excellence (LOEWE program). The authors thank Dr. Richard M. Twyman for revising the manuscript.

Author details

Christiane Elseberg^{1#}, Jasmin Leber^{1#}, Tobias Weidner^{1, 2} and Peter Czermak^{1, 2, 3, 4*}

*Address all correspondence to: peter.czermak@lse.thm.de

1 University of Applied Sciences Mittelhessen, Institute of Bioprocess Engineering and Pharmaceutical Technology, Giessen, Germany

2 Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Project Group Bioresources, Giessen, Germany

3 Justus Liebig University, Faculty of Biology and Chemistry, Giessen, Germany

4 Kansas State University, Department of Chemical Engineering, Manhattan, Kansas, USA

These authors contributed equally to this work

References

- [1] Li X, Bai J, Ji X, Li R, Xuan Y, Wang Y. Comprehensive characterization of four different populations of human mesenchymal stem cells as regards their immune properties, proliferation and differentiation. *Int J Mol Med* 2014;**34**:695–704. doi:10.3892/ijmm.2014.1821
- [2] Wuchter P, Wagner W, Ho AD. Mesenchymal Stromal Cells (MSC). *Regenerative Medicine—From Protocol to Patient*. Cham: Springer International Publishing; 2016, p. 295–313. doi:10.1007/978-3-319-27610-6_11
- [3] McGuirk JP, Smith JR, Divine CL, Zuniga M, Weiss ML. Wharton's jelly-derived mesenchymal stromal cells as a promising cellular therapeutic strategy for the management of Graft-versus-Host disease. *Pharmaceutics* 2015;**8**:196–220. doi:10.3390/ph8020196
- [4] Wang S, Qu X, Zhao R. Clinical applications of mesenchymal stem cells. *J Hematol Oncol* 2012;**5**:19. doi:10.1186/1756-8722-5-19
- [5] Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016;**10**. doi:10.1016/S0140-6736(16)31203-X
- [6] EMA (European Medicines Agency). Holoclar—ex vivo expanded autologous human corneal epithelial cells containing stem cells. Summary of the European public assessment report 2015. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/002450/WC500183406.pdf (accessed August 22, 2016).
- [7] Abdallah BM, Haack-Sørensen M, Burns JS, Elsnab B, Jakob F, Hokland P, et al. Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene despite of extensive proliferation. *Biochem Biophys Res Commun* 2005;**326**:527–38. doi:10.1016/j.bbrc.2004.11.059
- [8] Simonsen JL, Rosada C, Serakinci N, Justesen J, Stenderup K, Rattan SIS, et al. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. *Nat Biotechnol* 2002;**20**:592–6. doi:10.1038/nbt0602-592
- [9] Heathman TR, Nienow AW, McCall MJ, Coopman K, Kara B, Hewitt CJ. The translation of cell-based therapies: clinical landscape and manufacturing challenges. *Regen Med* 2015;**10**:49–64. doi:10.2217/rme.14.73
- [10] Jung S, Panchalingam KM, Wuerth RD, Rosenberg L, Behie LA. Large-scale production of human mesenchymal stem cells for clinical applications. *Biotechnol Appl Biochem* 2012;**59**:106–20. doi:10.1002/bab.1006
- [11] Chen AK-L, Reuveny S, Oh SKW. Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction. *Biotechnol Adv* 2013;**31**:1032–46. doi:10.1016/j.biotechadv.2013.03.006

- [12] Chabannon C, Caunday-Rigot O, Faucher C, Slaper-Cortenbach I, Calmels B, Lemarie C, et al. Accreditation and regulations in cell therapy. *ISBT Sci Ser* 2016;**11**:271–6. doi:10.1111/vox.12205
- [13] Oppermann T, Leber J, Elseberg C, Salzig D, Czermak P. hMSC production in disposable bioreactors in compliance with cGMP guidelines and PAT. *Am Pharm Rev* 2014;**17**. <http://www.americanpharmaceuticalreview.com/Featured-Articles/160358-hMSC-Production-in-Disposable-Bioreactors-in-Compliance-with-cGMP-Guidelines-and-PAT/> (accessed August 23, 2016).
- [14] Renner M, Anliker B, Flory E, Scherer J, Schüßler-Lenz M, Schweizer M, et al. Regulation for Gene and Cell Therapy Medicinal Products in Europe. In: Terai S, Suda T, editors. *Gene Therapy and Cell Therapy through Liver*. Tokyo: Springer Japan; 2016, pp. 105–23. doi:10.1007/978-4-431-55666-4_10
- [15] Unger C, Skottman H, Blomberg P, Sirac Dilber M, Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. *Hum Mol Genet* 2008;**17**:R48–53. doi:10.1093/hmg/ddn079
- [16] ISSCR (International Society for Stem Cell Research). Guidelines for Stem Cell Research and Clinical Translation 2016. <http://www.isscr.org/guidelines2016> (accessed August 23, 2016).
- [17] Torre ML, Lucarelli E, Guidi S, Ferrari M, Alessandri G, De Girolamo L, et al. Ex vivo expanded mesenchymal stromal cell minimal quality requirements for clinical application. *Stem Cells Dev* 2015;**24**:677–85. doi:10.1089/scd.2014.0299
- [18] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–7. doi:10.1080/14653240600855905
- [19] Wuchter P, Bieback K, Schrezenmeier H, Bornhäuser M, Müller LP, Bönig H, et al. Standardization of good manufacturing practice-compliant production of bone marrow-derived human mesenchymal stromal cells for immunotherapeutic applications. *Cytotherapy* 2015;**17**:128–39. doi:10.1016/j.jcyt.2014.04.002
- [20] EMA—Christine Bugge. Quality Guidelines—Standard operating procedure—Article 20 2012. www.ema.europa.eu/.../en_GB/document_library/Standard_Operating_Procedure_-_SOP/2009/09/WC500003014.pdf.
- [21] Justice C, Brix A, Freimark D, Kraume M, Pfromm P, Eichenmueller B, et al. Process control in cell culture technology using dielectric spectroscopy. *Biotechnol Adv* 2011;**29**:391–401. doi:10.1016/j.biotechadv.2011.03.002
- [22] Jossen V, Pörtner R, Kaiser SC, Kraume M, Eibl D, Eibl R. Mass production of mesenchymal stem cells—impact of bioreactor design and flow conditions on proliferation and differentiation. *Cells Biomater Regen Med* 2014:119–74. doi:10.5772/59385

- [23] Merten O-W, Flickinger MC. Cell Detachment. Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology. John Wiley and Sons, Inc.; 2009, pp. 1–22. doi:10.1002/9780470054581.eib195
- [24] Jayme DW, Smith SR. Media formulation options and manufacturing process controls to safeguard against introduction of animal origin contaminants in animal cell culture. Cytotechnology 2000;33:27–36. doi:10.1023/A:1008133717035
- [25] Jung S, Panchalingam KM, Rosenberg L, Behie LA. Ex vivo expansion of human mesenchymal stem cells in defined serum-free media. Stem Cells Int 2012. doi:10.1155/2012/123030
- [26] Tarle S, Shi S, Kaigler D. Development of a serum-free system to expand dental-derived stem cells: PDLSCs and SHEDs. J Cell Physiol 2011;226:66–73. doi:10.1002/jcp.22304
- [27] Hudson JE, Mills RJ, Frith JE, Brooke G, Jaramillo-Ferrada P, Wolvetang EJ, et al. A defined medium and substrate for expansion of human mesenchymal stromal cell progenitors that enriches for osteo- and chondrogenic precursors. Stem Cells Dev 2011;20:77–87. doi:10.1089/scd.2009.0497
- [28] Mimura S, Kimura N, Hirata M, Tateyama D, Hayashida M, Umezawa A, et al. Growth factor-defined culture medium for human mesenchymal stem cells. Int J Dev Biol 2011;55:181–7. doi:10.1387/ijdb.103232sm
- [29] Al-Saqi SH, Saliem M, Quezada HC, Ekblad Å, Jonasson AF, Hovatta O, et al. Defined serum- and xeno-free cryopreservation of mesenchymal stem cells. Cell Tissue Bank 2015;16:181–93. doi:10.1007/s10561-014-9463-8
- [30] Gottipamula S, Muttigi MS, Kolkundkar U, Seetharam RN. Serum-free media for the production of human mesenchymal stromal cells: a review. Cell Prolif 2013;46:608–27. doi:10.1111/cpr.12063
- [31] Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. ALTEX 2010;27:53–62.
- [32] Docheva D, Haasters F, Schieker M. Mesenchymal stem cells and their cell surface receptors. Curr Rheumatol Rev 2008;4:155–60. doi:10.2174/157339708785133479
- [33] Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2009;5:17–26. doi:10.1016/j.stem.2009.06.016
- [34] Merten O-W. Advances in cell culture: anchorage dependence. Philos Trans R Soc B Biol Sci 2014;370:20140040. doi:10.1098/rstb.2014.0040
- [35] Salzig D, Leber J, Merkewitz K, Lange MC, Köster N, Czermak P. Attachment, growth, and detachment of human mesenchymal stem cells in a chemically defined medium. Stem Cells Int 2016:Article ID 5246584. doi:10.1155/2016/5246584
- [36] Weber C, Pohl S, Pörtner R, Wallrapp C, Kassem M, Geigle P, et al. Expansion and harvesting of hMSC-TERT. Open Biomed Eng J 2007;1:38–46. doi:10.2174/1874120700701010038

- [37] Sensebé L, Bourin P, Tarte K. Good manufacturing practices production of mesenchymal stem/stromal cells. *Hum Gene Ther* 2011;**22**:19–26. doi:10.1089/hum.2010.197
- [38] Timmins NE, Kiel M, Günther M, Heazlewood C, Doran MR, Brooke G, et al. Closed system isolation and scalable expansion of human placental mesenchymal stem cells. *Biotechnol Bioeng* 2012;**109**:1817–26. doi:10.1002/bit.24425
- [39] Elseberg CL. Prozessintensivierung bei der Herstellung von stammzellbasierten (hMSC-TERT) Implantaten für die Zelltherapie – Anwendung der dielektrischen Spektroskopie, Dissertation. Technical University Berlin. ISBN: 978-3-8440-2858-4, 2014.
- [40] Schnitzler AC, Verma A, Kehoe DE, Jing D, Murrell JR, Der KA, et al. Bioprocessing of human mesenchymal stem/stromal cells for therapeutic use: current technologies and challenges. *Biochem Eng J* 2016;**108**:3–13. doi:10.1016/j.bej.2015.08.014
- [41] Mizukami A, Fernandes-Platzgummer A, Carmelo JG, Swiech K, Covas DT, Cabral JMS, et al. Stirred tank bioreactor culture combined with serum-/xenogeneic-free culture medium enables an efficient expansion of umbilical cord-derived mesenchymal stem/stromal cells. *Biotechnol J* 2016. doi:10.1002/biot.201500532
- [42] Sousa MFQ, Silva MM, Giroux D, Hashimura Y, Wesselschmidt R, Lee B, et al. Production of oncolytic adenovirus and human mesenchymal stem cells in a single-use, vertical-wheel bioreactor system: impact of bioreactor design on performance of microcarrier-based cell culture processes. *Biotechnol Prog* 2015;**31**:1600–12. doi:10.1002/btpr.2158
- [43] Weber C, Freimark D, Pörtner R, Pino Grace P, Pohl S, Wallrapp C, et al. Expansion of human mesenchymal stem cells in a fixed-bed bioreactor system based on non-porous glass carrier—Part A: inoculation, cultivation, and cell harvest procedures. *Int J Artif Organs* 2010;**33**:512–25.
- [44] Weber C, Freimark D, Pörtner R, Pino Grace P, Pohl S, Wallrapp C, et al. Expansion of human mesenchymal stem cells in a fixed-bed bioreactor system based on non-porous glass carrier—Part B: modeling and scale-up of the system. *Int J Artif Organs* 2010;**33**:782–95.
- [45] Tsai A-C, Liu Y, Ma T. Expansion of human mesenchymal stem cells in fibrous bed bioreactor. *Biochem Eng J* 2016;**108**:51–7. doi:10.1016/j.bej.2015.09.002
- [46] Cierpka K, Elseberg CL, Niss K, Kassem M, Salzig D, Czermak P. hMSC production in disposable bioreactors with regards to GMP and PAT. *Chemie Ing Tech* 2013;**85**:67–75. doi:10.1002/cite.201200151
- [47] Fossett E, Khan WS. Optimising human mesenchymal stem cell numbers for clinical application: a literature review. *Stem Cells Int* 2012:1–5. doi:10.1155/2012/465259
- [48] Hewitt CJ, Lee K, Nienow AW, Thomas RJ, Smith M, Thomas CR. Expansion of human mesenchymal stem cells on microcarriers. *Biotechnol Lett* 2011;**33**:2325–35. doi:10.1007/s10529-011-0695-4

- [49] Frauenschuh S, Reichmann E, Ibold Y, Goetz PM, Sittlinger M, Ringe J. A microcarrier-based cultivation system for expansion of primary mesenchymal stem cells. *Biotechnol Prog* 2007;**23**:187–93. doi:10.1021/bp060155w
- [50] Schop D, van Dijkhuizen-Radersma R, Borgart E, Janssen FW, Rozemuller H, Prins H-J, et al. Expansion of human mesenchymal stromal cells on microcarriers: growth and metabolism. *J Tissue Eng Regen Med* 2010;**4**:131–40. doi:10.1002/term.224
- [51] Elseberg CL, Leber J, Salzig D, Wallrapp C, Kassem M, Kraume M, et al. Microcarrier-based expansion process for hMSCs with high vitality and undifferentiated characteristics. *Int J Artif Organs* 2012;**35**:93–107. doi:10.5301/ijao.5000077
- [52] Ma T, Tsai A-C, Liu Y. Biomanufacturing of human mesenchymal stem cells in cell therapy: influence of microenvironment on scalable expansion in bioreactors. *Biochem Eng J* 2016;**108**:44–50. doi:10.1016/j.bej.2015.07.014
- [53] Clark JM, Hirtenstein MD. Optimizing culture conditions for the production of animal cells in microcarrier culture. *Ann N Y Acad Sci* 1981;**369**:33–46. doi:10.1111/j.1749-6632.1981.tb14175.x
- [54] Schirmaier C, Jossen V, Kaiser SC, Jüngerkes F, Brill S, Safavi-Nab A, et al. Scale-up of adipose tissue-derived mesenchymal stem cell production in stirred single-use bioreactors under low-serum conditions. *Eng Life Sci* 2014;**14**:292–303. doi:10.1002/elsc.201300134
- [55] Grein TA, Leber J, Blumenstock M, Petry F, Weidner T, Salzig D, et al. Multiphase mixing characteristics in a microcarrier-based stirred tank bioreactor suitable for human mesenchymal stem cell expansion. *Process Biochem* 2016;**51**:1109–19. doi:10.1016/j.procbio.2016.05.010
- [56] Eibes G, dos Santos F, Andrade PZ, Boura JS, Abecasis MMA, da Silva CL, et al. Maximizing the ex vivo expansion of human mesenchymal stem cells using a microcarrier-based stirred culture system. *J Biotechnol* 2010;**146**:194–7. doi:10.1016/j.jbiotec.2010.02.015
- [57] Polgár L. The catalytic triad of serine peptidases. *Cell Mol Life Sci* 2005;**62**:2161–72. doi:10.1007/s00018-005-5160-x
- [58] Cierpka K, Mika N, Lange MC, Zorn H, Czermak P, Salzig D. Cell detachment by prolyl-specific endopeptidase from *Wolfiporia Cocos*. *Am J Biochem Biotechnol* 2014;**10**:14–21. doi:10.3844/ajbbbsp.2014.14.21
- [59] FDA. FDA Proposes barring ceratin cattle material from medical products as BSE safeguard 2007. <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2007/ucm108825.htm> (accessed August 23, 2016).
- [60] Serra M, Brito C, Correia C, Alves PM. Process engineering of human pluripotent stem cells for clinical application. *Trends Biotechnol* 2012;**30**:350–9. doi:10.1016/j.tibtech.2012.03.003
- [61] Pattasseril J, Varadaraju H, Lock L, Rowley J. Downstream technology landscape for large-scale therapeutic cell processing. *Bioprocess Int* 2013;**3**:38–46. <http://www.bioprocessintl.com>

com/upstream-processing/bioreactors/downstream-technology-landscape-for-large-scale-therapeutic-cell-processing-340981/ (accessed August 23, 2016).

- [62] Hassan S, Simaria AS, Varadaraju H, Gupta S, Warren K, Farid SS. Allogeneic cell therapy bioprocess economics and optimization: downstream processing decisions. *Regen Med* 2015;**10**:591–609. doi:10.2217/rme.15.29
- [63] Cunha B, Peixoto C, Silva MM, Carrondo MJT, Serra M, Alves PM. Filtration methodologies for the clarification and concentration of human mesenchymal stem cells. *J Memb Sci* 2015;**478**:117–29. doi:10.1016/j.memsci.2014.12.041
- [64] van Reis R, Leonard LC, Hsu CC, Builder SE. Industrial scale harvest of proteins from mammalian cell culture by tangential flow filtration. *Biotechnol Bioeng* 1991;**38**:413–22. doi:10.1002/bit.260380411
- [65] Kretzmer G. Influence of stress on adherent cells. *Adv Biochem Eng Biotechnol* 2000;**67**:123–37. doi:10.1007/3-540-47865-5_4
- [66] Dong J, Gu Y, Li C, Wang C, Feng Z, Qiu R, et al. Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts. *Acta Pharmacol Sin* 2009;**30**:530–6. doi:10.1038/aps.2009.40
- [67] Wakeman R, Williams C. Additional techniques to improve microfiltration. *Sep Purif Technol* 2002;**26**:3–18. doi:10.1016/S1383-5866(01)00112-5
- [68] Gao D, Critser JK. Mechanisms of cryoinjury in living cells. *ILAR J* 2000;**41**:187–96. doi:10.1093/ilar.41.4.187
- [69] Grein TA, Freimark D, Weber C, Hudel K, Wallrapp C, Czermak P. Alternatives to dimethylsulfoxide for serum-free cryopreservation of human mesenchymal stem cells. *Int J Artif Organs* 2010;**33**:370–80.
- [70] Karlsson JOM, Toner M. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* 1996;**17**:243–56. doi:10.1016/0142-9612(96)85562-1
- [71] Dalimata AM, Graham JK. Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. *Theriogenology* 1997;**48**:831–41. doi:10.1016/S0093-691X(97)00305-1
- [72] Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW. Mesenchymal stromal cells derived from various tissues: biological, clinical and cryopreservation aspects. *Cryobiology* 2015;**71**:181–97. doi:10.1016/j.cryobiol.2015.07.003
- [73] Asghar W, El Assal R, Shafiee H, Anchan RM, Demirci U. Preserving human cells for regenerative, reproductive, and transfusion medicine. *Biotechnol J* 2014;**9**:895–903. doi:10.1002/biot.201300074
- [74] Tan KY, Reuveny S, Oh SKW. Recent advances in serum-free microcarrier expansion of mesenchymal stromal cells: parameters to be optimized. *Biochem Biophys Res Commun* 2016;**473**:769–73. doi:10.1016/j.bbrc.2015.09.078

- [75] Petry F, Smith JR, Leber J, Salzig D, Czermak P, Weiss ML. Manufacturing of human umbilical cord mesenchymal stromal cells on microcarriers in a dynamic system for clinical use. *Stem Cells Int* 2016:Article ID 4834616. doi:10.1155/2016/4834616
- [76] Smith JR, Pfeifer K, Petry F, Powell N, Delzeit J, Weiss ML. Standardizing umbilical cord mesenchymal stromal cells for translation to clinical use: selection of GMP-compliant medium and a simplified isolation method. *Stem Cells Int* 2016. doi:10.1155/2016/6810980
- [77] Dos Santos F, Campbell A, Fernandes-Platzgummer A, Andrade PZ, Gimble JM, Wen Y, et al. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnol Bioeng* 2014;**111**:1116–27. doi:10.1002/bit.25187
- [78] Hervy M, Weber JL, Pecheul M, Dolley-Sonneville P, Henry D, Zhou Y, et al. Long term expansion of bone marrow-derived hMSCs on novel synthetic microcarriers in xeno-free, defined conditions. *PLoS One* 2014;**9**:e92120. doi:10.1371/journal.pone.0092120
- [79] Rafiq QA, Brosnan KM, Coopman K, Nienow AW, Hewitt CJ. Culture of human mesenchymal stem cells on microcarriers in a 5 l stirred-tank bioreactor. *Biotechnol Lett* 2013;**35**:1233–45. doi:10.1007/s10529-013-1211-9
- [80] Czermak P, Pörtner R, Brix A. Cell and Tissue Reaction Engineering—Chapter 4 Special Engineering Aspects. In: Eibl R, Eibl D, Pörtner R, Catapano G, Czermak P, editors. *Cell Tissue Reaction Engineering*. Berlin, Heidelberg: Springer; 2009, pp. 122–36. doi:10.1007/978-3-540-68182-3_4
- [81] Varani J, Dame M, Beals TF, Wass JA. Growth of three established cell lines on glass microcarriers. *Biotechnol Bioeng* 1983;**25**:1359–72. doi:10.1002/bit.260250515
- [82] Salzig D, Schmiermund A, Pino Grace P, Elseberg CL, Weber C, Czermak P. Enzymatic detachment of therapeutic mesenchymal stromal cells grown on glass carriers in a bioreactor. *Open Biomed Eng J* 2013;**7**:147–58. doi:10.2174/1874120701307010147
- [83] Jing D, Sunil N, Punreddy S, Aysola M, Kehoe D, Murrel J, et al. Growth kinetics of human mesenchymal stem cells in a 3-L single-use, stirred-tank bioreactor. *BioPharm Int* 2013;**26**:28–38. <http://www.biopharminternational.com/growth-kinetics-human-mesenchymal-stem-cells-3-l-single-use-stirred-tank-bioreactor> (accessed August 23, 2016).
- [84] Rafiq QA, Coopman K, Nienow AW, Hewitt CJ. Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors. *Biotechnol J* 2016;**11**:473–86. doi:10.1002/biot.201400862
- [85] Pardo AMP, Bryhan M, Krasnow H, Hardin N, Riddle M, LaChance O, et al. Corning® CellBIND® Surface: An Improved Surface for Enhanced Cell Attachment. Technical Report 2005. <http://www.sigmaaldrich.com/technical-documents/articles/biofiles/evolution-of-cell.html> (accessed June 14, 2016).
- [86] Justice C, Leber J, Freimark D, Pino Grace P, Kraume M, Czermak P. Online- and offline-monitoring of stem cell expansion on microcarrier. *Cytotechnology* 2011;**63**:325–35. doi:10.1007/s10616-011-9359-4

- [87] Druzinec D, Weiss K, Elseberg C, Salzig D, Kraume M, Pörtner R, et al. Process Analytical Technology (PAT) in Insect and Mammalian Cell Culture Processes: Dielectric Spectroscopy and Focused Beam Reflectance Measurement (FBRM). In: Pörtner R, editor. *Animal Cell Biotechnology*. Vol. 1104. Humana Press; 2014, pp. 313–41. doi:10.1007/978-1-62703-733-4_20
- [88] Rosa F, Sales KC, Carmelo JG, Fernandes-Platzgummer A, da Silva CL, Lopes MB, et al. Monitoring the ex-vivo expansion of human mesenchymal stem/stromal cells in xeno-free microcarrier-based reactor systems by MIR spectroscopy. *Biotechnol Prog* 2016;**32**:447–55. doi:10.1002/btpr.2215
- [89] Weber C. Festbettbasierte Kultivierungsverfahren für die Herstellung zelltherapeutischer Implantate [Thesis]. Hamburg University of Technology. ISBN: 978-3-18-327817-6, 2010.
- [90] Weber C, Pohl S, Portner R, Wallrapp C, Kassem M, Geigle P, et al. Cultivation and differentiation of encapsulated hMSC-TERT in a disposable small-scale syringe-like fixed bed reactor. *Open Biomed Eng J* 2007;**1**:64–70. doi:10.2174/1874120700701010064
- [91] Weber C, Pohl S, Pörtner R, Pino Grace P, Freimark D, Wallrapp C, et al. Production Process for Stem Cell Based Therapeutic Implants: Expansion of the Production Cell Line and Cultivation of Encapsulated Cells. *Bioreactor Systems for Tissue Engineering II*. Berlin, Heidelberg: Springer; 2010, pp. 143–62. doi:10.1007/10_2009_25
- [92] Osiecki MJ, Michl TD, Kul Babur B, Kabiri M, Atkinson K, Lott WB, et al. Packed bed bioreactor for the isolation and expansion of placental-derived mesenchymal stromal cells. *PLoS One* 2015;**10**:e0144941. doi:10.1371/journal.pone.0144941
- [93] Elseberg CL, Salzig D, Czermak P. Bioreactor expansion of human mesenchymal stem cells according to GMP requirements. *Methods Mol Biol* 2014:199–218. doi:10.1007/7651_2014_117
- [94] Carmelo JG, Fernandes-Platzgummer A, Cabral JMS, da Silva CL. Scalable ex vivo expansion of human mesenchymal stem/stromal cells in microcarrier-based stirred culture systems. *Methods Mol Biol* 2014:147–59. doi:10.1007/7651_2014_100
- [95] Cunha B, Aguiar T, Silva MM, Silva RJS, Sousa MFQ, Pineda E, et al. Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells. *J Biotechnol* 2015;**213**:97–108. doi:10.1016/j.jbiotec.2015.02.023
- [96] Jing D, Punreddy S, Sunil N, Aysola M, Murrell J, Niss K. Characterization of human mesenchymal stem cells: expansion in a 3-L, single-use, stirred-tank bioreactor. *Bioprocess Int* 2013;**11**:30–6. <http://www.bioprocessintl.com/upstream-processing/upstream-contract-services/characterization-of-human-mesenchymal-stem-cells-340980/> (accessed August 23, 2016).
- [97] Nienow AW, Rafiq QA, Coopman K, Hewitt CJ. A potentially scalable method for the harvesting of hMSCs from microcarriers. *Biochem Eng J* 2014;**85**:79–88. doi:10.1016/j.bej.2014.02.005

- [98] Caruso SR, Orellana MD, Mizukami A, Fernandes TR, Fontes AM, Suazo CAT, et al. Growth and functional harvesting of human mesenchymal stromal cells cultured on a microcarrier-based system. *Biotechnol Prog* 2014;**30**:889–95. doi:10.1002/btpr.1886
- [99] Schnitzler A, Verma A, Aysola M, Murrell J, Rook M. Media and microcarrier surface must be optimized when transitioning mesenchymal stem/stromal cell expansion to stirred tank bioreactors. *BMC Proc* 2015;**9**:P57. doi:10.1186/1753-6561-9-S9-P57
- [100] Heathman TR, Glyn VAM, Picken A, Rafiq QA, Coopman K, Nienow AW, et al. Expansion, harvest and cryopreservation of human mesenchymal stem cells in a serum-free microcarrier process. *Biotechnol Bioeng* 2015;**112**:1696–707. doi:10.1002/bit.25582
- [101] Peng I-C, Yeh C-C, Lu Y-T, Muduli S, Ling Q-D, Alarfaj AA, et al. Continuous harvest of stem cells via partial detachment from thermoresponsive nanobrush surfaces. *Biomaterials* 2016;**76**:76–86. doi:10.1016/j.biomaterials.2015.10.039
- [102] Yang HS, Jeon O, Bhang SH, Lee S-H, Kim B-S. Suspension culture of mammalian cells using thermosensitive microcarrier that allows cell detachment without proteolytic enzyme treatment. *Cell Transplant* 2010;**19**:1123–32. doi:10.3727/096368910X516664
- [103] Song K, Yang Y, Wu S, Zhang Y, Feng S, Wang H, et al. In vitro culture and harvest of BMMSCs on the surface of a novel thermosensitive glass microcarrier. *Mater Sci Eng C* 2016;**58**:324–30. doi:10.1016/j.msec.2015.08.033
- [104] Çetinkaya G, Kahraman AS, Gümüşderelioğlu M, Arat S, Onur MA. Derivation, characterization and expansion of fetal chondrocytes on different microcarriers. *Cytotechnology* 2011;**63**:633–43. doi:10.1007/s10616-011-9380-7
- [105] Tostoes R, Dodgson J, Mason C, Veraitch F. A novel filtration device for point of care preparation of cellular therapies. *Cytotherapy* 2015;**17**:S26. doi:10.1016/j.jcyt.2015.03.396
- [106] Cunha B, Silva RJS, Aguiar T, Serra M, Daicic J, Maloisel J, et al. Improving washing strategies of human mesenchymal stem cells using negative mode expanded bed chromatography. *J Chromatogr A* 2016;**1429**:292–303. doi:10.1016/j.chroma.2015.12.052
- [107] Ullah I, Subbarao RB, Rho G-J. Human mesenchymal stem cells—current trends and future prospective. *Biosci Rep* 2015;**35**:1–18. doi:10.1042/BSR20150025
- [108] Freimark D, Sehl C, Weber C, Hudel K, Czermak P, Hofmann N, et al. Systematic parameter optimization of a Me2SO- and serum-free cryopreservation protocol for human mesenchymal stem cells. *Cryobiology* 2011;**63**:67–75. doi:10.1016/j.cryobiol.2011.05.002
- [109] Minonzio G, Corazza M, Mariotta L, Gola M, Zanzi M, Gandolfi E, et al. Frozen adipose-derived mesenchymal stem cells maintain high capability to grow and differentiate. *Cryobiology* 2014;**69**:211–6. doi:10.1016/j.cryobiol.2014.07.005
- [110] Lechanteur C, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. *J Transl Med* 2016;**14**:145. doi:10.1186/s12967-016-0892-y

- [111] Demirci S, Doğan A, Sisli B, Sahin F. Boron increases the cell viability of mesenchymal stem cells after long-term cryopreservation. *Cryobiology* 2014;**68**:139–46. doi:10.1016/j.cryobiol.2014.01.010
- [112] Zeisberger SM, Schulz JC, Mairhofer M, Ponsaerts P, Wouters G, Doerr D, et al. Biological and physicochemical characterization of a serum- and xeno-free chemically defined cryopreservation procedure for adult human progenitor cells. *Cell Transplant* 2011;**20**:1241–57. doi:10.3727/096368910X547426
- [113] Biofreeze. Freezing medium by Biochrom AG, Germany. 2010. http://www.biochrom.de/fileadmin/user_upload/service/produktinformation/englisch/BC_catalogue_36_37_Biofreeze.pdf (accessed June 8, 2016).
- [114] Thirumala S, Goebel WS, Woods EJ. Clinical grade adult stem cell banking. *Organogenesis* 2009;**5**:143–54. doi:10.4161/org.5.3.9811
- [115] Gurruchaga H, Saenz del Burgo L, Ciriza J, Orive G, Hernández RM, Pedraz JL. Advances in cell encapsulation technology and its application in drug delivery. *Expert Opin Drug Deliv* 2015;**12**:1251–67. doi:10.1517/17425247.2015.1001362
- [116] Levit RD, Landazuri N, Phelps EA, Brown ME, Garcia AJ, Davis ME, et al. Cellular encapsulation enhances cardiac repair. *J Am Heart Assoc* 2013;**2**:e000367. doi:10.1161/JAHA.113.000367
- [117] Wallrapp C, Thoenes E, Thürmer F, Jork A, Kassem M, Geigle P. Cell-based delivery of glucagon-like peptide-1 using encapsulated mesenchymal stem cells. *J Microencapsul* 2013;**30**:315–24. doi:10.3109/02652048.2012.726281
- [118] Stucky EC, Schloss RS, Yarmush ML, Shreiber DI. Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response. *Cytotherapy* 2015;**17**:1353–64. doi:10.1016/j.jcyt.2015.05.002
- [119] Gryshkov O, Pogozhykh D, Zernetsch H, Hofmann N, Mueller T, Glasmacher B. Process engineering of high voltage alginate encapsulation of mesenchymal stem cells. *Mater Sci Eng C* 2014;**36**:77–83. doi:10.1016/j.msec.2013.11.048
- [120] Freimark D, Pino Grace P, Pohl S, Weber C, Wallrapp C, Geigle P, et al. Use of encapsulated stem cells to overcome the bottleneck of cell availability for cell therapy approaches. *Transfus Med Hemotherapy* 2010;**37**:66–73. doi:10.1159/000285777
- [121] Diniz IMA, Chen C, Xu X, Ansari S, Zadeh HH, Marques MM, et al. Pluronic F-127 hydrogel as a promising scaffold for encapsulation of dental-derived mesenchymal stem cells. *J Mater Sci Mater Med* 2015;**26**:153. doi:10.1007/s10856-015-5493-4
- [122] Serra M, Correia C, Malpique R, Brito C, Jensen J, Bjorquist P, et al. Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells. *PLoS One* 2011;**6**:e23212. doi:10.1371/journal.pone.0023212
- [123] Pravdyuk AI, Petrenko YA, Fuller BJ, Petrenko AY. Cryopreservation of alginate encapsulated mesenchymal stromal cells. *Cryobiology* 2013;**66**:215–22. doi:10.1016/j.cryobiol.2013.02.002

- [124] Gurruchaga H, Ciriza J, Saenz del Burgo L, Rodriguez-Madoz JR, Santos E, Prosper F, et al. Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin. *Int J Pharm* 2015;**485**:15–24. doi:10.1016/j.ijpharm.2015.02.047
- [125] Chen B, Wright B, Sahoo R, Connon CJ. A novel alternative to cryopreservation for the short-term storage of stem cells for use in cell therapy using alginate encapsulation. *Tissue Eng Part C Methods* 2013;**19**:568–76. doi:10.1089/ten.tec.2012.0489
- [126] Swioklo S, Constantinescu A, Connon CJ. Alginate-encapsulation for the improved hypothermic preservation of human adipose-derived stem cells. *Stem Cells Transl Med* 2016;**5**:339–49. doi:10.5966/sctm.2015-0131
- [127] Krause U, Seckinger A, Gregory CA. Assays of Osteogenic Differentiation by Cultured Human Mesenchymal Stem Cells. In: Vemuri M, Chase LG, Rao MS, editors. *Mesenchymal Stem Cell Assays and Application*. Vol. 698. Humana Press; 2011, pp. 215–30. doi:10.1007/978-1-60761-999-4_17
- [128] Fink T, Zachar V. Adipogenic Differentiation of Human Mesenchymal Stem Cells. In: Vemuri M, Chase LG, Rao MS, editors. *Mesenchymal Stem Cell Assays and Application*. Vol. 698. Humana Press; 2011, pp. 243–51. doi:10.1007/978-1-60761-999-4_19
- [129] Solchaga LA, Penick KJ, Welter JF. Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells: Tips and Tricks. In: Vemuri M, Chase LG, Rao MS, editors. *Mesenchymal Stem Cell Assays and Application*. Humana Press; 2011, pp. 253–78. doi:10.1007/978-1-60761-999-4_20
- [130] Moreno-Navarrete JM, Fernández-Real JM. Adipocyte Differentiation. In: Symonds ME, editor. *Adipose Tissue Biology*. New York, NY: Springer; 2012, pp. 17–38. doi:10.1007/978-1-4614-0965-6_2
- [131] Köster N, Schmiermund A, Grubelnig S, Leber J, Ehlicke F, Czermak P, et al. Single-step RNA extraction from different hydrogel-embedded mesenchymal stem cells for quantitative reverse transcription–polymerase chain reaction analysis. *Tissue Eng Part C Methods* 2016;**22**:552–60. doi:10.1089/ten.tec.2015.0362
- [132] Chapter 2.6.27 Microbiological Examination of Cell-based Preparations 2016. http://www.gmp-compliance.org/enews_04922_European-Pharmacopoeia---Chapter-2.6.27-Microbiological-Examination-of-cell-based-Preparations-revised.html (accessed June 14, 2016).
- [133] Barkholt L, Flory E, Jekerle V, Lucas-Samuel S, Ahnert P, Bisset L, et al. Risk of tumorigenicity in mesenchymal stromal cell-based therapies—Bridging scientific observations and regulatory viewpoints. *Cytotherapy* 2013;**15**:753–9. doi:10.1016/j.jcyt.2013.03.005
- [134] Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, et al. Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;**12**:576–8. doi:10.3109/14653249.2010.507330

- [135] Capelli C, Pedrini O, Cassina G, Spinelli O, Salmoiraghi S, Golay J, et al. Frequent occurrence of non-malignant genetic alterations in clinical grade mesenchymal stromal cells expanded for cell therapy protocols. *Haematologica* 2014;**99**:e94–7. doi:10.3324/haematol.2014.104711
- [136] CellGro MSC Medium by CellGenix n.d. <http://www.cellgenix.com/products/product-lines/cellgror-serum-free-media.html> (accessed June 14, 2016).
- [137] Hartmann I, Hollweck T, Haffner S, Krebs M, Meiser B, Reichart B, et al. Umbilical cord tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and maintain their phenotypic and functional properties. *J Immunol Methods* 2010;**363**:80–9. doi:10.1016/j.jim.2010.10.008
- [138] Patrikoski M, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerström B, et al. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. *Stem Cell Res Ther* 2013;**4**:27. doi:10.1186/scrt175
- [139] CTS StemPro MSC SFM by Thermo Scientific n.d. <http://www.thermofisher.com/order/catalog/product/A1033201> (accessed June 14, 2016).
- [140] hMSC High Performance Media Kit XF by RoosterBio n.d. <http://www.roosterbio.com/collections/media/products/hmsc-high-performance-media-kit-xf-kt-016> (accessed June 15, 2016).
- [141] Human MSC medium, chemically-defined by AmCell Biosciences n.d. <http://amcell-bio.com/human-msc-medium-chemically-defined> (accessed June 14, 2016).
- [142] Lakhkar NJ, Day MR, Kim H-W, Ludka K, Mordan NJ, Salih V, et al. Titanium phosphate glass microcarriers induce enhanced osteogenic cell proliferation and human mesenchymal stem cell protein expression. *J Tissue Eng* 2015;**6**:1–14. doi:10.1177/2041731415617741
- [143] Mesenchymal Stem Cell Growth Medium DXF (Defined Xeno Free) by PromoCell n.d. <http://www.promocell.com/products/cell-culture-media/media-for-stem-and-blood-cells/mesenchymal-stem-cell-media/> (accessed June 14, 2016).
- [144] Mesenchymal Stem Cell Medium – animal component free by ScienCell n.d. <http://www.sciencellonline.com/mesenchymal-stem-cell-medium-animal-component-free.html> (accessed June 21, 2016).
- [145] MesenCult-XF Medium by STEMCELL Technologies 2016. <http://www.stemcell.com/en/Products/Cell-type/Mesenchymal-stem-cells/MesenCultXF-Medium.aspx> (accessed June 14, 2016).
- [146] MesenGro Human MSC Medium by System Biosciences n.d. <http://www.systembio.com/stem-cell-research/media-growth-factors/media> (accessed June 14, 2016).
- [147] Tan KY, Teo KL, Lim JFY, Chen AKL, Reuveny S, Oh SK. Serum-free media formulations are cell line-specific and require optimization for microcarrier culture. *Cytherapy* 2015;**17**:1152–65. doi:10.1016/j.jcyt.2015.05.001

- [148] MSC NutriStem XF Medium by Biological Industries n.d. <http://www.bioind.com/nutristem-msc-medium/> (accessed June 14, 2016).
- [149] MSC-GRO Serum-Free/Xeno-Free by Vitro BioPharma n.d. <http://vitrobiopharma.com/products/serum-free-xeno-free-complete-sc00b3-1/> (accessed June 14, 2016).
- [150] PowerStem MSC1 by PAN-Biotech n.d. <http://www.pan-biotech.com/en/serum-free-stem-cell-media/powerstem-msc1> (accessed June 14, 2016).
- [151] Heathman TRJ, Stolzing A, Fabian C, Rafiq QA, Coopman K, Nienow AW, et al. Serum-free process development: improving the yield and consistency of human mesenchymal stromal cell production. *Cytotherapy* 2015;**17**:1524–35. doi:10.1016/j.jcyt.2015.08.002
- [152] PRIME-XV MSC Expansion SFM by Irvine Scientific n.d. <http://www.irvinesci.com/products/91135-prime-xv-msc-expansion-sfm> (accessed June 14, 2016).
- [153] SPE-IV Media by ABCell-Bio n.d. <http://www.abcell-bio.com/index.php?babrw=root/DGAll/racine/3niveaux/defined-serum-free-media-inventor-and-manufacturer--syn-h-spe-iv--and-characterized-human-primary-cells-manufacturer-huvec-epc-huaec-msc-cd34-cd133/navigation-1/products/tissue-regenerat> (accessed June 14, 2016).
- [154] Stem Cell 1 by Cell Culture Technologies n.d. http://www.cellculture.com/?page_id=814 (accessed June 14, 2016).
- [155] StemMACS MSC Expansion Media Kit XF by Miltenyi Biotec n.d. <http://www.miltenyibiotec.com/en/products-and-services/macs-cell-culture-and-stimulation/media/stem-cell-media/stemmacs-msc-expansion-media-kit-xf-human.aspx> (accessed June 14, 2016).
- [156] StemXVivo Xeno-Free Human MSC Expansion Media by R&D Systems n.d. http://www.rndsystems.com/products/stemxvivo-xeno-free-human-msc-expansion-media_ccm021 (accessed June 14, 2016).
- [157] Rajaraman G, White J, Tan KS, Ulrich D, Rosamilia A, Werkmeister J, et al. Optimization and scale-up culture of human endometrial multipotent mesenchymal stromal cells: potential for clinical application. *Tissue Eng Part C Methods* 2013;**19**:80–92. doi:10.1089/ten.tec.2011.0718
- [158] Wuchter P, Vetter M, Saffrich R, Diehlmann A, Bieback K, Ho AD, et al. Evaluation of GMP-compliant culture media for in vitro expansion of human bone marrow mesenchymal stromal cells. *Exp Hematol* 2016;**44**:508–18. doi:10.1016/j.exphem.2016.02.004
- [159] TheraPEAK MSCGM-CD Mesenchymal Stem Cell Medium, Chemically Defined by Lonza n.d. <http://www.lonza.com/products-services/bio-research/stem-cells/adult-stem-cells-and-media/human-mesenchymal-stem-cells-media/therapeak-mscgm-cd-mesenchymal-stem-cell-medium-chemically-defined.aspx> (accessed June 14, 2016).