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Bioprocess Development for Human Mesenchymal Stem Cell Therapy Products

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Abstract

Mesenchymal stem cells (MSCs) are advanced therapy medicinal products used in cell therapy applications. Several MSC products have already advanced to phase III clinical testing and market approval. The manufacturing of MSCs must comply with good manufacturing practice (GMP) from phase I in Europe and phase II in the US, but there are several unique challenges when cells are the therapeutic product. Any GMP-compliant process for the production of MSCs must include the expansion of cells *in vitro* to achieve a sufficient therapeutic quantity while maintaining high cell quality and potency. The process must also allow the efficient harvest of anchorage-dependent cells and account for the influence of shear stress and other factors, especially during scale-up. Bioreactors are necessary to produce clinical batches of MSCs, and bioprocess development must therefore consider this specialized environment. For the last 10 years, we have investigated bioprocess development as a means to produce high-quality MSCs. More recently, we have also used bioreactors for the cocultivation of stem cells with other adult cells and for the production of MSC-derived extracellular vesicles. This review discusses the state of the art in bioprocess development for the GMP-compliant manufacture of human MSCs as products for stem cell therapy.

Keywords: bioreactors, quality-by-design, critical process parameters, stem cell potency, standardization

1. Manufacturing cell therapy products

Cell therapy is a growing clinical research and healthcare sector in which living cells are introduced into a patient in an attempt to ameliorate or cure a disease. Stem cell therapy is one of the most promising fields within this sector because the introduced cells have the capacity to differentiate, allowing the repopulation of diseased organs with healthy cells, or to allow even complete organ regeneration. This chapter will focus on one specific type of stem cell (MSCs), which are variously defined as mesenchymal stem cells, mesenchymal stromal cells, or (most recently) medicinal signaling cells [1]. These various definitions reflect the controversial origin and functionality of MSCs and uncertainty about their clinical potential [2, 3]. Following encouraging initial results, the unclear or disappointing outcomes of some MSC clinical trials have clouded the picture [4], but the pioneers

of this approach still regard MSCs as a promising therapeutic option [5]. One of the key issues in the deployment of MSCs is ensuring they are safe and effective, which requires a well-characterized manufacturing process.

In order to provide enough MSCs for cell therapy, donor cells must be isolated from tissue and then expanded *in vitro* to reach a population of $1-9 \times 10^8$ cells, which is the typical dose for adult treatment [6]. The success or failure of MSC therapy depends on this *in vitro* expansion process, which was first studied in detail following the failure of the MSC product Prochymal in phase III trials for graft versus host disease (GvHD) [4], whereas a similar product succeeded in phase II. One reason proposed for the contrasting outcomes of each trial was the substantial differences in the MSC expansion step at the manufacturing scale, highlighting the specialized and complex nature of MSCs [4].

1.1 Definition of MSCs and current approved products

MSCs are classified as advanced therapeutic medicinal products (ATMPs) under regulations in Europe and the US. Many countries follow the regulations laid down by the US Food and Drug Administration (FDA), which defines MSCs as cell therapy products, whereas the European Medicines Agency (EMA) defines MSCs as cell-based medicinal products and distinguishes between somatic cell therapy medicinal products (SCTMPs) and tissue engineered products (TEPs) [7]. This means that clinical studies and drug approval are covered by a specific regulatory framework applied at the national or regional level. Manufacturing must therefore be compliant with good manufacturing practice (GMP) regulations that have been tailored for ATMPs, following strict criteria for product specification and release for clinical use. However, the regulatory framework for MSC manufacturing is confounded by ambiguous product definitions reflecting regional differences in the way the regulations are implemented. For example, the EMA requires GMP compliance and manufacturing authorization for phase I material, whereas the FDA does not apply this requirement until phases II and III, and in Canada, GMP compliance is not strictly required at any phase [8]. Even so, various MSC products have been manufactured under these different regulatory jurisdictions and have proceeded through clinical development, in some cases gaining market authorization from the local regulatory agency [9]. Most of these products are allogenic, which means that MSCs from one or more healthy donors are expanded, processed, and stored and then applied to patients as an off-the-shelf product (**Table 1**). In 2016, the allogenic MSC product TEMCELL (developed by Mesoblast) was licensed to JCR Pharmaceuticals, which received market authorization in Japan under a fast-track protocol for patients with steroid-refractory acute GvHD. Mesoblast also conducted a phase III trial with this product in the US, involving 60 patients of the same indication, achieving the primary endpoints (NCT02336230). In 2018, ALOFISEL (Takeda Pharma), an expanded allogenic adipose-derived MSC product, was approved by the EMA to treat complex perianal fistula in patients with Crohn's disease. This was supported by a placebo-controlled trial involving 212 patients [10]. Stempeucel (Stempeutics), an expanded allogenic MSC product, received market authorization from the Drug Controller General of India to treat limb ischemia in patients with Buerger's disease. However, it is limited to 200 patients on a cost-recovery basis, and a postmarket surveillance study is required. Ninety patients have already received an injection of this MSC product in a phase II trial, achieving a significantly better outcome than standard care [11]. CARTISTEM (Medipost) is an allogenic culture-expanded umbilical cord blood MSC product to treat knee articular cartilage defects in patients with osteoarthritis, grade IV, and following approval for the South Korean market in 2012, its clinical outcomes have remained

	Product 1	Product 2
Exemplary products	ALOFISEL	Queencell
Indication	Crohn's disease, perianal fistula	Regeneration of subcutaneous tissue
Patients per year	23,000 (in EU) [*]	n.d.
Cell type	Allogenic MSCs	Autologous, patient-specific MSCs
Cell source	Adipose tissue	Adipose tissue
Cells per dose	1.2×10^8 MSCs	7×10^7 ^{**}
Therapeutic relevant cell properties ^{***}	Anti-inflammation, immune modulation	Regeneration, anti-apoptosis
Manufacturing type	Bulk manufacturing	Patient-specific batch
Batch size	Large (min. 100–1000 doses per batch)	Small (1 dose per batch)
Scalability of production	Scale up	Scale out, several batches in parallel
Product storage	Frozen, off-the-shelf	No storage
Stability under storage	Stable >6 month, frozen	Fresh, stable max. 24 hours

^{*}0.003% of all citizens (741 million) in Europe are putative patients.
^{**}Stromal vascular fraction contains MSCs and other cell types such as preadipocytes, endothelial progenitor cells, pericytes, mast cells, and fibroblast.
^{***}Following both products have different critical quality attributes (CQAs) and the manufacturing processes have different critical process parameters (CPPs).
n.d. not determined.

Table 1.
Indication and properties of MSC products impact their manufacturing.

stable over 7 years of follow-up studies [12]. Several autologous MSC products have also been approved in South Korea, meaning that the MSCs are isolated from the patient's own tissue and then manipulated/expanded in a patented process and re-injected into the patient 4–6 weeks later. NEURONATA-R (Corestem) and Cellgram-AMI (Pharmicell) are autologous bone marrow-derived MSCs indicated for amyotrophic lateral sclerosis and acute myocardial infarction, respectively. Two other MSC products derived from adipose-tissue have been approved (Anterogen): a mixture of autologous adipose-derived MSCs with other cells for subcutaneous tissue defects (Queencell) and a pure adipose-derived MSC product for Crohn's fistula treatment (Cupistem) [9]. NEURONATA-R has been designated as an orphan drug by the EMA and FDA.

This brief survey of the market shows that the promise of MSC therapy is materializing, with positive efficacy data in controlled clinical trials followed by regulatory approval for a small number of products.

1.2 The therapeutic properties of MSCs

Although MSCs have been used in cell therapy applications for many years, the fundamental biology of these cells and their precise therapeutic properties are not fully understood. MSCs were initially isolated from bone marrow (bm-MSCs) based on their plastic adherence, but today they are usually isolated from adipose tissue (ad-MSCs) or umbilical cord blood (uc-MSCs), which are more accessible [13]. MSCs are also found in various other adult, fetal, and perinatal tissues [14]. Regardless of their origin, MSCs are heterogeneous and polyclonal cells, with at least three

subpopulations defined based on morphology. Type I MSCs are spindle-shaped proliferating cells resembling fibroblasts. Type II MSCs are large, flat, epithelial-like cells, which are more senescent than type I cells and feature visible cytoskeletal structures and granules. Finally, type III MSCs are small round cells with a high capacity for self-renewal [15]. The heterogeneity of MSCs can be considered beneficial in that it ensures that some therapeutically active cells are present, but it reduces the maximum potential efficacy because some of the cells are inactive. However, even monoclonal MSCs become heterogeneous during expansion [16].

Despite the heterogeneity described above, the International Society of Cell Therapy has published a set of minimal criteria that must be met before cells can be defined as MSCs. Such cells must (i) show plastic adherence; (ii) be able to differentiate into cartilage, bone, and fat tissue *in vitro*; and (iii) express the cluster of differentiation (CD) surface markers CD73, CD90, and CD105, but not CD11b, CD14, CD19, CD34, CD45, or HLA-DR [17]. However, this standard set of markers does not distinguish between MSCs and fibroblasts or nonstem mesenchymal cells [18]. Several other markers may be more specific but are only detected in certain MSC isolates or subpopulations. These include stage-specific embryonic antigen-4 (SSEA-4), stem cell antigen-1 (SCA1), nestin, CD44, CD146, CD166, and CD271 [19]. A unique MSC surface marker has yet to be identified.

It is important to note that MSCs cannot be defined merely as a collection of surface markers because this says nothing about their therapeutic effect (**Figure 1**). Initially, the therapeutic potential of MSCs was believed to reflect their ability to migrate into damaged tissues, differentiate *in situ*, and replace damaged or dead cells. However, although MSCs can differentiate *in vitro*, their ability to differentiate *in vivo* has never been confirmed [20]. Current opinion is that MSCs migrate to injury sites and secrete chemoattractants that recruit tissue-specific stem cells,

Figure 1. Properties of MSCs and their mode of action. MSCs modulate the host immune systems, e.g., by secreting various trophic factors. Thereby, they reduce inflammation, promote neoangiogenesis, and prevent apoptosis and fibrosis. Further, they stimulate local stem cells to develop new tissue. TSG-6, tumor necrosis factor-inducible gene 6 protein also known as TNF-stimulated gene 6 protein; STC1, stanniocalcin 1; IL-4/6/10, interleukins 4, 6 and 10; CCL20, macrophage inflammatory protein-3; IDO, indoleamine 2,3-dioxygenase; PGE2, prostaglandin E2; VEGF, vascular endothelial growth factor; FGF-2, basic fibroblast growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; CXCL12, stromal cell-derived factor 1; MMP1/2/9, matrix metalloproteinase-1/2/9.

which in turn generate new tissues or exert positive immunomodulatory effects [1]. The MSC secretome comprises a pool of cytokines, chemokines, growth factors, and extracellular vesicles (carrying proteins, lipids, and various forms of RNA). This secretome differs widely among MSC isolates and subpopulations and can be used to functionally distinguish between several MSC types (e.g., type I, II, and III cells), revealing that the self-renewable type III cells are therapeutically the most effective [16].

The immunomodulatory properties of MSCs and their secretion of anti-inflammatory molecules and extracellular vesicles are an important therapeutic functionality [14]. MSCs are therefore logical candidates for the treatment of immune disorders, including GvHD, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and diabetes [21]. MSCs also secrete peptides and factors that promote the regeneration of damaged tissue by stimulating cell proliferation and migration, promoting angiogenesis, and suppressing apoptosis and fibrosis [14]. The regenerative capacity of MSCs has been used to treat Alzheimer's disease, bone and cartilage diseases, diabetes, myocardial infarction, and osteoarthritis [22]. Another advantage of MSCs is that they do not form teratomas *in vivo*, which ensures an outstanding clinical safety profile. Human MSCs achieve senescence without evidence of transformation into tumor cells [23].

1.3 The critical quality attributes of MSCs

The biological complexity and heterogeneity of MSCs hamper the translation of laboratory-scale experiments into industrial processes for cost-effective and reliable manufacturing. This can be addressed by developing MSC manufacturing processes that adhere to quality-by-design (QbD) principles [24]. QbD provides a rational framework and integrates scientific knowledge and risk analysis into process development. It is guided by a thorough understanding of the fundamental biology and engineering principles underlying an MSC product and its production process. QbD begins with a description of the desired product quality characteristics, known as the quality target product profile (QTPP). This is used to identify critical quality attributes (CQAs), which are physical, chemical, and biological attributes that define the quality of the product. The QTPP for MSCs describes properties such as identity, purity, and potency, which will be unique for each MSC product and dependent on the therapeutic indication.

1.3.1 Identity

For MSCs, identity often means the cell phenotype, but as discussed above, there is no agreement on a single definition. Identity is often demonstrated by confirming a typical morphology and/or karyotype [25] and by detecting the presence or absence of surface markers. The minimal criteria for MSCs (see above) have led to a misconception that cells meeting these criteria are equivalent in identity and therapeutic functionality. In polyclonal MSC populations, the presence of multiple cell types can be a clinical benefit as stated above [26], and this should be reflected in the identity attributes.

1.3.2 Potency

The functionality and potency of MSCs are closely linked to their therapeutic efficacy and thus the clinical outcome, but potency is used to demonstrate manufacturing consistency for batch release so a measurable property is required. Viability can fulfill the role of a potency indicator because only living cells can act as a therapeutic

entity. Potency can also be measured using *in vitro* functional assays that determine MSC activity directly or via an indirect metric that correlates to MSC activity *in vivo*. An assay that measures differentiation potential is only appropriate to describe MSC potency if the therapeutic aim involves engraftment of the cells or tissue formation (notwithstanding the controversy over the assumption that MSCs differentiate *in vivo*, as discussed above). The FDA mandates that potency is measured using quantitative biological assays [27], so the standard approach is to differentiate MSCs *in vitro* by cultivating them in differentiation medium and then testing them after 21 days [17]. Staining for differentiation markers is nonquantitative, so alternative methods such as postdifferentiation RNA or protein analysis [28, 29], or the online monitoring of differentiation by Raman spectroscopy [30], are more suitable.

If the therapeutic effect of MSCs is conferred by the secretome, then the differentiation potential may not be the primary determinant of potency. The profile of secreted factors would be a more appropriate measure, and this could be determined by multiplex enzyme-linked immunosorbent assays (ELISAs) or mass spectroscopy [31]. However, a clear link between the secretome profile and *in vivo* efficacy must be established, so that animal models or cell-based assays can be used to determine the limits of the relevant factors. This is a typical way to move from a complex and highly variable *in vivo* assay to a multiassay approach combining the quantification of viability, target-specific cytotoxicity or cytokine release, surrogate biomarkers (morphological phenotype or released factors that correlate with function), bioactivity (e.g., presentation of surface markers), cell-based assays, and genomic, transcriptomic, and proteomic profiles [32].

1.3.3 Sterility and purity

Impurities are unwanted components from within the process, whereas contaminants come from outside the process. Impurities during MSC manufacturing include unwanted cell types, particles (e.g., residual microcarriers, or plastics and fibers from manufacturing equipment and materials), or components of culture medium. Contaminants include bacteria, fungi, viruses, endotoxins, and mycoplasma. The heterogeneity of MSCs makes it difficult to detect unwanted cell types. MSC preparations should ideally be pure, but fibroblasts are often present as impurities. Cell-specific sorting based on the marker CD166 (which is expressed at higher levels on MSCs) and CD9 (which is expressed at higher levels on fibroblasts) may help to achieve sufficient purity [33]. In other cases, it may be sufficient if most of the cells in the final product (>98%) fulfill the ISCT minimal criteria based on MSC surface markers. All other impurities and contaminants must be measured and the maximum residual levels must be defined to ensure safety and efficacy. A final sterilization step is not possible when the product is living cells, so the entire MSC production process must be carried out under aseptic conditions.

From the QTPP list, CQAs must be identified, which directly influence the safety and efficacy of the MSC product. This means that a risk assessment is carried out to reduce the QTPP list to the most influential attributes based on impact and certainty. According to ICHQ8, a CQA is “A physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality.” Therefore, every process parameter “whose variability has an impact on a CQA” is a critical process parameter (CPP) that “should be monitored or controlled to ensure that the process produces the desired quality.” There is no precise delimitation of the degree of impact required to define a CPP, so the broad definition of a CPP is generally divided into parameters that have a substantial impact on the CQAs and those with minimal or zero impact. Each process step has multiple CPPs. For example, during the *in vitro* expansion

step, CPPs can be directly associated with the MSCs (e.g., cell density and cell age) or raw material attributes (e.g., medium, serum, and growth factors) or operational features of the culture vessel/bioreactor system (e.g., pH, temperature, dissolved oxygen, and agitation). The effect of each CPP on the CQAs must be quantified in a design space. With an appropriate control strategy, the CPPs are kept in their normal operational range, which ensures the production of high-quality MSCs that meet all the required CQAs. Based on the heterogeneity and the complexity of MSCs, each MSC product can have unique CQAs and the corresponding CPPs must be identified case by case.

2. Expansion of human MSCs *in vitro*

Therapeutic applications of MSCs require at least 1×10^8 cells per dose, which is many more than can be isolated by tissue aspiration. All MSC production processes must therefore include an *in vitro* expansion. Having generated or isolated the starting cell population, *in vitro* expansion is followed by harvest, concentration, purification, formulation, fill and finish, storage, and shipping. The manufacturing steps of MSCs are therefore similar to the production of recombinant proteins, but MSCs are more challenging due to the variability of the starting material, the complexity of living cells as a product, an incomplete understanding of their mechanism of action, and the inherent difficulties encountered during product characterization.

2.1 CPPs that affect MSC manufacturing

The properties of MSCs are strongly influenced by the environment because MSCs in nature interact with surrounding cells and tissues, with the extracellular matrix and with various bioactive molecules. Even in an artificial environment like a bioreactor or T-flask, MSCs are very sensitive to their environment, and the most influential factors give rise to CPPs. By identifying CPPs that affect MSC quality, the process can be designed to favor the recovery of MSCs with specific phenotypes of interest, in this case those with the greatest therapeutic efficacy [34, 35]. The CPPs affecting MSC quality are discussed in more detail below.

2.1.1 Cell density and age

During MSC isolation, the seeding density is important because all sources contain different quantities of MSCs. For example, only 1 in 100,000 bone marrow cells is an MSC, whereas in adipose tissue, the ratio is nearer to 1 in 100 [36]. If plastic adherence is selected as a strategy for MSC isolation, the number of adherent cells therefore differs according to the source if a similar number of tissue cells are seeded. Standardization during this step can be achieved by isolating MSCs using a strategy of surface marker sorting, allowing a defined number of cells to be seeded into the culture vessel. The seeding density selected for the *in vitro* expansion step is a CPP. MSCs can be seeded at a very low density (50–100 cells per cm^2) and will proliferate until they achieve confluence. This corresponds to a high expansion factor, but the process takes a long time and requires more rounds of cell division for each seeded cell, so the cells experience significant aging [37]. The aging of MSCs during expansion is a problem, because older cells lose competence to behave as stem cells and have a tendency to enter senescence or even to undergo transformation. The manufacturing of Prochymal provided a clear example of this issue: 10,000 or more doses were manufactured from one donor, and the corresponding expansion stress led to replicative senescence, in which the cells retained a typical

MSC surface marker profile but lost functionality [4]. Aging MSCs are more likely to activate a senescence-associated secretory phenotype and produce pro-inflammatory cytokines such as IL-1, IL-6, and IL-8, which inhibit the regenerative process. The duration of *in vitro* expansion must be considered not only because of senescence, but also due to the phenomenon of clonal impoverishment. MSCs are polyclonal, but prolonged expansion favors the growth of specific cell types or clones. Depending on the expansion time and expansion factor, the cell mixtures may completely differ in phenotype and also in potency. Therefore, although a high expansion factor in a short process time is desirable to achieve high product yields, *in vitro* expansion should never change the properties of MSCs to the extent that it compromises their functionality and potency.

2.1.2 Culture medium

Several basal media have been shown to influence MSC expansion and potency, including Dulbecco's modified Eagle's medium (DMEM), Iscove's modified Dulbecco's medium (IMDM), and MEM alpha (α MEM) [37]. One of the key components of these media is glucose, which is the main carbon source for MSCs. Glucose may be provided at physiological concentrations (1 g/L) or higher (up to 4.5 g/L), the latter variously described as having a negative effect on MSC proliferation and growth factor secretion [38] or no effect at all [39]. Glutamine as a second carbon source is present at concentrations of 2–4 mM and appears essential for MSC growth [40], but its impact on MSC properties is complex, with contradictory results [41–43]. Glutamine is unstable at 37°C and spontaneously degrades to form ammonia. GlutaMAX (dipeptide Ala-Gln) is recommended instead of glutamine to promote MSC expansion [44]. Lactate and ammonia are the most abundant waste products formed by MSCs, and both therefore have the potential to inhibit growth. It therefore follows that glucose, glutamine, lactate, and ammonia levels should be considered as CPPs for the production of MSCs. Several other amino acids may also be relevant, given that the amino acid metabolism of MSCs differs from that of commercial cell lines such as Chinese hamster ovary (CHO) cells [42].

Basal media formulations must be supplemented to achieve MSC expansion. The most important supplement is fetal calf serum (FCS), which is added to a final concentration of 5–20%. FCS strongly influences MSC growth and phenotype, but the specific effectors are unknown because the composition of FCS is variable and lot-dependent [45]. The use of FCS for the manufacture of clinical MSC products is discouraged nowadays, in line with the drive to eliminate all raw materials of animal origin. The complex, uncertain, and variable composition of FCS also makes it difficult to validate for GMP-compliant processes. Finally, the manufacturing process must accommodate steps to eliminate FCS from the final product to avoid potential immunogenicity and allergenicity [46]. FCS can be replaced with human serum and its derivatives, such as human platelet lysate, which promotes MSC growth [47]. However, the same lot-dependent quality issues described above for FCS also apply to human serum [48]. The most acceptable alternative is serum-free or preferably chemically-defined medium, the latter not only serum-free but also lacking any hydrolysates or supplements of unknown composition. MSCs grow well in several commercial serum-free media, including BD Mosaic MSC Serum-free (BD Biosciences), RoosterNourish (Rooster Bio), Mesencult-XF (Stemcell Technologies), StemPro MSC SFM Xeno-Free (Invitrogen), TheraPEAK MSCGM-CD (Lonza), and PPRF-msc6, STK1 and STK2 (Abion) [49]. Growth in chemically-defined medium has also been demonstrated [50]. However, although MSCs showed excellent growth in these serum-free media, they reached senescence earlier, and there were changes in morphology, surface marker profiles, and potency [51]. This does not mean that

serum-free and chemically-defined media should be avoided-it is still better to use these media for MSC expansion in order to meet GMP requirements-but further investigations are required to optimize the media composition. The development of serum-free media is mainly driven by companies, which tend not to disclose the precise composition, making it difficult for other researchers to build on the results. In serum-free and defined media, supplemental growth factors such as FGF2 and PDGF are needed to stimulate MSC proliferation, but they also influence MSC potency [18]. Accordingly, chemically-defined media would be preferable for the *in vitro* expansion of MSCs, but growth factor concentrations are important CPPs that affect MSC identity and potency and must be carefully controlled.

2.1.3 Conditions in the culture vessel

MSCs are aerobic cells and any culture vessel must therefore ensure an adequate supply of oxygen. However, the oxygen saturation in standard T-flasks (21% O₂) is far removed from nature (5–7% O₂) [34]. MSCs therefore tend to be oversaturated with oxygen, which can increase the concentration of damaging reactive oxygen species (ROS). Several studies have confirmed that hypoxia enhances MSC proliferation, stabilizes their cell fate, and prevents apoptosis by reducing the levels of caspase-3 [52]. However, rather than imposing hypoxia by preconditioning the cells, it may be better to impose hypoxia during the entire expansion phase, because this mimics their natural niche [53].

In addition to oxygen saturation, temperature and pH are CPPs in every process and can be monitored and controlled very easily. Typically, *in vitro* expansion is carried out at 37°C and neutral pH (7.2–7.4). Expansion at lower temperatures can be advantageous under certain circumstances because this reduces stress (ROS production and frequency of apoptosis) and may yield more potent MSCs. Although the expansion of MSCs has been achieved in the pH range 7.5–8.3 [54], it is unclear how significant variations in pH influence MSC metabolism and whether this affects the secretome. The optimal temperature and pH must be evaluated for each MSC product.

Other CPPs include the parameters grouped under the term hydrodynamics, referring to the potential impact of aeration and agitation. Aeration is required to supply oxygen to the MSCs, but as well as affecting the oxygen saturation, it also generates forces that cause physical stress. In T-flasks, aeration is achieved by the diffusion of oxygen through the surface of the medium, whereas bioreactors must be actively aerated by, e.g., bubbling gas into the liquid. The bursting gas bubbles (cavitation) generate strong forces that can damage cells, although the stress can be reduced by controlling the bubble size [55]. Agitation in bioreactors is generally achieved with impellers, which help to disperse gas (and therefore contribute to aeration) but also maintain a homogenous suspension of cells and nutrients. The creation of a homogenous environment is advantageous because it avoids gradients of pH, nutrients, or waste products, whose effect on MSCs is unpredictable. Homogenization can also be achieved using pumps or is facilitated by air bubbles. Agitation always generates shear forces, so it is necessary to balance the homogeneity of the cultivation system and the impact of the hydrodynamic forces on the MSCs. Although excessive shear stress is detrimental, hydrodynamic forces can also stimulate MSC growth and increase potency [43]. For these reasons, the mode and rate of aeration and the method and intensity of agitation are CPPs that must be carefully optimized for each process.

2.1.4 Growth surface, cell harvest, and storage

MSCs are anchorage-dependent cells, so the properties of the growth surface also have a significant impact on the process and must be investigated and selected

carefully. However, unlike the parameters discussed above, the growth surface does not have to be monitored or controlled during MSC production, so it falls outside the technical definition of a CPP. The expression of certain surface markers by MSCs reflects the stiffness of the growth surface, so it is clear that the surface affects the phenotype [56]. As stated above, the ability to adhere to plastic surfaces is one of the minimal criteria that define MSCs, and tissue-culture plastic is therefore the most commonly-used growth surface. Although all commercial tissue-culture plasticware has a polypropylene base, the surface is often treated differently, and this changes the behavior and properties of the adherent MSCs [37]. MSCs further grow on other surface materials, e.g., glass [57] or dextran [58]. When MSCs are cultivated in serum-free medium, cell growth often requires that the surface is coated with further adhesion-promoting factors, such as fibronectin, vitronectin, or the peptide RGD.

Given that MSCs are anchorage-dependent cells, the harvesting of cells at the end of the *in vitro* expansion step requires an efficient cell detachment method that ideally does not affect functionality or potency. In the laboratory, MSCs can be detached from T-flasks by adding trypsin or other proteases, but this nonspecific proteolysis can affect cell viability and eliminate some MSC surface markers [59]. Proteolytic cleavage is incompatible with the larger-scale processes in bioreactors because longer incubation times are required for the enzymes to work, and even then, the efficiency of cell recovery is low [60]. More importantly, any negative effects of the enzymatic treatment on cell viability and potency are amplified by the longer exposure time, which can inhibit MSC differentiation [61]. These issues can be addressed by adjusting the hydrodynamic conditions to favor cell detachment after limited enzymatic treatment [62]. Alternatively, enzymatic treatment can be circumvented completely by promoting cell detachment using dissolvable growth surfaces [63] or thermosensitive surfaces that release cells following a temperature shift [64, 65]. However, unlike enzymatic treatments, these novel surfaces do not break direct cell-cell bonds and may be unsuitable if single cell is required. The formation of aggregates can be minimized by carefully monitoring the cell density and selecting a harvest point that favors the recovery of single cell, but this must be balanced against the efficiency of expansion given the need to harvest at lower cell densities. The so-called harvest problem, balancing the efficient release of cells against the recovery of cells with desirable properties, has yet to be solved. This highlights the importance of well-defined CPPs at the harvesting stage.

All the approved allogenic MSC products described earlier are cryopreserved, allowing them to be offered as off-the-shelf products that can be stored until quality control and batch release are completed. The use of cryopreserved allogeneic MSCs is the only feasible therapeutic strategy for acute tissue injury syndromes such as stroke, sepsis, or myocardial infarction, because the patient is likely to die before sufficient quantities of autologous MSCs could be prepared. However, cryopreservation and thawing have a massive impact on the potency of MSCs [66]. Indeed, even without optimization, fresh MSCs are much more potent than frozen ones [35]. A rule of thumb is to freeze the cells slowly (e.g., 1°C/min) but to thaw them quickly (e.g., direct transfer from storage to a 37°C water bath). The impact of multiple freeze-thaw cycles must be evaluated carefully [67]. The composition of the freezing medium is also important because it often contains dimethyl sulfoxide (DMSO) and FCS as cryoprotectants, the first being cytotoxic and the second undesirable for the reasons already discussed above. Nontoxic alternatives lacking DMSO and FCS have been tested and may be more compatible with MSCs intended for clinical applications [68–70].

In summary, the expansion of MSCs in bioreactors involves multiple CPPs including (i) the source of the initial MSCs before expansion, (ii) the impact of cell

density and age, (iii) the effects of the culture medium, (iv) the properties of the bioreactor and aeration/agitation systems, and (iv) the method used for cell harvest and storage. The impact of these CPPs on the quality of MSCs can only be determined by designing robust assays for (i) *in vitro* senescence and genetic stability and (ii) relevant disease-specific mechanism of action and potency. It is clear that there is no one-size-fits-all MSC expansion process and that unique processes must be developed to match different therapeutic objectives. These processes may feature distinct CQAs, meaning that the CPPs may also differ on a case-by-case basis.

2.2 MSC manufacturing for clinical trials

For the 989 interventional clinical trials involving MSCs reported thus far (www.clinicaltrials.gov, search term: mesenchymal stem cell OR mesenchymal stromal cell, 2019/09/27), the MSCs were expanded *in vitro* and in most cases were transfused intravenously at typical doses of $1\text{--}2 \times 10^6$ cells per kilogram, never exceeding 12×10^6 cells/kg [3].

The manufacture of protein therapeutics is almost always carried out in bioreactors because they are scalable, controllable via integrated process analytical technology, and most process steps can be automated. This is not the case for MSC products, and a survey of GMP manufacturing at US academic centers has revealed major differences in the various process steps (cell isolation, expansion, and characterization). In the context of cell expansion, 80% of the centers surveyed above used T-flasks or cell factories, whereas only 20% mainly used bioreactors. A broad range of seeding densities was used for cultivation ($50\text{--}2500$ cells/cm²) and the cultivation time ranged from 1 to 28+ days. The cultivation medium was supplemented with FCS (lot-selected or not) or donor-pooled human platelet lysate (in-house product or commercial product) [71]. All of the centers expanded MSCs under GMP conditions, but with huge variations in the protocol. The production of MSCs in T-flasks is adequate for a small number of patients (30 T-flasks each with a growth surface of 175 cm² would be required per patient, assuming each patient is dosed with 416 million cells and the harvesting efficiency is 8×10^4 cells/cm² [72]). But for larger clinical trials with >100 patients, the resources required for cell culture would become unsupportable (assuming the conditions stated above, a trial with 140 patients would require 4200 T-flasks filling 32 standard 160-L incubators and 9 full-time personnel to handle the cells). Expansion in T-flasks might also be sufficient for autologous cell therapy, given that only a single patient is involved and it would not be necessary to produce more than 10 doses. However, even for small-scale manufacturing, an automated bioreactor system would offer several advantages over manual cultivation. Given that the entire manufacturing process must be aseptic, closed bioreactors provide much better insurance against contamination than an open culture system based on T-flasks. For allogenic MSC products, where up to 1 million doses are produced per batch, bioreactors are the only feasible manufacturing option (Figure 2).

2.3 The expansion of MSCs in bioreactors

When an MSC product advances from research to commercial manufacturing, the *in vitro* expansion process must also change. Research is driven by the freedom to test different conditions, but the tests are typically conducted on a small scale. In contrast, commercial products must be manufactured using a standardized process to ensure robustness, and the scale is generally larger. Bioreactors play a key role in large-scale manufacturing because they offer greater traceability due to the control and monitoring of CPPs. The expansion of MSCs in bioreactors allows the

Figure 2. Manufacturing of autologous and allogenic MSC products. Autologous MSC products are isolated from the patient's own tissue, whereas for allogenic MSC products a healthy donor from the same specie donates cells. In the isolation and expansion, there are few differences between the two types of MSC products. Most common sources are bone marrow, adipose tissue, and umbilical cord (blood), all three giving different amount of MSCs. The expansion for both MSC product types differs in scale. Storage is only needed for allogenic MSC products. If we expect that 0.4×10^6 MSC are isolated per donation and one dose to treat a single patient is about 120×10^6 MSCs, an expansion factor of at least 300-fold is needed. If more doses should be produced from one isolate, e.g., because the patient needs several treatments or in case of allogenic MSC products, the expansion factor dramatically increases.

precise control of the microenvironment, which has a profound influence on cell potency and therapeutic efficacy [73, 74]. For example, the dynamic cultivation of human MSCs in a bioreactor has been shown to induce the secretion of several beneficial growth factors, including BDNF, NGF, VEGF, and IGF-1 [75]. The use of bioreactors also means that the *in vitro* MSC expansion and harvesting steps can be automated, which improves the efficiency of both steps and reduces the amount of hands-on work. The elimination of operator-related errors and contamination risks makes the process more stable, avoiding batch-to-batch variability. Nutrient gradients and abrupt fluctuations in pH caused by manual medium exchange are also avoided. This enables the production of MSCs with consistent identity and potency (CQAs). Many different bioreactor types have been used for the *in vitro* expansion of MSCs, including fixed bed, fluidized bed, and stirred tank reactors, as well as newer innovations such as wave reactors, wall-rotating systems, and vertical wheel reactors [76]. However, most studies have involved only two types of reactor: stirred tank or fixed bed, and these are discussed in more detail below.

Stirred tank reactors are the most widely-used devices for large-scale MSC expansion. They are often used with microcarriers, which are small beads that increase the surface area available for cell attachment, although MSCs can also be grown in bioreactors as aggregates or spheroids. The expansion of MSCs growing on microcarriers is typically a batch-mode manufacturing process because the cells are harvested at a predetermined density. However, fed-batch processes involve a smaller inoculum (100 cells/cm^2 , equivalent to five cells per microcarrier) and can thus achieve better economy and a higher expansion factor [77, 78]. There should be minimal (if any) agitation at the beginning of the expansion phase to allow for cell attachment to the microcarriers (if used) or otherwise for the formation of aggregates. However, agitation is required following attachment in order to homogenize the suspension and avoid the formation of large clumps. As discussed above, agitation is an important CPP and the parameters must be optimized based on the unique combination of

system properties (e.g., impeller type/speed and microcarrier size/amount) to keep microcarriers or aggregates in suspension without causing shear damage, and these parameters must be optimized at different manufacturing scales [79].

Fixed bed reactors are also widely used for MSC expansion, and in this case, the cells are grown either on macrocarriers or as capsules (500 μm diameter), both of which form a stable bed at the reactor base. The production of homogeneous conditions in the bed can be frustrated by the development of channels and gradients in the bed, particularly in large-scale systems [80]. The shear forces in fixed bed reactors are low ($\sim 0.5 \times 10^{-5} \text{ N/cm}^2$) and consistent throughout the reactor with no peaks near the impeller; the shear forces also remain constant at all scales [81]. The *in vitro* expansion of MSCs has been reported in several types of fixed bed reactors [82–85]. One of the major drawbacks of fixed bed and other reactor types compared to stirred tank reactors is the challenge of efficient harvesting. For example, in the reports above, the recovery of viable cells is rarely better than 70%, so this is a key aspect of bioreactor design that remains to be addressed [60, 86].

2.4 Remaining challenges

The earlier sections highlighted several challenges that must be overcome to develop robust processes for the expansion of MSCs in bioreactors, which are summarized briefly below. Furthermore, our current understanding of the CPPs affecting MSC production is rudimentary at best, and more work is required to determine the impact of hydrodynamic factors on the CQAs. Precise online monitoring tools are needed to control CPPs effectively and to measure their influence on cell viability, potency, and secretory profiles. An increase in process understanding will facilitate process modeling, to fulfill the requirements of process analytic technology as a prerequisite for GMP manufacturing.

The major challenge for MSC therapy is the development of an *in vitro* expansion process that mimics the natural MSC niche, but nevertheless allows scaled up production for clinical trials without compromising CQAs such as cell functionality and potency. The development of a standardized process is frustrated by the heterogeneity of MSCs, which are isolated from different donors and different tissues, resulting in variable phenotypes and functions. The heterogeneity of primary MSCs can be avoided by working instead with induced pluripotent stem cells (iPSCs), which can differentiate into MSC-like cells with potent therapeutic properties [87]. However, well-controlled *in vitro* expansion processes in bioreactors can also help to reduce the batch-to-batch variation often encountered with MSCs, because parameters such as the seeding density, shear stress during cultivation, and cell density at harvest can be monitored and controlled effectively.

Polyclonal MSCs often show the most potent therapeutic effects, but clonal impoverishment occurs during lengthy expansion phases and this must be avoided if potency is compromised. However, even monoclonal MSCs become heterogeneous over time, generating subpopulations with different morphologies and surface marker profiles. The therapeutic outcome can only be predicted if the MSC pool does not change during expansion, and the well-controlled conditions in bioreactors can therefore help to ensure that the cell products remain homogeneous.

Ultimately, even bioreactor-based processes for MSC expansion are constrained by the inbuilt replication limit of MSCs, which leads to senescence after a certain number of generations. Stem cells by definition have an unlimited capacity for self-renewal, but this property is lost *in vitro*. The expansion stress that leads to replicative senescence generates MSCs that maintain their marker profiles but nevertheless lose functionality and therefore therapeutic potency.

The production of MSCs with standardized properties would be facilitated by the development of standardized validated potency assays so that results obtained in different laboratories are truly comparable. The ISCT has taken steps in this direction by publishing standards for the harmonization of potency assays. In a matrix assay approach, they propose to use quantitative RNA analysis for selected gene products, flow cytometry to detect functionally-relevant surface markers, and protein-based assays to map the secretome and determine the immunomodulatory potency of MSCs [88].

3. Additional processes that require MSCs

MSCs are typically the sole product of any MSC cultivation process, but in some applications, the MSCs are used as helper cells to deliver a different product or they are used as a vehicle to produce a specific cellular component. In each case, the CQAs differ significantly from the standard MSC manufacturing process and other CPPs must therefore be considered. We discuss two examples below.

3.1 Production of MSC-derived extracellular vesicles

MSCs are potent therapeutics, but researchers are seeking new ways to achieve the same therapeutic effect without the drawbacks associated with MSC manufacturing, such as the limited availability of potent cells, the complex transfusion process, and the entrapment of MSCs in nontarget organs [89]. As discussed earlier, the therapeutic effect of MSCs reflects the secretion of cytokines, growth factors, and other paracrine signaling molecules, particularly via the release of extracellular vesicles that interact directly with target cells and deliver their contents into the cytosol. The advantage of these vesicles over whole MSCs is their much greater stability, which means they can be manufactured, stored, and shipped without losing therapeutic efficacy [90, 91].

The large-scale manufacturing of extracellular vesicles requires the cultivation of MSCs, which secrete these vesicles directly in the culture medium. Scalable production methods are not yet available, and vesicles are currently produced in T-flasks or cell factories without process monitoring. Bioreactors could be used to scale up production, and given there is no need to harvest the MSCs, it would be possible to consider a wider range of bioreactor systems than the relatively narrow selection favored for MSC manufacturing. A fixed bed bioreactor has been used for the continuous production and harvesting of extracellular vesicles, which increased the yield 10-fold compared to T-flasks [92]. Stirred tank reactors with microcarriers might also be suitable, but they have not yet been used for vesicle production [57]. The cells would be exposed to shear forces caused by the impellers and air bubble cavitation, and this may influence vesicle production and potency [93].

The effect of different process parameters on the production of MSC-derived extracellular vesicles has been investigated at the laboratory scale. For example, hMSCs and their vesicles are primed by hypoxic conditions or changes in medium composition, such as the removal of FCS or the addition of priming factors like $\text{IFN}\gamma$ and $\text{TNF}\alpha$ [34, 35]. The yield of extracellular vesicles can also be increased by preparing spheroids that mimic *in vivo* conditions, for example by laying down an extracellular matrix and supplying appropriate signaling molecules [89]. Cell density, passage number, and cell origin also affect the vesicle yield. The immortalized cell line hMSC-TERT is more stable than primary MSCs, but the immortalization process has an impact on vesicle production, which must be investigated individually for each cell line because it is not related to the immortalization method [93].

There is currently no standardized large-scale production platform for primed hMSC-derived vesicles, but even if such a platform existed, a corresponding purification process would be required. The laboratory-scale purification of vesicles captured from the culture medium is currently based on a combination of ultracentrifugation, dead-end filtration, precipitation, and size exclusion chromatography, which are difficult to scale up [94, 95]. However, tangential-flow filtration can also be used for large-scale purification, washing, and buffer exchange, and this method should be investigated in more detail for vesicle purification [94, 96]. Extracellular vesicles are even more sensitive to process changes than MSCs, so the influence of multiple cell-dependent, culture, and process parameters on the potency of these vesicles must be determined.

3.2 Cocultivation of MSCs with other cells

The ability of MSCs to restore the activity of dysfunctional cells *in vivo* is the basis of their therapeutic efficacy, but the same interactions can also be exploited *in vitro*. One key example is the interaction between MSCs and pancreatic beta cells, which are widely used for drug screening and cell therapy in the context of diabetes. In both applications, large numbers of functional beta cells are required, but beta cells rapidly lose their functionality when expanded *in vitro*. The loss of beta cell functionality *in vitro* can be prevented by cocultivation with MSCs, which not only stimulate beta cell proliferation but also enhance their glucose-dependent secretion of insulin [97–99].

The major challenge of cocultivation is to balance the demands of two completely different cell types. In large scales, the distribution of cells becomes heterogeneous, which can lead to instability within the bioreactor and lower cell viability. A well-balanced and tightly controlled culture environment is needed to stabilize large-scale cocultures. Because secreted factors are important for the cocultivation of MSCs and beta cells, the hydrodynamic forces in bioreactors, which influence the distribution of secreted molecules, must be considered at an early stage [100]. Furthermore, the optimal cocultivation ratio of the cells must be determined. Established processes can be modified to achieve a new process setup for cocultivation, but it is often beneficial to separate cell expansion from cocultivation (i.e., first expand the pure cultures to generate the cells needed for the coculture and then combine them to improve the function of beta cells in a second process step). For the expansion step, it can be sufficient to improve the growth of beta cells using conditioned medium from the cultivation of MSCs. Alternatively, the expansion and functionalization of beta cells can be combined in one process step [101]. The CPPs for such a complex process can be difficult to identify, but the CQAs of the beta cells are most relevant if the aim of the process is to produce functionalized beta cells for drug screening or cell therapy. Even so, the potency of the MSCs must not be neglected because they are required to stimulate the beta cells. Accordingly, the MSCs must be expanded under controlled and standardized conditions that maximize their beneficial impact on beta cells. In the future, cocultivation bioreactor concepts for MSCs and beta cells must be tested to allow the completely aseptic expansion and cocultivation of both cell types.

4. Conclusions

MSCs are potent therapeutic agents, but their complexity and environmental sensitivity make the GMP-compliant manufacturing of MSC products extremely challenging. Given the range of tissue sources, isolation procedures, and expansion

protocols, it is unclear whether MSC products are similar enough across manufacturing sites and whether results can be considered comparable even within the same study. Moreover, the incomplete definition of MSCs makes it difficult to develop objective release criteria. These issues strongly argue for the harmonization and standardization of MSC manufacturing processes, release criteria, and potency assays. The regulatory standards for MSCs are still evolving, and different standards apply in different jurisdictions. MSCs are living cells and cannot be held to the same standards as chemical entities or biopharmaceuticals, both of which can be tested against rigorous and objective quality criteria. The regulations for MSCs should be more flexible, acknowledging that each MSC product is developed for a specific indication, and unique platform technologies, CQAs, and CPPs may therefore be necessary for each manufacturing process. One of the most important platform technologies is the use of bioreactors for cell expansion, because this is the only current strategy that can bring MSC therapy into routine practice. MSCs can also be used as production aids for other products, including beta cells for drug screening or diabetes therapy, and novel biological agents such as extracellular vesicles. In the future, they could even be used for commodity products such as artificial meat. But in all these applications, a robust and scalable manufacturing process will be necessary.

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Conflict of interest

The authors declare no conflict of interest.

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