Chapter 16
cGMP Production of MSCs

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Abstract Over the past decade, mesenchymal stromal/stem cells (MSCs) have evolved into an important cell therapy demonstrating potential utility in a range of clinical applications, including bone and cartilage repair, cardiac repair, and immune disorders. MSCs can be isolated from a variety of tissue sources, including bone marrow, adipose tissue, dental pulp, and placenta. Groups have developed different manufacturing processes with a goal of improving the quality of clinical-grade cells and the overall efficiency of the manufacturing process. Variations in cell source and manufacturing process may have a significant impact on the efficacy of the final MSC product. Moreover, this variability in cell source and manufacturing processes has made it challenging to compare the resulting MSC products and associated results from clinical trials that have been conducted to date. The development of consistent, well-controlled manufacturing processes along with the implementation of thorough quality control testing, including rigorous potency assays, will insure high quality and may help to clarify the impact of cell source and manufacturing process on the resulting MSC product. In addition to providing an overview of the current good manufacturing practice (cGMP) methods for MSC production, this chapter summarizes key FDA regulatory requirements, including those related to cell source, raw materials, and quality control testing.

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P. Hematti and A. Keating (eds.), *Mesenchymal Stromal Cells: Biology and Clinical Applications*, Stem Cell Biology and Regenerative Medicine,
DOI 10.1007/978-1-4614-5711-4_16, © Springer Science+Business Media New York 2013

Introduction

Mesenchymal stromal/stem cells (MSCs) are adherent, fibroblast-like cells that are characterized by the expression of certain cell surface markers and the potential to differentiate into bone, fat, and cartilage [1, 2]. Although bone marrow (BM) is the most common source of starting material, cells with characteristics similar to BM-derived MSCs have been isolated from other tissue sources, including adipose, umbilical cord blood, placenta, and dental pulp [3–6]. Given the ability of MSCs to differentiate into adipocytes, osteoblasts, and chondrocytes, initial clinical applications focused on the use of MSCs to regenerate tissues using engineered bone constructs [7]. However, MSCs are excellent candidates for other applications due to several characteristics, including their ability to migrate to the site of injury/inflammation, the potential to stimulate proliferation and differentiation of resident progenitor cells, and the propensity to promote recovery of injured cells and/or modulate the immune system through secretion of growth factors [8–15]. Recent clinical applications have focused on utilizing the immunomodulatory properties and paracrine effects of MSCs in cardiovascular disease, neurological disorders, and immune dysregulation disorders. MSCs have demonstrated encouraging clinical results in Crohn's disease [16] and graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell (HSC) transplantation, and these studies have now advanced to phase III clinical trials [17].

Many of these initial clinical trials have demonstrated significant promise in using MSCs as a therapeutic. However, efforts to repeat clinical observations have resulted in variable success. One major hurdle in comparing results from clinical studies is the potential variability in cell quality and characteristics between clinical sites. Due to the complex nature of cell therapeutics, it is important to recognize that the manufacturing process will likely have a significant impact on important cell properties that impact *in vivo* efficacy. In addition to variability in starting cell source, there is a wide range of cell culture media and culture practices that are currently employed in producing MSCs for clinical applications. It is therefore critical to establish a panel of quality control (QC) test methods that can be used to assess the impact of these variables on the safety and potency of the final MSC product. This chapter provides an overview of important considerations when producing MSCs for clinical applications. In addition to a brief overview of regulations for clinical production of MSCs, this chapter provides an overview of a number of manufacturing and testing considerations.

FDA Regulations and cGMP Compliance

A thorough understanding of applicable regulations and industry standards are essential when developing biotherapeutics. Regulatory requirements will often drive key decisions for manufacturing process development, selection of raw materials,

and development of QC testing plans for raw materials and final product. This section provides a brief overview of regulations that are applicable to MSC-based therapies in the USA.

In the USA, cell therapies are regulated by the Center for Biologics Evaluation and Research (CBER) division of the Food and Drug Administration (FDA). Although the regulatory requirements for cell therapies are expected to evolve as new therapies move through human clinical trials toward approval, the FDA has provided guidance documents and regulations covering several key areas of production and testing.

In May 2005, Part 1271 of Chapter 21 of the US Code of Federal Regulations became effective. Part 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products provides the basis for regulation of human cellular and tissue-based products (HCT/Ps). In addition to providing regulations for Donor Eligibility (Subpart C), Subpart D outlines Current Good Tissue Practices (cGTP). The regulations in 21 CFR 1271 Subpart D cover a broad range of requirements, including quality system, personnel, procedures, facilities, environmental monitoring and control, equipment, supplies and reagents, process changes/validation, and product labeling/storage/tracking [18]. The HCT/P regulations outlined in 21 CFR 1271 and cGMP regulations (21 CFR 210, 211, 610) are intended to be applied in a progressively more strict manner as therapeutics move toward the eventual filing of a Biologics License Application (BLA) [19]. However, the FDA expects that certain key requirements of the cGTP/cGMP regulations even will be met during early-stage human clinical trials [20].

In addition to the regulations outlined above, the FDA has issued several guidance documents that are applicable to HCT/Ps. The FDA issued a guidance in March 1998 that provides an overview of manufacturing and testing requirements for human somatic cell therapy and gene therapy products including procedures for cell collection, cell culture, cell banking systems, and release testing requirements for cellular therapy products [21]. The *International Conference on Harmonization* (*ICH*) has also issued several guidance documents that provide further details on testing requirements for cell therapeutics [22, 23]. Guidance documents are also available for issues related to the sourcing and testing of the initial cell material including donor eligibility determination and addressing xenotransplantation issues for cell therapeutics that were previously cultured *ex vivo* with live nonhuman animal feeder cells [24, 25]. Since HCT/Ps typically cannot undergo a terminal sterilization step, HCT/Ps must be manufactured following aseptic processing methods. Several documents are available providing general guidance for validation and cGMP compliance for aseptic processes [26]. In addition to guidance from the FDA, AABB (formerly the American Association of Blood Banks) and the Foundation for the Accreditation of Cellular Therapy, or FACT, have established standards to assist with meeting regulatory requirements [27, 28]. Several groups from academia and industry have published documents that provide guidelines for moving HCT/Ps into human clinical trials [29–31].

Cell Source

MSCs were originally isolated as an adherent cell population derived from bone marrow (BM) [1]. Subsequent studies have found that similar populations can be isolated from other adult and perinatal tissues, including adipose tissue (AT) [6], skeletal muscle [32], synovium [33], dental pulp [34], placenta [35], amniotic fluid [36], and umbilical cord blood (UCB) [37]. Several studies that have compared the properties of the cells derived from these diverse sources have found that the cells demonstrate very similar characteristics including cell marker expression, differentiation potential, and immunological properties [38–40]. However, a study that compared the gene expression profiles of MSCs derived from BM, AT, and UCB found that while MSCs derived from different donors using the same source material and expansion protocol exhibited consistent and reproducible profiles, MSCs from AT, BM, and UCB display differences in the transcriptome [41]. The impact of these differences on *in vivo* efficacy remains unclear. However, the results serve to highlight potentially important differences between MSCs derived from different sources. This section provides a brief overview of MSCs derived from BM, AT, and UCB. In addition, information is provided on donor screening and eligibility requirements that apply to all sources of starting cell material.

Bone Marrow

The starting BM for MSC production is typically obtained from a 25–100-mL BM aspirate from the posterior superior iliac crest of the donor. The procedure is performed in a clinical setting allowing for sterile harvest of the BM aspirate. In addition, donors typically go through a full medical screening process (see Donor Screening below) and a rigorous informed consent procedure, very similar to that of a blood donor.

Several important factors regarding the BM donation may have a significant impact on the quantity and quality of MSCs derived from the BM. The age, sex, and health of the donor, including factors such as smoking, may impact the quality of the BM harvest [42, 43]. Donor-to-donor variation has also been observed in the profile of cytokines and chemokines that are secreted by MSCs in response to stimulation with proinflammatory cytokines [44]. Freezing of BM prior to MSC isolation was also reported to have a negative impact on both MSC yield and immunosuppressive properties of the MSC in mixed lymphocyte cultures [45]. Finally, as discussed in section "MSC Isolation from Bone Marrow," the method that is used for isolating the mononuclear cell fraction from the BM may have a signification impact on the resulting MSCs.

Adipose Tissue

Although the bulk of the published literature concerns BM-derived MSCs, AT is also considered to be an easily obtainable source of starting cells for MSC production. AT-derived MSCs have been used in a few small clinical trials for Crohn's disease [46], steroid-refractory acute GVHD [47], enhancement of HSC engraftment [48], and as salvage therapy for refractory pure red cell aplasia after major ABO-incompatible HSC transplantation [49]. The procedure for producing MSCs from AT involves red blood cell (RBC) washing steps similar to BM processing with the density-gradient step essentially replaced by a collagenase digestion step. A number of factors including donor characteristics and anatomical location of AT harvest can impact the characteristics of the resulting MSCs [50].

Umbilical Cord Blood

UCB is the most recently established source of hematopoietic stem cells for clinical utility [51]. Although some investigators have had limited success [52, 53], it is also now generally accepted that UCB is a suitable starting material for MSC isolation and expansion [3, 54]. With efficiency of isolation varying among research groups with success rates in the range of 24–63% [3, 55], an effort has been made to optimize cell processing [55]. In general, the approach is very similar to that of marrow-derived MSCs. The mononuclear cell (MNC) fraction is isolated using a density-gradient centrifugation and then seeded into culture flasks (e.g., 1×10^6 MNC/cm^2). Within 24 h the non-adherent cells are removed, and the remaining adherent cells are carried through culture much like MSCs from other sources. Interestingly, one group demonstrated UCB-derived MSCs to have a greater proliferation capacity, becoming senescent later than adipose- and marrow-derived MSCs [56]. The same group was unable to show adipogenic potential of UCB-derived MSCs, though others have been able to demonstrate in vitro differentiation to fat cells [3, 54]. In fact, some researchers have isolated MSC-like cells from UCB and succeeded in coaxing to cell types representative of all three embryonic lineages [57–59]. The potential value of UCB-derived MSCs over other types remains to be determined, though their unique qualities suggest there may be some advantages [56].

Donor Evaluation

Donor evaluation is an important requirement for cell therapeutics derived from human tissue sources. Requirements for donor evaluation are outlined in the HCT/P regulations (21 CFR 1271 Subpart C) and FDA guidance documents on donor

Table 16.1 Examples of current FDA-licensed kits for Donor Testing[a]

Test	Methodology	Manufacturers
HBsAg	EIA	Bio-Rad Laboratories
	EIA	Abbott Laboratories
	ELISA	Ortho Clinical Diagnostics
Anti-HBc (IgG+IgM Ab)	EIA	Abbott Laboratories
	EIA	Ortho Clinical Diagnostics
HBV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti-HCV	EIA	Abbott Laboratories
	EIA	Ortho Clinical Diagnostics
HCV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti- HIV-1/2	EIA	Bio-Rad Laboratories
	ChLIA/EIA	Abbott Laboratories
HIV nucleic acid	PCR	Roche Molecular Systems
	TMA	Gen-Probe, Inc.
Anti-HTLV I/II	EIA	Abbott Laboratories bioMerieux
Treponema pallidum[a]	Olympus PK TP System Anti-TP(IgG & IgM)	Fujirebio Inc.
CMV antibody[a] (IgG+IgM Ab)	Solid phase red cell adherence	Immucor
	Solid phase EIA	Abbot Laboratories
West Nile virus	PCR	Roche Molecular Systems
Nucleic acid	TMA	Gen-Probe, Inc.

[a]See the FDA website for specific testing requirements and additional approved tests (www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/TissueSafety/ucm095440.htm)

eligibility [25]. A comprehensive donor evaluation is typically performed by a physician with expertise in the collection procedure at the time of initial evaluation. The donor evaluation typically consists of three components: donor questionnaire, medical examination, and testing for infectious disease markers. All potential donors fill out a questionnaire that screens donors for transmissible diseases on the basis of history [60]. The donor's medical history is reviewed including information on transfusion history, surgical history, pregnancies, vaccination history, family history, social history, and health habits including smoking, alcohol, and recreational drug use. A general medical examination is performed prior to donation and typically includes routine laboratory testing (CBC with differential and platelet count, PT/INR, standard blood chemistry panel, and ABO/Rh type).

A blood sample is also taken from the donor at the time of initial donor assessment and, if needed, at the time of collection for infectious disease testing as required in 21 CFR 1271 Subpart C FDA Donor Eligibility. Infectious disease testing is performed using FDA-licensed test kits as summarized in Table 16.1. The results from donor testing, donor eligibility assessment, and the informed consent for tissue donation are typically retained in a file that is coded to protect patient confidentiality while maintaining traceability of the final MSC product back to the original tissue source.

MSC Production Methods

Along with advances in clinical applications for MSC-based therapies, strides have been made in several key technical areas related to production, testing, and banking of MSCs. Producing MSCs for clinical applications requires addressing several key issues [61, 62]. In addition to addressing regulatory compliance issues, manufacturers of MSC for clinical applications must address issues related to source material, cell culture conditions, and media source/quality. Several studies have been performed to determine the optimal conditions for culturing MSC for clinical applications [63–66]. New media formulations that avoid the use of FBS have been described recently [67, 68]. Efficient procedures for MSC cryopreservation and conditions for transporting and holding cells for transplantation have also been evaluated [66, 69].

In addition to developing well-defined and reproducible manufacturing procedures, quality control (QC) test methods must be established to characterize and evaluate the final cell product. Characterization assays are especially critical for MSC products given the diversity of starting material, isolation methods, and culture methods [70]. Several groups have published reports on QC test methods that are currently used for both in-process testing and testing MSC products intended for human clinical trials [71, 72]. This section provides a brief overview of a typical manufacturing process for BM-derived MSCs including discussions regarding key process steps and parameters that potentially impact the quality and efficacy of the final MSC product.

Overview of MSC Manufacturing Process

A typical MSC manufacturing process consists of the following steps: isolation of MNC fraction from BM, MSC seed/master cell bank (MCB) production (optional), MSC expansion, and cryopreservation. Final formulation may take place prior to or after cryopreservation following the thaw. The overall process is depicted in the process flow diagram presented in Fig. 16.1. Different seeding and passaging strategies can be used in the MSC production process. For example, a low seeding density of MSCs may be subjected to a single expansion step without production of an intermediate cell bank. Seeding density and passaging schedule have an impact on the final MSC population and this is discussed in section "MSC Culture Method."

Raw Materials

Raw materials that are used in cGMP manufacturing processes should be sourced from vendors that have been audited for compliance with cGMPs or other appropriate quality standards. QC testing and documentation should be maintained for each

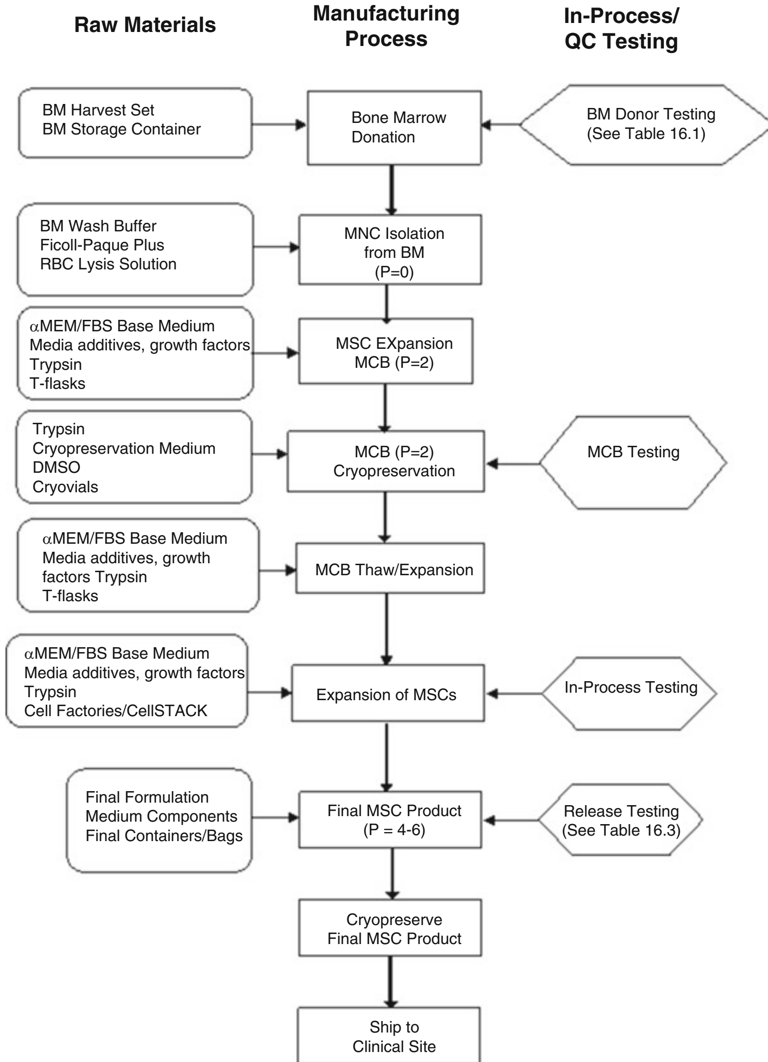

Fig. 16.1 Process flow diagram for MSC production from bone marrow

raw material, and traceability from final MSC product back to all raw materials should be maintained for each production lot. Raw materials should be reviewed to identify potential risks that may be introduced, for example, through the use of animal-derived raw materials. Table 16.2 provides an overview of common raw materials used in MSC production with recommendations for QC testing and documentation requirements.

Table 16.2 Biological source raw materials common for MSC production

Raw material	Use	Special QC testing and documentation considerations	Risk mitigation
Bone marrow	Starting material	Donor testing, medical history, and informed consent for donation	
Fetal bovine serum	Media component	9CFR 113 bovine pathogen testing Country of origin certificate Bovine source from low TSE-risk country	Consider irradiation or viral filtration Consider moving to serum-free media
Growth factors (e.g., FGF-2, TGF-β, PDGF)	Media supplement	Review manufacturing process for recombinant proteins to assess risk from mammalian cell lines and animal-derived materials	Utilize GFs produced in microbial expression systems or well-characterized mammalian cell lines
Porcine trypsin	Cell detachment	9 CFR 113 testing for porcine pathogens	Consider moving to recombinant enzyme
Human serum albumin	Final formulation	Derived from human serum collected from tested donors collected, complies with 21 CFR 640.80	Recombinant HSA
Final container	Product storage	Sterility, endotoxin – USP <161> Extractables – USP <661>/USP <88>	

MSC Isolation from Bone Marrow

MSCs are present in the mononuclear cell (MNC) fraction of the BM. The MNC fraction is enriched from the BM using density-gradient centrifugation. This step is typically performed using Ficoll-Hypaque density-gradient medium. cGMP-grade versions of density-gradient medium are commercially available. Following enrichment, the cells are washed with PBS or Hank's Balanced Salts Solution (no phenol red, calcium, or magnesium) prior to initial plating. Studies have demonstrated that modifications to the MNC isolation step can have a significant impact on the yield and quality of the resulting MSC product. For example, MNC isolation using 1.073 g/mL Ficoll produced an MNC fraction that was lower in CD45+ cells resulting in about a twofold increase in MSC yield after four passages with higher expression of CD90, CD146, and GD2 [73].

MSC Seed Bank Production

Following enrichment of the MNC fraction by density-gradient centrifugation, the washed cells are typically plated (passage 0) in cell culture flasks and incubated at 37°C with 5% humidified CO_2 using the selected MSC culture media (see section "Media Selection"). Twenty-four to forty-eight hours later, the non-adherent cells are removed (suctioned out) and the adherent cells are expanded in culture with media changed every 3–4 days.

At this point in the manufacturing process, the MSCs may either be expanded directly to final product or expanded to an intermediate stage (e.g., $P=2$) where they are harvested and cryopreserved to create a seed bank for future production trials. The creation of MSC seed banks allows future production campaigns to be performed with a starting cell source that has undergone testing for key attributes such as growth characteristics and biological activity. This allows for more uniform production campaigns and can be used to address key issues such as donor-to-donor variability in MSC properties. Cells from the seed bank ($P=2$) are typically expanded through several additional passages to generate the final MSC product ($P=4$–6) to be used in clinical trials. It should be noted that this product will be several passages older than MSCs that are expanded directly without creating an intermediate seed bank. While there are advantages to such an expansion strategy from a time/yield and logistics perspective, the overall impact of time in culture and passaging on cell quality and potency remains to be established. Limited studies suggest that there is an impact of time in culture on MSCs possibly related to the age of the donor [74, 75]. Other studies have shown that moderate time in culture (4–7 passages) does not affect the immunosuppressive activity of MSCs [45]. It is advisable, however, that investigators qualify their chosen MSC manufacturing approach for the intended clinical use (see section "Potency Assays").

Media Selection

Currently there is no standard method of culturing MSCs from any source/starting material, and there is no consensus among the investigators on the most efficient approach to producing MSCs. This is important since proliferation rate, differentiation potential, and immunophenotype of cells could change depending on the culture method. Nevertheless, clinical trials based on the use of MSCs generated at different academic centers have all showed that infusions of these cells are safe and potentially efficacious. The most commonly used media for MSC production appears to be fetal bovine serum (FBS)/alpha-minimum essential medium (αMEM). Considerations for the use of FBS in MSC culture along with several media options are presented below.

Fetal Bovine Serum

FBS has traditionally been utilized to expand human MSCs for both research and clinical applications. FBS is often added to alpha-MEM base media supplemented with glutamine with the FBS concentration ranging typically from 5 to 17%. Lot-to-lot variability is typically observed in the ability of FBS to support MSC expansion requiring screening of FBS lots and highlighting the potential impact on MSC quality and potency. Interestingly, the concentration of FBS can affect the subpopulation of MSCs that grow out in culture with serum deprivation resulted in selection of an Oct-4-positive early progenitor population [76]

The use of FBS in the production of cellular therapies generates several potential concerns including the introduction of the risk of transmission of zoonotic agents, bovine spongiform encephalopathy (BSE), and the introduction of antigens of animal origin that may be incorporated into the cell therapeutic (e.g., Neu5Gc) or present from residual contaminating FBS [77, 78]. The risk associated with BSE transmission may be reduced by selecting a FBS source from countries classified by the World Organization for Animal Health [Office International des Epizooties (OIE)] as negligible BSE risk or Geographical BSE-Risk (GBR) I, as classified by the European Food Safety Authority (EFSA) [79]. The potential risk of bovine pathogen transmission may also be mitigated by using only FBS that has undergone screening for bovine pathogens (9 CFR 113) and that has additionally undergone a viral inactivation step such as gamma irradiation.

The potential risks associated with BSE, pathogens, animal antigens, and variability drive the desire to identify other potential media for MSC clinical production. Alternatives to FBS that have been investigated include serum-free media, autologous serum, fresh-frozen plasma, and human platelet lysates [65, 68, 80–82].

Platelet Lysates

Among the current alternatives for FBS, media based on human platelet lysate have been studied the most extensively, including evaluation in human clinical trials [83]. One advantage of platelet lysate media is that it can be sourced from normal healthy donors that have passed all infectious disease testing. Platelet lysate media is typically produced using platelet concentrates collected from single donors by apheresis. The platelets are frozen, thawed, and then heat inactivated at 56°C for 30 min. After removal of the remaining platelets by centrifugation, the resulting platelet lysate is frozen in aliquots for future use in MSC culture. Despite its clear advantages, preparation of platelet lysate media requires additional time, and it may result in donor-to-donor (i.e., lot-to-lot) variability in MSC growth characteristics and potentially cell quality due to variability in growth factor content (e.g., platelet-derived growth factor – PDGF) [84].

Serum-Free Media

Several groups have developed serum-free media formulations that have demonstrated promise in MSC production. Meuleman et al. found that commercially available medium supplemented with a serum substitute demonstrated a significant increase in MSC yield compared to standard FBS/αMEM. In addition, the resulting MSCs were similar with respect to cell marker expression, differentiation potential, and the ability to support the growth of hematopoietic progenitors [85]. Chase et al. described development of a proprietary serum-free media that also demonstrated enhanced MSC growth over FBS/αMEM when the medium was supplemented with fibroblast growth factor-2 (FGF-2), transforming growth factor-beta (TGF-β) and PDGF [86]. MSCs produced using this medium showed similar cell surface marker expression by flow cytometry, differentiation potential, and gene expression profile relative to MSCs produced using standard FBS/αMEM. Although this initial version of media contained animal-derived components, a new xeno-free version is now commercially available [87, 88]. Additional *in vitro* potency studies and animal studies are needed to demonstrate whether the use of these serum-free media will have a significant impact on the *in vivo* efficacy of the MSCs. In addition, these media are proprietary formulations that contain undisclosed components. Care should be taken to identify potential risks from media components such as growth factors or other animal-derived components. For example, some growth factors may be produced using mammalian cell lines such as rodent cell lines (e.g., CHO, NS0) that inherently introduce the risk of retrovirus and retrovirus-like particle contamination [89]. Growth factors that are derived using such mammalian expression systems should utilize tested cell lines and have purification processes that have been validated for clearance of viral pathogens.

MSC Culture Method

MSCs are typically grown as adherent cells using standard tissue culture plasticware. Initial cultures of MSCs from the enriched MNC fraction or seed bank are typically expanded in T-flasks. Cells from T-flasks are then used to seed large-scale cell culture devices such as Cell Factories (Nunc) or CellSTACK (Corning). Cell Factories have demonstrated utility in producing MSCs for clinical applications [90]. Cell Factories/CellSTACK provide a convenient format for large-scale culture of adherent cells. Media may be prepared in disposable bioprocess containers, and bags and tubing sets can be used to allow the entire feeding and harvesting steps to be performed in a single-use, disposable, closed system. This format, therefore, provides the added benefits of decreased contamination risk and elimination of the need to perform cleaning validation as would be required for multiuse bioreactors.

Beyond the impact of donor characteristics, MNC isolation method, and media selection discussed above, a number of factors in MSC culture can impact the final

MSC characteristics. Seeding density is one important major factor that has a significant impact on the MSCs. Low seeding densities (10–50 cells/cm^2) have been shown to promote the growth of a subpopulation of MSCs that appears to represent early progenitors [91]. The resulting MSCs have an increased growth rate, thin spindle-shaped morphology, and have increased adipogenic potential relative to the later developing MSCs that have a wider morphology and greater chondrogenic potential.

Although several scalable formats including Cell Factories (Nunc) and CellSTACK have been used for MSC production, the relatively large doses (0.4–9 × 10^6 cells/kg) [92] of MSCs that are required for many indications suggest that other scalable production methods may be needed for future applications. Bioreactors offer a potential solution for large-scale production of cell therapeutics with the opportunity to provide greater control over cell growth conditions and potentially over cell quality. Most of the work aimed at growing MSCs in bioreactors is recent with a focus on growing MSCs on novel and commercially available microcarriers [93–96]. Although results to date have demonstrated modest levels of expansion, further optimization of seeding parameters, media formulation, feeding strategies, and bioreactor conditions will likely lead to further improvements in cell yield and manufacturing efficiencies.

Final Formulation and Cryopreservation

Following the final harvest, the MSCs are typically centrifuged, washed, and changed over to a formulation that is compatible with cryopreservation and administration to the patient. One formulation that has been used in previous clinical trials is PlasmaLyte A (Baxter, Deerfield, IL, USA) containing 5–10% human serum albumin and 10% DMSO. Alternative cryoprotectants have been evaluated with some success in reducing the required levels of DMSO by utilizing PEG and albumin [97]. The dose range for MSCs is typically 2–8 × 10^6 cells/kg or 1–6 × 10^8 MSCs/dose that is formulated as 25–100 mL of cells in a bag that is suitable for low-temperature storage. Bags of cells are typically frozen using a controlled rate freezer (−1°C/min) and stored in liquid nitrogen freezers in liquid or vapor phase at temperatures below −150°C.

MSCs have been thawed and immediately infused; however, they are often thawed and washed or diluted with an appropriate solution (e.g., Dextran 40, 5% human serum albumin) and then infused. Studies should be conducted to ensure that time limits are established for holding the final thawed product under defined conditions prior to administration. Previous studies have demonstrated a range of acceptable hold times depending on the formulation and hold temperature [66].

Manufacturing Controls

Cleanroom Environment

A key aspect of manufacturing cell therapeutics for clinical applications is the inability to perform a terminal sterilization step. This necessitates that the product be manufactured under strict aseptic conditions through the entire production process. The FDA has issued a guidance document that outlines key issues for aseptic manufacturing processes [98]. Key areas of focus that should be addressed for an aseptic manufacturing process include: clean room design, clean room cleaning practices, environmental monitoring practices, personnel gowning, personnel monitoring, and validation of aseptic processing methods. For cell therapy production, the clean room environment should, at minimum, meet class 10,000 (ISO class 7) clean room rating with a biosafety cabinet or other class 100 (ISO class 5) zone for performing open manipulations. Strict gowning practices, cleaning practices, and environmental monitoring (viable and nonviable) are critical for ensuring that the manufacturing environment is maintained in a controlled state during clinical production.

Process Qualification

Process validation is defined by the FDA as the "collection and evaluation of data, from the process design stage throughout production, which establishes scientific evidence that a process is capable of consistently delivering quality products" [99]. Initial process (performance) qualification (PQ) trials are typically conducted at the end of the initial process development studies and prior to initiating clinical production campaigns. Preclinical PQ trials typically consist of performing trials (3–5 runs) of the cGMP manufacturing process with full documentation and testing, including in-process testing. These trials allow final details to be worked out for manufacturing procedures and documentation and demonstrate that the manufacturing process is capable of producing material that will meet release testing requirements for clinical trials. Material from these initial PQ trials can typically be used as reference standard for future QC testing or for use in preclinical animal studies.

Process validation typically occurs throughout the product life cycle with data collected during production runs and process design experiments. The goal of this stage is to identify key process parameters and material attributes (e.g., donor variability) that impact process variability and product quality. Studies are then performed to demonstrate that the manufacturing process is capable of producing acceptable product within the limits established for these key operating parameters. Comprehensive process validation studies are required to be completed prior to commencing commercial distribution of the therapeutic [99].

Aseptic Processing Qualification

As discussed above, maintaining aseptic conditions during manufacturing is a critical aspect of clinical production for cell therapeutics. Demonstrating the ability to maintain aseptic conditions during the manufacturing process, especially during critical steps such as open manipulations, is therefore a critical component of process qualification. Media simulation studies are typically performed to validate aseptic processes [98]. These studies are performed using microbial growth media (e.g., soybean casein digest (SCD) medium) in place of cell culture medium with simulation of a full production run. Critical steps in cell production including seeding, feeding, harvest, and dispensing of the product into the final container should be included in the simulation runs. The final product containers containing SCD medium are incubated for 14 days, typically at two temperatures, with observation for any signs of microbial growth.

Quality Control Testing

Quality control testing is a critical component of the clinical production program. QC testing is typically performed at multiple points in the manufacturing process, prior to production (i.e., including donor material, raw material), cell (seed) bank, in-process samples, and final product release testing. Specifications are typically set for donor, raw materials, and final product based on key safety and performance requirements. In addition, data from PQ trials are used to establish process capabilities and set specifications for both in-process testing and final product release testing. Specifications are expected to address key attributes including identity, strength, quality, purity, and potency. Typical testing for donor tissue (see Donor Evalution) and raw materials (see Raw Materials) is discussed above. This section will cover QC testing requirements for the final MSC product.

Release Testing

Each lot of final MSC product will undergo testing to demonstrate that it meets preestablished specifications prior to release for clinical trials. Quality assurance is responsible for reviewing all production records, including QC testing, prior to release of final product. A summary of the typical final QC release testing performed on each lot of MSCs is provided in Table 16.3.

Identity Testing

Identity testing is typically performed using either short tandem repeat (STR) testing or human leukocyte antigen (HLA) testing. The identity tests create a genetic

Table 16.3 Quality control testing for MSCs

Characteristic	Test method	Specification
Identity Testing	Short tandem repeat testing HLA – high-resolution mapping of HLA-A, HLA-B, HLA-C, and HLA-DRB1	STR/HLA profile matches donor
Viable cell count	Viable count – Trypan Blue or 7-amino-actinomycin D (7-AAD)	>70%
Microbial and fungal contamination	21 CFR 610.12 sterility testing including bacteriostasis and fungistasis	No contamination detected
Mycoplasma	In vitro in Vero cells with culture method consistent with FDA PTC document	No contamination detected
Endotoxin	LAL kinetic turbidometric method – USP	<5 EU/kg/dose
MSC Antigen Expression	Flow cytometry for MSC markers: Positive: CD105, CD73, CD90 Negative: CD34, CD45, CD14, CD19, HLA-DR	 ≥95% expression ≤2% expression
Karyotype	G-band, 20 metaphase spreads	No clonal abnormalities
Residual FBS	ELISA assay for residual bovine proteins (e.g., BSA, transferrin)	Report level 6-log reduction
Bovine pathogens[a]	Testing for specific bovine pathogens according to 9 CFR 113	No contamination detected
Porcine pathogens[a]	Testing for specific porcine pathogens according to 9 CFR 113	No contamination detected
Human pathogens[a]	PCR or other appropriate assays for human pathogens – HIV-1 and HIV-2, HTLV-1 and HTLV-2, HBV, HCV, CMV, EBV	No contamination detected
Potency testing	Testing based on intended clinical indication	Specification to be established

[a]Testing is preferably performed on the raw material or human donor sample

fingerprint that can be used to relate the cell source back to the original donor. This is especially important if multiple cell lines are being produced in the same facility. STR testing is typically performed using commercially available kits [100]. HLA testing is performed by high-resolution sequencing of the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci. This technique is becoming more efficient as techniques utilizing next-generation sequencing methods are developed [101].

Viable Cell Count

Viable cell counts are typically performed by staining cells with reagents such as Trypan Blue or acridine orange (AO)/propidium iodide (PI) and performing manual counts with a hemacytometer or using an automated cell counter. Alternatively,

viable counts can be performed using 7-AAD or PI staining in conjunction with flow cytometry analysis of MSC cell maker expression [102].

Microbial and Fungal Contamination

Sterility testing is typically conducted using the direct transfer method in accordance with 21 CFR 610.12. The test article is inoculated into SCD and FTM and incubated at 20–25 and 30–35°C, respectively, for 14 days. Alternative strategies such as use of automated testing systems (e.g., BACTEC, BD, Franklin Lakes, NJ, USA) commonly used in the clinical setting may be employed if agreeable by FDA. Bacteriostasis and fungistasis testing described in United States Pharmacopeia (USP) <71> is also performed on the product at a minimum with the PQ to insure that the product components, or residual antibiotics if used in initial isolation, do not interfere with sterility testing.

Mycoplasma

Mycoplasma testing is conducted on both the cells and supernatant from the final product as well as the master cell bank, if that manufacturing approach is taken. Although PCR or chemical testing can be used as a screening assay for mycoplasma, the Points to Consider (PTC) culture method is preferred for release testing. The PTC method, which takes 28 days for completion, includes both a direct assay and an indirect assay [103]. The direct assay involves cultivation of the test article in agar and broth media under conditions suitable for growth of cultivatable mycoplasmas. The indirect method involves culturing the test article in Vero indicator cells followed by staining with a DNA-binding fluorochrome (Hoechst stain) to detect nuclear and extranuclear fluorescence. Appropriate positive controls are included in each arm of the assay.

Endotoxin

Endotoxin testing that is performed on the final production should conform with USP <85> Bacterial Endotoxins Tests. Testing is typically based on the Limulus amebocyte lysate (LAL) assay utilizing commercially available reagents and test kits (e.g., Endosafe, Charles River). Testing should include inhibition and enhancement test controls. A typical recommended specification for endotoxin is <5.0 EU/kg/dose.

MSC Antigen Expression

Flow cytometry is performed on the MSC seed bank and final product to verify appropriate expression of MSC markers. Most groups use the guidelines as proposed by the Mesenchymal and Tissue Stem Cell Committee of the ISCT [2].

This group defined criteria for MSC identification to include presence of CD105, CD73, and CD90 as positive markers (≥95%), and absence of CD45, CD34, CD14/CD11b, CD19/CD79α, and HLA-DR as negative markers (≤2%).

Karyotype

Karyotyping is typically performed using standard Giemsa/Trypsin/Leishman (GTL) banding (or simply G-banding) on 20 metaphase spreads. Analysis is performed in compliance with the Clinical Cytogenetics Standards and Guidelines published by the American College of Medical Genetics. Chromosome counts are performed on 20 cells with full band analysis performed on 5–10 cells [104].

Residual FBS

Levels of residual FBS in the final product are typically determined based on the level of residual bovine serum albumin (BSA). Levels of BSA can be measured using a commercially available ELISA kit. A typical target of reduction for therapeutics is <1 ppm residual FBS. However, acceptable specifications should be based on process capabilities and potential risk to the patient population. Cross-reactivity of the ELISA with human serum albumin will be a primary consideration if the final product is formulated with HSA. In that case, other components of FBS can be exploited to determine residual amounts of FBS in the final product (e.g., bovine transferrin) [105].

Potency Assays

The FDA requires that biological products meet requirements of safety, purity, and potency for biologics license application approval. A potency assay must be established prior to initiating phase III trials, and it must be validated before BLA submission. Potency is defined by FDA as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result" [21 CFR 600.3(s)]. The regulations allow potency assays to be *in vitro*, *in vivo*, or both as long as the assay(s) is designed specifically for the given product to assess potency as described above [106].

Since MSCs are used for a variety of clinical applications, the intended effect will undoubtedly vary. MSCs may be administered for an immunomodulatory effect (e.g., graft-versus-host disease), tissue or organ repair (e.g., meniscal repair), enhancement of engraftment following blood/marrow transplant, etc. Table 16.4 lists a few resources for potency testing of MSCs for various medical applications. Some approaches are more developed than others. Cytokine-based analysis is listed

Table 16.4 Examples of potency assays for MSCs

MSC application	Potency assay	References
GVHD treatment	MLR-based, cytokine-based	[107, 108]
Acute lung injury	*Ex vivo* lung model, cytokine-based	[109]
Crohn's disease	MLR-based	[110]
Connective tissue repair	PCR-based, trilineage potential	[111, 112]
Engraftment post-BMT	CFU-/LTCIC-based	[113, 114]
Cardiac regeneration	*In vitro* support of cardiac stem cell proliferation	[115]

below as a possible potency assay, and this approach is expected to grow given the expansion of research in this area [116].

There are several advantages to establishing a potency assay as early in the developmental pathway as possible. These include evaluating multiple candidate assays, evaluating the impact of media and production methods, generating data to support lot release specifications, and establishing a stability program. In the 2008 guidance, the FDA provides more practical benefits to early work on potency testing, as well as direction toward relevant biologics and cGMP regulations for consideration of potency assays [106].

Conclusion

MSCs can be produced from a variety of different cell sources with many variations in the initial isolation, cell expansion, and formulation/cryopreservation procedures. In addition, a variety of different test methods are used by groups to assess the quality of MSCs. This chapter provides a brief overview of some of the more common methods that are used for producing and testing MSCs for clinical applications. Clearly one of the major challenges facing the field of MSC-based therapeutics is the need to develop better analytical methods, including potency assays, to better assess how differences in production methods impact cell quality and *in vivo* potency.

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