

Platforms for Manufacturing Allogeneic, Autologous and iPSC Cell Therapy Products: An Industry Perspective



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Abstract As cell therapy processes mature from benchtop research protocols to industrial processes capable of manufacturing market-relevant numbers of doses, new cell manufacturing platforms are required. Here we give an overview of the platforms and technologies currently available to manufacture allogeneic cell products, such as mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs), and technologies for mass production of autologous cell therapies via scale-out. These technologies include bioreactors, microcarriers, cell separation and cryopreservation equipment, molecular biology tools for iPSC generation, and single-use controlled-environment systems for autologous cell production. These platforms address the challenges of manufacturing cell products in greater numbers while maintaining process robustness and product quality.

Keywords Allogeneic, Autologous, CAR-T, Cell therapy, iPSCs, Mesenchymal stem cells

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1 Introduction

Cell therapy is the practice of using living cells, either from a donor (allogeneic) or from the patient (autologous), as a therapeutic modality. Different cell types and modes of action are used in cell therapy, ranging from allogeneic mesenchymal stem cells (MSCs) that are delivered intravenously or intramuscularly to treat stroke or peripheral artery disease, to autologous genetically engineered immune cells delivered intravenously to eliminate cancer, to donor-derived induced pluripotent stem cells (iPSCs) differentiated into insulin-producing cells which are encapsulated and injected subcutaneously to treat diabetes. Although there is great promise in cell therapy and the related field of tissue engineering, manufacturing the required cells can be daunting. This new therapeutic modality is not only complex to manufacture but the cellular product is often more sensitive to ostensibly minor process changes or variations, which may result in an ineffective therapy. A deep understanding of cell biology, clinical mode of action, and process and manufacturing considerations are all critical for success. A significant amount of R&D and process development, as well as choosing the appropriate commercially relevant manufacturing platform are imperative for success. In this chapter we discuss challenges and potential solutions in the area of cell therapy manufacturing and how these therapies can be made available to the patients that need them.

The focus of this chapter is on three manufacturing/therapy modalities which are currently leading the field and are distinct in terms of their manufacturing methods and challenges. These are allogeneic cell therapies, autologous cell therapies, and induced pluripotent stem cell (iPSC)-based therapies.

2 Allogeneic Cell Manufacturing

2.1 Introduction

Multiple cell types are used as allogeneic therapies, including mesenchymal stem cells (MSCs), hematopoietic stem cells, iPSCs, and cancer cells. These cell types are used with the aim of treating a variety of clinical indications including cardiovascular disease, neurodegenerative disease, diabetes, autoimmune diseases, graft-versus-host disease, and tissue replacement, to name a few. A fundamental risk of allogeneic cell therapy is the potential to elicit an immune reaction which could destroy the donor cells, thus rendering them ineffective. However, there are some ways to overcome this risk; for example, by using cell types that are hypoimmune (i.e., do not elicit a significant immune response in the recipient), such as MSCs, and by encapsulating cells in such a way that protects them from the host immune system.

From a manufacturing perspective, the allogeneic approach holds significant advantages such as the ability to scale up manufacturing to reduce therapy cost, the ability to choose the donors with highest cell potency, and the ability to have an off-the-shelf frozen therapy that can be administered at any time to an incoming patient. However, the manufacture of allogeneic cell therapies (as with other CT modalities) is not simple and is still evolving. The main considerations for manufacturing are to achieve high quality cells (potent) in sufficient quantities to treat eventually millions of patients and at a cost per dose that is sustainable for a specific indication. In this section we review and discuss the following two main manufacturing paradigms: 2D manufacture and 3D bioreactor manufacturing. We discuss the advantages and disadvantages of each, as well as considerations regarding downstream processing and facility design.

2.2 2D Manufacturing

The standard method of growing cells in academic labs is in 2D plastic flasks. This method of growing both adherent and non-adherent cells, although differing from the natural *in vivo* environment, is well-accepted and generates sufficient cells for most lab uses, such as biological assays and small animal experiments. As early cell therapies were mostly developed in translational academic labs, this meant that early cell therapy products were developed in 2D platforms. However, because small culture flasks, such as T-75 and T-175, are too small to produce a sufficient number of cells for even small clinical trials, these cell culture methods were scaled up.

The 2D flask concept was expanded into 10-layer and 40-layer Nunc[®] Cell Factory systems, which have surface areas much larger than T-175 flasks (36- and 144-fold larger surface area per vessel, respectively). An additional improvement to the 2D culture method was the introduction of the Hyperstack[®] Cell Culture Vessel (Corning[®], Tewksbury, MA), which incorporated a gas permeable surface, eliminating the need for headspace and allowing an incremental improvement in



Fig. 1 2D platforms; Nunc[®] 10 layer Cell Factory (*left*), Corning[®] 32 layer Hyperstack[®] (*center*) and CellCube[®] (*right*)

efficiency. Ten-layer cell factory processing and manipulation is almost entirely manual, whereas 40-layer Cell Factory systems, because of their size and weight, are partially manipulated for fluid exchange and detachment (Thermo Fisher Scientific, Waltham, MA). There have also been attempts to design large-scale 2D manufacturing solutions, products such as the CellCube[®] Module (Corning[®], Corning NY) and the Xpansion[®] Multiplate Bioreactor System (Pall Corporation, Port Washington, New York), which are similar to cell factories and Hyperstacks but include perfusion capabilities and limited environmental control (Fig. 1). Several of these platforms are used today to produce cell therapies for clinical trials.

The manufacturing process using a 2D platform varies between different cell types, but usually involves seeding and passaging of cells with a seed train involving increasing numbers of 2D culture units which are kept in 5% CO₂ incubators at 37°C. Media are exchanged by removing the 2D vessel from the incubator, placing in a grade A clean space (e.g., laminar flow hood), and manually replacing the media. The timing of this media exchange is typically based on a predetermined interval, although the timing of passage and harvest for adherent cells is usually determined based on percent confluence of the cells on the surface as observed under a microscope (although only one of the cell factory layers can be viewed). At the end of the expansion process, cells are manually removed from the multiple vessels that constitute the batch and are pooled for downstream processing.

In general, scaling up 2D platforms is both fundamentally inefficient and logistically impractical. At the fundamental level, 2D expansion is relatively inefficient in terms of cell growth surface area to vessel-volume ratio. It is also not cost effective because the only way to produce more cells is to purchase more cell factories, cost increases linearly with manufacturing scale, undermining the primary economic motivation of large-scale manufacture (economies of scale). In addition, of course, the general lack of pH/DO (dissolved oxygen) monitoring or environmental control makes it difficult to envision using these platforms to grow more sophisticated cell therapy products.

The logistical 2D challenges, however, are more acute. Large-scale 2D cell manufacturing requires large GMP clean rooms equipped with many biosafety cabinets and incubators, which are expensive to build and maintain. The culture vessels need to be manipulated primarily manually using open processes, requiring large teams of highly-trained workers who can be difficult to find and retain. For these reasons, these platforms are limited in their ability to meet allogeneic manufacturing requirements in terms of quantity and cost. Using current methods, the size of a

2D-based manufacturing lot is capped at approximately 100 10-layer cell factories per batch, primarily because of clean room space and downstream processing time [1].

2.3 Bioreactor-Based Manufacturing of Adherent and Non-adherent Cells

Because bioreactors address many of the shortcomings of 2D manufacturing, their use in allogeneic cell therapy manufacturing is becoming increasingly commonplace. There are many bioreactors available and each has advantages and limitations for specific cell types and processes. Some of the more common bioreactor configurations are stirred-tank, packed-bed, and rocker-based. These bioreactors are manufactured by multiple companies at various scales, ranging from 250 to 2,000 L and above. In most cases the vessels used are single-use and are available in both bag configuration as well as hard plastic. Bioreactors can be used to culture single cells, cell aggregates, and adherent cells on different commercially available microcarriers.

The primary advantage of using bioreactors is increased efficiency in terms of the number of cells obtained from a given vessel volume (up to 80-fold increase over 2D). For example, the proposed floor space required to build a 2D manufacturing suite capable of producing batches of one trillion cells is nearly ten times as big as a comparable 3D suite (Fig. 2). Additional advantages include a reduced need for

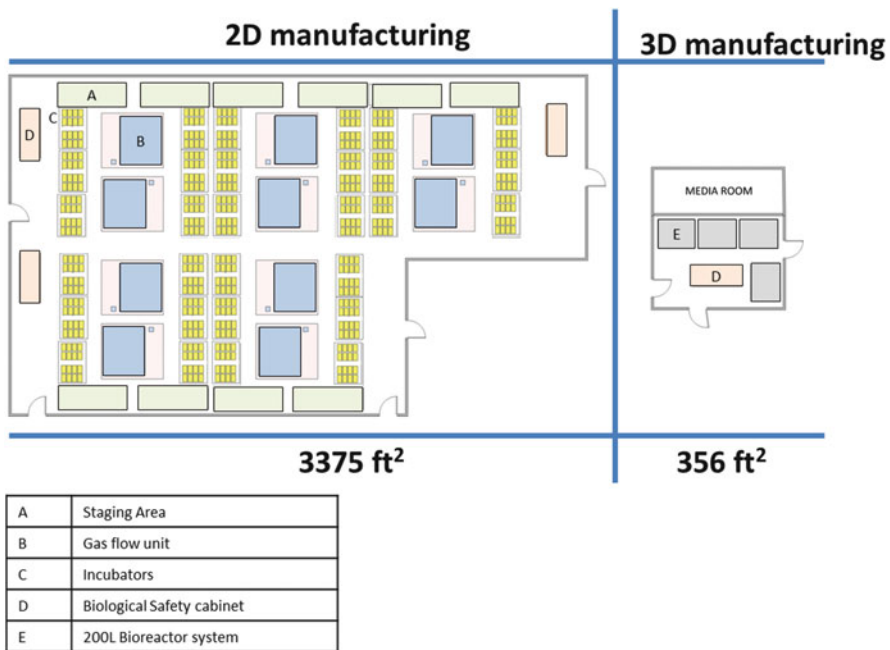


Fig. 2 Manufacturing footprint necessary for a batch size of one trillion cells; 2D vs 3D

clean room and incubator space, closed processing, automation, and environmental control (temperature, DO, pH, etc.). As opposed to a flask or cell factory, the interior of a bioreactor is easily accessible to probes and sampling lines which allow the user to monitor closely the cells and their environment [2]. In addition, media exchange, whether performed in batch mode or via perfusion, can also be automated. Finally, bioreactors are inherently scalable; large-scale reactors with volumes in the hundreds and thousands of liters and are relatively commonplace in the manufacturing of biotechnology products such as proteins, biologic drugs, and viruses.

The disadvantages of bioreactors are that they are expensive to purchase, they require skilled personnel to set up and monitor, and in some cases can introduce undesired fluid shear stress on cells. One of the main considerations when using a dynamic bioreactor culture, as opposed to a static 2D culture, is agitation and shear. Although cell expansion occurs in the bioreactor, earlier process steps such as isolation and seed train operation are often still done in 2D. As an example, 2D culture is generally required for isolating MSCs, which are selected based on plastic adherence, and one or two 10-layer Nunc[®] Cell Factory systems are needed to expand enough cells to seed a 50-L bioreactor [3]. However, it is certainly possible to develop bioreactor-based seed trains in which cells are cultured in successively larger bioreactors, either by harvesting and re-seeding cells, or by transferring microcarriers to larger reactors and adding new carriers and media. It is also possible to seed frozen cells directly into a bioreactor from an intermediate cell bank.

In some cases, cell therapy companies initiate clinical trials with cells from a 2D culture, and then wish to move to 3D bioreactor culture to supply the required cell numbers. In these cases, biological comparability between cells grown in both systems need to be shown. The effect of suspension culture on the biological activity of cells is a key question to consider before switching from 2D to 3D bioreactor platforms. There are several key differences between 2D and suspension culture, such as shear stress, altered culture conditions, and cell–cell interactions. Because of these differences, cells cultured in these two platforms may have a somewhat divergent biological profile. Particularly for MSC therapies, in which the mechanism of action is largely based on the secretome, changes to culture conditions may change critical attributes of the product. An example is increased secretion of VEGF in response to suspension culture conditions [4, 5].

2.4 Process Variables for Bioreactor Cell Culture

Considerations in developing and implementing a 3D bioreactor culture manufacturing system include:

- Choice of carrier type (adherent cells)

Cell carriers can be primarily classified into static carriers, such as Fibracel[®] carriers used in packed-bed bioreactors, and mobile microcarriers, which move in suspension in both stirred-tank and rocker bioreactors. Microcarriers are usually

several hundred microns in diameter, and are further classified into porous and nonporous microcarriers. Nonporous microcarriers are essentially solid plastic beads upon whose surface the cells adhere, akin to 2D surfaces. And as with 2D surfaces, these microcarriers are often coated with materials that promote adherence. Unlike 2D cell culture, however, in microcarrier cell culture the cells can form bridges between microcarriers, leading to microcarrier aggregation. Also, unlike 2D cell culture, the cell is in a constant state of motion and subject to shear stress that could potentially stress it, dislodge it from its surface, or change its biological behavior. Porous microcarriers offer cells additional surface area for attachment, and some degree of protection from shear forces in the bioreactor. Independent of the porous/nonporous classification, microcarriers can be made from either degradable or non-degradable materials, which primarily affects downstream processing.

- Choice of system and vessel (stir tank, packed bed, wave)

Stirred-tank reactors are characterized primarily by their central impeller, which keeps the bioreactor fluid in motion. This keeps microcarriers in suspension and promotes the even diffusion of gases and nutrients throughout the reactor. Packed-bed reactors are a subset of stirred-tank reactors. With the cells confined to a packed bed, media perfusion is relatively easy, although cell sampling and harvesting is more difficult. WAVE bioreactor systems are cheap to produce and scale up, and are good for suspension culture. However, perfusion and monitoring is more difficult, and the rocking motion of a wave reactor is insufficient to suspend microcarriers.

- Media feeding strategy

Media changes in bioreactors are usually done either through nutrient addition, total or partial media replacement, or perfusion. If a cell culture produces low and non-damaging levels of waste products, concentrated levels of nutrients (e.g., glucose) can be added over time to feed the growing culture. Waste metabolites such as lactate and ammonia often begin to accumulate, and either media replacement or perfusion is required. In stirred-tank reactors, media replacement is done by allowing carriers to settle, pumping media out and pumping it back in. This can be a lengthy process on a large scale, and also results in abrupt changes to the cells' environment. Perfusion, in which fresh media is gradually fed in as old media is gradually fed out, is the ideal way to feed/drain and still maintain a stable environment. Perfusion rate can be set based on a theoretical rate of nutrient consumption per cell, or based on real-time process variables (e.g., glucose concentration).

- In-process sampling strategy

Bioreactor samples consist of cells, the reactor media, or both. Bioreactor media are accessible in all bioreactor configurations, but in packed-bed bioreactors the cells are usually inaccessible for sampling. Cell samples are taken for tracking the reactor cell concentration over time, and sometimes for imaging and characterization (e.g., bioreactor processes which include differentiation). Bioreactor media are analyzed for metabolite concentration, both as an in-process control and for setting the perfusion rate. However, sampling the bioreactor has certain drawbacks. First, to get a representative bioreactor sample, an operator often needs to take a large volume, which can impact on the final yield. Sampling needs to be performed at least once a day, and processing of

sampled material can be time-consuming. Finally, repeated sampling can increase the risk of contaminating the bioreactor. For these reasons, the industry is trending away from sampling and toward in-process analytics, which can replace sampling. Examples include biomass probes which correlate with cell density and spectroscopy probes (Raman, NIR), which can relay the real-time concentration of multiple metabolites.

- Method of oxygen delivery

The goal of oxygen delivery is to keep up with cellular oxygen demand. Oxygen can be fed into a bioreactor system either via the headspace above the liquid or via a sparging tube at the bottom which bubbles gas up through the bioreactor. The volumetric mass transfer coefficient k_La describes the efficiency of oxygen transfer in a system, and is a function of vessel size/volume, media, temperature, agitation speed, and oxygen delivery (headspace, sparging, or both). The k_La of a given combination of conditions can be measured without cells, and that data can be used to choose an optimal set of conditions for cell culture.

- Optimization of seeding and agitation

Bioreactor agitation rates are sometimes set based on k_La measurements, as mentioned above, but are also optimized to keep microcarriers in suspension. Bioreactor seeding can be done either under agitation, without agitation, or with a combination thereof. Agitation is important to distribute the seeded cells evenly, but too much agitation kills off a large number of them. Protocols that work for one cell type can be used as a starting point for developing a protocol for a new cell type, but the work must largely be done empirically.

- Determination of optimal DO and pH levels

Given the level of control bioreactors have over DO and pH levels, it is important to test the effect of both on the cells. These tests should not be conducted in 2D because in 2D an initial DO or pH level generally drifts in one direction over the course of culture. In bioreactors, a PID loop is used to maintain DO or pH at a given set point. It is important to find not only the optimal set point but also the boundaries within which the cells are unaffected.

2.5 Downstream Methods and Challenges

Downstream processes for allogeneic products include cell harvesting, washing, concentrating, formulating, final fill finishing into vials or bags, verification of lack of visible particulates, and finally cryopreservation. Although each of these process steps in itself can be considered straightforward, the nature of the cell product combined with all these steps makes the downstream process quite complex. Two main considerations for downstream processing of allogeneic cell therapies are the time window, which is limited because the cells are adversely affected by prolonged periods in a suboptimal environment (temperature, nutrients, substrate, etc.) and that the current methods for conducting the multiple downstream unit operations for large numbers of cells are not fully developed and available. A



Fig. 3 (Left) TFF system for cell therapy downstream processing (source: Lonza Walkersville Inc.). (Right) Sep continuous centrifugation systems

discussion of some of the considerations for each of the downstream unit operations follows.

- Cell harvesting

Harvest methods differ depending on cell culture method (single cells, cell aggregates, cells attached to microcarriers). Adherent cells on plastic microcarriers need to be detached from the substrate using enzymes in a manner akin to 2D cell harvest, although the cells then need to be separated from the plastic carriers. Cells grown on degradable microcarriers are harvested by dissolving the microcarriers themselves, leaving behind a cell suspension which is then collected for further processing. Cell aggregates might need to be dissociated using various enzymes as well. Because enzymes are often used in the harvesting step, it is critical to have a good understanding of their effect on cell viability, and whether they cleave surface markers critical to the product's function or identity.

- Concentration and washing

Washing is required to remove enzymes, fetal bovine serum (FBS), and other unwanted residuals; cells need to be washed in a way that does not harm them. Cells need to be concentrated to bring them up to the therapeutic dose density. This will vary primarily based on the route of delivery (e.g., I.V., I.M.), but typical densities include $10\text{--}20 \times 10^6$ cells/mL. Use of a standard centrifuge is an option, although this is a volume-limited manual open process that very quickly becomes unfeasible at large scale. Two available technologies are tangential flow filtration (TFF) and continuous centrifugation (Fig. 3). In TFF, cells pass over a filter, the medium passes through, and the cells are retained. This can be done both to concentrate and to wash the cells. The downside is that the cells are required to circulate repeatedly over the filter, and this can kill cells and introduce particles. In continuous centrifugation the cells are pumped into a flow chamber which suspends cells using centrifugal force and allows the medium to flow out; this can also be done both to concentrate and to wash.

This is the principle of the kSep[®] system (kSep, Morrisville, NC). This step can be challenging for larger volumes or higher numbers of cells.

- **Formulation**

Cells must be formulated with the appropriate excipients, including appropriate cryopreservant fluid to prevent damage when freezing cells. In many cases DMSO at 5–10% is used for this purpose, although there is also a trend toward DMSO-free cryopreservants. Human serum albumin is also a common ingredient in cryopreservation media, used as a regulatory-approved replacement for FBS.

- **Final fill finish**

Homogeneous delivery of cells to bags or vials is also a challenge. At high concentrations, cell solutions become sticky and cell aggregation occurs. There are currently no real robust turnkey solutions for this processing step, which means that it can be a difficult matter. The main risks are inaccurate dosing and excessive hold time. Cells need to be homogeneously maintained in solution and a system that allows sufficient bagging or vialing speed must be used. Bags or vials also need to be appropriate for cryopreservation, in most cases in liquid nitrogen.

- **Visual test**

As per regulatory requirements, each dose must be “essentially free” of visible particulates (50–100 μm). Therefore each vial/bag needs to be visually inspected by a human, usually using a black and white background for observation, to ensure that no such visible particles (e.g., cellulose, plastic, metal) are present in the dose. Automated systems that are capable of such visual analysis do not yet exist.

- **Cryopreservation**

Cryopreservation, typically at -196°C in liquid nitrogen or nitrogen gas, is accomplished using a controlled rate freezer (CRF) to allow optimal freezing of cells. A CRF has the flexibility to run custom protocols to optimize the freezing protocol for any cell type.

All of these downstream steps need to happen within a given time window, which may vary between cell types but usually does not exceed 6–8 h to minimize cell damage. In addition, the ability to conduct some unit operations for large numbers of cells or doses is extremely challenging because of the lack of automated and scalable hardware.

2.6 Summary: Allogeneic

Allogeneic cell therapies, with their potential for scaling-up, off-the-shelf availability, and reduction in CoGS, represent an extremely promising approach to a

wide host of diseases and conditions. However, current methods of manufacturing need to evolve and the correct approaches must be adopted by translational researchers and pharmaceutical companies to enable future commercial manufacturing at scale. Clearly, the use of 3D bioreactor scalable platforms for manufacturing is a central aspect in this move toward commercialization. However, hand-in-hand development of downstream and point-of-care delivery methods must be developed. A thorough understanding of critical quality attributes (CQAs) of the cell therapy, mode of action, and commercial goals (dose, patient number), and matching these as early as possible to the appropriate manufacturing methods, are absolutely critical for success. Moreover, one must consider that changes to the manufacturing process, once in the clinic, especially a move to a different manufacturing platform (e.g., 2D to 3D bioreactors), are a significant hurdle that may pose comparability issues.

3 Autologous Cell Manufacturing

3.1 Introduction

Autologous cell therapies utilize multiple cell types (e.g., Mesenchymal Stem Cells (MSC), Natural Killer (NK) cells, Dendritic Cells (DC), T cells, hematopoietic stem cells, and myoblasts) to treat a variety of clinical indices [6, 7]. Although the following is not an all-inclusive list, MSCs have been utilized to improve liver function, regenerate tissue, and regulate immune response, and have been shown to be effective in treating liver cirrhosis, liver failure, multiple sclerosis (MS), osteoarthritis, and Graft vs Host Disease [8–13]. NK cells are used in various autologous immune enhancement therapies to regulate cellular immune responses against malignant tumors and treat recurring gliomas, breast cancer, and other types of cancerous tumors [14–16]. Dendritic cells pulsed with tumor-associated antigens (TAAs), thus becoming antigen-presenting cells (APC), are able to target and kill specific tumors through cell-mediated cytotoxicity by stimulating an antitumor immune response. There are also several dendritic cell (DC) studies to determine the effectiveness of using DCs as a vaccine against various types of cancers and other autoimmune diseases, such as Type 1 diabetes [17]. In addition, there have been many recent successful treatments of critical patients suffering from advanced leukemia using autologous CAR-T cells [7, 18–22], which have increased demand for such therapies across the world.

3.2 Autologous Processes: Isolation, Expansion

The majority of autologous immunotherapies are first investigated at a small research scale for a select few individuals (less than 20) with less than 500 mL of culture per patient. Thus, many autologous procedures involve steps to isolate, modify, activate, expand, harvest, and test cells in traditional 2D tissue culture vessels, such as well plates, dishes, flasks, or Nunc[®] Cell Factory systems, with manufacturing timelines ranging from 1 to 3 weeks.

Current CAR-T manufacturing is labor intensive, requiring a large number of manual, open process steps [23, 24] including Ficoll gradient cell separation, cell activation, vector introduction (which can be viral or non-viral), cell expansion of target cell types, optional removal of undesirable cell types, and finally harvest. MSCs used in autologous cell therapies are typically derived from three main sources: fresh bone marrow of a patient's Iliac crest (BM-MSCs), adipose tissue through liposuction (AT-MSCs), or previously cryopreserved umbilical cord (UBC-MSCs). BM-MSCs and UBC-MSCs are commonly isolated using density gradient separation (e.g., Ficoll separation), whereas isolation of AT-MSCs involves the digestion of the fatty tissues, usually in a collagenase digestion procedure. Isolation of cells of interest from peripheral blood mononuclear cells (PBMCs) occurs via density gradient isolation or magnetically labeled antibody selection. In all these processes there are several "open steps," which are labor-intensive, time consuming, and introduce a risk of contamination into the process.

Methods for the *in vitro* expansion of these cells include co-culturing of the cells of interest with an irradiated feeder cell line, the use of protein-rich media formulated with human plasma/platelet lysate or FBS, and cytokine media supplementation. Ideally, an optimized, chemically defined medium specific to the cell process is used to avoid issues with serum and platelet lysate, including lot-to-lot variability, serum availability, quality assurance, and quality control standards. The amount of additional media supplements added to chemically defined media should also be limited, as many autologous cell processes require the use of costly cytokines, activation agents, and growth hormones. Determining the optimized media and feed strategy in the early stages of a product's life cycle streamlines future scaling up or scaling out processes for commercial manufacturing.

3.3 Challenges for Commercializing Autologous Cell Therapies

Although there are some closed and even automated cell expansion systems used in autologous cell therapies, which are discussed later in this chapter, the activation and expansion of many autologous cell processes occur in standard, open tissue culture vessels, including well plates, cell culture dishes, T-flasks, cell factories,

cell bags, and other gas-permeable rapid expansion cultureware such as G-Rex™ vessels (Wilson Wolf, St Paul, MN). Depending on the therapy, a few of these lab-scale vessels are adequate to provide over one billion cells per batch of the population of interest. For example, after approximately 2–3 weeks, the G-Rex system is able to produce up to 2×10^9 viable donor-derived virus-directed cytotoxic T lymphocytes targeted against the Epstein–Barr virus (EBV-CTLs) from 1×10^7 PBMCs [25]. However, these systems require multiple manual manipulations during the inoculation, activation, feeding, splitting, washing, and harvesting steps of a process. With each step in these systems, the risk of contamination and the overall costs associated with labor, lab usage, and materials increase.

As the number of patients requiring treatment increases, the overall autologous batch size for each patient is not expected to exceed more than a few liters because of the limited amount of starting material obtained from a patient to initiate the therapy and the time sensitivity of the cells to retain their optimal levels of functionality and efficiency. Thus, scaling up autologous immunotherapy processes to larger batch sizes is typically not needed and scaling out the process for multiple batches (i.e., one patient per batch) at a given time requires a thorough assessment of space, labor, and financial abilities and restraints. Many small-scale vessels (e.g., flasks, dishes, plates, cell factories, etc.) are limited by the available clean room area needed, and costs associated with required labor, increased materials, and risks of contamination or lot failure. In addition, the need to segregate strictly between patient materials requires extensive cleaning procedures between patient samples when common equipment is used. Therefore, when considering the best strategy for increasing throughput of patients treated as well as dose size per batch, the answer likely lies not in the use of larger vessels but rather in closing and automating manufacture. In addition to being labor intensive, 2D manufacturing methods are not robust and present a high risk of product contamination because of both the length of culture time and the number of manual washes, feeds, and cell manipulations that occur within the manufacturing process.

In addition to contamination risks, current autologous cell manufacturing methods are also cost-prohibitive for all but a select few patients [21, 26]. One of the main reasons for this high cost is that the production of engineered autologous cells for therapy differs significantly from traditional manufacturing of biological products such as monoclonal antibodies or recombinant proteins. Traditional commercial biological manufacturing models center on process scale-up such that a single but larger batch of product can be packaged and shipped to treat many patients [26]. This scale-up allows for a decrease in manufacturing cost through economies of scale.

However, in the case of autologous cell therapy, the product is only manufactured for a single patient with a limited number of doses, making the requirements for the commercial manufacturing of these cell products out-scalable, not up-scalable. Therefore, the concept of increasing the batch size to allow for increased financial efficiency via reduction in cost/unit of product does not apply [27]. Thus, to make these life-saving therapies universally available, other

cost-saving methods must be explored. Switching manufacturing from a manual process to an automated process can allow much-needed cost reduction [6, 24, 27]. Automation would allow preprogrammed bioreactor systems to perform the necessary steps of manufacturing with minimal labor input. This would greatly reduce labor costs by reducing both the number of personnel needed and the space and time required to manufacture a dose.

Autologous therapies must also be extremely robust. Patients seeking autologous therapies have often exhausted all traditional therapies, thus leaving autologous therapy as a last option for successful disease treatment. As these powerful therapies become more mainstream and thus more widely available, they are likely to become viable treatment options for patients during earlier phases of their disease. In both instances, because cells being produced as autologous therapies are being manufactured “on demand” for patients in need, there is little margin for error. A contamination or mistake in the culture process means that a patient in critical need has to both donate more cells for manufacture and wait longer to receive treatment. Moreover, there is variation in starting material based on biological and clinical differences between patients. The ability to have a robust process increases the chances of manufacturing a high quality and potent therapy for each individual patient. Clearly, an automated and controlled process is desirable from both a clinical and a cost perspective. An automated process would allow for consistent execution of process steps, resulting in decreased process-to-process variation [6]. Additionally, it follows that automation would be paired with a closed system, thereby making manufacturing both more affordable and more robust.

3.4 Commercialization Solutions for Autologous Cell Therapies

3.4.1 Streamlining: Closed Systems, Disposable Components

One method to reduce the risk of contamination of autologous cells is shifting as much of the procedure as possible from an open process to a closed system. A closed system is a sterile environment where multiple steps of a manufacturing process can occur without disrupting the sterile environment. The number and type of steps which can be transferred to a closed system is determined by the culture system and by the components being used.

There are a few devices used to help decrease the risks associated with the isolation of cells, by performing the process steps in a closed system, as opposed to “open” processes in which a sample is handled in vessels which have removable lids/caps. The Pall Purecell Select™ system is a closed system isolation method designed to isolate mononuclear cells from whole blood through gravity filtration. With this system, a patient’s blood sample is injected by syringe into the input bag, the entire device is suspended from an IV pole or hook, and the sample then passes through a filter which retains cells of interest and removes unwanted cells to waste.

Subsequently, the filter is washed with reverse flow, and the cells of interest are collected into a cell collection bag with an aseptic syringe luer lock adapter. Another example of a closed isolation system is the Miltenyi CliniMACS™. The CliniMACS™ system utilizes magnetic microbeads labeled with various types of antibodies for either positive or negative selection of target cell populations. The Miltenyi Prodigy™ also uses magnetic bead selection and is capable of cell expansion.

One of the simplest closed method systems for cell expansion involves the use of sterile cell expansion bags. Many companies sell cell expansion bags along with weldable tubing and sterile connections that allow cell inoculation, feeding, expansion, and harvesting under sterile conditions. Such systems allow the reduction in the number of open process steps, thereby reducing contamination risk. Cell expansion bags meant for static culturing are typically made of gas permeable materials and can be tissue treated for 2D cultures. Other cell expansion bags may be untreated and non-gas permeable for use in controlled suspension bioreactor culture systems (e.g., GE WAVE). Use of such a system was utilized in 2013 for the manufacture of a CD19 autologous CAR-T cell under Good Manufacturing Practices (GMP) which were used in a Phase I clinical trial treating a pediatric B cell malignancy [28].

Clearly, more advanced culture systems which would allow increased automation and closing of all process unit operations, thus allowing out-scaling, reducing Cost of Good (COGS), limiting manpower needs and clean room space, as well as better monitoring and control over the whole process are desirable. Such culture systems are currently being developed.

In small-scale, scale-out manufacturing, the use of disposable components is preferable. This allows scale-out and minimizes the risk of contamination. Although the use of disposable components would at first glance involve additional cost for the process, the elimination of costs to clean and sterilize components as well as to clean rooms would offset the cost of disposable process components. Furthermore, utilizing disposable components would significantly decrease the downtime of the autologous manufacturing facility, which is one of the most cost-prohibitive aspects of autologous manufacturing.

3.4.2 Biofeedback

Another caveat of autologous cell manufacturing revolves around the patients themselves and the cells which they supply. Patients seeking treatment have likely undergone many alternative treatments and therapies previously. They may have undesirable medication or undesirable cell types in their system when cells are harvested for manufacturing, which may make cell manipulation and/or expansion difficult. The ability to monitor critical process parameters such as culture temperature, pH, dissolved gasses, nutrients, metabolites, confluency, and biomass provides valuable insight into better cell health and more efficient cell growth [26, 29].

There are several offline devices and assays that provide these types of measurements through absorbance and fluorescence measures or conversion of electronic signals from the free flow of ions when presented with a sample of the culture supernatant. However, to reduce the risk of contamination from manual manipulation and sampling of a culture, other methods, such as PreSens[®] microsensor optodes, Lonza CytoSMART[™] Live cell Imaging System, or similar non-invasive culture monitoring devices, are recommended.

A system which incorporates biofeedback based on culture health and other real-time monitored analytes would create a dynamic culture system which could potentially improve expansion and yield of difficult-to-manipulate cells.

3.4.3 Available Systems for Commercializing Autologous Therapies

Autologous cell manufacturing must address three main issues: (1) the ability to scale-out manufacture to be able to treat significant numbers of patients, (2) improved process control and robustness both to minimize risk of contamination and to minimize failure caused by poor performance of cells, and (3) cost reduction of the autologous manufacturing process to make these therapies available to more than just a select few. Because traditional scale-up strategies cannot be utilized to reduce the cost of manufacturing for therapeutic autologous cells [27], other options must be explored. Utilizing an automated and closed bioreactor system would allow scale-out as well as significant reduction both in personnel and facility requirements, translating into cheaper manufacturing costs. Multiple companies are working to develop such a system specifically to address the needs of autologous manufacturing.

The ideal autologous cell manufacturing system would allow multi-step processing sequences that include a maximum of process steps including initial cell isolation (Ficoll, magnetic bead, or adherence), automatic feeding and washing, ability to incorporate downstream processes such as magnetic bead selection and/or electroporation of cells, harvest of cells, and finally concentration of cells. Additionally, a system that includes biosensors coupled with biofeedback-based process adjustment would allow for valuable process control. The ideal autologous manufacturing system would also accommodate multiple cell types, including not only suspension CAR-T cells but also adherent cell types such as mesenchymal stem cells and dendritic cells. Finally, a method to quantify cell concentration and/or confluence such as via an in-system camera would be very beneficial to gain valuable insight into cultures without repeated disruption through sampling or removing the culture chamber or bag for visualization under a microscope.

Two systems which incorporate many of the desired components of the future autologous cell manufacturing system are the CliniMacs Prodigy[®] by Miltenyi and the Cocoon[™] system by Octane Biotech Inc. The CliniMacs Prodigy[®] features a closed system capable of automatic cell separation via density gradient, cell washing, positive and negative cell selection using magnetic beads, expansion of suspension cells, and final cell concentration [30]. The CliniMacs Prodigy[®] system

has several fixed components of the system such as the fractionation chamber and magnetic separation capabilities. There are multiple tubing sets which have been designed specifically for isolation and expansion of certain cell types. The technician would manually attach the selected GMP tubing set to the system using sterile welding and run a predesigned program to generate the cells of interest. This system incorporates a small microscope for visualization of cells within the system during culture. One drawback of the system is that, although it does incorporate sampling ports for offline biofeedback sampling, it does not incorporate such sampling automatically and throughout the run. Furthermore, although development is underway to translate processes for adherent autologous cell types such as MSCs to the system, traditionally the Prodigy has been limited to suspension cell types only.

A second system which is gaining attention in the automated cell culture realm is the Cocoon™ system by Octane Biotech Inc. This system features a closed and fully automated cell culture system that can successfully culture and expand both adherent and suspension cells. The Cocoon™ system incorporates a small input chamber which can be used both for sample loading and for low volume processes that are volume dependent, such as viral transduction for CAR-T cell generation. After loading or other minimal volume-requiring activities, cells are then automatically transferred to proliferation chambers designed specifically for the cell type being cultured. The Cocoon™ system features inline monitoring and control of fluid oxygen, pH, and CO₂. Feeding, washing, and concentration of cells are accomplished through completely automated software, enabling the technician to invest little to no hands-on time after sample loading until time of harvest. One feature of the Cocoon™ system which makes hands-off perfusion possible is the fact that, in addition to a 37°C culture chamber, the Cocoon™ system also has a 4°C chamber incorporated which allows up to six different media and reagents to be pre-loaded and fed via the preprogrammed expansion protocol. Each protocol can be designed specifically to user specifications, making this technology flexible yet robust.

3.5 Centralized Manufacturing vs Point-of-Care

As autologous cell therapies increase in prevalence, one strategic question that arises is the concept of whether these cells should be manufactured at centralized/regional facilities or whether manufacturing should be shifted to “point-of-care,” meaning the cells would be harvested bedside in the hospital where the patient is being treated, engineered and expanded, and then returned to the patient with all manufacturing occurring at the same facility where the patient is seeking care. Both centralized manufacture and point-of-care manufacture have benefits and drawbacks.

Centralized manufacturing would allow the cell engineering to occur in a state-of-the-art facility designed and constructed specifically for the manufacture of autologous cell therapies. Additionally, the staff that support such a centralized

location would be well-experienced in performing techniques critical for engineering and production of the therapeutic cells and would have full quality control and quality assurance support. One major drawback of centralized manufacturing revolves around the logistics of how the cells themselves would be transported, both from the patient for *ex vivo* manipulation and then back to the patient post-manufacture [29]. Same-day or next-day shipping is a viable option in developed countries such as the United States. However, expedited shipping of cells would not make this therapy available to patients who are in need worldwide because of the limitations of viable shipping options. A second method to address the shipping of cells would be to evaluate cryopreservation of cells for shipment to patients. This could prove to be a viable option; however, additional studies to evaluate the stability and efficacy of cryopreservation for some autologous cell types is necessary [6]. One final consideration is that centralized manufacturing facilities would likely be limited to the number of patients that can be treated at a given time, which could limit the total number of patients treated [6, 7].

The second manufacturing strategy revolves around point-of-care autologous cell engineering. In short, this would make the production of the cells possible in many hospitals worldwide. A key component around which this strategy revolves is the use of a closed, disposable system equipped with automation of a maximum number of process steps. Having a system with disposable components would reduce the downtime of manufacturing by reducing the requirement for cleaning and sterilization of tools. In addition to a reduction of downtime, the facility would not require dedicated space for cleaning and sterilization of reusable system components. Moreover, the use of a closed system would significantly reduce facility needs by allowing open cell manipulations to occur within an approved biosafety cabinet, which would need to be cleaned after use and then utilized for the next patient. Reduction of facility needs would greatly decrease the financial burden on the hospital utilizing the closed system, thus resulting in more hospitals or point-of-care sites capable of performing autologous cell therapy treatments on-site. Finally, a fully-automated system would decrease the need for intensive training of on-site technicians in specialized *ex vivo* autologous cell engineering. This would both decrease costs by decreasing specialized labor requirements and increase the number of sites willing to incorporate these powerful autologous cell therapies into their repertoire of available life-saving therapies, thus making them available to patients within their region.

No matter which manufacturing method is implemented, full sample and product traceability is crucial. There should be multiple fail-safes to ensure the patient sample is properly handled from time of collection through time of patient treatment. Some of the closed systems previously discussed in this chapter, including the CliniMacs Prodigy[®] and Octane Cocoon[™] system, are equipped with barcode scanners that allow the user to link information about all materials used in a process to the batch generated. This is one example of how the equipment used in a process can increase lot traceability across a process, as well as prevent potential user error.

3.6 Regulatory Considerations

One final consideration for the expansion and use of autologous cell therapies is how this therapeutic, cellular product is regulated by the Food and Drug Administration (FDA) and equivalent regulatory agencies worldwide moving forward. Currently, the FDA is responsible for ensuring that autologous cell therapy products are safe, pure, potent, and effective [31]. Historical FDA oversight involved products which are mass produced in large batches or lots of a single product which can be used by multiple patients. A small portion of this lot can be tested for safety, purity, and effectiveness, leaving a large portion of the product available for treatments. Autologous cell therapies by definition are problematic under current regulatory definitions (21 CFR 1271 [31]) for a variety of reasons, one of which is that variations in the starting material, a specific patient's cells, makes a pre-determined standard of purity of final cellular product defined by frequency of specific cellular markers difficult because of inherent variations in these markers among different patients [7]. Furthermore, quantifying a baseline for potency of therapeutic autologous cells is also difficult because the cells themselves are dividing and growing rather than remaining static [32]. Specifics of how certain aspects of Phase I, II, and III clinical trials for certain autologous therapies would be accomplished given the limited number of starting materials and the difficulty with administering an effective placebo further complicate the regulatory situation of autologous cell therapies [6]. As this powerful and quickly-developing therapy modality moves forward, it is clear that one size does not fit all with regard to regulation of all cell therapy products.

3.7 Summary

Autologous cell therapies hold much promise for successful treatment of many diseases and conditions including multiple forms of cancer. To make these life-saving therapies accessible to all patients in need, the processes must become more standardized, robust, and cost efficient. One method of achieving these goals is to develop autologous cell therapy processes with the following attributes:

- (1) A closed system, with limited/no open process steps from initial inoculation through final formulation
- (2) An automated process to increase process robustness and limit the risk of human error in a process with limited starting materials
- (3) Incorporated dynamic biofeedback
- (4) Disposable system components
- (5) Cost effectiveness

Finally, it is also important to assess the benefits and disadvantages of manufacturing any cell therapy product in either centralized or point-of-care

facilities. Although centralized facilities have dedicated space and staff focused on delivery of a high quality product, complications surrounding delivery of both the initial tissue sample and final cell product may arise. Moreover, manufacturing cell products at the point-of-care may increase the risk of human error in the handling, documentation, or process troubleshooting for the product.

4 Induced Pluripotent Stem Cell (iPSC) Manufacturing

4.1 Introduction

The isolation of human embryonic stem cells (hESCs) from the inner cell mass of 8-day-old blastocysts [33] introduced the concept of pluripotency (i.e., the ability of cultured cells to form all cell types of the body that are derived from ectoderm, endoderm, or mesodermal lineages). This remarkable accomplishment dramatically changed the fields of developmental biology, in vitro differentiation, and regenerative medicine. In 2007, Dr. Shinya Yamanaka successfully converted adult human cells to induced pluripotent stem cells (iPSCs) [34]. The iPSCs have similar characteristics to embryonic stem cells (ESCs) and by definition have the ability to self-renew indefinitely and become any cell type in the body. Initially, retroviruses expressing four transcription factors (Oct3/4, Sox2, Klf4, c-Myc) were used in the reprogramming process, which was readily replicated worldwide and improved upon by numerous investigators. Similar to ESCs, human iPSCs are pluripotent and can be readily derived from any individual. iPSCs have become an important scientific tool and are spurring advancements in basic research, disease modeling, drug development, and regenerative medicine. Equally important, this discovery unlocked many new opportunities for using iPSCs in both allogeneic and autologous cell therapy applications. iPSC-based therapy is a newly developing field and builds on several key technical advances that have enabled the widespread use of embryonic stem cell (ESC)-based technology [35–38] for drug discovery and basic biology. Companies such as Geron, Asteris, Ocata (formerly known as Advanced Cell Technology), Biotime, Viacyte, and Johnson & Johnson have developed products from ESC and several have initiated early-stage clinical trials [39], and several patients have been treated with no deleterious side effects [40]. These results have led companies such as HealiOS and Megakaryon to initiate plans to generate products using iPSCs. Recently, a study involving one patient treated with retinal pigment epithelium (RPE) cells derived from iPSCs was carried out using cells manufactured in a cGMP environment using autologous cells (<http://stemcellstm.alphamedpress.org/site/misc/News159.xhtml>). The huge potential of iPSCs for therapeutic purposes stems from the fact that differentiated cells (from blood or skin) can be taken from a donor, turned into iPSC, expanded as needed, and then differentiated into the required cell type. This means that a future in which tissue replacement (e.g., cardiomyocyte replacement after acute myocardial infarction) or

even organ replacement (e.g., kidney replacement) can be facilitated by use of this method. The cells utilized can be either from the patients themselves or from a donor. The advantage of using a patient's own cells is that there is no risk of immune rejection, but a disadvantage is an extremely expensive and not off-the-shelf therapy. An advantage of securing cells from a donor is that this is a less expensive approach which is potentially also off-the-shelf but requires lifelong immunosuppression for the patient (similar to donor organ transplantation).

Here we briefly highlight some of the key considerations regarding the manufacture of iPSCs, differentiation of iPSCs into cell therapy products, and characterization of iPSCs and their derivatives during the manufacturing process.

4.2 *iPSC Generation*

Initially, iPSCs were generated through reprogramming with retrovirus constructs [34, 41] which permanently integrated into the cell genome. This method is not preferred for clinical cell therapy applications. Moreover, these cells were usually generated and expanded using a feeder layer system which has lot-to-lot variability, regulatory and safety concerns, and scalability issues. Later, alternative reprogramming methods were established including: (1) non-integrating SeV reprogramming where Sendai-viral particles were used to transfect the target cells with replication-competent RNAs that encode the original set of reprogramming factors (OCT4, SOX2, KLF4 and cMYC), (2) non-integrating episomal-based reprogramming using plasmids (e.g., Epstein–Barr virus-based episomal plasmid DNA replication system) encoding reprogramming factors OCT4, SOX2, KLF4, LMYC, and LIN28A in combination with different enhancers (e.g., P53 knock-down (shP53)), and (3) mRNA reprogramming where the cells are transfected with in vitro-transcribed mRNAs encoding OCT4, SOX2, KLF4, and cMYC with additional reprogramming factor LIN28A [42–44]. The main factors used to compare each of these reprogramming methods are safety, efficiency, cell line stability, reliability, and ease of establishing a GMP-compliant process [42]. SeV reprogramming, although efficient and reliable, lacks GMP compatibility because a cGMP grade reprogramming reagent is not available. In comparison with SeV reprogramming, episomal-based reprogramming is an integration-free, reliable, and cGMP-compliant method that can be used for different starting materials (bloods cells and fibroblasts). RNA-based reprogramming has been shown to be fast, highly efficient, and have zero footprint. However, this method suffers from difficulty in successful reprogramming of fibroblast cells and, most importantly, insufficient reproducibility by different groups. Although the method of derivation, starting materials, and morphology of iPSCs can be different, a method-specific difference in the quality of iPSC lines with respect to marker expression profiles, differentiation capacity, DNA methylation, or genetic instability has not been observed [42].

As highlighted by Daley and colleagues [42], there are currently safer alternative reprogramming methods compared to the original viral transfection, but the choice

of reprogramming method depends on the specific applications or requirements of each research lab. As the field of pluripotent stem cells is rapidly growing and further methods and technologies are evolving (e.g., using a gene-free, small molecule-based reprogramming method), it is important to shift the focus to establishing methods to manufacture clinical quantities of pluripotent stem cell-derived products. We have recently reported the development of a robust, reproducible, and cGMP-compliant manufacturing process to generate clinical-grade iPSCs from cord blood CD34+ cells for use in further manufacturing of therapeutic cellular products [45]. The next section briefly describes some of the main design considerations in establishing this iPSC manufacturing process [46].

4.3 iPSC Manufacturing Process Design Consideration

The use of non-integrating plasmid DNA to carry the reprogramming transcription factors into the somatic cells (e.g., CD34+ cells derived from newborn umbilical cord blood or adult peripheral blood mononuclear cells) has been previously reported [43, 44]. However, switching to integration-free methods and potentially clinically-compliant methods to generate cGMP-compliant human iPSCs is often inefficient and technically challenging. To establish a robust and reliable cGMP iPSC manufacturing process, we took three major stages [46], focusing on: (1) establishing an iPSC generation process using a non-integrating episomal-based technology (stage 1.0 – proof of principle), (2) process optimization and protocol development based on the critical attributes of the process (stage 2.0), and (3) tech transfer of the manufacturing process into a cGMP cell therapy suite (stage 3.0).

A number of challenges must be considered in the development of a cGMP iPSC manufacturing process. These challenges include: (1) iPSC derivation challenges (including safety of the reprogramming method, efficiency, donor-to-donor variability, and choice of starting materials), (2) iPSC manufacturing challenges (including development of a cell culture system for generation and expansion of iPSCs, sensitivity and robustness of the iPSCs, cryopreservation, and revival of the iPSCs), and (3) safety and QC challenges (including standard safety concerns such as sterility, normal karyotype, residual plasmid clearance, in-process controls to evaluate the quality of iPSCs, and critical attributes of the final iPSC products). Other challenges are labeling and packaging, storage and warehousing of the final product, facilities, human resources, and training. Equipment and utilities requirements should also be considered during the design considerations for developing a cGMP manufacturing process. Finally, regulatory issues applicable to the tissue acquisition and iPSC manufacturing and testing need to be carefully evaluated from the early stages of the process [45, 46].

4.4 iPSC Directed Differentiation Processes

From the cell therapy applications perspective, human pluripotent stem cells, including iPSCs and hESCs, have the potential to be used in allogeneic applications. However, iPSCs are the only source of pluripotent stem cells that can be used for autologous cell therapy applications by taking a patient's own tissue (e.g., peripheral blood or skin biopsy), isolating the appropriate population of cells (peripheral blood mononuclear cells – PBMCs or fibroblast cells from skin biopsy), generating patient-specific iPSCs, and differentiating the iPSCs into specialized cells, which could undergo a gene correction method prior to transplantation into the patient. The concept of autologous iPSC transplantation has been tested in animal models [47, 48], demonstrating the feasibility of this approach. The first clinical trial involving one patient treated with retinal pigment epithelium (RPE) cells derived from iPSCs was recently carried out using cells manufactured in a cGMP environment using autologous cells (http://www.cdb.riken.jp/en/news/2014/researches/0915_3047.html).

Pluripotent stem cells are not directly transplanted into human subjects because of their proliferation and tumorigenic capability. iPSCs must undergo a differentiation process, which is usually stage specific and guided by specific chemicals and cytokines that direct the cells through the differentiation process based on the appropriate signaling pathway identified in research labs. Functional insulin-secreting beta islet cells have been generated from iPSCs using a multistage directed differentiation process [49, 50]. As reviewed recently by Li and colleagues, significant progress has been made toward the differentiation of pluripotent stem cells into highly homogeneous neural progenitors with a larger proportion of mature dopaminergic neurons with improved survival and integration after transplantation [51]. Other diseases have also been targeted by investigating the potential of pluripotent stem cells to generate specialized cells with transplantation capacities [52–55]. The directed differentiation process often requires very accurate control of cell fate in each differentiation stage. Varying parameters, including the type of cell culture system prior to the start of differentiation, induction time at each step of the differentiation, type and concentration of the cytokines or chemicals, differentiation medium composition, mode of culture (2D vs 3D), and physiological conditions (e.g., oxygen concentration) play important roles in the outcome of the differentiation process.

4.5 Characterization of Pluripotent Stem Cells and Their Derivatives

In parallel to developing a process to generate human iPSCs or iPSC-derived products, it is critical to establish an appropriate final product testing platform to evaluate identity, safety, purity, and viability of the final product. However,

establishing a characterization platform for iPSCs or iPSC-derived products may be very challenging, considering the absence of specific guidelines for characterization of these cells. The field of pluripotent stem cells and their application for cell therapy is still emerging, but there are growing efforts to address these unmet needs. We have established a platform for characterizing iPSCs by focusing on the criticality of the assay (i.e., indicating safety, identity, or purity) according to the existing regulatory guidelines for cell therapy products [45, 56]. Importantly, one critical feature of a release assay is the ability to qualify the assay or availability of an existing standard, GMP-compliant quality control assay. The assay qualification is performed according to the current Good Manufacturing Practices, the International Conference on Harmonization Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines [57]. Depending on the nature of the assay, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) are determined during the qualification studies. Aside from standard safety assays, including plasmid clearance, karyotype analysis, sterility, mycoplasma, and endotoxin tests, we have developed and qualified some of the iPSC-specific assays including: (1) flow cytometry to evaluate the expression of four PSC-specific markers (SSEA-4, Tra-1-60, Tra-1-81, and Oct3/4), (2) quantitative PCR for evaluation of residual plasmid clearance used for reprogramming, and (3) cell count and viability. Short Tandem Repeats (STR) have also been incorporated into the release assays to confirm that the final iPSC product matches the initial donor cells used in the reprogramming process. According to FDA regulations, release of allogeneic master cell banks for clinical use requires extensive testing for the presence of viral contaminants. Therefore, master cell bank viral testing needs to be included in the release testing, but the viral testing panel for hiPSCs should be adjusted based on the cellular characteristics of pluripotent stem cells and should be comprised of both *in vitro* and *in vivo* assays [45]. In addition to the release testing, we have also incorporated additional characterization assays (classified as For Information Only (FIO)) in the testing panel for iPSCs, including evaluation of hiPSC colony morphology, plating efficiency of hiPSCs post-thaw, and embryoid body (EB) formation. The EB formation has been used to demonstrate the identity and potency of hiPSCs by investigating spontaneous differentiation into three germ layers (i.e., ectoderm, mesoderm, and endoderm) and evaluating the results through immunofluorescence at the protein level or qPCR analysis at the transcript level. Post-thaw plating efficiency was evaluated based on alkaline phosphatase (AP) staining. AP, a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions, has been widely used for evaluation of undifferentiated pluripotent stem cells including both embryonic stem cells and iPSCs [34, 58–60]. Considering that iPSCs could very likely be used as starting material for derivation of a variety of cell therapy products, an additional subset of analytical methods should be incorporated into a routine testing process to provide data in an unbiased way, such that if collected in a database over time the users would be able to monitor potential variability of critical characteristics of iPSCs. This variability lies in the biological changes associated with the manufacturing process at or after

implantation as they respond to the environment. We propose the use of a transcriptome analysis, a SNP-CHIP/CGH array, and whole genome sequencing as three basic tests to complement the standard tests for pluripotency, differentiation ability, and composition that are routine [56].

In the case of iPSC-derived specialized cells and products, it is crucial to develop two critical assays associated with safety and potency of the final product. Considering that iPSCs have the potential to proliferate almost indefinitely as well as the potential to generate tumors, it is necessary to develop a safety assay to ensure that the iPSCs are eliminated from the final product lot through the directed differentiation process. The assay needs to be sensitive enough to detect very small quantities of iPSCs at the gene level. Moreover, a safety assay must be developed to detect the functionality of the final product developed from iPSCs. For instance, Pagliuca et al. use a glucose stimulated insulin secretion (GSIS) assay to evaluate the functionality of iPSC or hESC-derived beta cells generated in a 3D-differentiation process. This test evaluates the capacity of the PSC-derived beta cells to respond to multiple, sequential high-glucose challenges as well as depolarization with KCl [49].

4.6 Summary

In summary, human pluripotent stem cells and iPSCs, in particular, hold great potential to be used as starting material for derivation of a variety of cell therapy products through directed differentiation processes. The directed differentiation process is usually a stage-specific process requiring tight control of the differentiation process from pluripotent stage into multi-potent and eventually into specialized cells with specific functions. The manufacturing of cGMP-grade iPSCs and their products requires compliance with cGMP regulation and implementation of appropriate in-process controls and final characterization tests to ensure that safe and high quality materials are generated. Recent advances in the development of cGMP manufacturing processes for the generation of clinical quantities of iPSC products as well as the outcome of ongoing clinical trials using PSC-derived products should have a major impact on the commercialization and routine use of iPSC-derived cell therapy applications.

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