HHS Public Access

Author manuscript

Cytotherapy. Author manuscript; available in PMC 2025 July 24.

Published in final edited form as:

Cytotherapy. 2024 November ; 26(11): 1275–1284. doi:10.1016/j.jcyt.2024.05.024.

Bioprocessing considerations for generation of iPSCs intended for clinical application: perspectives from the ISCT Emerging Regenerative Medicine Technology working group

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Abstract

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Approval of induced pluripotent stem cells (iPSCs) for the manufacture of cell therapies to support clinical trials is now becoming realized after 20 years of research and development. In 2022 the International Society for Cell and Gene Therapy (ISCT) established a Working Group on Emerging Regenerative Medicine Technologies, an area in which iPSCs-derived technologies are expected to play a key role. In this article, the Working Group surveys the steps that an end user should consider when generating iPSCs that are stable, well-characterised, pluripotent, and suitable for making differentiated cell types for allogeneic or autologous cell therapies. The objective is to provide the reader with a holistic view of how to achieve high-quality iPSCs from selection of the starting material through to cell banking. Key considerations include: (i) intellectual property licenses; (ii) selection of the raw materials and cell sources for creating iPSC intermediates and master cell banks; (iii) regulatory considerations for reprogramming methods; (iv) options for expansion in 2D vs. 3D cultures; and (v) available technologies and equipment for harvesting, washing, concentration, filling, cryopreservation, and storage. Some key process limitations are highlighted to help drive further improvement and innovation, and includes recommendations to close and automate current open and manual processes.

Keywords

induced pluripotent stem cells; reprogramming; cell manufacturing

Introduction

Induced pluripotent stem cells (iPSCs) are playing a big role in shaping the future of cell and gene therapy industry. The Emerging Regenerative Medicine (ERM) group as part of the International Society for Cell & Gene Therapy (ISCT) Process Development and Manufacturing (PDM) Committee, comprising experts from industry and academia with foundational knowledge in implementation of emerging technologies for use in bioprocessing and manufacturing, has come together to create a high-level, step-by-step guide for the manufacture of iPSC cell banks for use in the development and manufacture of iPSC-based cell and gene therapeutics (Figure 1). From starting material to cell banking, this manuscript provides an overview of considerations, available technology, best practices, and limitations to support end users in their clinical design and development activities.

Intellectual Property Considerations

The iPSC intellectual property (IP) landscape for therapeutic applications is complex, with additional licensing considerations required when moving from research use to use in clinical manufacturing [1]. Since the filing of the first patent on reprogramming by Yamanaka and colleagues in 2006, the number of patents surrounding iPSC technologies have dramatically increased. For example, a study from 2019 estimated that a total of 1516 patent applications for iPSC technologies were filed in the United States, 895 in Japan, and 420 in Europe [2].

For therapeutic sponsors developing an iPSC-based therapy, the process for navigating intellectual property constraints and ensuring freedom to operate (FTO) requires careful consideration and may be lengthy in process. FTO should be considered early in a

development program due to the potential of identifying limitations that may result in needing to pursue alternative technology strategies. For example, a shift in iPSC platform may be needed to de-risk the potential for exorbitant licensing costs or refusal to grant licenses associated with each step in the manufacturing process. Considerations that impact FTO may include: choice of reprogramming factors, methods used to deliver reprogramming factors, choice of somatic donor cell type, culture conditions, method to identify and characterize iPSC, methods to introduce genome editing in iPSC, differentiation method, cell expansion method, therapeutic target, and target geographical location for clinical studies and commercialization [3]. In cases where cells undergo somatic genetic modification, IP considerations related to the gene editing technology, the delivery system and, in specific instances, the resultant gene mutation also must be considered and may be complex. For example, for CRISPR/Cas9 technology the first patents were filed in 2012 by MIT, the Broad Institute, University of Vienna, and the University of California, Berkeley; however, the patent landscape continues to evolve and vary by jurisdiction and application [4]. Finally, FTO and IP considerations will need to be assessed based on the intended use of the method or technology and may require licenses for each stage of development, clinical use, or commercialization.

A comprehensive strategy for iPSC-based therapies should include upfront assessment of current patent landscape and licensing considerations that could hinder a program's progression along the development path [5]. To limit infringement of existing third-party rights to the licensed technology, FTO should be considered relative to a sponsors' intended development strategy, target jurisdiction(s) where the therapeutic is anticipated to be implemented, and at each stage from research through clinical development and commercialization. Multiple licenses will be required to support the entirety of a workflow and clinical program. Enlisting a patent specialist or attorney is recommended to navigate this complex patent landscape effectively.

Starting Material and Raw Materials

Starting material for iPSC generation

The next key consideration when evaluating the use of iPSCs is donor tissue sourcing, also referred to as starting material. Legally, the donor must meet requirements for donation, procurement, and testing in the country where they donate. Where the final iPSC-derived therapeutic product is intended for use in multiple jurisdictions, consideration also needs to be given to differences in regulatory requirements for use of donor tissue in each jurisdiction, as these are not always consistent. Variation will be observed depending on region-specific risk factors of donor origin, such as the presence of adventitious agents. For example, the US places restrictions on material from donors of European origin because of potential risk associated with transmissible spongiform encephalopathies (TSEs). Additionally, certifications, method types, and extent of sensitivity of those tests often also vary across jurisdictions. The EU requires donor test methods to be EU-approved in vitro diagnostics, and the US requires FDA-approved methods. Finding test methods approved across jurisdictions is possible, but the need to include multiple tests might be necessary to ensure sufficient coverage. It is therefore important to keep this in mind early

in the therapeutic development process, and particularly important with tissues intended for allogeneic therapy applications, as iPSC-derived allogeneic therapies may use a single donor for the entirety of the product life cycle. Representative guidances and regulations to support these considerations include:

- Commission Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells (Europe)
- Guidance for Industry Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps) (US)
- FDA 21 CFR 1271 Human Cells, Tissues, and Cellular and Tissue-based Products (US)
- ARGB Appendix 12 - Guidance on TGO 108: Standard for Human Cell or Tissue Products - Donor Screening Requirements (Australia)
- Guidance Document for Cell, Tissue, and Organ Establishments - Safety of Human Cells, Tissues, and Organs for Transplantation (Canada)

In addition to establishing compliance with defined regulations and guidances, developers will also need to comply with unique regional requirements, such as those imposed by institutional review boards (IRBs), Office for Human Research Protection (OHRP) and Stem Cell Research Oversight (SCRO) in the US.

Other common and critical elements of the donor selection process include region-specific donor compensation and informed consent. Donor consent typically includes documented instructions outlining the intended use of the donated tissue, including immortalization, genetic editing, genetic testing (i.e., next generation sequencing), and commercial use; the benefits, if any, to the donor or others; the risks and potential discomfort of the donation; confidentiality of donor information; compensation (if any) and a statement demonstrating voluntary participation of the donor in the program. The consent process is different for autologous and allogeneic therapies. For autologous, eligibility requirements are comparatively minimal provided it is clearly stated that the collection of the blood or blood components is intended solely for autologous use. For example, FDA does not require testing for relevant transfusion-transmitted infections (RTTI) of autologous donations. For allogeneic therapies, additional important aspects of sourcing donor tissue include donor eligibility determination, which is based on the results of donor screening and testing. This activity requires review of the donor medical records along with a physical exam, and clinical laboratory testing for relevant communicable disease agents or diseases depending on tissue type. This may include human immunodeficiency virus, hepatitis B virus, hepatitis C virus, transmissible spongiform encephalopathies, human T-lymphotropic virus, *Treponema pallidum, Chlamydia trachomatis*, and *Neisseria gonorrhoea*, along with other endemic diseases in the country or region of donation, such as West Nile and Zika in the US [6].

Raw material considerations

The selection of raw materials, also referred to ancillary materials, refer to the reagents used in the manufacture of the therapeutic product but not intended to be part of the final product. The strategy for selection of such materials should be driven by a risk-based approach that assesses potential impact on final therapeutic product quality, performance, and safety to the patient [6]. Regulators expect the highest quality grade of raw material to be selected and qualified for clinical manufacture, but starting with highly qualified raw materials compliant to good manufacturing practice (GMP) may not always be possible or available early in development. However, raw material characterization, control, and knowledge is expected to evolve and mature as an understanding of critical process parameters progresses throughout clinical development. The production of iPSCs deemed suitable for clinical manufacture has been demonstrated by an increasing number of iPSC-based therapies receiving clinical trial approvals in multiple jurisdictions. As a result, access to high-quality raw materials for manufacturing iPSCs is becoming more readily available. An approach strongly recommended once a candidate process has been selected is a raw material risk assessment [7]. This approach is designed to identify and assess potential risks across a matrix of considerations that results in an overall risk score. If done prospectively, this approach provides opportunity for early intervention to mitigate high-risk materials that could slow the path to regulatory approval.

Other necessary considerations for raw materials are evaluation of consistency, animal origin and supply continuity. First, collection and curation of critical documents and rationale for the raw material choices made are necessary to offer compelling justification for its use in the manufacturing process. Whenever possible, it is best to use animal origin free (AOF) reagents that do not contain or use in the manufacturing processes any primary and secondary raw materials that are derived directly from animal (including human) tissue or body fluid. The most challenging standard of AOF is the tertiary level, which requires raw material manufacturers to ensure that none of the components used to manufacture the raw materials are derived from an animal source [8]. It is therefore critical to request and review supplier Certificates of Analysis, Certificates of Origin, and any other supporting statements from the supplier that clarify the quality and testing of those materials. In some cases, suppliers may have a regulatory support package (e.g., drug master file or equivalent) that therapeutic developmers can reference in their own regulatory filing. Collecting this information early and proactively for all parts of the iPSC manufacturing process from iPSC derivation through to cell bank generation (and subsequently in therapeutic product manufacture) is highly recommended to minimize delays during clinical development. Second, assurance of continuity of supply of raw materials by suppliers and provision of necessary technical, quality, and regulatory support through the different stages of the cell therapy product life cycle is critical. It is prudent to have a supply agreement with the vendor to be notified of any changes made to the starting material such as media, cytokines, etc. It is recommended that upfront identification of critical raw materials and qualification of materials from more than one source can help avoid later delays. A robust supplier and material management plan, including appropriate supply agreements, should be part of the developers' quality management system. Lastly, the shelf life of different components used in production may vary, requiring upfront staging of procurement and storage.

Somatic Reprogramming Methods for Generating iPSCs

Reprogramming systems

Two of the most commonly referenced combinations of reprogramming factors used to generate iPSCs include the Yamanaka factors: Oct4, Sox2, Klf4, c-Myc [9], and the Thomson factors: Oct4, Sox2, Nanog, Lin28, both named by the principal investigator that identified them [10]. The initial discovery of these transcription factors relied on viral vectors for integration into the genome and have now been replaced by transient methods that have been referred to as foot-print free methods. There are now a wide range of approaches to introduce, replace, or enhance the performance of these factors that can be grouped into the following categories: plasmid, Sendai virus, self-replicating RNA (srRNA), mRNA, and chemical based methods (Table 1). The most recent approach is the complete replacement of transcription factors with a chemically defined method using a complex cocktail of small molecules to reprogram human adipose-derived mesenchymal cells and fibroblasts [11,12]. Although the efficiency for iPSC generation across methods varies, most methods can generate appropriate numbers of clones required to support autologous or allogeneic approaches. Method selection for generating clinical iPSCs is typically driven by familiarity, ease of use, manufacturing costs, regulatory considerations, accessibility to licenses, and associated licensing fees for the reprogramming technology. For autologous workflows, efficiency of reprogramming is critical to ensure high success rates from a wider type of donor cells. Generation of iPSC intermediates is therefore a part of the process. For allogeneic workflows where iPSC cell banks are the starting material, genetic stability and regulatory considerations are typically most important due to the broader downstream patient impact. For example, if a master cell bank (MCB) is generated and continually used as the cell source, then banked cells have likely undergone a higher passage number that may impact genomic stability and possible introduction of adventitious agents.

Starting somatic cells

There is no clear consensus on the best somatic cell source to use for reprogramming, which is largely dependent on access, efficiency of the chosen reprogramming method and associated processes. Historically, the most popular cell type has been fibroblasts due to the ease in which iPSCs can be generated from human skin. However, the use of dermal fibroblasts has the risk of previously existing sun exposure-induced DNA damage; mitigation here includes selecting skin from areas that are typically not exposed to the sun. Keratinocytes have been cited as an alternate option [13] but have not been widely adopted. The use of hemopoietic sources is thought to reduce the risk of somatic mutation, and CD34+ cells from bone marrow, cord blood [14] or peripheral blood [15] that are minimally cultured or manipulated have all been successfully reprogrammed by different methods. Of these, peripheral blood is the most convenient source of cells due to ease of accessibility via blood banks [16]. Blood-derived cell types, such as T cells, can also be successfully reprogrammed [17], with the added advantage that the T cell receptor serves as a unique identifier to enable easy tracking and tracing. A sample of the donor starting material should be retained for support future identity testing.

Media/Matrix

Traditional culture media for iPSC expansion relied on murine embryonic feeders (MEFs) and media containing fetal bovine serum (FBS) [18]. Cell culture conditions have now evolved to include a number of commercially-available, highly-qualified, and GMP-compliant formulations that include: feeder-free with MEF-conditioned media [19], defined feeder-free media [20,21] and xeno-free media, along with defined culture matrices like Vitronectin [22] and laminin fragments [23] for use in adherent culture expansion of iPSCs. It should be noted that the critical component in all these media formulations is basic fibroblast growth factor (FGF2), which, given its short half-life, must be supplemented either directly or via addition of fresh media. Several approaches to stabilize FGF2 have been used, and commercial products are now available to ease the need for daily media exchanges [24]. Considerations for medium and matrix selection are highlighted above, and should also include compatibility with the developers' process and automation requirements.

Bioprocessing equipment

Current methods of iPSC generation are highly manual and feature open processes that are not well-suited for operations under GMP compliance. Initial clonal isolation is a key step requiring a high level of expertise and high degree of manual manipulation involving careful dissection of the colony. Automated systems are evolving and now exist to overcome this constraint, but to date, none of these systems are yet GMP-compliant and easy to deploy for clinical-use iPSC expansion. Generally, automation and bioprocessing equipment is a critical unmet need and should advance to support the generation of iPSCs.

Post-reprogramming Colony Selection

Like other steps, an upfront strategy is required for colony selection, including method of cloning (clone picking vs. singularization of cells to form colonies), proof of clonality (molecular methods such as T cell receptor profiling of T cell-derived iPSCs or visual microscopy), number of clones per donor, and size of the bank (based on the testing requirements, intended use and downstream commercial-use risks). Compared to the use of bulk iPSCs, clonal selection provides a means to choose discrete clones to confirm suitability and de-risks the possibility of a few clones with suboptimal differentiation potential or abnormal karyotype that may lead to heterogeneity. It is critical to ensure that the best iPSC clones from the seed stock are selected for generating working cell banks (WCBs) and MCBs.

Table 2 describes emerging technologies for selecting most promising colonies. Mechanization of colony picking will be relatively more important for autologous iPSCs because reprogramming is performed for each batch of product. For allogeneic iPSCs, the need to invest in such equipment is less likely to make economic sense because the need and frequency of iPSC clone generation is usually significantly lower compared to autologous iPSCs.

Clone selection based on characterization studies (described below) and feasibility studies for the intended application should be performed on a seed bank (for allogeneic) or

cryopreserved intermediate (for autologous) for each candidate clone. These intermediates are ideally generated as soon as the clonal line demonstrates stable growth and, where applicable, are clear of reprogramming vectors used in their generation. Suitable identity tests are required to unequivocally identify the donor source, and where genetically modified, the specific modified source. For allogeneic approaches, comprehensive characterization should be completed on all cell banks using a risk-based approach based on the banking strategy and size of the MCB.

Morphology features

The morphology of iPSCs is unique and with experience, a trained eye can determine whether colonies are healthy and undifferentiated. Adherent iPSC colonies typically display a “tight” and “sharp-edged” colony morphology with low adherence to the matrix-coated culture vessel. A healthy iPSC colony is completely “fenced-in” by a prominent wall of ~20-30um filaments of actin stress fibers dotted with large (4-5um) paxillin-positive “cornerstone” focal adhesions (FAs) at their distal ends, creating a strikingly unique multicellular architecture controlled by actomyosin contractility anchored by these cornerstone FAs [25]. It is acknowledged that such subjective qualitative assessment is not ideal for clinical manufacturing and continued advancements in image recognition technologies can reduce the subjectivity and skill, making the process more reliable and scalable. New emerging technologies such as machine learning and artificial intelligence utilizing imaging-based analysis algorithms can assist in determining the quality of undifferentiated iPSC colonies [26].

iPSC marker expression and differentiation potential

Confirmation of a cell line as a bona fide iPSC line is typically performed by assessing their ability to express extra- and intra-cellular markers including OCT4, SOX2, and NANOG, TRA-1-60 and/or SSEA-3/4 when in the undifferentiated state [27–29]. iPSCs should be further characterized to confirm their ability to differentiate by performing a trilineage differentiation assay to confirm capacity to form three germ layers (ectoderm, mesoderm, and endoderm). The markers used to identity each of the germ layers can vary but often include TUJ1 for early ectoderm, alpha-smooth muscle actin (α-SMA) for early mesoderm, and alpha-fetoprotein (AFP) for early endoderm [29].

The detection of residual reprogramming factors represents another common test to confirm the establishment of iPSCs. The role of the reprogramming factors is critical for the derivation of iPSCs, then factors are typically lost by dilution as cells divide and are no longer needed. The rate of loss for these factors is dependent on the delivery mechanism (e.g., viral, RNA or plasmid), efficiency of cargo uptake, and passaging methods. For example, Sendai virus-based reprogramming methods can take multiple passages to be diluted but can be accelerated using a variety of culturing methods such as limiting dilution [30]. The detection of residual reprogramming factors following the establishment of iPSCs is typically achieved using high sensitivity methods, such as quantitative or digital droplet PCR. Ultimately, it is important to establish quantitative methods to confirm loss of these factors to mitigate concerns associated with residual components that might impact patient safety.

Single cell seeding and clonality maintenance

Single cell passaging is critical for cell bank generation, particularly for clinical banks, in order to support the use of consistent and reliable iPSCs for therapeutic applications. Key drivers include: clonal purity and maintenance of genetic and phenotypic, genetic stability, better standardization of processes (both quality control and manufacturing), and the ability to scale uniformly. For iPSCs to survive as single cells, it is necessary to inhibit cell detachment-mediated apoptosis. This is typically achieved via the inhibition of Rho-associated protein kinase - myosin pathway (ROCK-myosin II) by the small molecule Y-27632, a selective inhibitor of ROCK, or another specialized cloning supplement. iPSC identity markers (TRA-1-81, SSEA3, OCT4, NANOG, SOX2) typically remain initially unaffected when the colonies are seeded at single cell density [31,32]. ROCK inhibitor-treated cells thus remain pluripotent for some time but display reduced expression of markers of the undifferentiated state, especially SOX2 [25].

Automation

Multiple technology platforms that were originally developed for automated colony detection and cell picking in various bioprocessing workflows can also be applied to iPSC colony handling (Table 2). One example is the StemCellFactory automated manufacturing platform, developed by a diverse consortium of publicly funded EU laboratories. Another example is the CellQualia instrument, a fully automated and closed system that includes passaging and harvesting, although reprogramming and clonal selection are not part of the system. In general, common machine learning algorithms can be trained on various conserved iPSC colony features as described above. Implementation of such automated multifunctional robotic platforms to recognize, select, and expand iPSC colonies is achieved by various independent devices that are integrated into a central control system which orchestrates process execution and data handling [33].

Machine learning deployed for label-free detection of iPSC colonies can also be used to eliminate undesired differentiating colonies using targeted laser-based systems on patterned substrates. These systems generally require precise tuning and optimization to minimize damage to neighboring cells. Such automated, trained laser-based colony pruning approaches allow for label-free selective 2D density management of iPSC cultures [34].

Expansion of iPSCs

Prior to full-scale expansion, the chosen clone(s) should be evaluated for the ability to differentiate efficiently and consistently into the cell type of choice, particularly for allogeneic approaches where there may be more flexibility to identify suitable clones. The critical task of determining which clones to bring forward can be aided by utilizing a quality-by-design (QbD) approach of identifying target therapeutic product profiles early in development and ensuring iPSCs and iPSC cell banks can efficiently and consistently differentiate into the cell type(s) of interest [35]. Although reagent and regulatory considerations for expansion of iPSCs for clinical use may share similar considerations to the differentiation of iPSCs into the specific cell type(s) of interest, differentiation protocols are outside the scope of this discussion.

Choice of strategy for the expansion of iPSC is based on multiple factors such as cost, cell line sensitivity, reagent quality, required cell yield and optimal seeding condition for downstream differentiation. While some lines expand well in suspension, many cell lines demonstrate increased cell death and reduced expansion rates compared to adherent culture. Nonetheless, there are two general approaches for both adherent and suspension culture: (i) low-cost, low-complexity consumables, for example, tissue culture flasks or cell factories for adherent cultures or spinner flasks for suspension cultures, or (2) higher-capital investment and more complex and commonly more regulatory-friendly tools, for example, hollow fiber bioreactors for adherent cultures or other suspension bioreactors that may also monitor process parameters like pH, dissolved oxygen, and glucose/lactate levels, all of which may help control and/or maintain iPSC stemness. Approaches for 2D and 3D expansion of iPSCs are highlighted in Table 3.

Platforms and technology

The simplest approach to iPSC expansion is standard culture conditions such as plates and flasks (Table 4). While these require manual handling and more operator time, they require less development and so might be more suitable when only one bank is needed. In general, adherent iPSC culture using these low-cost consumables is more commonly used than the suspension equivalents. This is due to the proficiency of most early-stage development labs in adherent culture processes, as well as the adherent characteristic of most cell lines.

Suspension culture bioreactors are more complex and include rocking-motion and stirred-tank approaches. The latter is considered better-suited to the needs of iPSCs as it promotes formation of aggregates and can also be used with microcarriers [36]. Automated stirred-tank bioreactors have been successfully used for large-scale production of mammalian cells and are currently being explored for use with iPSCs [37–39]. Such automated bioreactors can offer real-time traceable monitoring and closed-loop control of process parameters like agitation, temperature, pH, dissolved oxygen, and media feed rates critical for maintaining optimal growth conditions. On-line or in-line process analytical technology tools offer real-time monitoring and controlling of the process while reducing off-line sampling and manual feeding, decreasing the risk of contamination while minimizing the risk of batch failures. The use of QbD principles together with defining critical process parameters can be used to control iPSC aggregate sizes while ensuring that pluripotency is maintained within the iPSC aggregate [37]. Ultimately, the choice of any expansion platform needs to be based on the yield of high-quality iPSCs needed for downstream processing.

Banking Strategy and Cryopreservation

A prospective banking strategy should reflect the intended use of the bank for clinical development and therapeutic product manufacture and will differ based on autologous or allogeneic use. The banking strategy also informs the manufacturing process in order to prepare cells for banking, such as the number of expansion steps and technology used to achieve the target cell number. A prospective strategy is necessary to ensure the banks are of an appropriate size and that other resources such as storage, including off-site contingency storage, are available. General considerations are summarized in Table 5.

Autologous iPSC-derived products will require modest levels of frozen stocks, but these are more likely to be defined as frozen intermediates rather than formal cell banks. For allogeneic iPSC-derived products, the first consideration is whether a multi-tier iPSC bank is needed (i.e. seed stock, WCB, and MCB), or whether the iPSCs are used only to prepare a MCB of cells capable of lasting for the duration of the therapeutic product life cycle [40]. Whenever possible, use of a single iPSC bank is recommended, as transitioning from one iPSC line to another made from a different donor necessitates significant testing to demonstrate comparability for each new line.

There are numerous other considerations that impact the required size of the cell bank. For full life cycle use from development through to commercial manufacturing of the therapeutic cells, these considerations include, but are not limited to: cell dose, dosing frequency, size of the patient population for the given indication, targeted market penetration, likelihood of indication extension, geographical market area, quality control requirements (e.g. release testing, stability program, retains), likelihood of process changes requiring comparability, and redundancy in cell bank. This strategy will also have other impacts on the cost of goods, as the cost to test each bank under regulatory frameworks (e.g. ICH Q5A and Q5D) is significant. These considerations are complex and likely require overestimation to avoid estimates that are found to be too low later [41]. However, the upper limit to the bank size also needs to be considered as this may impact both overall costs and has potential technical constraints. For example, there will be a maximum number of vials that can be filled at one time due to constraints in recommended exposure time of iPSCs with dimethyl sulfoxide (DMSO) cryoprotectant prior to freezing that negatively impacts viability. For context, it has been demonstrated that approximately 10^9 cells can be banked at one time [42] and that as many as 300-400 vials can be generated for a single MCB [43,44].

Cell preparation and dissociation

The first step in preparing for cryopreservation is determining when cells are ready for harvest. Culture confluency and/or cell counts are common in-process considerations, and harvesting iPSCs during their logarithmic growth phase (approximately 3 days after passage) is recommended [45,46]. Dissociation of iPSC colonies takes place by detaching cell colonies from the culture surface and generating suspensions of single cells or small aggregates for banking. Dissociation can be achieved chemically or mechanically as outlined in Table 6, and cells will vary in response depending on whether they are in aggregate or single cells [47]. The entire iPSC dissociation procedure should be performed as quickly as possible to maintain cell survival, viability, and stability [44]. Gentle manipulation and minimal exposure to enzymes are necessary to avoid disrupting the aggregates. This can be facilitated by the use of non-enzymatic detachment solutions, such as those containing EDTA [48].

Once iPSCs have been dissociated, further processing is needed to concentrate the final product and formulate in the appropriate buffer. Although this is relatively easy to perform at small scale, performing such steps at large scale required in clinical processing can be challenging. For example, processing liters of product via manual centrifugation is highly labor intensive and thus not amenable to scale-up. Commercially available counterflow

centrifugation devices can perform media exchange and concentrate at mid-range (~20 L input). For larger volumes, continuous counter flow centrifugation systems can wash, concentrate, and formulate in a single step, but the technology is complex, expensive per operation and requires significant development to optimize. A key limitation of these technologies is the ability to replicate them in scaled-process development models (such as what might be needed for process development), potentially requiring studies to be performed at full-scale.

Cryopreservation

Cryopreservation of cell banks is the final iPSC banking step that needs to be optimized in the clinical bioprocessing process. Parameters to maximize post-thaw yield and ensure iPSC quality include: cryoprotectant, freezing device, volume, and storage container [49]. DMSO-containing cryopreservation solutions are commonly used, and advancements in alternative DMSO-free cryoprotectants such as sucrose, glycerol, isoleucine, and albumin have shown promising results [50,51]. Addition of DMSO to washed/concentrated cells can be achieved in automated systems provided that the consumables are compatible with DMSO. Following addition of cryoprotectant, cells are ready for vial filling. Filling into vials can be done with a higher level of accuracy, consistency, and cell viability when using automated filling systems, which minimizes variability, risk of error, potential for contamination and duration, including exposure time to DMSO-containing cryoprotectants while at room temperature.

Finally, cryopreservation is performed using slow, controlled temperatures in a liquid nitrogen control rate freezer, or newer models that are liquid nitrogen-free, or vitrification [49,52]. Freezing via a controlled-rate freezer helps improve consistency in cell recovery post-thaw, especially when temperature mapping is utilized to ensure uniform cryopreservation; this becomes especially important with larger cell banks. Once the cells have been properly frozen, they can be stored long term in the vapor phase of a liquid nitrogen tank or in a −150°C freezer [45].

Regulatory considerations

The generation of an iPSC cell bank is typically performed under GMP, which implies that strict controls are in place in compliance with appropriate regulations and guidances, robust quality systems, and industry best practice to ensure the cell bank is suitable for use in downstream clinical applications. However, there can still be a broad range of procedures and process implemented across manufacturing institutions to substantiate the claim that a cell line is GMP-compliant. Considerations also may be different depending on whether the iPSCs will be used in autologous or allogenic therapies. For autologous workflows specifically, derivation of iPSC intermediates need be carried out under GMP. However, iPSCs for allogeneic workflows can be considered a raw material which means there may be greater flexibility in the source requirements, which could be either an original iPSC line derived under GMP to generate MCB or an existing iPSC line derived under research conditions re-derived under GMP to generate a MCB. The latter approach would result in iPSC with larger passages with greater risk for genome instability and chances of karyotypic abnormalities, therefore requiring more extensive characterization.

Seeking out early engagement and alignment with regulatory agencies is highly encouraged. The final item to consider is the regulatory expectations for MCB testing and the incorporation of adventitious agent testing under compliance to 21 CFR 1271.90(a); test results must be confirmed to be free of adventitious agents in order for the iPSC bank to be used for generation of clinical cell therapies.

Conclusions

As can be seen above, the production of an iPSC-derived cell therapy is a relatively long process involving multiple manipulations and materials. The frequency of manual interventions, process duration, and open culturing systems increases the risk for contamination, in addition to the potential risks introduced by raw materials and product contact materials used during cell bank manufacture. However, given that iPSC generation and bank production is significantly upstream of administration of the final cell product into a patient, there is substantial opportunity to perform adequate process control steps and testing to reduce risk to the patient (e.g., testing for adventitious agents). This ability to implement control and testing measures represents one of the distinguishing characteristics afforded by an iPSC-derived therapy.

Although iPSCs as a starting material have the potential to be the next frontier in cell therapy, iPSC-based therapies currently under development rely on conventional methods with suboptimal manual manipulations. Rapid advances are being made in optimization and integration of clinically-minded bioprocessing methods and tools to address current gaps. However, these new technologies create novel opportunities and also challenges due to the continually evolving landscape and drive to identify the best solutions. This is further compounded by changing and more stringent global regulatory requirements being somewhat addressed by advancements in global regulatory convergence. We see automated platforms that support more regulatory-friendly manufacture, with integrated analytics and use of machine learning and artificial intelligence to assist with decision making having the potential to revolutionize the field.

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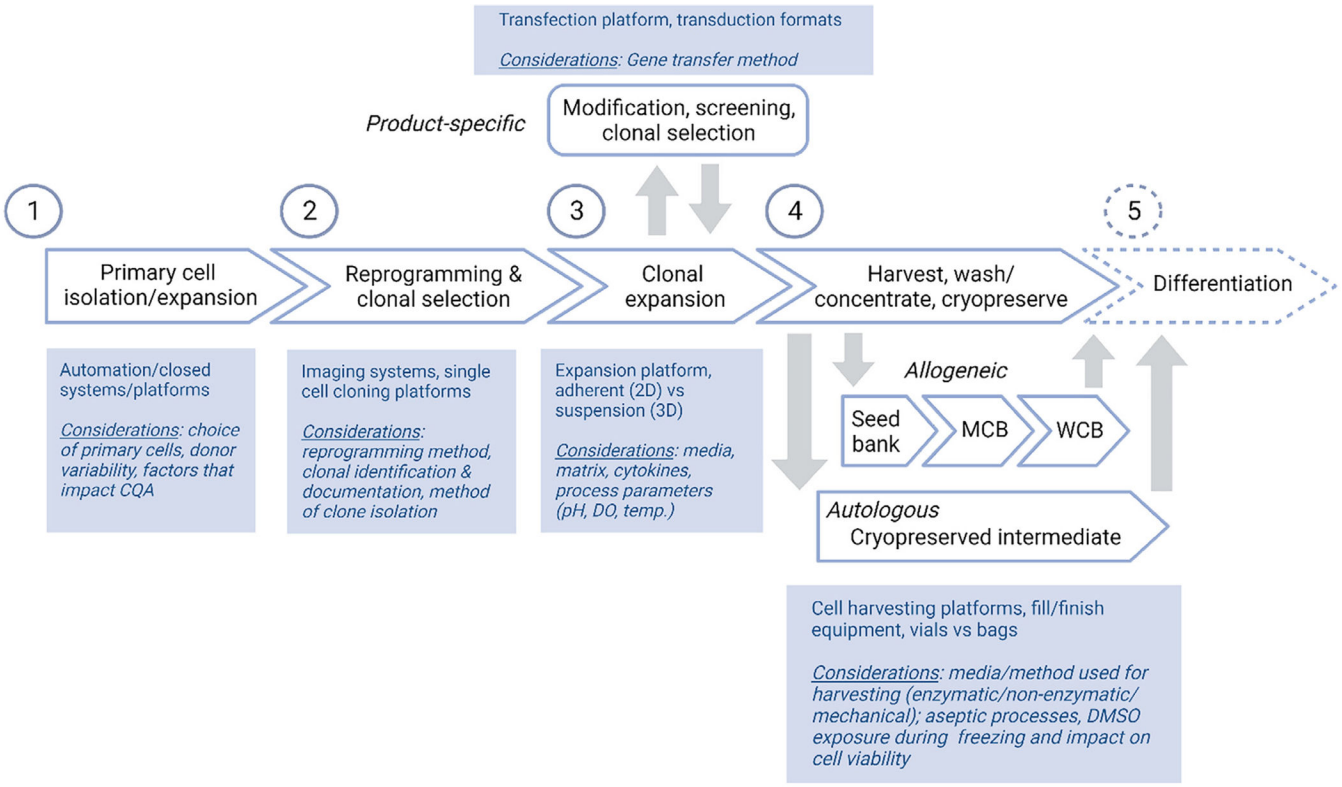

Fig. 1.

iPSC generation for clinical manufacturing. Schematic depicting a common paradigm broken down by high-level process steps to derive, expand, and bank iPSCs. A non-exhaustive and representative set of considerations are provided for each step. Made with biorender.com.

Abbreviations: CQA: Critical Quality Attributes; 2D/3D: two- or three-dimensional platforms; MCB: Master Cell Bank; WCB: Working Cell Bank; DMSO: dimethyl sulfoxide.

Table 1

Representative approaches for the generation of iPSCs.

Approach	Description	Advantages	Disadvantages	Representative Publications
Plasmid DNA	Episomal plasmids used to deliver a combination of reprogramming factors and enhancers; EBNA1/oriP backbone ensures retention long enough to induce reprogramming	Requires a single round of transfection, long term stability of plasmid DNA, amounts needed are low, a single manufacturing lot of plasmid could provide unlimited supply, GMP manufacture of plasmid DNA is possible, demonstrated precedent for regulatory path	Manufacture of GMP plasmid DNA requires an upfront investment, IP considerations can limit application unless license is negotiated, dependency on delivery device and reduced viability	[53]
Sendai virus	Non-integrating RNA viral vectors that deliver a combination of reprogramming factors or enhancers	Foot-print free, broad host range, high efficiency, high viability, easy access to high-quality grade material, demonstrated precedent for regulatory path	Rate of loss of residual RNA can be slow and require further passaging, product shelf life is relatively short and IP considerations might limit application unless license is negotiated	[54]
srRNA and mRNA	Synthetic, capped srRNA or mRNAs with modified nucleobases to encode transcription factors coupled with enhancers and inhibitors of the interferon response	Demonstrated capacity for single cell reprogramming, high efficiency in human fibroblasts, higher rate of loss of residual RNAs	Currently, not broadly applicable across cell types, requires multiple rounds of introduction for mRNA	[55,56]
Chemically defined	Small molecule replacement of key transcription factors including inhibitors of the JNK pathway	Enables possibility for more control over the process inclusive of raw materials, does not depend on delivery devices, viruses, or lipophilic reagents	Requires further development to demonstrate suitability for clinical manufacture	[11]

EBNA1, Epstein Barr Nuclear Antigen 1; oriP, origin of DNA replication; IP, Intellectual Property; JNK, c-Jun N-terminal kinase pathway; mRNA: messenger RNA; srRNA: self-replicating RNA

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Table 2

Representative list of automated colony handling technologies.

Devices	Features
Traditional Clone pickers designed for hybridoma clones cultured in semi solid agar, adapted to mammalian cells	
ClonePix2 *Molecular Devices*	Developed for semisolid media, primarily used for cell line development. 10x faster than limiting dilution and FACS, software and integrated robotics enables a picking speed of > 10,000 clones per day.
RapidPick *Hudson Robotics*	Liquid, semi-solid robotic arm only. Automated selection and growth of cells grown on a colony plate; speed is 2400 colonies/hour.
UniPicker *Unimed Biotech*	Nanoliter micropicking capillary: 20um-1000um suite for single cell, colony, cell cluster, & tissue. Freely configurable tool that can automatically scan, analyze, pick & transfer single cell and cell colonies.
Scirobotics Pickolo *Tecan SciRobotics*	Add-on for Tecan's Freedom EVO®. Automated/interactive colony selection, 600 colonies per hour, fixed and disposable tips, automated documentation.
Clone pickers specifically designed for mammalian cells, in particular, stem cells	
Cell Celector *ALS/Sartorius*	Image-integrated liquid-phase picking. Single cell and colony picking platform liquid-phase picking.
CellX *CellX Technologies*	Image-integrated liquid-phase picking. Robotic system with integrated colony picking and fluid management.
StemCell Factory *Fraunhofer*	Fully automated platform composed of different (commercially available) robots for automation of somatic cell isolation, iPSC generation, expansion, and differentiation. Has software for automating entire process, uses AI to make decisions at certain points.
CellQualia *Sinfonia Tech Inc.*	Intelligent cell processing system equipped with process analytical technology to monitor culture status in real time. Stabilizes process by enabling manufacture of cell products based on QbD concepts.

Table 3

Approaches for 2D and 3D expansion of iPSCs.

	Formats	2D vs 3D
Scale out	Culture flasks	2D
	Closed automated systems	2D or 3D
	Single use mini bioreactors	3D
Scale up	Multilayer systems	2D
	Bioreactors for adherent cells	2D
	Hollow fiber systems	2D
	Vertical wheel bioreactors	3D
	Wave bioreactors	3D
	Stir tank bioreactors	3D

Table 4

Considerations for low-cost vs. higher-cost expansion technologies.

	Footprint	Labor requirements	Contamination risk	Automation	Process intensification
Low-cost options: Shake or spinner flasks, T flasks, multitrays	+++	+++	+++	+	+
Higher-cost options: Adherent and suspension bioreactors	+	+	+	+++	+++ [57]

Table 5

Considerations for developing an iPSC banking strategy.

	Technique	Advantages	Disadvantages
Filling	Vials	• Better for smaller volumes • More opportunity for automation	• If manually filled, can take a long time
	Bags	• Better for larger volumes	• Can affect aggregates making thaw recovery difficult
	Manual	• Cost effective • Better for small harvest volumes • Flexible	• Open manipulation • User variable • Slower output
	Automated	• More consistent • Faster output • Closed	• High cost • Made for large volumes. • Equipment can malfunction
Cryo-formulation	DMSO	• Gold standard • Readily available	• Long exposure can lead affect viability. • High concentrations can affect cell health
	DMSO-free	• Minimizes risk to cell health	• Not as well understood. • Not as readily available
Cryo-mechanisms	Vitrification	• Decreases chances of osmotic shock • Faster	• Increases chances of contamination • Difficult to scale at commercial level
	Controlled rate freezing	• More consistent • Higher cell recovery	• Dependent on if cells are aggregates. • Requires extensive optimization
Thaw	Automated	• Controlled temperature • Repeatable	• Expensive • Risk of malfunction
	Manual	• Inexpensive • Flexible	• Increased risk of contamination • Inconsistent

Table 6

iPSC dissociation techniques and considerations.

	Principle	Advantages	Disadvantages
Enzymatic dissociation	An enzyme, or a combination of enzymes, when applied to cell colonies acts to degrade the cell surface proteins which tethers the cells to the culture surface [48]	• Straightforward process • Several GMP-grade enzymatic dissociation agents commercially available • Can be scaled up to large scale automated systems such as bioreactors (in some cases will require specific process optimization due to the large volumes and associated cost)	• Can be expensive • Requires screening study to optimize process for scale up • Enzyme inactivation strategy to be assessed as well to avoid too large volumes for downstream processing • Can induce genetic and epigenetic aberrations in iPSC cultures [58]
Non-enzymatic chemical dissociation	Use of enzymatic chemical dissociation methods such as EDTA-based reagents [59]	• Can offset both the complexity of mechanical digestion and the genetic instability associated with enzymatic digestion • Cost-efficient	• Efficiency can be low depending on cell type and process
Mechanical dissociation	Involves physical disruption of the cell colonies by trituration with pipette tips [60] or cutting colonies into small pieces with an appropriately-sized needle [58]	• Method has proven successful to dissociate encapsulated iPSCs [61] • Automates systems available that eliminate the non-desirable parts of iPSCs colonies. • Superior genetic and epigenetic stability	• More susceptible to user-variability and sterility issues • More complex process • Not compatible (to date) with large scale bioreactor culture