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Review article

A risk-based approach for cell line development, manufacturing and characterization of genetically engineered, induced pluripotent stem cell–derived allogeneic cell therapies

Jennifer L. Dashnau[1,*], Qiong Xue[2], Monica Nelson[1], Eric Law[1], Lan Cao[2], Derek Hei[3]

[1] *Century Therapeutics, Inc., Philadelphia, Pennsylvania, USA*
[2] *Takeda Pharmaceuticals, Cambridge, Massachusetts, USA*
[3] *Clade Therapeutics, One Kendall Square, Cambridge, Massachusetts, USA*

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ABSTRACT

Advances in cellular reprogramming and gene-editing approaches have opened up the potential for a new class of *ex vivo* cell therapies based on genetically engineered, induced pluripotent stem cell (iPSC)-derived allogeneic cells. While these new therapies share some similarities with their primary cell-derived autologous and allogeneic cell therapy predecessors, key differences exist in the processes used for generating genetically engineered, iPSC-derived allogeneic therapies. Specifically, in iPSC-derived allogeneic therapies, donor selection and gene-editing are performed once over the lifetime of the product as opposed to as part of the manufacturing of each product batch. The introduction of a well-characterized, fully modified, clonally derived master cell bank reduces risks that have been inherent to primary-cell derived autologous and allogeneic therapies. Current regulatory guidance, which was largely developed based on the learnings gained from earlier generation therapies, leaves open questions around considerations for donor eligibility, starting materials and critical components, cell banking and genetic stability. Here, a risk-based approach is proposed to address these considerations, while regulatory guidance continues to evolve.

Introduction

Over the past two decades, important advances have been made in key technologies that have enabled the evolution of new classes of *ex vivo* cell therapies. The discovery of induced pluripotent stem cells (iPSCs) and improvements in gene-editing technologies have expanded the cell therapy candidate space to include essentially any human cell type with enhanced potency and safety owing to select genetic modifications [1]. As iPSC-derived cell therapies have progressed into human clinical trials, methods for manufacturing and controlling these therapies have evolved along with the regulatory landscape [2].

Although the starting cell source and method of genetic modification creates a wide range of potential scenarios for cell therapy production, there are many common aspects of the manufacturing processes. In general, production of an *ex vivo*–modified cell therapy is initiated with the selection of the starting cell material, including donor screening, infectious agent testing and harvest of the starting cell material. Cell therapies can be produced from a variety of cell/tissue sources, including whole blood, peripheral blood mononuclear cells, bone marrow, cord blood, adipose tissue and skin biopsies. The starting cell source can be further categorized into either autologous or allogeneic sources, depending on whether cells are sourced from the same individual intended for treatment or a healthy donor intended to treat multiple patients. Many of the current approved cell therapies are derived from autologous cell sources. The use of an autologous cell source addresses potential issues of recipient immune response but faces other challenges in commercialization, including donor-to-donor variability and greater cost of goods. Allogeneic cell therapies provide several advantages including the ability to produce large batches of drug product and that can be more thoroughly characterized with the disadvantages of requiring either patient immunosuppression or other methods to address the immune response. Gene editing of the cell source is one potential method of addressing the immune response to allogeneic cell therapies through either suppression of human leukocyte antigen (HLA) molecule expression (e.g., beta 2 microglobulin, class II major histocompatibility complex transactivator) and/or expression of immunomodulatory molecules (e.g., CD47, PDL1, HLA-E/G) [3,4].

In primary cell–derived autologous and allogeneic processes, the manufacturing process starts with the collection of starting cell

* Correspondence: Jennifer L. Dashnau, PhD, MBA, Analytical Development and Quality Control, Century Therapeutics, Inc., 3675 Market St., Suite 200, Philadelphia, PA 19104.
E-mail address: jdashnau@centurytx.com (J.L. Dashnau).

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material from a patient or healthy donor. Depending on the cell or tissue source, cells may be further selected or purified. Following selection or purification, cells may be genetically modified, expanded and harvested to yield a drug substance; this drug substance is further formulated and filled to produce the drug product. The drug product may be administered fresh (i.e., without cryopreservation) to the patient shortly after manufacture, or cryopreserved and later thawed for administration.

In contrast to the processes used to manufacture primary cell–derived autologous and allogeneic products, the process for iPSC-derived allogeneic therapies can be divided into three main stages related to the cell source, cell line development and manufacturing. In the first stage of the process, the donor material (i.e., the cell source) is subjected to a reprogramming process by introducing reprogramming genes (e.g., Oct-4, KLF4, SOX-2 and c-Myc [OKSM]) [5] that are transiently expressed by either viral or non-viral reprogramming vectors. Reprogrammed cells may be expanded and banked to create a parental iPSC bank, which serves as a renewable cell source with nearly infinite expansion capacity. The ability of iPSCs to be expanded in an undifferentiated state allows for more extensive genetic modification and larger-scale manufacturing in later steps of the process.

The second stage of the process consists of activities related to cell line development. During cell line development, reprogrammed iPSCs (i.e., the parental cell line or parental bank) may be further genetically modified to introduce product-specific modifications using viral vectors, non-viral gene editing components (e.g., plasmid DNA, mRNA, RNA replicons), through enzyme (e.g., CRISPR/Cas9), or transposon mediated gene editing [6]. Subsequent genome modification disrupts endogenous genes or introduces transgenes relevant to function of the product. Throughout this paper, iPSCs that have undergone product-specific genome modification and their resulting drug products are referred to as "genetically engineered iPSCs" and "genetically engineered iPSC-derived allogeneic cell therapy products."

Following genetic modification, the modified cells may be subjected to a clonal isolation step (i.e., cloning), in which the clones are expanded and multiple clones may be screened to select a single clone with the appropriate performance characteristics for use in the manufacturing process. The selected clone may then be expanded to produce a preliminary seed bank, or cell substrate, that may be further expanded to create a single master cell bank (MCB) for the product. The MCB undergoes extensive testing and qualification before being used for production of drug product. Cell line development, which includes activities related to generation of the iPSC cell source, gene editing and cell banking, is performed once over the lifetime of the product, before generation of the MCB, which marks the start of the manufacturing process.

The manufacturing process for an iPSC-derived allogeneic product starts at MCB vial thaw. In some cases, a two-tiered banking strategy may be used, in which case working cell banks (WCBs) may be expanded from the MCB. The genetically modified iPSCs from the MCB or WCB are then typically expanded in an undifferentiated state to achieve the desired scale of manufacturing and further processed to induce the intended cell phenotype; processing may include various unit operations, including differentiation and maturation or activation to produce a fully active drug substance. The drug substance may then be formulated, filled and cryopreserved to yield the drug product intended for administration. Figure 1 provides a high-level overview of major steps typically found in the process when starting with primary cells or iPSCs. While this figure captures some examples, there are many potential variations in the process depending on the starting material used and intended drug product profile. Additional, more detailed examples of cell therapy processes can be found in the cell therapy process maps developed by BioPhorum [7].

As with any new class of therapies, understanding the ever-evolving regulatory landscape is a challenge, and engaging in discussions with regulators early in development is important. The level of risk for an allogeneic therapy developed to treat multiple patients may be perceived as greater than that of an autologous therapy intended to treat a single patient. In addition, more extensive genome modification may introduce additional risks to product quality and safety depending on the editing approach used. The use of risk-based

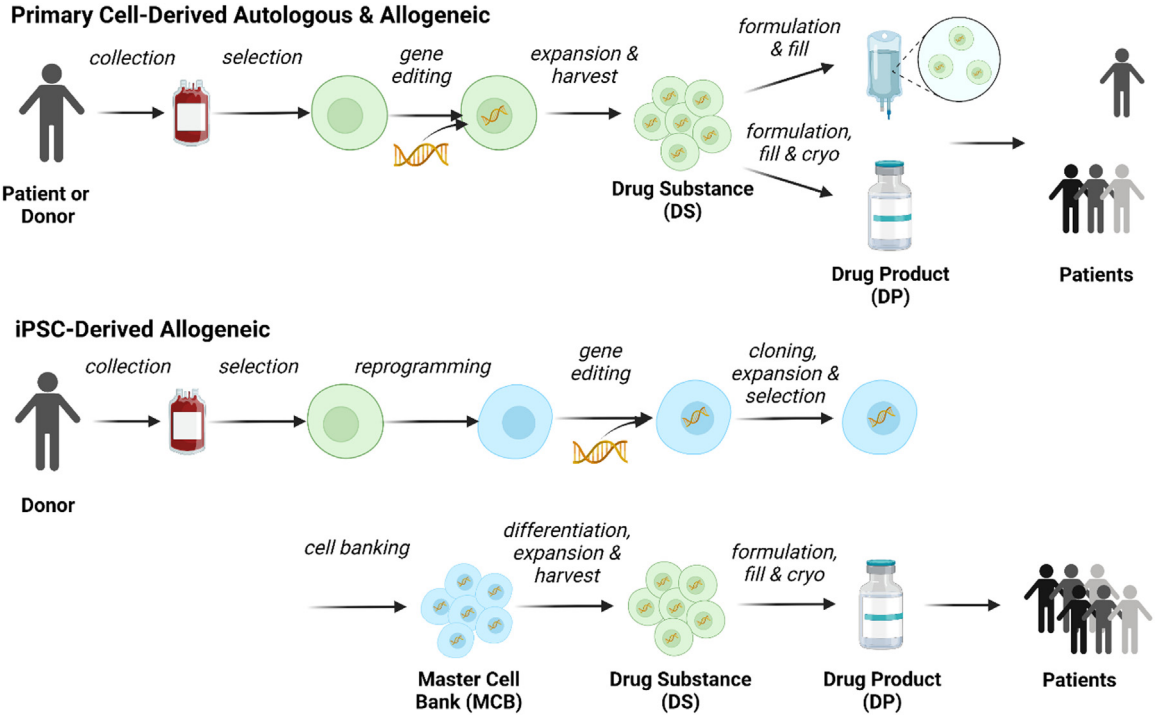

Figure 1. Overview of manufacturing processes for primary cell–derived autologous and allogeneic therapies, and iPSC-derived allogeneic cell therapies. Created with BioRender.com

approaches is widely recognized by regulatory authorities as an important tool in designing manufacturing and testing plans for cell and gene therapies [8,9]. One of the key hallmarks of allogeneic cell therapies is the ability to subject the drug product to in-depth characterization before administration. This is a key advantage of allogeneic cell therapies and allows for significant risk mitigation through additional testing and evaluation of the cell therapy in animal models. The intent of this paper is to frame key considerations and strategies in applying a risk-based approach in the development of manufacturing and testing procedures for genetically engineered, iPSC-derived allogeneic cell therapies.

Current regulatory guidance

As health authorities build familiarity with cell therapy manufacturing processes and controls, regulatory guidance has evolved to reflect this experience. A brief overview of the current regulatory landscape in the United States and European Union is provided, focusing on guidance relevant to genetically engineered iPSC-derived allogeneic cell therapies.

Food and Drug Administration (FDA) guidelines

In the United States, the Center for Biologics Evaluation and Research (CBER) within the FDA has regulatory authority over all biological products for human use under applicable federal laws. Since the early 1990s, the CBER has issued a number of guidance documents that provide manufacturers with current thinking or recommendations regarding the production, quality control and administration of cellular therapy products. Early guidance, such as "The Guidance on Human Somatic Cell Therapy and Gene Therapy," captured important considerations on areas such as cell collection, cell culture, cell banking procedures, raw materials, product quality and characterization testing. In addition, "The Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products" established expectations on donor eligibility determination, including donor screening and testing, for allogeneic cellular therapies in the context of enforcing the Current Good Tissue Practice requirements found in 21 CFR Part 1271, subpart C.

However, the field of cell and gene therapy has expanded and advanced significantly in recent years. In 2021, CBER received 299 Investigational New Drug (IND) applications for gene and cell therapy clinical studies, a huge jump from the 163 submitted in 2016 [10,11]. By 2030, the number of durable cell and gene therapy product-indication approvals in the United States are estimated to rise by approximately five per year over 2022 to 2025, reaching a total of 54 to 74 by 2030 [11]. In response to the rapidly evolving scope and landscape of gene and cell therapies, the agency has finalized and published guidance documents specifically relevant to genetically engineered cell therapies beginning in 2020. These newer guidance documents provide additional considerations for IND applications and testing of retroviral vector-based human gene therapy products. A list of select current relevant guidance from the FDA is provided in supplemental materials and Table 1, "Select FDA guidance documents relevant to cell sourcing, cell line development, quality control, characterization and manufacture of genetically-engineered cell therapies."

EMA guidelines

In Europe, medicinal products for human use are governed by the European Medicines Agency (EMA) Directive 2001/83/EC and the European Commission regulation of 726/2004. Specifically for the manufacture and quality of genetically engineered cell therapies, the regulatory framework is based on a series of guidance documents issued by the EMA and European Commission on Advanced Therapy Medicinal Products (ATMPs), which comprises medicines for human use that are based on genes, tissues or cells. Early guidance, such as "Gene therapy product quality aspects in the production of vectors and genetically modified somatic cells" and "Guideline on human cell-based medicinal products," covered the quality aspects in the production of the gene transfer vectors and genetically modified somatic cells such as fibroblasts and myoblasts, and addressing the approach toward a risk analysis to be used to justify product development. In addition, detailed guidelines on Good Manufacturing Practice (GMP) specific for ATMPs were established for implementation by both market authorization holders, importers to the European market and manufacturers of investigational medicinal products. In recognition to the increase in clinical experience on ATMP development (with chimeric antigen receptor [CAR] T cells in particular) and the emergence of novel technologies such as iPSCs and genome editing, the EMA has revised the overarching guidance governing the quality, non-clinical and quality requirements of gene modified cells using these technologies, particularly in the areas of starting materials, manufacturing process control and characterization/release of the finished product. Recently, the EMA issued a question-and-answer guidance document clarifying how GMP principles should be considered and applied to starting materials for ATMPs of biological origin. The document elaborated minimal requirements in the fields of quality management system, risk management product development, production and quality control toward the definition of GMP principles. An overview of ATMPs is available on the EMA website [12] and a list of select current relevant guidance from the EMA is provided in the supplemental materials and Table 2.

Table 1
Select FDA guidance documents relevant to cell sourcing, cell line development, quality control, characterization and manufacture of genetically engineered cell therapies.

Guidance for Industry Document Titles	Published Date
Guidance for Human Somatic Cell Therapy and Gene Therapy	March 1998
INDs for Phase 2 and Phase 3 Studies Chemistry, Manufacturing, and Controls Information	May 2003
Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products	August 2007
Current Good Manufacturing Practice for Phase 1 Investigational Drugs	July 2008
Potency Tests for Cellular and Gene Therapy Products	January 2011
Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up	January 2020
Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy IND Applications	January 2020
Human Gene Therapy Products Incorporating Genome Editing: Draft Guidance for Industry	March 2022
Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Therapies: Draft Guidance for Industry	March 2022

FDA, Food and Drug Administration; IND, Investigational New Drugs.

Open questions

The current guidance documents developed by the FDA and EMA provide sponsors a solid foundation for the development of cell and gene therapy products. However, the recommendations and requirements in these guidance documents have been largely influenced by experiences gained to date with autologous and primary cell–derived allogeneic therapies. As a result, there are open questions around considerations for donor eligibility, starting materials and critical components, cell banking and genetic stability given the differences in cell line development and manufacturing processes used for genetically engineered, iPSC-derived allogeneic therapies.

Donor eligibility

Allogeneic cell therapies can be derived from a variety of starting cell sources, including specific blood cell types such as hematopoietic stem cells, natural killer cells and T cells derived from peripheral

Table 2
Select EMA guidance documents relevant to cell sourcing, cell line development, quality control, characterization and manufacture of genetically engineered cell therapies.

Guideline document titles	Document reference	Date for coming into effect
Gene therapy product quality aspects in the production of vectors and genetically modified somatic cells	Directive 75/318/EEC as amended, reference number 3AB6A	July 1995
Guideline on human cell-based medicinal products	EMEA/CHMP/410869/2006	September 2008
Reflection paper on stem cell-based medicinal products	EMA/CAT/571134/2009	January 2011
Reflection paper on design modifications of gene therapy medicinal products during development	EMA/CAT/GTWP/44236/2009	December 2011
Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products	EMA/CAT/CPWP/686637/2011	February 2013
Guideline on potency testing of cell-based immunotherapy medicinal products for the treatment of cancer	EMA/CHMP/BWP/271475/2006 rev.1	September 2016
EudraLex – The Rules Governing Medicinal Products in the European Union – Volume 4 Good Manufacturing Practice: Part IV – Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products	C(2017) 7694 final	ATMP manufacturers should comply no later than 22 May 2018
Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells	EMA/CAT/GTWP/671639/2008 Rev. 1 – corr	Effective from June 2021
Draft Guideline on Quality, Non-Clinical and Clinical Requirements for Investigational Advanced Therapy Medicinal Products in Clinical Trials	EMA/CAT/852602/2018	To be determined
Questions and answers on comparability considerations for ATMP	EMA/CAT/499821/2019	Not applicable
Questions and answers on the principles of GMP for the manufacturing of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs	EMA/246400/2021	Not applicable

ATMP, advanced therapy medicinal product; EMA, European Medicines Agency; GMP, Good Manufacturing Practice.

blood mononuclear cells, bone marrow, adipose tissue, umbilical cord blood or iPSC cell lines. Regardless of the cell source, a similar process of donor screening, consent and testing is typically performed to establish donor eligibility for allogeneic therapeutic applications. In the United States, donor material is required to meet key eligibility requirements outlined in regulations and related guidance documents [13–16]. Similarly, in the European Union, multiple directives address standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells [17–20]. Donor eligibility expectations for autologous use are discussed elsewhere [20,21] and are outside the scope of discussion below.

Donor screening

The first stage of procuring donor material for allogeneic cell therapies involves donor screening. Donor screening is conducted using a standard Donor History Questionnaire, such as those developed by the AABB Donor History Task Force and recognized by the FDA for standard blood donations to address the requirements outlined in the aforementioned regulations [22]. The Donor History Questionnaire document, which is designed for self-administration by the donor with follow-up by medical establishment personnel or may be administered by a qualified physician during standard medical examination, addresses a range of questions related to medical history and risks associated with infectious agents including transmissible spongiform encephalopathy. This questionnaire represents the minimum requirements for donor screening as part of donor eligibility determination. However, additional donor screening requirements may be included based on the intended application of the resulting cell therapy product. For example, questions may be added to assess the donor's medical history with respect to potentially inherited diseases that could be relevant for the intended application. Other characteristics such as age and sex also may be considered when identifying a potential donor.

Donor consent and compensation

Once potential donors have been screened, information regarding the donation process and intended use of the donor material is provided to the donor as part of informed consent. The Informed Consent document should be reviewed and approved by an institutional review board or ethics committees and should cover key requirements related to potential commercial use of the resulting cell material. When identifying potential donors for a given cell therapy product, care should be taken to address donor consent requirements, which may differ across intended markets. In the case of primary cell–derived allogeneic therapies, the use of multiple donors over the lifetime of the product may provide some flexibility in designating select donors for individual markets. However, for iPSC-derived allogeneic therapies where a single donor is used for the lifetime of the product, requirements for all intended markets should be considered during initial sourcing of donors. One key point that should be considered is donor compensation versus reimbursement for expenses associated with the donation process. Many jurisdictions have strict requirements forbidding donor compensation. However, reimbursement of reasonable expenses associated with the donation process may be allowed.

Donor testing

Donor testing is performed to detect the presence of relevant communicable disease agents or diseases as required by regulatory agencies. Donor testing must be performed on blood samples collected within a window of 7 days before or following donation. However, it should be noted that some countries require repeat testing post-donation to ensure that the donor did not test negative due to a recent infection without sufficient time for seroconversion. Donors are generally tested for HIV type 1 and 2, hepatitis B virus, hepatitis C virus and *Treponema pallidum* (syphilis). In addition to these general requirements, additional testing based on the type of donor material may apply. For instance, donors of viable, leukocyte-rich cells must also be tested for human T-lymphotropic virus types I and II and cytomegalovirus. Finally, some countries require testing for additional disease agents, such as hepatitis A, hepatitis E and human parvovirus B19, at the donor level [23]. If a product is intended for global acceptance, it is recommended to perform a complete review of requirements across all intended markets prior to initiating donor collections. For all disease agents tested, testing must be performed by a qualified testing lab (e.g., Clinical Laboratory Improvement

Amendments–certified) using viral nucleic acid (e.g., nucleic acid test) and/or antibody (e.g., enzyme immunoassay) tests, which have been appropriately licensed, approved or cleared for use in the intended market. In the United States, the FDA has published and maintained a list of approved donor screening assays for infectious agents [24].

Testing for other viruses outside those included on the list of relevant communicable disease agents or diseases (e.g., West Nile virus) may be considered relevant based on the risk of transmission, severity of effect and availability of screening measures or tests. For instance, a more extensive set of virus testing including viruses such as human herpes virus-6/7/8 is required for MCBs [25]. Therefore, it is tempting to include additional virus testing at the donor stage to de-risk potential viral concerns at later stages of cell line development or manufacturing. While these viruses are prevalent in the human population, infection may be localized to certain cell types [26,27]. Therefore, inclusion of such testing at the donor level may exclude a large number potential donors, where not warranted.

Another consideration is that donor testing requirements may vary depending on regional regulatory requirements and the status of potentially transmissible diseases at the time of donation [23,28]. Therefore, ongoing review of regulatory guidance should be monitored to ensure donor testing programs remain up to date. For example, donor testing of blood and blood components for Zika virus is no longer required, given decreased prevalence in the potential donor population in the United States [29]. In contrast, donor testing for West Nile virus is now routinely performed due to the fact that this virus is now endemic throughout most of the country [30]. More recently, guidance around the handling of severe acute respiratory syndrome coronavirus 2 was updated [31]. In this case, testing of asymptomatic donors was not required but the addition of screening questions to the Donor History Questionnaire was recommended to identify potential for increased risk. It is recommended to retain donor samples to allow future testing for potential newly identified testing requirements.

In some situations, it may be appropriate to perform more extensive donor testing to assess other potential risks, such as mutations associated with oncogenes or diseases that are relevant for intended therapeutic area (e.g., cardiac diseases for a cell therapy to be used for cardiac indications). With increased access and affordability of next-generation sequencing (NGS), this technique may be used as a broad screen for mutations. Alternately, a more focused approach using genomic screening arrays can be used to identify mutations in recognized oncogenes (e.g., p53) and tumor suppressor genes by comparison to established oncogene databases, such as the Catalogue of Somatic Mutations in Cancer, Cancer Gene Census [32] and OncoKB [33], or disease panels. While this extensive screening is not required during donor screening and selection, having this information early in the process can avoid progressing donor materials with high-risk mutations into genetic engineering.

In addition, testing for potentially beneficial characteristics, such as blood type, HLA type or other genetic polymorphisms may also be considered. For example, use of material from HLA-homozygous donors, such as CiRA iPSC seed stocks [34], in allogeneic cell therapies may reduce the likelihood of immunologic rejection due to HLA-mismatch in situations in which HLA-mismatch will not be addressed through genetic engineering [35]. This additional testing may be performed at the time of donor selection or retrospectively using retained donor samples.

Typically, for allogeneic therapies, multiple donors are screened during product development. In the case of primary cell–derived allogeneic therapies, multiple donors are required for manufacturing over the lifetime of the product, since a single donor can supply a finite number of drug product batches. Therefore, it may be important to understand donor-to-donor variability to improve manufacturing consistency. For iPSC-derived allogeneic therapies, a single donor can typically be used for the full product lifetime, owing to the greater expansion capacity of iPSCs. However, during cell line development, donor variability may impact susceptibility of cells to genetic engineering, ability to differentiate iPSC lines into specific lineages as well as the function and safety of the drug product derived from donor material. Therefore, multiple donors may be screened during cell line development before selecting a single donor or clone to advance to manufacturing. It is recommended to establish specifications for incoming donor material as either a formal ancillary material specification document or for critical quality attributes in a quality target product profile. Development of these documents allows for clear identification and communication of key requirements for donor material procurement.

Starting Materials and Critical Components

Starting materials for genetically modified cells are defined as the "human or animal cells and the tools (e.g., vectors, mRNA) used to genetically modify them" as per the EMA guideline on quality, nonclinical and clinical aspects of medicinal products containing genetically modified cells [36]. The components used for genetic modification of cells may include one or combination of a few: a viral or nonviral vector encoding the gene of interest, the genetic sequences for modification of the cell genome (e.g., a regulatory guide RNA) with an mRNA expressing the modifying enzyme or a ribonucleoprotein (e.g., Cas9 protein pre-complexed with gRNA), the repair template (e.g., linear DNA fragment or a plasmid), or the components to produce them. Considerations for these starting materials and critical components are discussed to follow.

Vectors and plasmids

Once donor material has been identified, attention is placed on the *ex vivo* genetic modification of the donor cells. Here, the selection of the appropriate vector expression systems to introduce exogenous gene(s) of interest (e.g., CAR) becomes the priority. There are two main types of expression vectors that have been successful in cell therapies: viral and non-viral vectors. Viral vectors are composed of recombinant viruses, such as lentiviruses, gammaretroviruses or adeno-associated viruses, which serve as packaging cassettes for the gene template of interest for site-specific insertion post-genome editing. Non-viral vectors are composed of plasmid DNA complexes (naked or in lipid or polymer systems) carrying the gene of interest, introduced by non-viral delivery methods, such as electroporation.

The vector expression system is considered a critical component for genetically engineered allogeneic cell products. Consequently, it is important to fully understand its history and derivation, as well as ensure significant characterization of the final vector(s) is performed. Vector/plasmid production should be well documented, capturing the source for the vector construct, the production cell line, and the culturing materials used during cell transfection and amplification. Post-production, the resulting vector product should be tested for titer/concentration, purity (both desired and undesired products), safety and efficacy. In addition, full sequencing of vectors 40 kb or smaller should be performed to ensure the correct genetic sequence and should include information on the transgene insert, flanking regions, regulatory elements (e.g., promoter, enhancer and polyadenylation signal), pertinent restriction endonuclease sites and open reading frames.

Health authorities recommend use of the greatest-quality vector expression system for genetic engineering; however, there remains a question as to whether this requires vector production to be performed under full GMP (or current GMP [cGMP] in the United States) compliance or if following the select principles of GMP in vector production is sufficient. FDA guidance states "a vector used to transduce cells *ex vivo* and which furnishes a pharmacological activity for the

treatment of disease is a critical component" and "a separate DS section should be provided for vectors used for *ex vivo* modification of cells," which would suggest cGMPs apply to such material [25]. In recent draft guidance from the FDA, additional clarification is provided which recommends that although the principles of cGMP may be appropriate for Phase 1, for later Phase studies and licensure, genome editing components should be manufactured according to cGMP standards (21 CFR Parts 210 and 211) [37].

Meanwhile, EMA guidance suggests that the principles of GMP shall apply from the bank system used to produce these materials onwards [36]. In a recent document, the EMA has clarified what the principles of cGMP mean and to which stages of the process these principles apply [38]. Using the example of an *ex vivo* autologous CAR-T manufacturing process, GMP principles are said to apply to establishment of cell banks for plasmid and/or vector expansion, plasmid and vector manufacturing, while GMP is applied to manufacturing of genetically modified cells. The principles of GMP include aspects related to the "the quality management system, documentation, raw materials, cell banks, production, specification, testing and control, storage and other aspects of handling and distribution as appropriate having regard to the relevant risks for the quality, safety and efficacy of the finished product." While the example used may not be fully relevant for allogeneic processes, the document provides room for interpretation and the use of a risk-based approach for selection of applicable GMP principles.

When the *ex vivo* modification of cells is part of the routine GMP manufacture of each batch, the approach described in FDA and EMA guidance is appropriate to summarize the various active ingredients used for the transfection of the starting donor material. However, for iPSC-derived allogeneic products, genetic modification of the cell source often is performed once in the lifetime of the product before the generation of the GMP MCB. The MCB is manufactured before initiating Phase 1 studies and additional batches are not manufactured to support later Phase studies or licensure. In this case, the genetically engineered MCB serves as the starting material for manufacturing of each batch and the only active ingredient that is cultured and differentiated to the final drug product. The GMP principles applied to single-use vectors or plasmids used upstream of the MCB may be less extensive than when these materials are used for routine manufacturing. Here, a risk-based approach is appropriate. Select principles of GMP should be applied and documented, focusing on the potential for microbial and viral contamination, cross-contamination with other vectors or genetic material, replication-competent virus (where applicable), process residuals and product-related impurities. Aspects related to ensuring process consistency, such as those related to process and analytical validation, in process testing, or stability, are less applicable for one-time production of these components.

Gene-editing components

Besides the vector or plasmid, other components of genome editing such as nucleases and guide RNA (gRNA) serve a critical role in the *ex vivo* modification of cell therapy products. Nuclease and gRNA may be introduced to cells directly as a ribonucleoprotein to induce gene knockouts or in combination with plasmid carry transgenes of interest, which may be integrated via homology directed repair. These materials are key for the success of the genetic engineering steps but, unlike the vector or plasmid, are not intended to furnish a pharmacologic activity or be part of the final product. Due to their importance, it is equally essential to source and/or produce these gene editing components with the highest standards for safety, purity and activity, whenever possible, when intended for direct introduction outside the vector expression system. As discussed previously for vector and plasmids, the principles of GMP applied to these materials may be more extensive when these materials are introduced as part of the manufacturing process than when used during cell line development. Once more, a risk-based approach should be followed to ensure the chosen materials are produced, controlled and characterized under the appropriate principles of GMP.

Cell Banks

Unlike primary cells, iPSCs are continuous cell lines that have an infinite capacity for growth. This expansion capacity allows for cell banks to be created at multiple points during cell line development or the manufacturing process. For instance, a bank of reprogrammed, but non-engineered, iPSCs may be created to serve as a parental cell bank for genetic modification. Cells also may be expanded to create an MCB that is of sufficient quantity to support continued manufacture of a product. Creation of a clonally derived MCB, especially following completion of genetic engineering, allows for the use of a single, well-characterized, starting source for each production batch over the lifetime of a given product. This consistent starting material can significantly reduce batch-to-batch manufacturing variability and allow for more extensive qualification and characterization testing throughout the process.

Recommendations on the derivation and characterization of cell substrates used for the production of an MCB and ultimately, the final product, is provided in International Conference on Harmonisation (ICH), FDA and EMA guidance [25,36,39]. While this guidance was originally developed for biological and biotechnological products, the principles found in this guidance may be applied to genetically modified, iPSC-derived allogeneic products (e.g., allogeneic CAR T products) with MCBs.

Source, history and generation of the cell substrate

Activities conducted during the research and development of the cell substrate can contribute to the risks associated with the use of this material for manufacturing. Therefore, information on the source, history and generation of the cell substrate is important to support an assessment of potential risks to quality and safety of the product. For genetically engineered, iPSC-derived allogeneic products, the risks associated with the cell source should be evaluated. In addition to providing information on the donor and critical components used for genetic modification, information on the generation of the cell substrate should be provided. This information typically includes the methods for isolation, selection, reprogramming, genetically engineering and expanding the cells, as well as raw materials used in the process, and finally analytical testing performed for characterization. This information aids in assessing and reducing the risks associated with product- or process-related impurities (e.g., residual gene-editing components and unintended cell types), cross-contamination (e.g., vector/plasmid contamination), microbial contamination (e.g., sterility, mycoplasma, endotoxin) and introduction of adventitious agents (e.g., human and animal-associated viruses) or transmissible spongiform encephalopathy.

Clonality

The cell substrate used for production of a genetically modified, iPSC-derived MCB may be generated from a single-cell clone, which allows for the selection of cells exhibiting the desired functional attributes, increases cell substrate homogeneity and has the potential to improve product consistency and quality. Guidance on the clonal derivation of cells lines for allogeneic cell therapy products is limited, but insights may be gleaned from practices used for clonal derivation of mammalian production cell lines used in manufacture of recombinant DNA-derived products [40]. Isolation of single-cell clones from a bulk cell population is typically performed through one of three methods: limiting dilution, fluorescence-activated cell sorting or use

Table 3
Recommended qualification and testing for cell banks.

Attribute	Test	Analytical methods	Purpose
Identity	Short tandem repeat (STR)	STR profile for donor identity	Release
	Transgene insertion or expression	Genotype (e.g., qPCR, ddPCR) and/or phenotype (e.g., flow cytometry, ELISA) detection of transgenes for engineered cell line identity and/or cell type	Release
Purity	Cell phenotype	Phenotype (e.g., flow cytometry, RT-PCR) detection of cell markers associated with iPSCs	Release, Stability
Impurities	Residual reprogramming components	PCR assay(s) for detection of residual reprogramming components (vectors, plasmids)	Release
	Residual gene editing components	Product-specific assay(s) (e.g., PCR, Western blot, ELISA) for detection of residual gene editing components (nucleases, guide RNAs, vectors, plasmids)	Release
Quantity	Enumeration	Cell count by dye exclusion or flow cytometry	Release, Stability
	Viability	Cell viability by dye exclusion or flow cytometry	Release, Stability
Biological activity	Pluripotency	Genotype or phenotype level detection of genes or cell markers associated with iPSCs; see Cell phenotype	Release, Stability
	Differentiation potential	Embryoid body formation and/or directed differentiation	Characterization
	Doubling time	Growth profiles	Characterization
Genetic safety	Transgene copy number	PCR-based assay for quantitation of transgene copy number	Release
	Off-target integration	Evaluation of off-target insertion of transgenes (e.g., PCR-based, target locus amplification, next generation or whole genome sequencing)	Characterization
	Structural variants (e.g., inversion, duplication, translocation)	Evaluation of structural variants (e.g., karyotype by G-banding, optical mapping, or whole genome sequencing)	Characterization
	Small variants (e.g., insertion, deletion, copy number variants)	Genome screening arrays (aCGH/SNP) or whole genome sequencing	Characterization
Microbiological safety	Sterility	USP <71> and <61>, Ph.Eur. 2.6.27 and 2.6.1, JP <4.05> and <4.06>; or appropriately validated rapid methods	Release
	Mycoplasma	USP <63>, Ph.Eur. 2.6.7, JP <G3>; or qualified PCR methods	Release
	Endotoxin	USP <85>, Ph.Eur. 2.6.16, JP <4.01>; or appropriately validated rapid methods	Release
Viral safety	Retrovirus and animal (e.g., murine, porcine, bovine) viruses	Based on risk assessment of starting and raw materials	Release
	Replication competent retrovirus (RCR)	Culture or PCR-based tests for replication competent virus as used in manufacturing process	Release
	Human viruses	PCR-based tests for cytomegalovirus (CMV); human immunodeficiency virus (HIV) 1 and 2; human lymphotrophic virus (HTLV) 1 and 2; human herpes virus (HHV) 6, 7, and 8; JC virus; BK virus; Epstein–Barr virus (EBV), human parvovirus B19; hepatitis A virus (HAV); hepatitis B virus (HBV); hepatitis C virus (HCV); human papillomavirus (HPV)	Release

aCGH, array comparative genomic hybridization; ddPCR, droplet digital polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; iPSC, induced pluripotent stem cell; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; SNP, single-nucleotide polymorphism.

of automated clone picking systems (e.g., ClonePix) that rely on cell immobilization in a semi-solid matrix or growth on 2-dimensional substrates. These techniques may be followed by cell imaging at time of isolation and early stages of colony formation to provide additional assurance of clonality. However, use of these imaging systems for assurance should take into account optical considerations related to illumination, focal plane and resolution, which may impact ability to accurately detect single cells. Documentation of the process and procedures used for clonal derivation is recommended to allow for evaluation of residual risks associated with non-clonality and inform the selection of an appropriate control strategy for the MCB and drug product. While establishing clonality after reprogramming and genetic modification improves the homogeneity of the MCB with respect to off-target integration and larger structural variants, the MCB and drug product will continue to accumulate small variants with additional passages as would any somatic cell.

Cell bank system and procedures

The generally accepted approach for cell banking is to establish a two-tiered system consisting of an MCB from which additional WCBs are produced to provide cells directly for the manufacturing process. However, single-tiered systems consisting only of an MCB also may be used in cases where a large number of aliquots is not required for production. The decision on whether to create a tiered banking system should take into account the amount of material needed to support the lifetime of the product. Regardless of which approach is taken, for both the MCB and subsequent WCBs, cell banks are typically prepared through progressive expansion of cell cultures until the appropriate bank size is achieved.

MCB and WCBs, which serve as the starting material for manufacturing, should be produced under cGMP to ensure traceability and reduce the risk of microbial, viral and cross-contamination. Assurance of traceability and avoidance of contamination largely focus on documenting the source and quality of ancillary materials (e.g., media, cytokines, growth factors, small molecules) used during cell banking, procedures in place for controlling contamination and procedures for tracking of the cell bank.

Cell bank qualification and testing

Expansion of cell substrate into cell banks allows for sufficient material to perform extensive qualification and characterization testing. The purpose of testing is to confirm the identity, purity and suitability of the cell substrate for manufacturing. While the testing to be performed may vary for a specific product, general guidance on the qualification and testing of cell banks is provided in ICH Q5D. Quality control considerations for clinical-grade iPSC lines have been previously described [41]. However, for genetically modified iPSC-derived MCB and WCBs, additional characterization may be recommended, especially as related to evaluating genetic safety and off-target editing events. A summary of recommended qualification and characterization testing for cell banks is provided in Table 3, based on considerations for genetically modified iPSC-derived banks.

In processes in which a fully gene-edited, clonally derived MCB is produced following cell line development, characterization of the

Table 4
List of potential methods used for assessing genetic safety and tumorigenicity risk.

Process step(s)	Risk	Potential methods
Genetic modification	Off-target integration and structural variants associated with genetic modification	Whole-genome sequencing (e.g., *de novo* assembly, short-read alignment, long-read mapping) for identification of off-target integration and associated structural variants [43,44]
		Target capture sequencing/amplicon next-generation sequencing (NGS) to detect select off-target integration sites and associated structural variants
		Target locus amplification (TLA) to detect off-target integration sites and associated structural variants [45–47]
	Structural variants (greater than 50 bp in length)	Karyotype by Giemsa banding [48]
		Single-nucleotide polymorphism (SNP) arrays [49–51]
		Array comparative genomic hybridization (aCGH) [52,53]
		Fluorescence *in situ* hybridization (FISH) [54,55]
		Optical genome mapping [57,58]
Cell expansion	Small variants (<50 bp in length) acquired during cell expansion	Short-read whole-genome sequencing to detect substitutions, insertions and deletions
Drug product	Benign teratoma formation associated with residual undifferentiated stem cells	Flow cytometry to detect undifferentiated or unintended cell phenotype [58]
		PCR to detect residual undifferentiated cells [59,60]
		Colony formation assay [61]
	Tumorigenicity associated with transformed cells	Cell proliferation assay [62–64]
		In vivo tumorigenicity study

MCB may reduce the need to perform certain testing on the drug product. For instance, in recent FDA draft guidance, it is recommended drug product testing include on-target editing efficiency, characterization of editing events occurring at the on-target site, off-target editing frequency, chromosomal rearrangements, residual genome editing components and total number of genome-edited cells [37]. These recommendations reflect considerations relevant for autologous and primary cell–derived allogeneic therapies. In these processes, where gene editing is performed as part of per-batch manufacturing, bulk cell populations may vary in the level of these attributes. However, for iPSC-derived allogeneic therapies originating from a single cell clone selected after gene-editing, these attributes are not expected to change from the MCB to drug product.

Cell substrate stability

In addition to the qualification and testing described in the previous section, as outlined in ICH Q5D, cell substrate stability should be evaluated to demonstrate: (i) consistent production of the intended product; and (ii) retention of production capacity under defined storage conditions.

Evaluation of cell substrate stability during cultivation for production is addressed through limit of *in vitro* cell age or "end of production" (EOP) studies conducted under pilot or commercial scale conditions before registration. In these studies, at least two timepoints are examined cells with a minimal number of population-doubling events (i.e., the MCB) and cells that have been expanded at or beyond what will be experienced during manufacture (i.e., WCB or beyond). As noted in ICH Q5D, the test articles used for assessment depend on the nature of the cell substrate, the cultivation methods and the product. Recommendations for cell lines containing recombinant DNA expression constructs and non-recombinant cells lines are provided, which suggest the primary focus of these studies is on confirming integrity or invariability of the protein coding sequence to ensure consistent production of the intended product. Nucleic acid testing, analysis of purified product or monitoring of other traits such as biochemical, immunological, genotypic or phenotypic markers are all suggested.

For genetically engineered, iPSC-derived allogeneic products in which the modified cell is the product as opposed to a purified nucleic acid or protein, the structure of limit of *in vitro* cell age studies is not well-defined, owing to lack of clarity on how to define "EOP" for cell-based products. In processes used for production of biological or biotechnological products, EOP is marked by the end of cell cultivation typically the harvest of cells that is performed before downstream purification of the product. For cell-based products, however, there is no clear delineation; all manufacturing steps through drug product are cell-containing. "EOP" may be defined as the end of iPSC expansion before differentiation. In this case, studies are designed to evaluate the suitability of the iPSCs for differentiation and are structured to assess the number of population doublings incurred at or beyond the levels required for the start of differentiation. If a WCB is to be introduced, the number of additional population doublings evaluated for the extended passage condition should also factor in the expansion required to create a WCB. The EOP material is then evaluated for differentiation potential (i.e., the ability to form the intended cell type) and sequence integrity (i.e., the ability to express the intended modifications). In addition, the genetic stability of the expanded passage cells may be confirmed through genetic characterization techniques.

Retention of production capacity under intended storage conditions is typically evaluated during production, i.e., when a cryopreserved MCB vial is thawed for preparation of a new WCB. Qualification of the WCB is usually sufficient evidence for banked cell stability. In the case of a single tiered bank where only an MCB is produced, extended passage cells may be generated and placed on long term stability in place of the MCB to preserve MCB vials for manufacturing.

Genetic Safety

Genetic safety risks may arise from multiple steps of the cell line development and manufacturing process for genetically engineered iPSC-derived allogeneic therapies. Donor selection, genetic modification and extended cell expansion may contribute to increased risk for presence and/or development of genetic "variants of concern," which may lead to cellular transformation (i.e., tumorigenicity) or unintended cellular structures (e.g., teratomas). The main genetic variants of concern may be grouped into three broad categories, including off-target integration of transgenes, structural variants (e.g., deletions, insertions, duplications, translocations and inversions greater than 1 kb in length) and small variants (e.g., insertions, deletions, substitutions less than 50 bp in length).

Donor screening for pre-existing oncogenic mutation and selection of starting material is the first step to control genetic safety risk for an allogenic cell product. For primary cell–derived allogeneic

products, tumorigenicity risk is primarily associated with changes introduced during genetic modification, which may include random insertion by a viral vector, as well as off-target and structural effects caused by genetic editing tools. While the tumorgenicity risk associated with lentivirus or gamma retrovirus transduction has been shown to be low with primary cell-derived products, the risk associated with genetic editing is yet to be fully understood. For iPSC-derived allogeneic products, the risk of tumorgenicity may be associated with genetic modification as described above, but may also be related to genetic changes and malignant transformation of iPSCs acquired during cell expansion. In addition, residual iPSC impurity in the final products can form benign teratomas, adding another safety risk [42].

Genetic safety assessment is not required for release of autologous cell therapy products, due to the limited material for testing, stringent timeline between manufacturing to infusion and limited number of patients treated. However, allogeneic cell products should be characterized for genetic safety risk, with select assays considered for routine batch release where additional quality control of the drug product is warranted. A list of potential methods which may be used to assess variants and risks associated with the process is provided in Table 4 [43–64]. The combination of multiple, orthogonal methods may improve detection of genetic variants and assist in verifying results. Regardless of the methods chosen for evaluating genetic safety, sponsors should clearly document the procedures used for assessment. This includes information on the analysis pipeline, since the approaches used for alignment, variant calling and threshold or cutoff setting may impact the results of the analysis. In addition, sponsors should consider how the results will be evaluated to assess risk and what additional studies or long-term follow up may be required in the case impact cannot be fully assessed.

Off-target integration and structural variants associated with genetic modification

When applying genome-editing approaches to permanently modify the human genome, there is the risk that off-target cleavage may occur at unintended locations, disrupt the function or regulation of genes in an unpredictable manner and lead to adverse events [36]. In addition, homology of off-target sequences to the intended locus or editing of multiple loci can cause simultaneous double-strand break (DSB) formation at different loci, leading to chromosome rearrangement, including deletion, addition or translocation. Therefore, an assessment of off-target activity and structural variation is expected for genetically engineered cell therapy products.

Off-target activity may be evaluated by biased and unbiased approaches. *In silico* prediction is a commonly applied, biased approach used during target site, nuclease and gRNA selection to reduce the potential for off-target activity [65–68]. The algorithmic models used for *in silico* prediction are based on (i) alignment, in which guide RNA is aligned to a reference genome to identify off-target events based on sequence homology; or (ii) scoring, in which machine or deep learning approaches are used to score and rank guide RNA [69]. However, given the limited sensitivity and high false-positive rate of *in silico* prediction, off-target activity must be verified under the actual conditions and system of use through empirical, unbiased approaches [70].

Unbiased methods for detection of off-target activity are grouped into three main categories including those based on detection of DSBs, single-stranded breaks/base edits and translocation or other chromosomal aberrations. Detection of DSBs includes *in cellula* and *in situ* methods. Methods using an *in cellula* approach, such as GUIDE-seq [71] and IDLV capture [72], identify DSBs through insertion of exogeneous sequences, which are used as primer binding sites during amplification using linker-mediated PCR. In the case of *in situ* methods, such as BLISS [73], BLESS [74] and SITE-seq [75], adapters are ligated to open DSB ends for transcription and sequencing. Methods to monitor single-stranded breaks/base edits approaches, including Digenome-seq [76], are emerging and rely upon *in vitro* nicking, base modification and DNA end-repairing followed by whole-genome sequencing (WGS). Translocation approaches, based on nested polymerase chain reaction (PCR) with primers specific for regions between a known target and unknown fused site, include TC-seq [77], UDiTaS [78], AMP-seq [79] and LAM-HTGTS [80]. Comprehensive review of unbiased approaches is covered elsewhere [70]. However, these approaches come with limitations as DSBs are short-lived, challenging to assess in repetitive genomic regions and can be difficult to distinguish relative to background mutations [70,81].

For genetically engineered, primary cell–derived therapies, genetic engineering is performed as part of the manufacturing process for each batch. Therefore, monitoring of off-target events as part of drug product release testing may be expected. To develop methods for monitoring off-target events that are amenable to execution and potential validation in quality control laboratories, a three-stage approach may be applied to assess the risk of off-target activity and confirm off-target integration events. This approach involves (i) discovery, (ii) risk assessment and (iii) confirmation of select, high-risk off-target events. During the discovery stage, biased *in silico* and unbiased empirical methods are used to identify potential sites for off-target activity. An assessment is then performed to determine the level of risk associated with the potential sites. For any potential sites deemed high-risk, targeted amplicon NGS to confirm occurrence under the actual process conditions is performed. Confirmed off-target events at known tumor-suppressor genes, large deletions extending into neighboring genes that can impact gene expression and translocations and inversions near active promoters that may cause activation of the translocated or inverted gene are considered to have the highest potential impact to patients. This approach, using a sensitive method for detection of off-target events at select sites is amenable for the detection of low frequency events in heterogeneous, bulk-edited drug products.

However, for genetically engineered, iPSC-derived allogeneic therapies, genetic engineering is commonly performed one time as part of cell line development before the establishment of a clonally derived master cell bank. As a result, drug products are not expected to contain different off-target events from one batch to the next. Therefore, for these types of products, an alternate approach may be applied for evaluating off-target events. WGS has not typically been applied in the analysis of off-target events in primary cell–derived allogeneic therapies given the difficulty in detecting low-frequency off-target events in a bulk, or non-clonal, population of cells [82]. However, when applied to clonal populations, WGS provides a genome-wide, unbiased method to characterize off-target events including insertions, deletions, single-nucleotide variants and structural variants such as inversions, rearrangements, duplications and large deletions [43,44] For iPSC-derived allogeneic therapies, WGS may be performed during master cell bank characterization, as opposed to drug product release, to confirm off-target events and evaluate structural variants, thus obviating the need for the aforementioned three-stage approach for release.

While WGS-based approaches, which include *de novo* assembly, short-read alignment and long-read mapping, may be useful for characterization, these techniques have limitations [83] and may not be amenable for higher-throughput applications such as screening multiple clones during single cell clone selection or in the case of product release testing where methods require validation. Alternative, complementary methods may be leveraged; given their different applications, use of multiple complementary techniques may be useful. Target locus amplification is a cross-linking-based technique used to generate contiguous DNA sequence of >100 kb surrounding a single primer pair complementary to a locus-specific sequence. This technique, in combination with next generation sequencing, can be used

for detection of insertion sites and translocations without prior locus information [45–47]. Interphase and metaphase fluorescence *in situ* hybridization, with the capability of detecting translocations even in the presence of large resections, may be used in addition to these high-sensitivity, high-throughput methods [54,55,84]. Optical genome mapping, which functions much like a higher resolution digital karyotype, may also be used to resolve structural variants [56,57]. Finally, array comparative genomic hybridization [52,53] and single-nucleotide polymorphism microarrays [49–51] may be useful for evaluating copy number variants [85].

Accumulation of small variants associated with passage-induced genomic instability

Passage-dependent accumulation of mutations can occur during the *ex vivo* expansion of primary-cell and iPSC-derived allogeneic products. The accumulation of genetic changes during cell culture is a potential risk for highly expanded cell products and is a function of the cell culture conditions and number of population doublings. For instance, induced pluripotent stem cells have been shown to accumulate single-base pair substitutions at a rate of approximately 3.5 ± 0.5 mutations per genome per population doubling under atmospheric oxygen conditions (20% O_2). This rate is reduced to 2.1 ± 0.5 mutations per genome per population doubling when iPSCs are cultured under low oxygen conditions [86,87]. Therefore, monitoring may be performed as part of process development and characterization to understand the frequency and consistency of small variant accumulation during cell line development and manufacturing activities, depending on the nature of the process and cell culture conditions.

Studies of human genome variation using population-scale sequencing have identified millions of single-nucleotide polymorphisms, short insertions and deletions, and tens of thousands of structural variants with unknown implication in the general population. While much of the variation upwards of 95% is common across the population, on average, individuals carry approximately 250–300 loss-of-function variants in annotated genes and 50–100 variants implicated in inherited disorders [88]. Given the large number of variants inherently present in the genome, a differential assessment performed at multiple points of the cell line development and manufacturing process is recommended to focus analysis on small variants generated as a result of cell line development and manufacturing.

Ideally, an initial assessment of small variants is performed before reprogramming and genetic modification to establish a reference profile associated with the cell source (i.e., donor); the variations present in the donor may be considered acceptable based on healthy donor screening, with the exception of known high-risk variants (e.g., p53 mutations). Following reprogramming and genetic modification, which involve a large number of population doublings, an evaluation of acquired variants relative to the donor should be performed as part of comprehensive cell bank qualification (i.e., during MCB qualification and EOP studies). By testing at this stage of the process, the impact of cell-line development activities on accumulation of variants can be evaluated. It also serves to establish a baseline for evaluating accumulation of variants during the manufacturing process. Variants identified through the differential assessment may be assessed for potential risks associated with cancer or intended use as part of MCB characterization.

Where possible, an assessment also may be performed at the end of the manufacturing process on the same material going into IND-enabling non-clinical studies to establish linkage between genetic characterization data and potential risks that may manifest during animal studies (e.g., tumorigenicity). This approach helps address situations in which the risk associated with new variants is unable to be assessed by available literature and comparison with publicly available risk databases. If it is not possible to use GMP material for IND-enabling studies or in the case of major process or facility changes, it may be appropriate to repeat the differential assessment of drug product relative to MCB to confirm the change does not negatively impact accumulation of small variants. At this time, it is not recommended small variants be monitored for routine release of drug product given limited understanding of how the presence of such variants may translate to increased risk.

Tumorgenicity and teratoma risk related to cellular transformation and pluripotency

Tumorgenicity risk associated with iPSC-derived cell therapy products can arise from two aspects: unintended transformation of pluripotent cells during expansion and teratoma formation from residual undifferentiated iPSC cells. It should be noted that core pluripotency factors play critical roles in both pluripotency and oncogenesis. The genes (e.g., c-Myc, Lin28, Nanog) involved in epigenetic reprogramming are largely transcription factors with potent oncogenic properties [89]. Therefore, iPSC cells and iPSC-derived cells are potentially more prone to malignant transformation than primary differentiated cells.

In vitro assays can be developed to detect transformed cells and residual undifferentiated iPSCs down to very low levels. The digital soft agar colony formation assay [61] and cell proliferation assay [62–64] are well established for detecting anchorage-independent cell growth and uncontrolled cell proliferation, respectively, potentially associated with malignantly transformed cells *in vitro* [90]. The digital soft agar method is highly sensitive and can detect at approximately 0.02% HeLa cells in a cell population and 0.00001% HeLa spike in when complementing the 30-day culture method with digital image analysis. Meanwhile, the cell proliferation assay is able to detect up to 0.001% HeLa spike in a cell population over 30-day culture. On their own, these methods may be used to assess the potential risk of unintended transformation in a cell therapy, or may be used as complementary methods to assess the findings obtained from other structural or small variants approaches, such as NGS or WGS.

With respect to teratoma risk, previous studies have reported a minimum 1×10^4 human embryonic stem cells is sufficient to cause teratoma in immunocompromised mice [91]. Similarly, teratoma formation can frequently be observed within 4 to 6 weeks in animals administered 1×10^6 undifferentiated iPSC cells [92–96]. When iPSCs are used in a manufacturing process, the process should be designed to limit their levels in the drug product and the level of residual undifferentiated cells in the drug product should be determined to reduce potential safety risks.

Determination of residual iPSC in drug products requires highly sensitive methods with a well characterized lower limit of detection (LLOD). Flow cytometry methods recognizing stem cell markers (e.g., Oct3/4, SSEA4, Nanog, TRA-1-60, TRA-1-81) can be used to confirm the appropriate cell phenotype and detect unintended and undifferentiated cell phenotypes. Flow cytometry–based residual iPSC analysis can be optimized to a LLOD of 0.01%. However, for greater intended cell doses, this LLOD may be insufficient. In cases in which a lower limit of detection is required, the use of PCR-based methods may be preferred over flow cytometry for detecting undifferentiated cells [97]. For example, a quantitative real-time PCR method targeting Lin28 is able to detect 0.002% of residual undifferentiated iPSC cells (one iPSC in 5×10^4 cells) in the final cell population [59]. It is also noted that a different target for the PCR based method may be required for optimal detection of residual iPSCs depending on the final differentiated cell type [98].

In vitro assays can be developed to detect transformed cells and residual undifferentiated iPSCs down to very low levels. Despite the availability of *in vitro* methods, an *in vivo* tumorgenicity study is still

required as a non-clinical study to assess the risk in animals. The tumorgenicity study may be designed in a way to assess the tumorgenicity risk based on the detection limit of *in vitro* assays; i.e., through administration of a dose which is representative of the number of transformed or residual undifferentiated iPSCs that can be detected in the *in vitro* assay format. In addition, the tumorgenicity study can also help assess the safety risk related to genomic modification, which may cause gene/region mutations with unknown function and are usually challenges to translate to risk in patients.

Concluding Remarks

With genetically engineered iPSC-derived cell therapies entering clinical phase evaluation [99], there is growing need for international guidelines that address chemistry, manufacturing and controls considerations unique to these product types. Existing regulatory guidelines for cell and gene therapies are generally limited. Where guidelines do exist, recommendations are largely shaped by earlier generation autologous and primary cell–derived allogeneic therapies, which may not fully apply given fundamental differences in manufacturing processes.

Recommendations on donor eligibility, screening and testing are fairly comprehensive and provide a framework on which sponsors may build their donor sourcing and selection strategy. Within these guidance documents, the minimum requirements for cell sourcing are clearly outlined. However, differences in requirements and accepted testing exist across regions, which presents challenges for genetically-engineered iPSC-derived therapies. Given the one-time nature of cell line development and intent to use these "off-the-shelf" therapies to treat patients across many markets, decisions must be made very early in product development to ensure that the process used to select the donor will comply with regulations across all future intended markets. Efforts to internationally harmonize donor requirements across regions could alleviate these considerations, simplify product development and ensure broader access to these therapies in the future.

Increasing focus has been placed on the control of starting materials and critical components used in genetic modification, as evidenced by language present with recent FDA and EMA guidelines. It is recommended that vector expression systems and other critical components such as plasmids, nucleases and gRNAs are produced "under the principles of GMP" and documented similar to drug substances. Implicit in both guidance documents is a requirement to demonstrate manufacturing consistency potentially through the use of banking systems in addition to quality control. However, processes to produce genetically engineered iPSC-derived cell therapies have evolved; in some cases, resembling traditional biological products more than earlier cell therapies as relates to cell line development. Given the expansion capacity of iPSCs, gene editing may be performed once during cell line development, with fully edited cells being expanded to create a single MCB which will supply the full lifetime in the product. Consistency of vector or critical component production, in this situation, becomes irrelevant. Additional clarity on the select "principles of GMP" which should apply in these processes is warranted.

Cell banking, as relates to the handling of parental iPSC banks and genetically engineered MCB and/or WCBs, is a consideration which has many implications for genetically-engineered iPSC-derived therapies, but is less relevant to earlier generation cell therapies. While existing cell and gene therapy guidance documents touch on the requirements for banking systems as associated with vector production, fewer recommendations are available for cell banks. Here again, existing guidance applicable to biological products may be leveraged; these products also commonly use cell banks as the starting material for the manufacturing process. The recommendations in existing guidance documents for biological products are largely applicable to genetically engineered iPSC-derived therapies. However, key differences also exist between the processes. Biological products are typically purified from the MCB/WCB, while for cell therapies, the cell is the product. Therefore, additional clarity around the requirements for MCB/WCB qualification and design of EOP studies is warranted.

Finally, existing guidance does not speak to the expectations for establishing genetic safety. With advancements in analytical approaches available to evaluate genetic variants of concern, there are increasing opportunities to gain potentially useful insights which may inform genetic safety risk assessment. However, there is lack of consensus across industry and regulatory agencies on what constitutes a variant of concern, which methods should be applied to assess these attributes and how to interpret findings with respect to potential impact to patient safety. For genetically engineered iPSC-derived therapies, with the potential to treat a large number of patients, this evaluation is critical and requires input from sponsors and regulatory bodies to establish a standard paradigm and set of expectations.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the manuscript: JLD, QX, MCN, EL, LC and DH. Drafting of the manuscript: JLD, QX, MCN, EL and DH. Revision or review of the manuscript: JLD, QX, MCN, EL and DH. All authors have approved the final article.

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