Trends in Biotechnology

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Review

Bioprocess Technologies that Preserve the Quality of iPSCs

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Large-scale production of induced pluripotent stem cells (iPSCs) is essential for the treatment of a variety of clinical indications. However, culturing enough iPSCs for clinical applications is problematic due to their sensitive pluripotent state and dependence on a supporting matrix. Developing stem cell bioprocessing strategies that are scalable and meet clinical needs requires incorporating methods that measure and monitor intrinsic markers of cell differentiation state, developmental status, and viability in real time. In addition, proper cell culture modalities that nurture the growth of high-quality stem cells in suspension are critical for industrial scale-up. In this review, we present an overview of cell culture media, suspension modalities, and monitoring techniques that preserve the quality and pluripotency of iPSCs during initiation, expansion, and manufacturing.

Highlights

Maintenance of pluripotent status and self-renewal capability are essential for the production of clinical-grade iPSCs for cell therapies.

Media and matrix formulations and matrices have evolved through consideration of the signaling pathways that help to sustain pluripotency in iPSC lines and overall process scalability.

Matrix- and feeder-free iPSC suspension culture systems overcome the limited scalability of static matrices while supporting iPSC growth and pluripotent status.

Development of iPSC monitoring techniques, *in silico* models, and quality-by-design strategies that incorporate real-time data would enable robust process scalability.

Recent Advancements in, and Challenges to, Stem Cell Manufacturing

Cell therapies involving stem cells (such as tissue transplantation or drug discovery) are used to treat a variety of clinical indications, primarily in the fields of oncology, cardiology, immunology, and neurology [1]. Thus, a major focus in the stem cell research field is to advance strategies for cell growth while maintaining control of cell differentiation. Traditionally, embryonic stem cells (ESCs) were the ideal cell type for cell therapy due to their inherent pluripotency, that is, the ability of a cell to differentiate into any specialized cell type. The discovery of these cells initiated vast opportunities for regenerative medicine and treatment for a diverse range of pathological disorders. Regardless of stem cell origins, ESCs must undergo self-renewal to maintain pluripotency while proliferating, during which differentiation into a defined cell type is suppressed [2].

In 2007, Yamanaka and colleagues [3] led a major technological breakthrough in the stem cell field by successfully converting human somatic cells to stem cells with a similar gene expression profile and pluripotency to human ESCs (hESCs). These cells became known as human iPSCs (hiPSCs) [3]. Addressing ethical concerns by avoiding the use of embryos to extract stem cells, iPSCs are a more favorable platform for research and clinical use [4]. Given their inherent self-renewal capability, pluripotency, and relatively low immunogenicity [5], iPSCs represent a promising unlimited source of patient-derived cells for human genetic disease modeling [6] and toxicity studies [7], which lower the overall costs of, and risks associated with, drug development and clinical trials [8]. Due to its multifaceted capabilities, iPSC technology remains a promising scientific tool for personalized cellular therapy and regenerative medicine [9].

Current clinical cell therapies and tissue regeneration for humans require 10^8–10^{10} of clinical-grade stem cells grown with a current Good Manufacturing Practice (cGMP) process [10]. However, due to the dependence on a supporting matrix and sensitive pluripotent state of iPSCs, cell culture expansion to the necessary extent remains a significant challenge. Final iPSC quality during harvest depends on the metabolic state of the cell; more specifically, maintenance of pluripotent status and self-renewal are essential to producing clinical-grade iPSCs for

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cell therapies. Therefore, methods that measure intrinsic markers of cell differentiation state, developmental status, and viability are most useful for large-scale manufacturing. Furthermore, existing platforms are being optimized to meet cGMP standards in effort to efficiently scale bioprocesses to clinical manufacturing settings. As depicted in Figure 1 (Key Figure), in this review, we highlight recent developments in iPSC cell culture methods, including media, suspension modalities, and monitoring techniques, that preserve iPSC quality and pluripotency to the extent necessary for clinical manufacturing. Additionally, we discuss technologies that, if further developed, could improve iPSC bioprocess efficiency and yield.

Isolating IPSCs from Heterogeneous Cell Populations

Myriad signals can activate stem cell differentiation and, thus. subtle changes in cell culture conditions or stresses to the cells can result in heterogeneously differentiated cell populations. This is a serious safety concern, because differentiated cell contamination could give rise to potential tumor or teratoma formation in cell graft recipients. Yet, spontaneous stem cell differentiation can occur during cell culture, as observed in mesenchymal stem cells (MSCs) in response to extended culture in extracellular matrices [11]. To mediate this type of response and preserve

Glossary

Animal component free (ACF): finished product does not contain any ingredient that is either an animal (including human) tissue or body fluid or that is isolated or purified from an animal tissue or body fluid.

Chemically defined media (CDM): a growth medium suitable for the *in vitro* cell culture of human or animal cells in which all of the chemical components are known.

Extracellular matrix (ECM): a 3D network of extracellular macromolecules, such as proteins, glycoproteins, and polysaccharides, that provides structural and biochemical support to surrounding cells.

Xeno-free media (XFM): for therapeutic applications, a chemically defined medium devoid of animal- or human-derived components.

Key Figure

Technologies that Preserve and Monitor Induced Pluripotent Stem Cell (iPSC) Quality

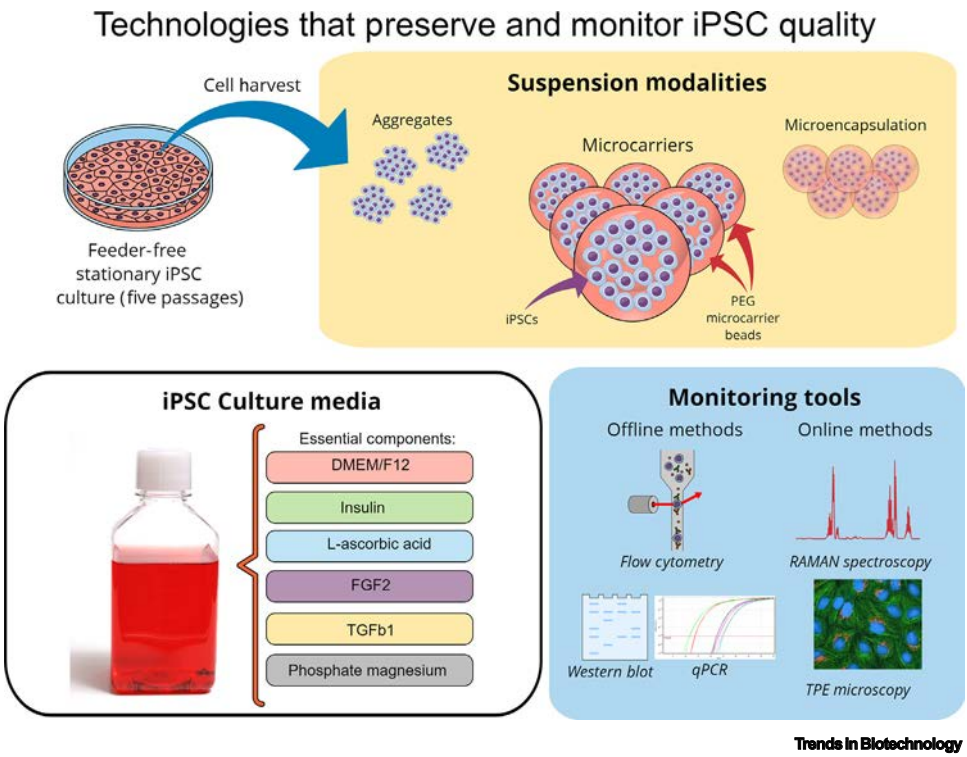

Figure 1. The figure provides a review of recent developments in iPSC cell culture methods, including media, suspension modalities, and monitoring techniques, that preserve iPSC quality and pluripotency to the extent necessary for clinical manufacturing. Abbreviations: FGF, fibroblast growth factor; PEG, polyethylene glycol; TGF, transforming growth factor; TPE, two-photon excitation.

PSCs for future use, cell-sorting methods that separate pluripotent cells from differentiated ones have been applied. Fluorescence activated cell sorting (FACS) and microwell adhesion are popular high-throughput methods used to isolate cells based on a defined pluripotency signature, and the selectivity of pluripotent marker-positive stem cells is enhanced by the preceding cell culture conditions. Regarding iPSCs, when supplemented with a small-molecule cocktail of four inhibitors (SMC4 medium), a 55-fold increase in SSEA4/Tra181-positive iPSC clones was observed post sorting compared with clones derived from cells sorted in conventional reprogramming medium [12]. Although FACS is highly automated and standardized, sorting individual cells to generate stable clones of pluripotent cells remains labor intensive and time consuming. Therefore, methods that can generate pluripotent cell populations as pooled cultures are preferred because pools can maintain the long-term stable expression of pluripotent markers. For this purpose, magnetic-activated cell sorting (MACS) methods using cell surface marker antibodies have been applied to generate pools of iPSCs [13]. In this case, one round of sorting heterogeneous cell pools with TRA-1-60 and SSEA4 antibodies enriched the population of TRA-1-60- and SSEA4-positive cells by 28% and 11%, respectively. Additional rounds of MACS further enriched the population of cells expressing the pluripotent markers, establishing MACS as an alternative to the clonal derivation of iPSCs. In general, MACS is a preferable method over FACS, because it can easily and quickly be carried out on multiple samples simultaneously while imposing less shear stress on cells.

Although cell-sorting methods are effective in characterizing stem cell populations based on their morphology and surface indications, currently widespread adoption of cell-sorting methods for the isolation of animal or clinical study-grade iPSCs is impeded. This is mainly due to the high cost of GMP-grade antibodies combined with the limited availability of clinical-grade FACS instrumentation and expertise. Therefore, scalable platforms for generating reliable, uniform, and safe populations of clinical-grade iPSCs are necessary for future cell therapy clinical trials.

Development of Optimal iPSC Culture Matrices and Medium

A key initial step to control the quality of iPSCs during expansion is to utilize well-characterized materials during the bioprocess, where cell culture media and matrix have pivotal roles. Cell culture medium is essential for maintaining healthy, proliferating cells in culture by providing a favorable balance of nutrients, minerals, and pH. Cell culture matrices are utilized as scaffolds for cells to adhere and proliferate upon, and matrices are often coated with feeder cells or growth-supporting factors to further enhance cell adhesion and growth. The use of a fully characterized cell culture system is vital for a well-controlled bioprocess, especially for the production of clinical-grade biological samples. Over the past decade, iPSC media formulations and matrices have evolved through consideration of the signaling pathways that help to sustain pluripotency in iPSC lines and overall process scalability.

When it comes to designing media for iPSCs, one strategic approach is to identify intrinsic growth factors involved in pluripotency-dependent signal transduction pathways. Various pathways regulate pluripotency gene levels in stem cells, such as transforming growth factor (TGF)-β superfamily-activated cascades, receptor tyrosine kinase signaling [downstream of basic fibroblast growth factor (bFGF)], pathways involving insulin-like growth factors (IGFs) [14], and so on. Interestingly, proteins and growth factors that are sufficient at preserving pluripotency in mESCs [i.e., bone morphogenic proteins (BMP) and leukemia inhibitory factor (LIF)] differ from those for hESCs [14]. iPSCs may also have differing growth factor requirements for pluripotency and self-renewal maintenance. For example, bFGF has been identified as a critical supplement for sustaining hESC self-renewal *in vitro*, with concentrations ranging from 40 ng/ml to 100 ng/ml in feeder-free cultures [15]. Also implicated in hPSC self-renewal is canonical Wnt/b-catenin

signaling, although supplementation with Wnt3a alone is not sufficient to maintain undifferentiated hESC without feeder cells [16]. Given that these metabolic studies were conducted on hESCs and not iPSCs, more in-depth characterization of iPSCs is needed to design media that considers the unique requirements of each new cell line developed for clinical applications.

Feeder cell-based matrices prevent spontaneous stem cell differentiation and improve ESC and/or iPSC attachment by providing specific stemness-supporting factors and producing an **extracellular matrix** (ECM; see Glossary)-rich environment [17]. The most commonly used feeder cells to support PSCs are proliferation-inactivated mouse embryonic fibroblasts (MEFs), because they produce various proteins critical to pluripotency maintenance, such as TGF-β1, activin A, BMP-4, pleiotrophin (heparin-binding growth factor), and so on. Yet, technical challenges arise when it comes to the large-scale production of iPSCs under feeder conditions due to the limited proliferation capacity of feeder cells, reduced efficiency to support iPSC pluripotency after repeated passages, and high risk of contamination during iPSC isolation [17]. In addition, animal-derived feeder matrices can pose an increased risk of transferring zoonotic pathogens and unknown viruses to host cells, which can cause immune system rejection [17]. Thus, iPSC culture methods have focused heavily on transitioning to animal-component and cell-free (termed 'feeder-free') culture systems through use of ECM proteins [18,19], conditioned medium [20,21], or synthetic biomaterials [22].

Along with media, iPSC cell culture matrices have been improved over the past decade to meet cGMP standards step by step. A recent iPSC derivation study revealed that the long-term use of animal-derived serum and many xeno-containing molecules can affect cell morphology, expansion potential, gene expression, and cytokine profile [23]. This led to the formulation of **xeno-free media** (XFM), shortly followed by **animal component-free** (ACF) media to support iPSC expansion. Table 1 lists the most well-characterized matrices and media currently available for stem cell line expansion, research and development, and clinical use. Among the types of ACF media developed, Essential 8™ (Thermo Fisher Scientific) medium is the most utilized basal media for iPSC culture because it contains eight of the most essential elements for stem cell proliferation: DMEM/F12, L-ascorbic acid, phosphate magnesium, sodium selenium, FGF-2, insulin, $NaHCO_3$, and transferrin, TGF-β1, or Nodal [24].

Development of feeder-free iPSC expansion methods have fostered the possibility of future automated manufacturing. In fact, scalable automated production of undifferentiated iPSCs was shown to be feasible under feeder-free conditions using a CompacT SelecT™ cell culture system (The Automation Partnership, UK) [25]. In this case, aggregate hiPSCs automatically passaged with chemically defined medium (CDM) supplemented with Activin A and FGF-2 maintained their characteristic morphology and pluripotency marker expression [25]. Biomaterials have also been explored as enhanced matrices for feeder-free iPSC culture systems. For

Table 1. Common iPSC Media Used for Research and Development, Cell Line Development, and Clinical Applications

Grade	Vendor	Brand	Application	Refs
CDM	STEMCELL™	mTeSR™1	Research and development	[93]
XFM	STEMCELL™	mTeSR™3D; TeSR™2	Cell line development	[83]
	Gibco™	StemPro® SFM; StemPro® SFM XENOFREE	Cell line development	[94,95]
ACF	STEMCELL™	TeSR™-E8™	Clinical	[96,97]
	Gibco™	Essential 8™	Clinical	[24,98]
	Lonza	L7™ hPSC	Clinical	[99]

example, one study found that an optimal elasticity of hydrogel-based matrices exists (25 kPa) at which cells maintain pluripotency [26]. Further investigation revealed that dual-chain vitronectin-derived oligopeptides grafted onto hydrogels (storage modulus of 25 kPa) supported the long-term growth of hESCs and iPSCs for more than ten passages [27]. ECMs, such as fibronectin, laminin, and vitronectin, or oligopeptides derived from ECMs have a specific cell-binding domain, which makes them essential components for supporting iPSC growth in feeder-free matrix systems. 3D bioprinting and cell/tissue printing techniques also unlock possibilities when it comes to future process scaling and automation. Recently, the effectiveness cell printing technologies was demonstrated when iPSCs were adapted and expanded on feeder-free chitosan or polyurethane membranes coated with fibronectin [22]. Here, iPSCs embedded into thermoresponsive polyurethane (PU) hydrogel matrices showed enhanced viability [22]. However, further optimization of polymer-based feeder-free matrices is necessary because differences in pluripotency markers (i.e., OCT4 and NANOG) varied in PU hydrogel cultures compared with control MEF feeder cultures.

Cell culture dynamics have a pivotal role in determining not only the ultimate behavior of iPSCs, but also the cost of the overall bioprocess. Although large-scale growth of hPSCs in 2D static cultures has been demonstrated in single-use multilayered plate bioreactors with the capability to monitor and feedback-control pH and dissolved oxygen (DO) [28], scale-out of hPSC production on 2D or 3D static matrices remains a cost-, labor-, and space-intensive approach. Static culture conditions are also known to induce unfavorable gradients of media components, waste metabolite products, paracrine factors, and gases. With the major consensus being that dynamic suspension culture is the optimal approach to achieve the density of hPSCs required for clinical applications, newer strategies for efficient and scalable expansion of iPSCs in suspension are currently underway. Successful matrix-based studies can be utilized, for example, to design optimal suspension culture modalities. The following sections of this review provide insight into the work that has been done in this area.

Key Considerations for iPSC Suspension Modalities (Aggregates, Microcarriers, and Microencapsulation)

Aggregates

Matrix-free iPSC suspension culture systems overcome the limited scalability of static matrices while supporting iPSC growth and pluripotent status [29]. Due to the adherent nature of iPSCs, 3D aggregates (or spheroids) spontaneously form when they are seeded and expanded in suspension. iPSC aggregates closely resemble embryoid bodies (EB) and, therefore, can be a useful modality for direct lineage differentiation after expansion [29]. The growth and pluripotent status of iPSC aggregates mainly depend on the microenvironment, aggregate size distribution, and cell culture vessel size. Culture conditions have been optimized where hiPSCs have been expanded as undifferentiated suspension cell aggregates under E8™ feeder-free conditions in spinner flasks for more than ten passages [30]. Since then, stirred suspension cultures have been scaled-out to 3000-ml single-use bioreactors (1000-ml working volume) to produce large quantities of hiPSC aggregates (up to 2×10^9 cells), all while preserving the expression of pluripotent state markers, including TRA-1-81, SSEA-4, OCT4, and SOX2 [31].

However, there are limitations to expanding iPSC aggregates in dynamic suspension cultures, noted by reduced expansion rates compared with static suspension cultures and heterogeneous aggregate size formation [32]. If not controlled, formation of large agglomerations of cells (>800 μm) can lead to decreased cell viability, spontaneous differentiation, nutrient and oxygen diffusion gradients, and an overall slower expansion process [30,33]. Aggregate size can be controlled in iPSC cultures via optimization of impeller type and agitation speeds. One example

of a novel bioreactor system that reduces aggregate size while expanding iPSCs is the single-use Vertical-Wheel bioreactor (VWBR), where agitation is provided by a vertical impeller and, therefore, efficient homogenization of the vessel is achieved [34]. Using this system, aggregates with an average diameter of ~350 μm were generated (2.3×10^6 cell/ml maximum density) while maintaining pluripotency [34].

Media supplements can also influence aggregate formation. For instance, short-term treatment with retinoic acid (RA) was shown to further sustain pluripotency during expansion of hiPSC aggregates [35]. Retinoids support iPSC self-renewal via increased expression of pluripotency-dependent transcription factors (Nanog and Oct4) and activation of the phosphatidylinositol-3-kinase (PI3K) signaling pathway [35]. Additionally, CDM supplemented with Rho-associated coiled-coil kinase (ROCK) inhibitor Y27632 promotes iPSC aggregate formation in various suspension vessel types, starting from single-cell inoculation [32,36,37]. ROCK inhibitor supports the survival of stem cell aggregates by diminishing dissociation-induced apoptosis and enhancing cloning efficiency [38]. However, recent studies suggest that prolonged exposure to ROCK inhibitor alters the metabolism of iPSCs [39]. Thus, media supplementation, inoculation strategies, and bioreactor settings during expansion are critical parameters to optimize to boost bioprocess yields.

With further optimization, large-scale manufacturing of iPSC aggregates sufficient for clinical applications can be realized. Both the nutrient transport mechanisms and aggregate size distributions need to be improved in this modality to preserve the pluripotency and viability of the cell aggregates at high densities.

Microcarriers

Efforts in enhancing stem cell culture expansion in bioreactor systems has advanced from the implementation of microcarriers. Microcarriers offer the advantage of providing a surface area to support the growth and attachment of iPSC while maintaining the benefits of a dynamic suspension culture system. Microcarriers can be cell seeded and, if biodegradable, can be utilized to ultimately transport cells to desired damaged tissues during treatment. A variety of biodegradable materials has been used to produce microcarriers for cell line expansion in general, including dextran [40], collagen [41], gelatin [42], poly-lactic-co-glycolic acid (PLGA) [43], poly-l-lactic acid (PLLA) [44], polystyrene (PS) [45], and hydroxyapatite (HA) [46]. Similar to larger static matrices, microcarriers provide even further enhanced support for PSC growth depending on their surface charge properties and when coated with ECM proteins, such as vitronectin, fibronectin, and laminin [45,47–49]. Stirred microcarrier culture systems have been shown to facilitate the expansion and scale up of anchorage-dependent stem cells while providing the tools necessary for monitoring and controlling stem cell health and differentiation [50], improving the yield of PSCs through enhanced oxygenation and metabolite mass transport and reduced microenvironment toxicity [45,51].

However, considerations are necessary when using microcarriers for the expansion of hiPSCs. Microcarrier limitations depend on their diameter (100–400 μm), density (typically ~1 g/ml), and chemical composition, which can affect cell attachment and, thus, expansion capability. Due to the limited surface area of beads, the achievable peak density of hiPSCs appears to be limited by the bead:cell ratio. Cells grown adhered to microcarrier beads also need to be enzymatically dissociated and filtered, which may sacrifice cell viability and pluripotency depending on the method used. To mitigate this problem, biodegradable microcarriers are being developed. Recent progress in the development of xeno-free dissolvable microcarriers has enabled substantially enhanced cell recovery rates in spinner flasks compared with conventional PS microcarriers, while increasing cell proliferation after 5 days [52].

Stirred tank bioreactors also introduce hydrodynamic shear stress to the microcarrier beads, which can impact overall hiPSC health and pluripotency. This can be overcome through optimization of bioreactor process parameters (i.e., stir rate) to minimize effects of shear stress on iPSCs. For example, Gupta and colleagues demonstrated that long-term attachment, pluripotency, and expansion of iPSCs on microcarriers in spinner flasks are reliant on maintaining an optimal agitation speed of 25 RPM [53]. This parameter optimization approach would need to be applied to iPSC expansion at larger scales (i.e., in bioreactors).

The cost of fabricating microcarrier beads is another consideration, because the process can become expensive depending on the material and additives used. Therefore, it is important that the microcarrier materials are cost-effective, (if possible) recyclable, and possibly sterilizable after each production run. With a well-controlled bioprocessing system, microcarrier-based iPSC culture methods are now being developed for the continuous expansion and recovery of human iPSCs for cellular therapies and tissue engineering, as outlined in Figure 2.

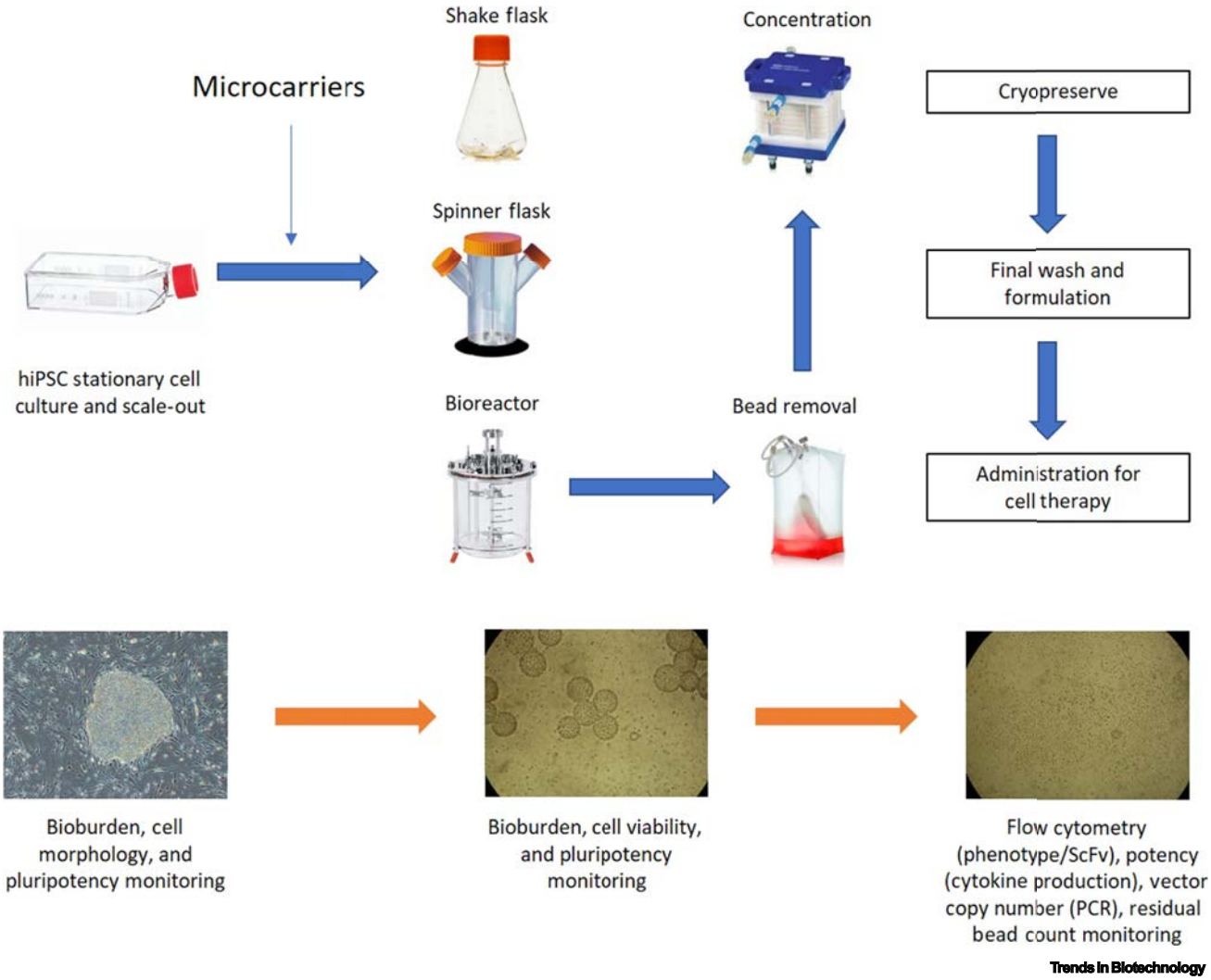

Figure 2. Current Human Induced Pluripotent Stem Cell (hiPSC) Manufacturing Bioprocess Using Microcarriers. A typical process for hiPSC manufacturing includes stationary culture, scale-up process, downstream process, and formulation [22]. Microcarriers are generally introduced during the scale-up process and removed during the downstream process [45].

Microencapsulation

Unlike microcarriers, which attach cells to the surface of beads, microencapsulation involves capturing cells within spherical capsules through which nutrients, oxygen, and other growth factors necessary for cell growth can diffuse through. The spherical capsules comprise semipermeable materials or membranes that can protect cells against agglomeration and shear forces while in suspension culture systems [54]. Biomaterials used to generate microcapsules include alginate, agarose, nylon, collodion, polystyrene, acrylate, polylysine–alginate hydrogel, cellulose acetate–ethyl cellulose, and polyester membranes [55,56]. Cells are commonly captured via emulsification or extrusion methods to form protective vessels that allow cells to proliferate [57]. In general, polymeric microcapsules have certain advantages over gel-based microencapsulation methods, including being bio- and GMP compatible [58]. More cells can be packed per unit volume of capsule material, and intraparticle diffusion limitations are less severe in polymeric capsules due to the presence of a liquid cell suspension in the intracapsular space.

Stem cells cultured in microencapsulation systems can either maintain pluripotency or be induced to differentiate depending on the composition of their capsule, and the growth factors present in their microenvironment [59]. The disadvantages to using microencapsulation for the large-scale production of iPSCs are similar to those for microcarriers in terms of cost of fabricating and limited surface area. Moreover, some stem cell types, such as hMSCs, have trouble proliferating while encapsulated (e.g., in alginate) without the addition of peptides or proteins (i.e., fibronectin) that improve cell attachment [60]. By contrast, recovery of encapsulated cells can pose a more difficult challenge to overcome during harvest if cell attachment is enhanced.

Table 2 summarizes the advantages and disadvantages of each suspension cell culture modality for iPSC growth in dynamic environments.

iPSC Monitoring Techniques, *in silico* Modeling, and Quality by Design

The sensitive pluripotent nature of hiPSCs is impacted by changes in the cellular microenvironment, metabolism, and signaling pathways, thereby creating a major challenge in the development of cell therapy and tissue-engineering methods that regulate stem cell differentiation [61,62]. Recent developments in cell-aggregate suspension cultures [30] and microcarrier systems [50,63] have provided hope for one day achieving the large-scale production of hiPSCs for use in various clinical applications. However, future utilization of larger scale bioreactors (>5 l) requires the development of novel bioreactor monitoring techniques that allow for process optimization of iPSC production while controlling stem cell differentiation.

There are several offline analytical methods currently available for bioreactor monitoring, many of which determine vital process variables, such as cell count, cell viability, and concentrations of

Table 2. Advantages and Disadvantages of iPSC Suspension Culture Modalities

Modality	Advantages	Disadvantages
Aggregates	• Least expensive • Controllable culture parameters • Simple harvesting method	• Agglomeration of cells • Lower cell viability • Slower recovery
Microcarriers	• High-density cell expansion • Homogeneous media composition • Controllable cell culture parameters • Continuous exchange and monitoring of nutrients and waste	• Costly (biomaterials, additives, manufacturing of microcarriers) • Hydrodynamic shear stress • Requires cell dissociation during harvest
Microencapsulation	• Controllable chemical gradients • Eliminates shear stress	• Costly (biomaterials, additives) • Difficult cell recovery

components in the media [64]. Dye exclusion assays (i.e., Trypan Blue exclusion microscopy) and flow cytometry are regularly used to measure cell concentration and viability, while some flow cytometry assays can also quantify PSC populations [65]. Recent efforts to improve the throughput of iPSC characterization show that incorporating fluorescence cell barcoding (FCB) into flow cytometry enables the identification of pluripotency as well as cell heterogeneity [66]. Although barcoding methods are not practical for continuous platforms, they are useful offline quality-control (QC) tests. Mass spectrometry (MS) and high-performance liquid chromatography (HPLC) have been used in conjunction to conduct metabolomics and proteomics studies, identifying metabolic markers and pathways that indicate or influence pluripotency [67,68], as reviewed recently by Dahan and colleagues [69]. These discoveries reveal the need for *in silico* modeling to predict and monitor iPSC differentiation at the metabolite level. Interestingly, glycolytic and methionine metabolism were found to regulate stem cell differentiation in hPSCs [70,71] and, thus, modeling the behavior of key metabolites involved in these pathways may be a suitable starting point. In the meantime, improvements in HPLC and MS systems have been made to facilitate online and at-line measurements, such as sampling automation, yet these technologies remain limited by their maintenance costs and time required to process the samples. Western blots, qPCR or RT-PCR, and immunohistochemistry (ELISA) are common assays that determine hiPSC metabolic function and differentiation status at specific time points by detecting the expression of ESC marker genes at comparable levels to native ESCs [72–74]. Overall, there are several effective offline characterization methods for determining iPSC pluripotency and quality throughout the manufacturing process. However, sample collection and preparation for non-automated offline analytical testing, along with the costs and maintenance of instruments and reagent kits, limit process scalability, increase manufacturing costs, sacrifice samples, and risk culture contamination. Additionally, the time delay between sample collection and data output significantly limits their usefulness as process control techniques.

In effort to enhance process scalability, the biopharmaceutical sector recently began adopting a Quality by Design (QbD) strategy, where process and product management are based on scientific knowledge and risk assessment [75]. This way, more agile manufacturing processes can be developed utilizing sensors or other analytical technologies that detect variations in real-time, allowing for rapid response by data-driven process controls that will mitigate a potential run failure before it occurs. QbD frameworks operate by linking measurable molecular and cellular characteristics of cell populations to the final product quality. In the case of iPSCs, effective QbD strategies would require real-time measurements of critical parameters that impact stem cell pluripotency and self-renewal.

Online sensors are the promising when it comes to real-time bioreactor process monitoring, including current state-of-the-art DO and pH probes. Previous studies have shown that hiPSC differentiation into cardiomyocytes can be induced by combining the effects of hypoxia with bioreactor hydrodynamics [76] and, therefore, DO level is a critical process parameter in maintaining the quality of iPSCs during manufacturing. Spectroscopic methods, such as Raman and NIR, have also served as useful online probes to monitor metabolites (glucose, lactate, AAs, etc.) and, therefore, can provide useful information when developing chemometric models and machine-learning algorithms that will predict and regulate iPSC pluripotency in real-time [77,78]. In addition to standard process characteristics, reproducible and steady formation of hiPSC aggregates is vital for process scalability. In this regard, certain optical techniques show potential as monitoring tools for cell health and differentiation status in real-time. For example, two-photon excitation (TPE) fluorescence microscopy to optically measure the autofluorescence of NAD(P)H and FAD has proven to be an effective technique for monitoring cell differentiation in 3D tissue constructs [78–81]. When combined with fluorescence-lifetime imaging microscopy

(FLIM), TPE can be used to determine the differentiation state of MSCs via the optical redox ratio and fluorescence lifetimes of NAD(P)H and FAD. Confirmed using LC-MS/MS measurements of NAD+/(NADH + NAD+), a decrease in the optical redox ratio value was observed for MSC cultures undergoing differentiation, which serves as an example to the potential capability of a TPE-FLIM probe designed for bioreactors (Figure 3) [82]. To utilize TPE-FLIM as an iPSC monitoring tool, a robust and flexible TPEM probe must be developed that can access cells within the bioreactor and collect online data; however, no such product currently exists. Recently, an *in situ* microscopic imaging device was developed for real-time visualization and monitoring of hiPSC aggregation in continuous stirred tank bioreactors [83]. Although control of aggregate size is significant to maintain pluripotency, a direct indication of differentiation progress could only be achieved with installation of a fluorescence microscopy module. Undoubtedly, there is still a need for new innovative tools that combine microscopy imaging and fluorescence detection to assess the pluripotent state of hiPSCs in dynamic cultures.

Quality Control Measures and Concerns

To realize the full potential of iPSC-derived therapeutics, iPSCs are required to be manufactured under clinical-grade GMP standards. This is a complex process, where the characterization and demonstration of comparability among iPSC cell lines, passages, and critical quality attributes (CQAs) are essential and well documented. CQAs are defined as biological, chemical, or physical properties that should remain within an appropriate limit to maintain or control the quality of the final product. With the development of automation, closed cell culture systems, and validated testing protocols, the objective to industrialize iPSC line manufacturing is now closer than ever. The International Stem Cell Banking Initiative (ISCBI) recently provided recommended guidance for clinical-grade hiPSC registration, including: (i) pluripotency tests; (ii) differentiation tests both *in vitro* and *in vivo*; (iii) karyotype analysis to show genetic stability; (iv) cell identity determination; (v) gene expression profiling via a stem cell array; and (vi) microbiological tests [84].

Current GMP-grade culture systems for iPSC expansion require multiple media changes and passages (sometimes daily), leading to significant scalability, reproducibility, and cost challenges. For these reasons, the current financial costs of personalized iPSC production are unaffordable for most patients and, therefore, the generation of iPSC banks would be beneficial. The Global Alliance for iPSC Therapies (GAiT)[i] was formed with the intent to support the creation and global

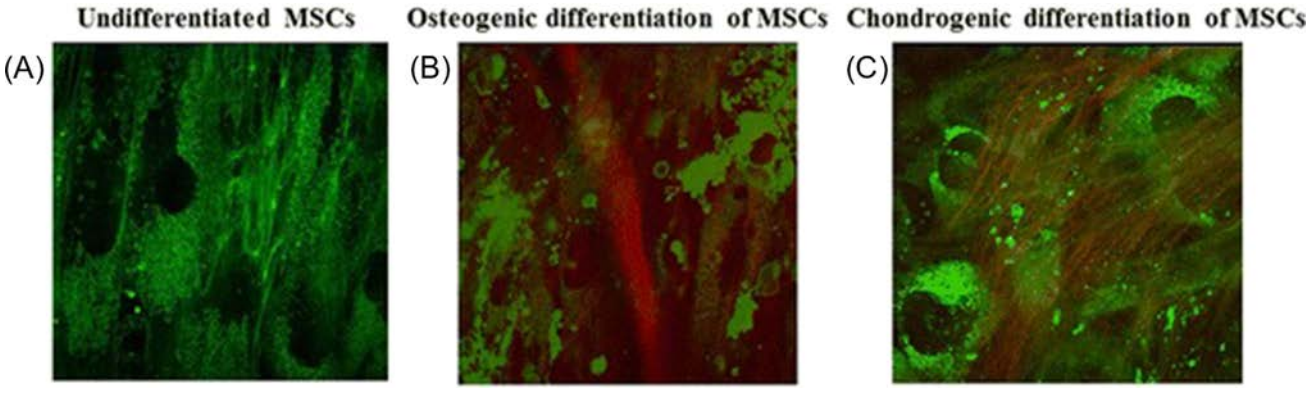

Figure 3. Two-Photon Excitation for Stem Cell (SC) Differentiation Monitoring. An example of a potential method for identifying SC differentiation in bioreactors via the ratio of endogenous fluorophores is displayed. (A) Overlapped two-photon excited autofluorescence (green) and second harmonic generation (SHG) (red) images of undifferentiated mesenchymal SCs (MSCs), and MSCs during (B) osteogenic and (C) chondrogenic differentiation on Day 21. Image size is 130 × 130 μm (512 × 512 pixels) Reproduced, with permission, from [82].

harmonization of iPSC banking for clinical applications. Alvarez-Palomo and colleagues summarized key considerations and standard operating procedures for creating clinical-grade iPSC banks [85].

One major QC concern in the iPSC field is genomic stability. With limited long-term clinical data available, stringent guidelines need to be set for genetic stability testing [85]. Previous clinical trials involving iPSCs were suspended due to observed single nucleotide variations (SNVs) and copy number variants (CNVs) upon cell reprogramming [86]. Therefore, whole-genome sequencing (WGS), including SNPs and comparative genomic hybridization (CGH) arrays, are recommended as QC tests before releasing iPSC lines into clinical settings [85]. Genetic transfection platforms for hiPSC generation also pose huge safety concerns for genome integration. Nongenetically modified PSCs generated by peptides show low efficiency and mRNA/miRNA transfection requires multiple steps, which creates batch-to-batch and lot-to-lot variabilities [87,88]. Needless to say, genetic marker validation throughout the cell therapy bioprocess is essential for the development of safe and effective treatments for patients.

Concluding Remarks and Future Perspectives

Over the past decade, hiPSC bioprocess technologies have significantly improved [89], yet progress for availability in clinical applications and new product release remains slow. The main challenges are derived from underlying biological science and limitations of current large-scale manufacturing platforms [90]. Existing hiPSC process development and manufacturing methods are labor intensive, which largely limits process scalability and leads to unpredicted lot-to-lot variability. Consensus across the sector agrees that the optimal approach to manufacturing iPSCs would be to expand suspension cultures, thereby increasing cellular growth capacity. When choosing a suspension modality for scale-up, a process that combines the growth profile of pluripotent iPSCs and their subsequent differentiation cell type should be considered, because several studies have found various beneficial matrices to grow specific types of high-quality iPSC. Compared with the repeated batch processes, fully automated perfusion with feedback control of the culture environment would allow for continuous exchange of nutrients and result in significantly higher cell yield [91]. Due to the stress sensitivity of hiPSCs, current perfusion systems have only been successfully used on mouse iPSCs. Yet, successful expansion of hiPSCs while using currently available ACF media (Essential 8™) and microcarrier suspension beads show the capability of the industry to achieve scalable, high-quality PSC densities in the future. Furthermore, additional opportunities exist for further advancing the iPSC process development field (see Outstanding Questions).

With new technologies and modalities becoming available, process-friendly characterization methods and monitoring tools are needed to control the quality of iPSCs during manufacturing. Due to the importance of genome and metabolome stability for hiPSCs, a genome-scale model can be useful for identifying key process parameters that impact pluripotency and guide the optimization of growth phenotypes during the manufacturing process [92]. In addition, a more sensitive cell quality testing tool combining image and fluorescence microscopy would further enhance PATs that monitor stem cell differentiation in real time. With the development of these analytical tools, further media optimization, and QbD strategies for scale-up, the goal of large-scale manufacturing of clinical-grade iPSCs will be realized over the coming decade.

Author Contributions

A.P. and B.K. conceived and wrote the review, as well as generating the tables and figures. A.P. critically revised the manuscript and produced the graphical abstract. All authors read and approved the final manuscript.

Outstanding Questions

What growth factors and proteins are unique to iPSCs in regard to sustaining pluripotency and self-renewal? How do these differ from hESCs or mESCs?

Can 2D or 3D scaffolds used for successful iPSC static cultures be incorporated into dynamic cultures to improve yield?

How can the challenges in obtaining real-time assessment of intrinsic pluripotency markers of iPSCs in dynamic cultures be addressed?

How feasible would it be to develop *in silico* models that accurately predict stemness changes in dynamic cultures based on metabolite profiling?

How can the issues of human error, lot-to-lot variability, and difficulty in scale-up be overcome in culturing large quantities of iPSCs suitable for clinical applications?

Resources

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