Progress and challenges in large-scale expansion of human pluripotent stem cells

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Review

Progress and challenges in large-scale expansion of human pluripotent stem cells

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ABSTRACT

The constant supply of high cell numbers generated by defined, robust, and economically viable culture processes is indispensable for the envisioned application of human pluripotent stem cells (hPSCs) and their progenies for drug discovery and regenerative medicine. To achieve required cell numbers and to reduce process-related risks such as cell transformation, relative short batch-like production processes at industry- and clinically-relevant scale(s) must be developed and optimized. Here, we will review recent progress in the large-scale expansion of hPSCs with particular focus on suspension culture, which represents a universal strategy for controlled mass cell production. Another focus of the paper relates to bioreactor-based approaches, including technical aspects of bioreactor technologies and operation modes. Lastly, we will discuss current challenges of hPSC process engineering for enabling the transition from early stage process development to fully optimized hPSC production scale operation, a mandatory step for hPSCs' industrial and clinical translation.

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1. Introduction

1.1. The need for large-scale production of human pluripotent stem cells and their progenies

Human pluripotent stem cells (hPSCs), including human embryonic (hESCs) and induced pluripotent (hiPSCs) stem cells, represent a unique cell source for the, in principle, unlimited production of functional human cell types *in vitro*. In this regard, hPSCs hold great promise for revolutionizing drug discovery, drug safety assays, *in vitro* disease modeling, and ultimately cell-based therapies (Fig. 1) [1,2].

The evolutionary conservation of mammalian genomes has resulted in numerous drugs that were discovered by assays employing ubiquitous cell lines and further validated in typical rodent models to lack efficiency or cause detrimental side effects after clinical translation [3,4]. Systematic research indeed revealed limitations of animal models regarding their predictability of drug function and toxicity in man. Underlying reasons include substantial species-specific differences in (i) cell and tissue physiology (such as liver metabolism, beating rate of the heart, *etc.*), (ii) inflammatory response, (iii) structure and specificity of the immunological system, and (iv) others [5]. This underscores the necessity for using human cells, ideally tissue-specific cells, for drug discovery, validation, and safety pharmacology [1,6,7].

Moreover, for many sporadic and rare diseases caused by genetic mutations, such as cystic fibrosis [8], hereditary pulmonary alveolar proteinosis (hPAP) [9], or Huntington's disease [10], novel drug candidates should ideally be screened and validated in human cells carrying the respective mutation(s). In contrast to immortalized cell lines, which are typically used for high throughput screening (HTS) drug discovery assays, this deems straightforward by using disease-specific *in vitro* models based on patient-derived hiPSC lines differentiated into functional cell types relevant to the respective disorder [8].

Beyond drug discovery and disease modeling *in vitro*, first patients have recently received hPSC progenies aiming for novel approaches in regenerative medicine. For treating age-related macular degeneration in the eye, both hESC- [11] and hiPSC-derived [12] retinal pigment epithelial cells were readily applied. The implantation of hESC-derived insulin-producing cells in patients with type 1 diabetes was announced by the company ViaCyte, and early hESC progenies were readily transplanted to the left ventricle of a first heart failure patient *via* a tissue engineering approach [13,14].

At present, functional hPSC progenies are mainly generated by protocols in laboratory scale and quality. However, the envisioned routine application of these cells will require appropriate large-scale production processes, ultimately by standardized and economically viable procedures and technologies.

Rough estimations suggest that for replacing disease-induced loss of hepatocytes, pancreatic β-cells, or cardiomyocytes, approximately $1–10 \times 10^9$ functional cells per patient will be required. Even higher needs were calculated for the visionary production of "in vitro blood", since approximately 2.5×10^{12} red blood cells are required per patient in transfusion medicine [15].

It is worth noting that equivalent cell numbers are readily required ahead of treating patients; for example, for pre-clinical studies in large animals such as pigs or non-human primates, which represent more physiologically and functionally relevant models of human diseases such as heart failure compared to rodents [16–18].

The need for developing well-defined large-scale hPSC expansion and differentiation processes is not dictated by cell number requirements alone. Another impelling necessity is to comply with the currently evolving regulatory framework for hPSC-derived therapeutics, including the application of relevant "current good manufacturing practice" (cGMP) guidelines [2,19,20].

Taken together, many of the envisioned clinical and industrial applications of hPSCs will depend on the constant, controlled production of billions of cells. In principle, bioprocesses for the production of recombinant proteins by common mammalian cell lines, which have been established in >1000 L scale, may serve as a blueprint [2,16,18,21]. In this scenario, the established bioreactor systems provide effective technologies to replace laborious and poorly controlled research-type processes.

By combining process automation, monitoring, and control with scalability, bioreactor systems are applied to reduce operator-dependent variability, paving the way for more robust and cost-effective hPSC production [22–24].

However, due to their intrinsic potential, hPSCs may switch from pluripotency toward (uncontrolled) differentiation not desired during the cell expansion phase. Moreover, subsequent differentiation into desired lineage(s) is a highly complex process altered by a multitude of overlapping parameters, which also includes effects of the proceeding expansion strategy. Therefore, hPSC processing is substantially more challenging than long-standing strategies for the cultivation of transformed and relative unpretentious cell lines typically used in industry and thus requires a high degree of innovation in process development and control [2,16,18,21,25].

2. Culture platforms for hPSC expansion

2.1. 2D culture systems and process scale-out

Undifferentiated hPSCs are conventionally maintained and expanded at two-dimensional (2D) conditions with cells adhering to the matrix-covered surface of culture plates or flasks. The cumbersome co-culture of hPSC colonies grown on mitotically inactivated fibroblasts ("feeder cells") – as initially described for their routine maintenance [26,27] – has been largely replaced by semi- or fully-defined matrices such as Matrigel [26], recombinant proteins (such as laminins [28]), or synthetic polymers [29].

To generate larger cell amounts, scale-out of the 2D approach has been suggested simply by multiplying culture dishes or by using multi-layered flasks marketed as "Cell Factories" or "Cell Stacks" [30]. Thus, the term "scale-out" refers to keeping a manufacturing lot size constant but multiplying the number of parallel unit operations (see Fig. 2A) [31]. However, although some degree of process automation for 2D culture has been published [32,33], the approach remains relatively cost-, space-, and labor-intensive. The method also restricts the online monitoring and control of key process parameters including vital cell counts, dissolved oxygen (DO), pH, and glucose and growth factor concentrations. It should be mentioned though that a culture system for large-scale 2D processing of hPSCs based on multilayered plates was recently introduced, which allows pH and DO monitoring and feedback-based control [34].

However, 2D cultivation typically relies on static culture conditions known to induce the formation of undesired gradients, including media components, metabolic waste products, paracrine factors, and gases. Together, despite its advantageous simplicity, 2D cultivation raises a number of issues that limit the strategies' utility for the systematic development of hPSC mass production [24,25,35].

2.2. 3D culture systems and process scale-up

The field is recently reaching consensus that three-dimensional (3D) culture (synonymously termed suspension culture) is a potent approach to achieve the extensive hPS cell number requirements

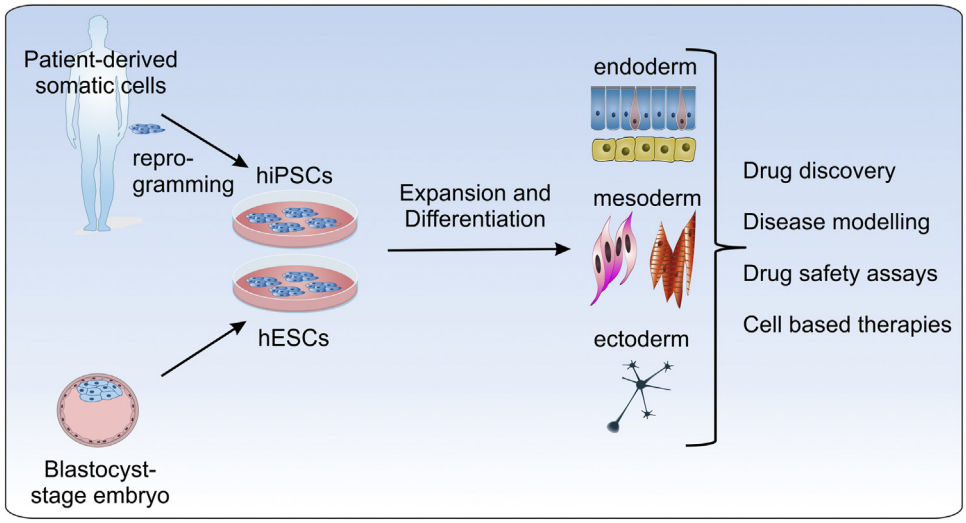

Fig. 1. Possible industrial and clinical applications of human pluripotent stem cells demanding large-scale cell expansion. Human PSCs are derived from the inner cell mass of the blastocyst (hESCs) or reprogrammed from the somatic cells (hiPSCs). The expansion and subsequent differentiation of these cells in large-scale culture platforms are mandatory for reaching the required cell numbers for cell-based therapies, in vitro disease modeling, high content screening for drug discovery, and drug safety assays.

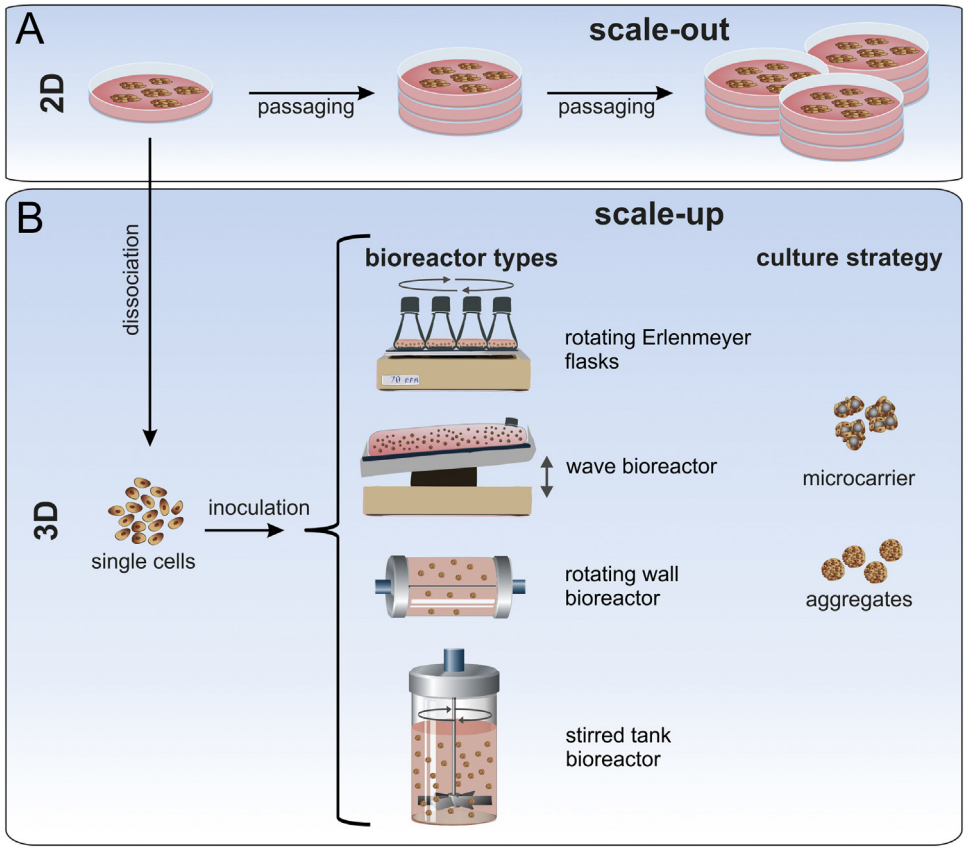

Fig. 2. Expansion strategies for human pluripotent stem cells. The expansion of hPSCs can be performed following two different approaches: (A) Adopting the standard 2D cultivation methods, *i.e.*, the scale-out approach based on increasing the culture surface *via* increasing the number of cultivation vessels; however, this is cost-, space-, and labor-intensive; (B) Using 3D cultivation methods within automated bioreactor systems, *i.e.*, the scale-up approach based on, *e.g.*, rotating wall bioreactors, wave bioreactors, shaking Erlenmeyer flasks, or stirred tank bioreactors depending on the desired final cell yield, which allows to increase the overall manufacturing scale. Regarding the culture strategy within the bioreactors, hPSCs can be cultivated attached to microcarriers or as self-assembling aggregates. Figure adapted from [16].

outlined above [2,36]. While 2D culture is inappropriate to simulate hPSCs' "natural microenvironment", cultivation in suspension culture based on matrix-free cell-only aggregates (see Section 3.2.1) may be a closer match of hES cells' "*in situ* niche" *i.e.*, equivalent to the inner cell mass of the early human embryo at the blastocyst stage [37] from which these cells originate (Fig. 1).

The overall suspension culture environment in combination with the microenvironment in spherical, multicellular aggregates seems to support hPSC viability and proliferation at the pluripo-

tent state while overcoming the necessity of external matrix supplementation [38,39] (Fig. 2B). Notably, aggregate-based hPSC cultivation also provides a useful starting point for directly switching from pluripotency to directed lineage differentiation, which can be induced and controlled by replacing the respective expansion media with lineage-specific differentiation media [40–43].

The use of bioreactor systems facilitates the development of dynamic suspension cultures, thus overcoming environmental inhomogeneity typical of static conditions [44,45]. Reactor type-specific mixing technologies (i) promote the control of hPSC aggregation (*i.e.*, after single cell-based process inoculation), (ii) avoid the formation of gradients by supporting homogeneous distribution of all culture components, and (iii) improve the mass transfer of gases and nutrients into cells/aggregates, thereby facilitating the development of large-scale hPSC culture processes at (relative) high cell density [18]. In addition, bioreactor systems are equipped with technologies for monitoring and (feedback-based) control of the culture environment, including basic and more specific parameters such as temperature, pH, and the concentration of nutrients (*e.g.*, glucose), metabolites (*e.g.*, lactate, ammonia), and gases (*e.g.*, O_2 and CO_2), which substantially facilitate controlled process up-scaling.

Therefore, in contrast to the scale-out approach, the use of bioreactor systems allows process "scale-up" defined by the increase in the overall manufacturing scale [31]. Several types of bioreactor systems, most of which have been previously developed for culturing conventional mammalian cell lines, are potentially applicable to hPSC cultivation and scale-up. In addition to simple Erlenmeyer flasks, these systems include wave bioreactors (also known as "cell bags") and rotating wall, stirred tank, packed bed, hollow fiber, bubble column, and airlift bioreactors described in detail elsewhere [19,22,31,35,39,46–48] (Fig. 2B). Since rotating wall and stirred tank bioreactors are the most used systems for hPSC production [18,48,49] (summarized in Table 1), these will be discussed in more detail.

3. Process engineering for hPSC suspension culture

3.1. Rotating wall bioreactors

In rotating wall bioreactors (RWBs), the cylindrical culture vessel is maintained in continuous rotation along its longitudinal, horizontal axis, creating a microgravity environment under laminar flow conditions. The rotation supports mixing and thus homogeneity of the overall culture, and, depending on the imposed rotational speed, a dynamic suspension of the immersed cells/aggregates in a quasi-periodic circular motion can be achieved. RWBs were initially used for "embryoid body" (EB)-based differentiation of hESCs by Gerecht-Nir and coworkers [50]. A 3-fold increase in EB generation efficiency compared to static control conditions was reported, and a strong impact of the vessel type on EB size and agglomeration tendency was observed. The system was improved *via* the implementation of a perfusion system combined with a dialysis chamber, leading to improved culture homogeneity and process control [51]. Although RWBs provoke low shear stresses on cells/aggregates, the limited volume capability and the complexity of the technical solutions adopted for the horizontal rotation of the reactor vessel impede systems' scalability, thereby limiting their universal use for large-scale hPSC production [24,50].

3.2. Stirred tank bioreactors

Stirred tank bioreactors (STBRs) are widely used in the biopharmaceutical industry for the large-scale production of recombinant proteins from common mammalian cell lines such as Chinese hamster ovary (CHO) or baby hamster kidney fibroblasts (BHK) cells [21]. STBRs typically consist of a permanent glass or single-use plastic vessel equipped with an internal impeller. In addition to efficient mixing, the impeller induces the desired uplift-current against gravity, thereby keeping cells, cell-aggregates, and potential microcarriers in suspension [31,39,52].

Integrated probes allow for the precise assessment of the culture environment, including pH, temperature, and DO. STBRs are also compatible with non-destructive cell- and medium-sampling *via* system-integrated ports without process interruption. Additional process monitoring and control parameters include the vital cell density assessment (*e.g.*, *via* bioreactor-integrated probes for impedance measurement or external analyzers) and the analysis of metabolites such as glucose, lactate, and ammonia concentrations measured by external analyzers [22,39]. Finally, STBR technology enables relatively linear and straightforward process up-scaling (using stepwise increments in reactor vessel dimensions) [18,25] and supports the flexible, automated modulation of operation modes [18], thus providing substantial new options for hPSC process modulation and improvement further outlined below.

Generally, to enable the transition of conventional 2D-dependent hPSC cultivation into STBRs in 3D, two alternative technologies have been applied. One approach relies on cell immobilization on microcarriers [53–55], while the other approach of matrix-free cell-only aggregation has been explored by us and other researchers [44,56,57]. By using both technologies, major progress was made in hPSC cultivation in STBRs in the recent years as outlined in Table 1; these achievements and the remaining challenges are discussed in the following sections.

3.2.1. Proof-of-concept studies for hPSC cultivation in suspension culture

While the successful propagation of human PSCs passaged as semi-dissociated cell clumps on coated microcarriers was reported [53–55] in parallel to respective studies with mouse PSCs [58,59], the expansion of hPSCs as self-assembling aggregates was initially hindered by human PSCs' sensitivity to rigorous dissociation into single cells, in contrast to their less sensitive mouse counterparts [60,61]. However, following the finding that the small molecule Y27632 (a Rho-associated coiled-coil kinase (ROCK) inhibitor) temporarily permits survival of single cell-dissociated hESCs [62], we and other researchers showed the robust multipassage expansion of undifferentiated hPSCs rigorously propagated as single cells in suspension [44,56,57]. In addition to Y27632 supplementation, it was demonstrated that hPSCs' re-aggregation into matrix-independent aggregates in suspension culture also depends on other parameters, particularly on the culture medium and the applied inoculation density [44,56,57]. Following the successful expansion of hPSCs in stirred spinner flasks (a simple STBR platform with limited monitoring/control abilities) in 2010 [63], continuous modification and improvement of distinct process aspects were published (Table 1).

3.2.2. Process control by controlling hPSC aggregation

The mechanical and hydrodynamic conditions in STBRs are important for keeping cells homogenously suspended and to avoid excessive agglomeration of aggregates but without hydrodynamic-induced damage of PSCs [64]. Therefore, for controlling aggregate formation and their further growth, the first parameters that were optimized in STBR included the impeller design, the stirring speed, and the hPSC inoculation density.

A key aim of these studies was to ensure overall aggregate homogeneity. In particular, the formation of extensive aggregate dimensions must be avoided since diameter exceeding ~300 μm is known to result in cell necrosis due to the limited nutrient and gas diffusion into the tissue/aggregate center [64]. Eventually, uncon-

Table 1
Overview of studies optimizing expansion of hPSCs in suspension in bioreactor vessels. The studies are sorted according to their date of publication.

Cell Type	Bioreactor Type	Working volume	Culture System	Culture media	Operation mode	Parameter optimized	Max. cell density [cells/mL]	Max. cell Yield [cells]	Peak in cell density [day]	Refs.
hESCs	Rotating wall bioreactor	55 mL	Embryoid bodies	FCS containing medium	Repeated batch	Rotate speed. inoculation density	36×10^6	19.8×10^8	28	[50]
hESCs	Rotating wall bioreactor	55 mL	Embryoid bodies	hESC KO-medium	Perfusion	Feeding strategy, medium dialysis	ND	ND	ND	[51]
hESCs	Spinner flask	50 mL	Microcarrier	MEF-CM	Repeated batch	Microcarrier coating	3.5×10^6	1.75×10^8	5	[54]
hESCs	Spinner flask, Stirred tank bioreactor	100 mL, 300 mL	Microcarrier	hESC KO-medium	Repeated batch (semi continuous)	Feeding strategy, DO	1.22×10^6, 2.26 x 10^6	1.22×10^8, 6.78×10^8	11, 10	[55]
hESCs	Spinner flask	100 mL	Aggregates	mTeSR1	Repeated batch	Rapamycin and RI addition	0.45×10^6	0.45×10^8	6	[63]
hESCs	Spinner flask	50 mL	Aggregates	KO-SR medium, mTeSR1	Repeated batch	Medium, stirring speed, heat shock treatment	2.4×10^6	1.2×10^8	7	[45]
hiPSCs	Stirred tank bioreactor	100 mL	Aggregates	mTeSR1	Repeated batch	Impeller design, stirring speed, inoculation density	2.0×10^6	2.0×10^8	7	[65]
hESCs	Spinner flask*	60 mL	Aggregates	mTeSR1, StemPro hESC SFM	Repeated batch	Medium, inoculation density	1×10^6	0.60×10^8	3	[66]
hiPSCs, hESCs	Spinner flask	100 mL	Aggregates	HFF-CM	Repeated batch	Medium, dissociation, stirring speed, inoculation density, DO	1.4×10^6	1.4×10^8	10	[77]
hiPSCs	Spinner flask	45 mL	Aggregates	E8	Repeated batch	Medium	$\sim 1.7 \times 10^6$	0.77×10^8	5	[78]
hiPSCs, hESCs	Spinner flask	50 mL	Microcarrier	mTeSR1	Repeated batch	Feeding strategy	6.1×10^6	3.05×10^8	7	[72]
hESCs	Spinner flask	60 mL	Microcarrier	KSR medium, KSR-XF, BRASTEM	Repeated batch	Medium	2.78×10^6	1.67×10^8	6	[92]
hESCs	Spinner flask	100 mL	Aggregates	mTeSR1	Batch	Stirring, inoculation density (3^2-factorial)	0.24×10^6	0.24×10^8	6	[74]
hESCs	Spinner flask	60 mL	Microcarrier	Not exactly stated	Repeated batch	Microcarrier type and coating	2.07×10^6	1.24×10^8	10	[76]
hiPSCs	Spinner flask	50 mL	Microcarrier	E8	Repeated batch	Carrier-coating, inoculation density, stirring (3^2-factorial)	1.4×10^6	0.7×10^8	10	[75]
hiPSCs	Stirred tank bioreactor	100 mL	Aggregates	mTeSR1, E8	Perfusion	Feeding strategy, Medium (xeno-free, fully defined)	3.01×10^6	3.01×10^8	7	[85]

Abbreviations: MEF: Mouse embryonic fibroblasts; CM: Conditioned medium; KO: Knock-out; RI: ROCK inhibitor (Y-27623); KO-SR: Knock-out serum replacement; SFM: Serum-free medium; HFF: Human foreskin fibroblasts; KO-SR: Knock-out serum replacement; XF: xeno-free.

* Authors state transition into 500 mL spinner flasks; however, no details on the working volume or cell densities, *etc*., are given.

trolled differentiation – particularly in large hPSC aggregates – might also occur. The regular dissociation of aggregates into single cells at every passage is therefore an integral part of the established suspension culture strategy; this contradicts uncontrolled hPSC differentiation and supports sustained culture homogeneity and quality [45].

In the first published trials of hPSC expansion in STBRs, relative high cell densities of ~2–3.5 × 10^6 cells/mL were readily achieved [54,65]. The robustness of both microcarrier- and aggregate-based hPSC expansion processes was underlined, and maintenance of pluripotency and karyotypic stability over several passages was confirmed [54,66].

However, in STBRs both the direct interaction of the cells with the vessel components and the complex hydrodynamic conditions may induce critical stresses. In particular, the geometry and the position of the impeller in combination with the agitation rate and the position of analytical probes can lead to complex, spatially and temporally modulated fluid shear stresses and heterogeneous mass transport, resulting in localized areas of turbulence and subsequently detrimental stresses [24,67]. Such non-physiological environment may change receptor-ligand binding properties, thereby affecting cells' metabolism and phenotype [47], reducing the viability [57,65,68], and disrupting the sensitive equilibrium of pluripotency versus differentiation, eventually inducing undesired culture heterogeneity [18,57]. Effects of shear stress on pluripotency and differentiation properties have been described for mouse pluripotent stem cells [69–71]. Furthermore, differentiation induction has been observed in microcarrier-based hPSCs cultures due to inappropriate culture agitation [72].

For these reasons, the optimization of the agitation speed and the impeller type in combination with the inoculation density are crucial settings for controlling aggregate size and development [65]. In this context, several works based on experimental and computational fluid dynamics techniques have been performed for characterizing and optimizing the 3D fluid dynamics in STBRs [73–77]. Consequently, modulations of bioreactor designs have been proposed [65,78–80]. Nevertheless, the influence on stem cell expansion and pluripotency of mechanical and hydrodynamic conditions arising from complex combination of factors – such as impeller/vessel geometries and imposed rotating conditions – remains challenging to be assessed, in particular because of the interplay of the complex mechanical and biological properties of the system [64,67]. Moreover, as the shear stress at the impeller tip increases with the bioreactor scale [47], the investigation of these hydrodynamic aspects will become even more important when hPSC expansion will be scaled-up beyond current dimensions of 100–300 mL (Table 1) toward 1–2 L scale (as recently published [40]) and subsequently towards dimensions of 10–1000 L envisioned for the "off-the-shelf" therapeutic cell production in future [16,18,48].

3.2.3. Process control by operation modes

Recently, work on hPSC expansion in STBRs aimed at more sophisticated operation modes to test the impact of alternative feeding strategies [55,81]. Most upstream stem cell culture processes can be categorized into the following four operation modes: batch, repeated batch, fed batch, and continuous feeding with cell retention, the later also known as perfusion [82] (Fig. 3A–D).

3.2.3.1. Batch. In batch mode (discontinuous processes), nutrients are provided only at process initiation (Fig. 3A). No subsequent supplementation is performed after process inoculation besides the addition of acid or base, anti-foam detergents, or the modulation of gassing, as required. Thus, batch is the simplest operation mode, entailing the lowest risk for contamination. However, modest cell yields are expected given by the limited nutrient concentrations that are tolerated by most cell types before inducing detrimental effects by hyperosmosis [82]. Since PSCs depend on the regular (nearly daily) supply of growth factors (that is bFGF and TGFß for human PSCs and LIF for mouse PSCs) to maintain stemness [49], strict batch processing is essentially incompatible for prolonged stem cell expansion. Batch processes, however, were described for hPSC cultures inoculated at very low cell densities of 2–8 × 10^4 cells/mL, which seems to be compatible with modest nutrient supply [83], but is inappropriate for the production of relevant cell numbers for most applications.

3.2.3.2. Repeated batch. For achieving higher cell yields, repetitive, more advanced processing strategies have been established. In the field of stem cell bioprocessing, the term "repeated batch" (sometimes also called "semi-continuous", *e.g.*, in [55]) is often used to describe a process that is initiated with a batch phase (typically lasting for 48 h), followed by repeated feeding cycles without cell harvest (Fig. 3B). This operation mode, which is also typical for the conventional PSC cultivation in 2D in a dish, was successfully applied by several groups using both microcarrier- [54,55,81,84,85] and aggregate-based suspension culture [45,63,65,66,80,86]. Thus, this is the most used feeding strategy for hPSC expansion to date. It is worth to highlight that some groups including us have applied very short cycles of repeated batch feeding, *i.e.*, performing partial medium replacement after every ~2 h culture interval, to achieve "perfusion-like" conditions termed "cyclic perfusion" [42,55].

3.2.3.3. Fed batch. In fed batch processes, in contrast to batch cultivations, one or more nutrients – usually glucose and amino acids – are supplemented by a concentrated feed stream during the cultivation, resulting in an increased culture volume over time (Fig. 3C). This nutrient replacement enables to achieve higher cell densities than that in batch cultures [82]. Although fed batch processes have been successfully applied for hematopoietic stem cell expansion [87], to our knowledge, no studies have used this strategy for hPSC cultivation. The method applicability might be hindered not only by the accumulation of toxic metabolites but also by the limited stability of growth factors mandatory for maintaining pluripotency [88].

3.2.3.4. Perfusion. Perfusion is characterized by the continuous replacement of medium from the reactor by fresh medium while retaining cells in the vessel by specific systems (Fig. 3D). Perfusion is often described as the superior operation mode for biopharmaceutical production processes enabling highest cell densities and productivity. However, perfusion feeding also represents the highest level of operational complexity, medium costs, and contamination risk [82]. Beside the advantage that cells in perfusion are constantly provided with fresh nutrients and growth factors, potentially toxic waste products are washed out, ensuring more homogeneous conditions in the reactor. Moreover, compared to repeated batch processes, perfusion processes support process automation and improved feedback control of the culture environment, including DO, pH, and nutrient concentrations [82]. Perfusion cultures may be optimized toward a relatively stable, physiological environment that also supports the self-conditioning ability of hPSCs by their endogenous factor secretion and thus eventually reducing supplementation of expensive medium components [88,89].

Lastly, in contrast to repeated batch cultures, which currently require manual withdrawal/replenishment of medium, no process interruption is mandatory in perfusion processes, thus minimizing operational errors [82].

Despite these advantages, only few studies have been published on the application of perfusion for PSC cultivation. By using a perfusion system based on matrix-attached monolayer culture, early

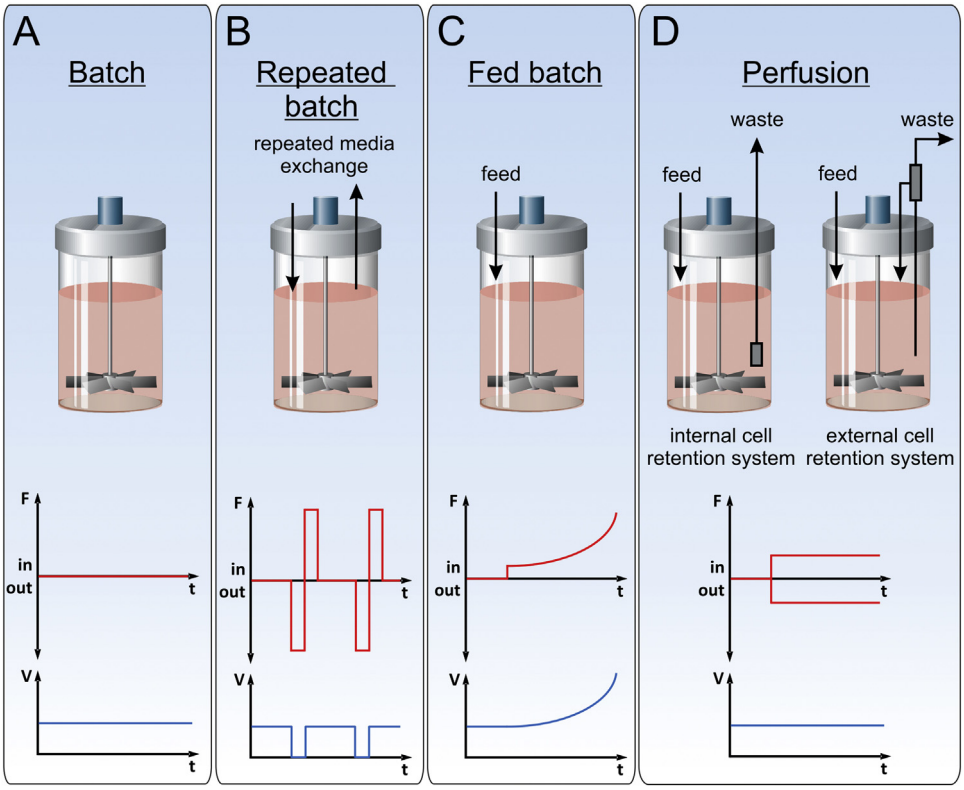

Fig. 3. Bioreactor operation modes. As feeding strategies in hPSC expansion, the following bioreactor operation modes can be adopted: (A) Batch – nutrients are provided only at process initiation. No subsequent feeding is performed afterwards and the working volume V remains constant; (B) Repeated batch – in this semi-discontinuous mode, after an initial batch phase, part of the medium is cyclically refeed with fresh medium without harvesting the cells, and the working volume V, except during the medium replacement passages, remains constant; (C) Fed batch – after an initial batch phase, during the cultivation the cells are supplied with fresh nutrients (normally *via* a concentrated feed medium) *via* the feed steam. Since the medium is not removed, this results in an increased working volume V over time; (D) Perfusion – after an initial batch phase, fresh medium is continuously added to the culture, while spent medium is continuously removed at the same flow rate; thus, the working volume V is kept constant. The cells are maintained inside the bioreactor via an internal or an external cell retention system. For each mode, the evolution in time of the medium feeding (graphs F-t) and of the working volume (graphs V-t) is provided. Figure adapted from [82,88].

work on human ESC suggested the supportive effect of perfusion [90]. This was confirmed for mouse PSCs expanded in STBRs in suspension cultures on microcarrier using ultrasonic separation or gravimetric control for cell retention [91,92] and for mouse PSCs grown as aggregates [60,92]. For human PSCs, cyclic perfusion-like conditions have been established using microcarrier and gravimetric control for cell retention [55]. Moreover, perfusion has been established using hPSC aggregate culture with a filtration system for cell retention more recently [93].

In the latter study, it was shown that perfusion resulted in much more homogeneous culture conditions and avoided zig-zag-like patterns of the main process parameters such as pH and DO, typical of repeated batch processes. In consequence, perfusion enabled significantly higher cell densities of $\sim 3 \times 10^6$ cells/mL on average using the same medium throughput (*i.e.*, 1x process volume/day) and process duration (7 days), thereby outperforming repeated batch controls by 50% with regard to the average cell density of 2×10^6 cells/mL and the respective overall cell yield [93] (Fig. 4A–C). Interestingly, investigations on hPSC physiology and global gene expression patterns revealed distinct changes in the cells' energy metabolism in a time-dependent manner in both repeated batch and perfusion processes [93]. The study suggests a suspension culture-induced switch from glycolysis to oxidative phosphorylation but notably in the absence of hPSC differentiation. This highlights the plasticity of hPSC energy metabolism and provides novel physiological and molecular targets for process monitoring and further optimization.

3.2.4. *Control of specific process parameters*

First attempts on controlling pH and DO in stem cell bioprocessing have been reported. For controlling pH, only a limited number of studies have been reported so far for mouse PSCs [68,94].

More but also quite controversial studies have been published on the impact of oxygen modulation. As oxygen solubility in aqueous solution is relatively low, *i.e.*, the saturation concentration is ~7 mg/L at 37 °C, continuous oxygen supply is necessary to ensure a stable concentration throughout the expansion processes of fast-proliferating cells [82]. However, to limit the excessive formation of free radicals, DO levels of 20% to 50% of air saturation are commonly used in mammalian cell cultures [82,95].

In uncontrolled 2D systems, a beneficial effect of hypoxia (defined by ~2–5% DO in respective studies) on hPSC expansion and pluripotency has been reported [96–98]. However, in these studies, the gas composition was modified without monitoring or controlling the dissolved oxygen tension resulting in the culture. This, to our experience, strongly differs from the gassing conditions. These studies are therefore difficult to interpret and results might be misleading, as also suggested by the work of Serra et al. [55]. By using a controllable bioreactor system, this study reported three times higher cell densities when DO was controlled at 30% compared to 5% air saturation. However, more studies using DO-control based on online DO-monitoring might be required in order to conclude about optimal DO set points for hPSC expansion.

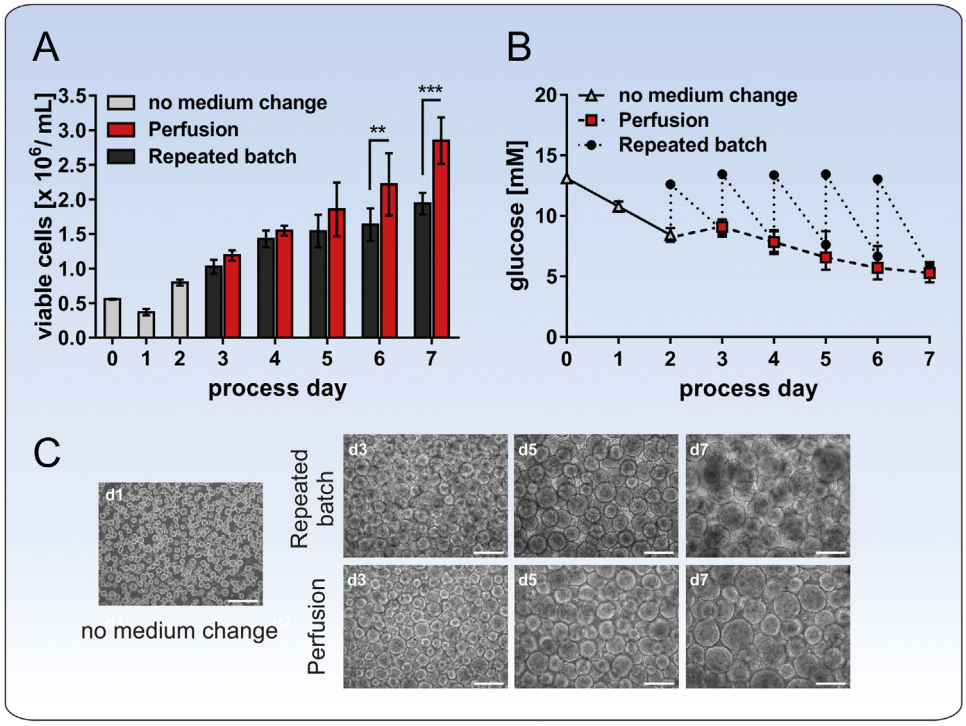

Fig. 4. Comparison between repeated batch and perfusion operation modes for influence on hPSC expansion. (A) Comparison of growth kinetics during repeated batch and perfusion operation modes culturing hCBiPS2 cells for 7 days in a stirred tank bioreactor. Cells were seeded at 0.5×10^6 cells/mL on d0. During the first 48 h, cultures were maintained without any medium exchange. From day 2, in repeated batch culture, the entire medium was replaced daily, while in perfusion culture, the perfusion was initiated (4.2 mL/h), keeping the daily and overall medium throughput equal for both feeding strategies. Up to 4.6-fold increase in repeated batch culture and up to 6.7-fold increase in perfusion culture could be achieved. (B) Glucose concentration patterns in the repeated batch and perfusion cultures of hCBiPS2 cells for 7 days in the stirred tank bioreactor. (C) Impact of feeding strategies on aggregate formation and size distribution. hCBiPS2 were detached from monolayer cultures and injected as single cell suspensions on day 0 within the stirred tank bioreactor. At day 7, the cells from both processes were harvested and analyzed. On process days 1, 2 (before the first repeated batch medium change and perfusion start), and days 3–7, the morphological features of the aggregates were assessed by light microscopy, as exemplary shown (scale bars: 200 μm). Figure adapted from [93].

3.2.5. Progress towards cGMP-compliance

Aiming at clinically compliant hPSC processing, substantial progress was made in recent years. This includes the use of xeno-free and chemically defined culture media, cGMP-compliant cell banking, and the use of cGMP-compliant single-use bioreactors.

In 2011, Chen et al. reported "essential 8" (E8), a xeno-free and completely defined culture medium, for hPSC cultivation [99]. This medium was shown to support hPSC maintenance in an aggregate-based suspension culture [80,93] and xeno-free microcarrier-based cultivation in STBRs [84]. Further, xeno-free culture media were successfully used in hPSC suspension culture systems, including BRASTEM [100] and the StemMACS™ iPS-Brew XF (unpublished data by our group).

Aiming at establishing cGMP-compliant strategies for hESC cell banking, Chen and coworkers developed a promising method to generate cell banks of several hESC lines [66]. In this study, suspension culture using serum-free and defined media for hESC expansion in spinner flasks was used, and feasibility for mass hESC production further supporting future clinical translation was demonstrated. hESCs were passaged for at least 20 times whereby $>1 \times 10^{13}$ fold expansion of the inoculated cells was (theoretically) calculated and pluripotency and a normal karyotype were maintained. Furthermore, the cells harvested from this system were cryopreserved in serum-free medium and thawed into either adherent or suspension culture to continue passaging and expansion, providing a GLP- and cGMP-compliant method for generating hESC banks [66].

Finally, successful translation of an expansion process into single-use stirred tank bioreactor was shown by us [93], representing another important step toward clinical applications. Whereas STBRs made of stainless steel or glass require cost-intensive and elaborated cleaning and validation procedures at every process cycle, fully instrumented single-use stirred tank bioreactors, which have been developed from 100 mL to 1000 L scale, overcome these time- and cost-consuming procedures [22,31,101]. Single-use culture vessels are therefore of great interest for the envisioned clinical translation of hPSCs as they support the development of GMP-compliant upstream processes with reduced risk of cross-contaminations and increased product safety [2,102].

4. Final remarks and outlook

Published studies discussed in this paper clearly indicate that suspension culture of hPSCs in stirred tank bioreactors is a promising approach for generating relevant cell numbers under more controlled conditions. However, the field of hPSC mass cultivation and differentiation is still at its infancy. Process scalability and standardization are challenging tasks reflecting the complexity of both pluripotent stem cell properties and large-scale cell processing, particularly with respect to process standardization and quality requirements raised by industrial and clinical translation.

Moreover, compared to conventional mammalian cell lines, which can typically be grown in relatively homogenous suspension of single cells or small clumps, hPSCs raise substantially higher challenges. This reflects the fact that the aggregate size constantly changes throughout the entire culture process (Fig. 4) and the potential for cell differentiation is a constant "threat."

Regarding the successful up-scaling of hPSC production in stirred tank bioreactors, it becomes fundamental to define the key operational parameters that allow a direct transfer of agitation

conditions established at the (currently relative low) optimization scale toward an increasing process scale.

Therefore, parameters such as the impeller type and dimension, impeller's location along the longitudinal reactor axis, and the agitation speed – all in combination with the cell inoculation density – must be considered and optimized for successful translation from laboratory to production scale. For example, when increasing the bioreactor scale, impeller dimensions must also be adapted. This results in increased shear stress at the impeller tips, which could affect cell viability and pluripotency. Therefore, an in-depth analysis of the impact of the increased impeller dimensions and the interplay with the agitation speed is required in future.

Because of this complexity, impeller-free technologies assuring laminar mixing flow regimen at low shear stress conditions might provide an interesting solution as recently demonstrated with a novel bioreactor type [103].

Moreover, since the aggregate development patterns and kinetics, the average (overall) aggregate size, and the degree of aggregate homo- vs. hetero-geneity have been shown to substantially alter lineage differentiation and process efficiency [43,60,61,104], novel, process-integrated tools aiming at better control of aggregate dimensions are urgently requested for hPSC processing.

In case of large-scale perfusion processes, large quantities of culture medium will be required. Therefore, further media optimization aiming at more cost-effective formulations will become crucial. Substituting costly growth factors with small molecules or more stable protein analogues may pave the way. An alternative strategy may include the implementation of a dialytic membrane into the perfusion circuit as described by Come et al. for RWBs [51]. The aim of this approach is to maintain hPSC-released pluripotency- and proliferation-supporting growth factors inside the bioreactor but, in parallel, enabling steady replacement of consumed glucose and removal of toxic metabolites by the molecule size-selective features of the membrane.

Regarding the cGMP- and regulatory-compliant hPSC production, the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the biopharmaceutical industry are promoting initiatives aimed at defining and implementing "Quality by Design" (QbD) and "Process Analytical Technologies" (PAT) approaches [101], which might have important impact on hPSCs bioprocessing. The QbD approach aims to ensure the quality of medical products by employing statistical, analytical, and risk-management methodology into the design, development, and manufacturing process, as defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [105]. PAT is defined by the FDA as "a system for designing, analyzing, and controlling manufacturing through timely measurements (*i.e.*, during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality" [106]. Therefore, critical sources of variability in a production process must be known and understood; variability itself should be managed by the process design and the product's quality should be reliably predictable [101].

For hPSC expansion the implementation of online measurement and the control of process parameters such as DO, pH, vital biomass, and nutrient/metabolites, which has only recently been introduced to hPSC cultivation, will be fundamental [22]. This technology could provide the missing link for allowing laboratory-scale hPSC processes to become measurement-driven operations [107], an indispensable step for relating process conditions to hPSC production quality. More sophisticated tools for online process monitoring and control, which might help implementing the QbD and PAT requirements, are currently under development. These technologies include automated 3D microscopy of aggregates [108], label-free and noninvasive monitoring of the cell's pluripotency state [109], and Raman spectroscopy and NMR-based metabolomics for the monitoring of multiple culture parameters [110,111]. The latter has been recently applied to monitor lineage-specific hPSC differentiation [112]. Integrated into controlled and automated bioreactors, these nondestructive and noninvasive measurement technologies could substantially expand the options for hPSC monitoring, including the online observation of, *e.g.*, hPSCs' proliferation, differentiation, and maturation kinetics, ultimately leading to robust hPSC production at the required standards. This standardization, guaranteeing higher reproducibility and safety in the hPSC production process, will boost the rapidly developing and highly competitive cell therapy industry [113–115], moreover enabling the attraction of capitals and investors.

Contributors

CK reviewed the current state of the art and prepared the figures. CK, DM, and RZ wrote the manuscript.

Disclosure statement

The authors declare no competing financial interests. All authors have approved the manuscript.

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