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Quality criteria for in vitro human pluripotent stem cell-derived models of tissue-based cells

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ABSTRACT

The advent of the technology to isolate or generate human pluripotent stem cells provided the potential to develop a wide range of human models that could enhance understanding of mechanisms underlying human development and disease. These systems are now beginning to mature and provide the basis for the development of in vitro assays suitable to understand the biological processes involved in the multi-organ systems of the human body, and will improve strategies for diagnosis, prevention, therapies and precision medicine. Induced pluripotent stem cell lines are prone to phenotypic and genotypic changes and donor/clone dependent variability, which means that it is important to identify the most appropriate characterization markers and quality control measures when sourcing new cell lines and assessing differentiated cell and tissue culture preparations for experimental work. This paper considers those core quality control measures for human pluripotent stem cell lines and evaluates the state of play in the development of key functional markers for their differentiated cell derivatives to promote assurance of reproducibility of scientific data derived from pluripotent stem cell-based systems.

1. Introduction and background

The discovery of the ability to culture human pluripotent stem cells (hPSCs) in vitro [1] raised the exciting possibility to utilize their capability to generate all of the three germ layers required to build the human body (Fig. 1). Furthermore, the development of reprogramming technology to generate human induced PSC (hiPSC) lines from somatic cells from any candidate donor [2], has made it possible to generate a diverse range of genotype-bespoke tissue cultures for cutting-edge, basic and applied biomedical research, safety and efficacy studies, or to design

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novel in vitro diagnostic assays and eventually precision therapeutic solutions [3–6].

Furthermore, the capacity to generate such in vitro models from bespoke patients with genetic disorders and syndromes is now showing promise to deliver valuable models of disease conditions, including cardiac disease [7], neurodevelopmental disorders [8,9] and diabetes [10,11].

The generation, characterization and use of many different human stem cell lines from many different donors has shown that variation between cell lines and even clones from the same donor [12,13], can confound interpretation of outcomes in disease modeling assays and potentially pose a risk in the use of the cells in cell therapies. Furthermore, individual stem cell lines show phenotypic variations from one culture preparation to another and may also change due to the appearance of genetic variants [14]. Such variations may be introduced by the culture conditions and indicate that careful control of culture quality is essential for reliable experimental work. It also indicates the need for good method practices, including careful documentation of specific human stem cell line features [15] as captured in Good In Vitro Method Practices (GIVMP) for the development of in vitro methods [16]. Acceptance criteria for hPSC use in therapy and as experimental models, which also include assessment of microbial contamination and cell authenticity, have also been specifically considered for hPSCs [17,18].

It is also important to carefully select markers and functional assays for each specific human stem cell-derived cell type and use phenotypic and/or genotypic characterization methods, which together will promote the development of in vitro cell- or tissue-based test systems that are fit for their specific purpose. Such selection must also include practical considerations such as the complexity, speed and cost of the respective analytical tools used.

Whilst the generic challenges such as microbial contamination, genetic variation, as well as stability, identity and cross-contamination of cell lines are well-described, there are special challenges for stem cell and tissue-based systems [18,19]. These challenges include lengthy differentiation protocols and differentiation into mixed cell types, which can still complicate the achievement of reproducible models with workable acceptance criteria and tolerances.

Here we have outlined the key techniques for the characterization and quality control (QC) of undifferentiated hPSC cell lines and reviewed the range of methods that may be applied to some representative differentiated hPSC derivatives. We also consider the technology readiness level of each differentiation method and its biomarkers for application in toxicology. Furthermore, we discuss critical issues to consider, in order to promote assurance of reproducibility of scientific data derived from hPSC-based systems.

2. Selection of hPSC lines, maintenance and quality criteria

It is now feasible to avoid the time and expense of deriving hiPSCs *de novo* by sourcing cells from the increasing number of professional hPSC resource centers [20]. When hPSC lines are not derived by the local laboratory, it is vital that cells are procured from trusted biobanking sources and are quality controlled prior to supply, rather than obtained from laboratories which do not perform such testing on released cell lines.

Below key parameters involved in hPSC quality control are discussed followed by a summary of minimal testing recommended for hPSC banks. For more detailed information and discussion on the techniques described in the following sections the reader is guided to a number of expert reviews on cell banking, quality control and characterization of hPSC lines including the International Stem Cell Banking Initiative [21], Andrews et al. [17], Oshea et al. [22] and Sullivan et al [23].

2.1. Cell line authentication

Historically, important authentication technologies, such as

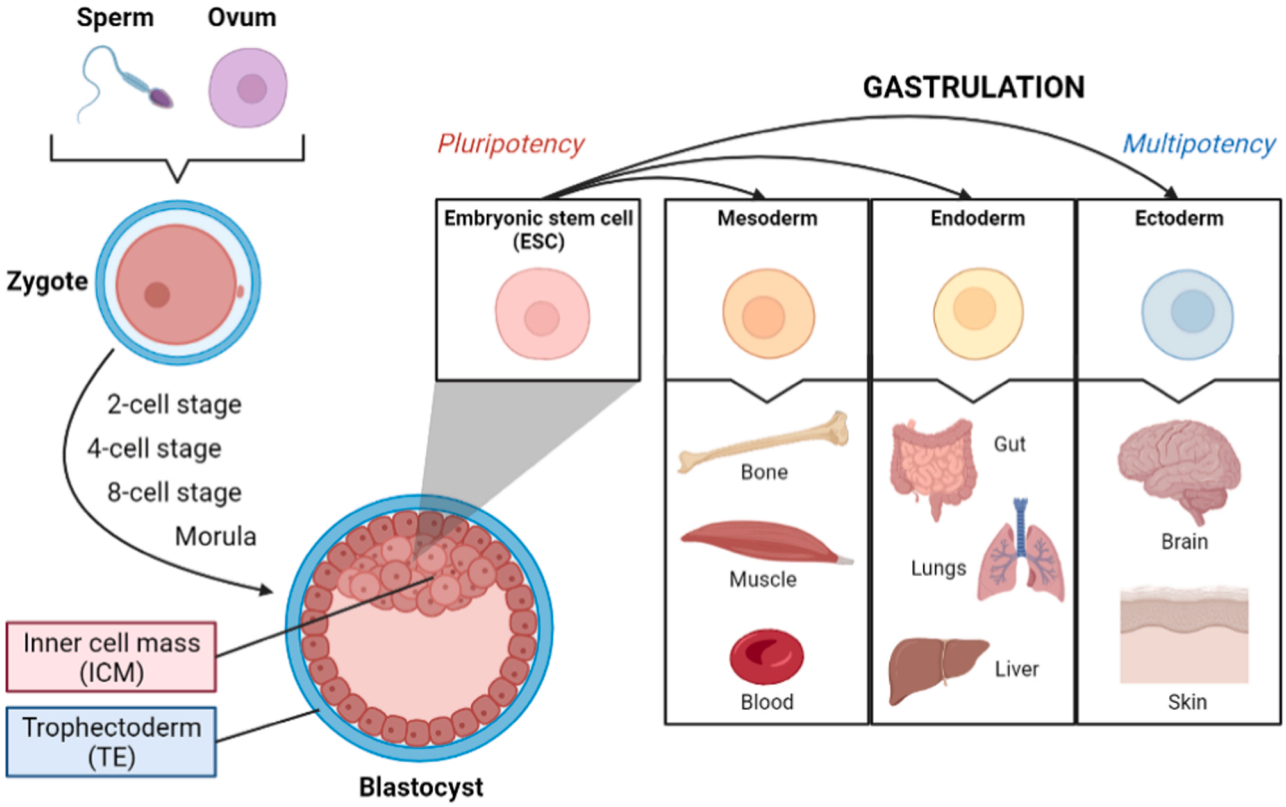

Fig. 1. Early development of human embryonic stem cells and formation of stem cell endodermal, mesodermal and ectodermal germ layers. Created in BioRender.com.

Table 1
Generic minimum QC tests with examples of helpful methodologies and acceptance criteria.

Characteristic	Type of analysis	Exemplar analytical method/s	Acceptance criteria
Pluripotent stem cell features	Stem Cell/ undifferentiated status/ self-renewal markers (e.g., Oct4, SSEA4, SSEA3, Tra 1–60 and Tra 1–81)	1. Flow cytometry 2. PCR	Predominant in stem cell population (e.g., 80–85% % of cells)
	Pluripotency method	1. Embryoid body formation and antibody and or PCR positive for germ layer markers 2. Directed differentiation method 3. Teratoma assay	Evidence of ability to form all three germ layers
Viability	Growth and replication capability	1. Membrane dye exclusion (e.g. trypan blue) 2. Cell attachment and colony growth	1. Majority of cells are viable e.g. > 70% of cells 2. Optimal near confluent culture (50–70%) within 5–7 days
Cell line authenticity	Short Tandem Repeat profile	Multiplex PCR for a number of variable number tandem repeat loci	Profile matches that of original donor cells or early passage hPSCs
Genetic integrity	Chromosome analysis	Giemsa banding karyology, qPCR-based genetic screening	Matches original donor (typically 46 chromosomes) Detection of most common karyotypic abnormalities reported in hPSCs
	Reprogramming vectors in hiPSCs	qPCR	Vector expression not detected
	Non-integrating reprogramming methods (e.g., SeV)	qPCR to assess SeV RNA clearance	SeV RNA not detected
Microbiological status	Mycoplasma	1. qPCR 2. Mycoplasma broth and agar sub-culture	1. Test sample negative with a positive limit of detection control 2. No turbidity or colonies observed at prescribed time point (typical maximum of 21 days)
	Viral pathogens	qPCR for a range of serious or prevalent human pathogenic viruses	1. Test sample negative with a positive limit of detection control
	Bacteria and fungi	1. Microbiological broth and agar subculture 2. 16s RNA qPCR[a]	1. No broth turbidity or agar colonies observed at prescribed time point. 2. No fungal/bacterial RNA detected

[a] methods for detection of multiple microbial species using common 16sRNA targets in qPCR are under development and will hopefully provide valuable QC tests in the future.

karyotyping, isoenzyme analysis, immunotyping and human leukocyte antigen typing, have not proven to have sufficient resolving (discriminating) power to enable unambiguous authentication of human cells to the individual donor-genotype level. The unintentional switching of lines or cross-contamination, is a significant threat to the quality of research work and currently the most commonly used technique is short tandem repeat (STR) analysis. This uses PCR to identify differences in STR alleles that are variable between cell lines from different individual donors.

2.2. Phenotypic characterization

2.2.1. Stem cell biomarkers

Pluripotent stem cells consistently express a common set of markers [24] that are typically characterized with fluorochrome-conjugated antibodies recognizing surface or intracellular stem cell-associated biomarkers (e.g., Oct4, Nanog, SSEA4, SSEA3, Tra 1–60 and Tra 1–81), using either immuno-histochemistry or flow cytometry. Flow cytometry is a sensitive methodology for the analysis of stem cell populations and their differentiated derivatives as a whole, whilst immuno-histochemistry permits detailed identification and location of markers on specific cells.

2.2.2. Pluripotency

Whilst surface and nuclear biomarkers are valuable to confirm hPSC culture identity, they are not sufficient to confirm the pluripotent nature of these cells which requires a pluripotency assay. Recognizing that undifferentiated cells have retained pluripotency is crucial for scientifically robust stem cell research. In early hPSC studies, pluripotency was assessed by teratoma assays in which immunologically compromised mice are injected either subcutaneously or under the kidney capsule to allow the stem cells to differentiate. Whilst this is a powerful research tool it has proved problematic for application in routine quality control (QC) [17] is clearly unacceptable from a 3Rs perspective (i.e. to replace, reduce and refine the use of animals in experimentation), and for some time alternative in vitro assays have been available [25]. Commonly used in vitro assays include embryoid body (EB) formation, directed differentiation of monolayer cultures and molecular analysis of early stage differentiating cells or even undifferentiated cells to reveal stem cell features [26]. These assays aim to demonstrate that hPSCs are capable of producing cell types representative of all three embryonic germ layers (endodermal, mesodermal and ectodermal, see Fig. 1).

2.3. Microbial contamination

The most frequent causes of contamination in cell cultures are bacteria, mycoplasma, yeast and fungi. It is a standard part of current good practice in stem cell banks to carry out routine microbiological controls of the stem cell lines [17,21] and to work with appropriate controlled environments and procedures to reduce the probability of contamination in stem cell cultures. Inoculation of cell culture samples into traditional culture media or testing samples using 16 s RNA PCR can be used to detect bacteria and fungi; however, specialist growth methods and/or staining techniques are needed in order to identify mycoplasma contamination. Contamination of hPSCs with serious viral pathogens appears to be very rare in the experience of stem cell banks. However, it is considered an important safety precaution (for lab worker safety) to test the donors and/or the derived hPSC lines for viruses such as hepatitis C, hepatitis B, human immunodeficiency virus, Epstein-Barr virus and other viruses (including SARS-Cov-2), taking into consideration the historical risks to which donors or cells have been exposed [17].

2.4. Genetic state and stability

It is well established that stem cell cultures can suffer from the appearance of variants with a range of types of genetic change, from point mutations to chromosomal amplification, deletion, inversion and translocation [24]. Genetic status and stability can be assessed by a range of techniques including karyology, comparative genomic hybridization, shallow or deep DNA sequencing and array-based single

Table 2
hPSC-derived endodermal cells/tissues.

Cell type	Key morphological, biochemical and functional parameters (or endpoints[a]) to characterize the model	Technology readiness level (i.e., Low, Medium or High)[b]	References for exemplary protocols for model and measured endpoints
Gastric	The foregut, from which the stomach derives, is marked by the expression of *Sox2*, whereas the midgut and hindgut express Cdx2	Low	[82]
	Contains 'chief' cells, mucus neck cells, parietal cells, mucus pit cells		[83]
Pancreatic islet-like cells	High % of PDX1 and NKX6.1 positive cells High % of insulin expression `Single hormone (e.g. insulin, glucagon, somatostatin) positive Glucose-responsive, insulin-secreting	High (currently undergoing clinical trials)	[42–88]
Intestinal	CDX2+ cells Contains intestinal mesenchyme and epithelium (enterocytes, goblet cells, Paneth cells, tuft cells, enteroendocrine cells) Crypt-villus architecture Posterior HOX genes Becomes more adult-like after transplantation	Medium	[89–91]
Liver (Hepatocytes)	Stable and long-term expression of mature hepatic markers (CYP3A4, ALB); significantly decreased expression of immature markers (AFP), HTS-LiverTox-verified,	Medium/High	[93]
	Direct comparison with primary hepatocytes, elevated levels of albumin secretion and urea synthesis,	Medium	[94]
	sustained metabolism of Terfenadine and production of Fexofenadine over long term culture		
	iPSC-hepatocytes have shown 65% sensitivity and 100% specificity in the long-term drug toxicity assessment; high levels of albumin secretion, urea synthesis and CYP3A4 activity	Medium	[95]
	Elevated expression levels of HNF4a, ALB, TTR, robust CYP (1A2, 2B6, 3A4) induction, lobular organization	Medium	[96,97]
	Long term expression of ALB, CYP3A4, RBP4 and decreased AFP levels	Low	[98]
	Increased expression of ALB, HNF4a, CYP3A4, CYP2C19, CYP2D6, ASGPR1; albumin secretion in vitro and in vivo	Low	[99]
	Expression of adult isoform of HNF4a, expression of ALB, LXR, UGTA1, FAH, no expression of AFP; high levels of urea secretion	Low	[100]
Liver (Cholangiocytes)	Mature biliary markers Appearance of cystic and ductal structures Bile acids transfer, responses to secretin, somatostatin	Medium	[101,102] [104]

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Table 2 (*continued*)

Cell type	Key morphological, biochemical and functional parameters (or endpoints[a]) to characterize the model	Technology readiness level (i.e., Low, Medium or High)[b]	References for exemplary protocols for model and measured endpoints
Bronchus and lungs	Epithelial functions (e.g. presence of CFTR) **Proximal lung progenitors**FOXJ1, SOX2, ARL13B	High	[63]
	Distal lung progenitors FOXP2, SOX9, SPC		[104,105]

'low': available protocol(s) is(are) reliable but still at an early stage of development (i.e., lacking well developed key parameters and/or assay).
'medium': available protocol(s) is(are) reliable and reproducible but mainly suitable for research;
'high': available protocol(s) is(are) reliable and reproducible and potentially suitable for industry or regulatory use.

[a] An 'endpoint' is any feature or parameter to analyse to characterize the cellular model.

[b] Column 'Technology readiness level' describes the degree of development of currently available cell culture protocols.

nucleotide polymorphisms (SNPs). Karyology and array SNP are probably the most common techniques used in stem cell banks, but PCR for detection of common chromosomal changes is also becoming more frequently used.

The sustained expression of reprogramming vectors following generation of an iPSC can affect the capacity of a cell line to differentiate and thus, it is important to test hiPSCs to ensure such expression does not persist. This is known to be a feature of a small proportion of hiPSCs generated by non-integrating Sendai virus vectors [25]. Detection of retention of reprogramming vectors can be performed by antibody-based detection or qPCR.

Epigenetic changes are important for normal stem cell functionality, given that during cell differentiation, methylation silences pluripotency genes and demethylation activates expression of certain genes for differentiated cell types. A range of other epigenetic-related changes including histone modification, can also influence the epigenetic landscape of stem cell genomes and their differentiated derivatives. In the future, analysis of such changes may provide valuable quality control tools.

Minimum scientific selection criteria for taking up stem cells for specific applications include the verification of expression of key stemness markers, demonstration of pluripotent capacity (e.g., teratoma assay, embryoid body formation, directed differentiation), microbial contamination data (including mycoplasma testing data) and negative viral safety testing data (of the cell line or human donor).

Questions relating to each of these features should be directed to any cell line suppliers, and sourcing cell lines from well qualified professional sources with a track record in operating best practice, is highly recommended [26]. It is also important to remember that there are important non-scientific factors that will influence the ability to use cell lines, including confirmation of ethical provenance of donor tissue and its use for derivation of cell lines as well as ensuring absence of any donor constraints on use of the cells. In addition, it is also vital to ensure that the cell user is not restricted by conditions of any materials transfer agreement. Such information should be discussed with the provider of the cell line.

Suppliers and user laboratories should be operating a biobanking process which meets best practice for hPSCs. Whilst the core process for biobanking is broadly the same for all applications, there will be different specific criteria for cells to be used for research purposes only [27] compared to those used for developing cell-based medicines [17,18]. A core quality control testing regime for hPSC banks is outlined in Table 1. More detailed evaluation criteria for different applications can be found in [17,30,31] and a detailed description of development of acceptance criteria used can be found in [18].

Certain quality control criteria should also be checked periodically in the user laboratory for each subsequent cell bank (frozen batch) of cells and for extended passage cells. Priorities in such quality control should include testing for mycoplasma, cell identity and genetic stability [16,18]. Of the various methods available to test genetic stability, probably the most commonly used for hPSC banking are Giemsa banding karyology, array single nucleotide polymorphisms and qPCR for the most frequent chromosomal variants found in the cultures. These different methods are known to have different benefits and disadvantages and are not necessarily equally efficient at detecting certain kinds of genetic change [22]. Selection of a single method will often involve a compromise between local requirements for sensitivity, specificity, speed, cost and availability of suitable equipment.

3. Criteria for hPSC-derived test systems

When working with hPSC-based models it is paramount to assess the quality of the starting culture. In particular, verification of cell viability by e.g., Alamar Blue assay [32], trypan blue exclusion [33] and cell counting before cryopreservation and after thawing of cryopreserved hPSCs should always be performed to ensure consistent seeding of viable

Table 3
hPSC-derived mesodermal cells/tissues.

Cell type	Key morphological, biochemical and functional parameters (or endpoints[a]) to characterize the model	Technology readiness level [b] (i.e., Low, Medium or High)	References for exemplary protocols for model and measured endpoints
Cardiac cells	With maturation, the shape of the cardiac myocytes changes from round to elongated/ anisotropic and the sarcomeric structure becomes well-organized and shows increased sarcomeric length.	Medium	[106] [48]
	Some proteins in the sarcomere structure undergo an isoform switch, also observed in human iPSC–derived cardiac myocytes, e.g. *TNNI1* is expressed in human embryonic cardiac myocytes, but *TNNI3* (slow skeletal troponin T) is expressed in adult hearts.		[107] [108] [109]
	Similarly, isoform switching of myosin heavy chain (MHC) occurs during development. In humans, β-MHC is the predominant isoform in ventricular myocardium, and human PSC–derived cardiac myocytes show increased expression of β-MHC and decreased expression of α-MHC during maturation.		[110]
	Electrophysiological properties change during cardiac myocyte maturation: the resting membrane potential in adult cardiac myocytes in heart tissue is ≈−90 mV but in human PSC–derived cardiac myocytes it is less negative, probably because of the lower expression of the I_{K1} ion channel.		[111]
	Calcium handling and excitation–contraction coupling are important determinants of the contractile properties of cardiac myocytes.		
Kidney I Renal proximal tubular cells	**Morphology:** cobble stone cells **Markers (1)** proximal tubular: LRP2 (aka megalin) and PTH1R (parathyroid hormone 1 receptor) **(2)** epithelial: TJP3 (aka ZO3) or OCLN (occludin) to indicate polarization of the monolayer	Low to medium	[112]
	Functional endpoints: (1) cAMP increase in response to parathyroid hormone **(2)** albumin uptake by megalin facilitated endocytosis **(3)** ABCB1 (aka Pgp) efflux via calcein-AM assay Note: polarization is very critical for proximal tubule; staining of these markers needs to be expressed at the tight junction (rather than cytoplasmic or nuclear)		
Kidney II Renal podocytes	**Morphology:** large cells with typical foot-processes	Low to medium	[113,114]
	Markers: SYNPO (synaptopodin) NPHS1 (aka nephrin), NPHS2 (aka podocin) and WT1 (Wilms tumour 1)		[115,120]
	Functional endpoints: Uptake of albumin		
Kidney III Renal organoids	**Morphology:** organoid tubule with multiple renal cell types	Low to medium	[117]
	Markers: Podocytes: NPHS1, SYNPO, WT1, PODXL; Proximal tubule: *Lotus tetragonolobus* lectin (LTL), LRP2, CUBN, HNF4A; loop of Henle: UMOD; collecting duct (AQP2); endothelial cells (PECAM1, SOX17)		[121,122]
Blood	Red blood cells	Low (Generating mature RBCs from hiPSCs is still an inefficient process)	[123,124]
Immune cells	**Dendritic cells** Expression of CD141, TLR3, and chemokine receptor XCR1 Protrusions and veils of cytoplasm	Medium	[125]
	Macrophages Lipopolysaccharide (LPS) co-receptor CD14 and the pan-leukocyte marker CD45	Medium	[126]
	T cells CD45RO expression Production of IFN-γ, TNF, and IL-2 upon the stimulation Anti-tumor activity	Medium	[127]
	Natural Killer (NK) cells $CD3^{-}CD56^{+}$ cells, low affinity Fcγ receptor	High (NB iPSC-derived NK cells	[128] [129]

(*continued on next page*)

Table 3 (*continued*)

Cell type	Key morphological, biochemical and functional parameters (or endpoints[a]) to characterize the model	Technology readiness level [b] (i.e., Low, Medium or High)	References for exemplary protocols for model and measured endpoints
	CD16 to mediate ADCC, can generally be classified into two major subsets: $CD56^{bright}CD16^{+}$ and $CD56^{dim}CD16^{+}$. The $CD56^{dim}CD16^{+}$ subset of NK cells is predominantly found in the blood and is highly cytotoxic, while the $CD56^{bright}CD16^{-}$ subset is the main subtype found in the lymph nodes and has only weak cytotoxic potential.	have progressed into clinical trials. Experience with key marker detection and assay validation, has meant that this process can be quickly progressed. New results for two early-stage studies have reported testing two different types of	[130,131]
	NK cell activation is regulated by a balance of signals received by the multitude of membrane bound activating and inhibiting receptors. The inhibitory signals such as KIR and NKG2A can detect MHC molecules from the MHC class I recognizing receptors to decrease NK cell activity. NK cytotoxicity can be induced through sufficient stimulation of activating receptors, including the stress ligand receptor NKG2D, and natural cytotoxic receptors (NCR) NKp30, NKp44, and NKp46.	experimental lymphoma treatments that utilize natural killer cells. Eight of 11 lymphoma patients who received one NK cell therapy, FT516, have shown evidence of a response, as have 10 of 14 given another known as FT596. No serious neurological side effects were reported, and only two Grade 1 or Grade 2 cases of the immune-related adverse events typically associated with other forms of engineered cell therapy were observed. The respective clinical trials numbers are NCT04630769, NCT04363346, NCT04245722, and NCT04555811)	NCT04630769 (https://clinicaltrials.gov/ct2/show/NCT04630769?term=NCT04630769&draw=2&rank=1)
	Critical parameters include:	`	NCT04363346 (https://clinicaltrials.gov/ct2/show/NCT04363346?term=NCT04363346&draw=2&rank=1)
	- Cytotoxicity against K562 cells at an 8:1 E:T ratio $\geq$ 50%		NCT04245722 (https://clinicaltrials.gov/ct2/show/NCT04245722?term=NCT04245722&draw=2&rank=1)
	- Flow cytometry for $CD3^{-}CD56^{+}CD45^{+}$		`NCT04555811 (https://clinicaltrials.gov/ct2/show/NCT04555811?term=NCT04555811&draw=2&rank=1)
Connective tissue (fibroblasts)	**Markers for fibroblasts** Vimentin (VIM), ALCAM, HSP47, Platelet-derived growth factor-beta (PDGFRB) **Functional assays (in vitro)** Scratch assay, Collagen contraction assay	Medium	[132,133]
Cartilage and bone	**Gene expression on Days 7-21 Chondroprogenitor lineage** Sry-type HMG box, SOX9, and type II collagen **Fully differentiated chondrocytes** Col2b, aggrecan, Runx2, type X collagen **Morphology**: Cellular outgrowths and cartilaginous nodules on Days 14-21 **Bone specific markers:** Alkaline phosphatase (ALP) activity Collagen type I gene expression	Medium	[134]

'low': available protocol(s) is(are) reliable but still at an early stage of development (i.e., lacking well developed key parameters and/or method)
'medium': available protocol(s) is(are) reliable and reproducible but mainly suitable for research;
'high': available protocol(s) is(are) reliable and reproducible and potentially suitable for industry or regulatory use.

[a] An 'endpoint' is any feature or parameter to analyse to characterize the cellular model.

[b] Column 'Technology readiness level' describes the degree of development of currently available cell culture protocols.

cells and reliable maintenance of stock cultures.

Moreover, evaluation of cell proliferation and growth rate (e.g., nucleic acid content with propidium iodide, 7-aminoactinomycin-D (7-AAD), Hoechst 33258, $Ki67^{+}$ cell percentage, incorporation of bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxiuridine (EdU)) should be carried out to enable determination of the likelihood of genetic change, cell culture phenotypic drift, ageing and cell senescence that may all occur as a consequence of extended passaging. It is also important to evaluate the possible impact of viral and/or mycoplasma infections, or issues with cross-contamination, which may all be responsible for inherent variation in the cultured cells (e.g., decrease of growth rate and/or cell metabolism assays) [34].

Appropriate characterization of the phenotypic identity of hPSC derivatives is important especially when working with heterogeneous/mixed cell cultures. This entails knowing cell differentiation capacity over time and the expected final proportions of different cell subpopulations, which should be verified by (quantitative) analysis of specific cell population markers (at gene and/or protein level) and by assessing cell functionality whenever appropriate (e.g., by analysis of spontaneous electrical activity of neuronal cell cultures, spontaneous

Table 4
hPSC-derived ectodermal and reproductive cells/tissues.

Cell type	Key morphological, biochemical and functional parameters (or endpoints)	Technology readiness level (i.e., Low, Medium or High)	References for exemplary protocols for model and measured endpoints
Neural stem cells (NSCs)	Expression of NSC markers (protein and gene expression) (e.g., nestin, Sox1, Sox2, Pax6, etc.)	Medium/High	[134–136]
Glutamatergic neurons	Expression of glutamatergic neuron markers (protein and gene expression) (e.g., VGLUT1, VGLUT2, GluR1, TBR1, TBR2, CTIP2, SATB2, GRIA (glutamate receptor, ionotropic, AMPA), GRIK (glutamate receptor, ionotropic, kainite), GRIN (glutamate receptor, ionotropic, NMDA), etc.)	Medium/High	[137–139]
	Electrophysiology, spike and burst patterns	Medium	[140,141]
	Neurotransmitter release	Low	[142]
GABAergic neurons	Expression of GABAergic neuron markers (protein and gene expression) (e.g., GABA, GAD1/2, GAD67, vGAT (SLC32A1), etc.)	Medium	[138,143]
	Electrophysiology, spike and burst patterns	Medium	[144–146]
	Neurotransmitter release	Low	[140,141,147]
Dopaminergic neurons	Expression of dopaminergic neuron markers (protein and gene expression) (e.g., TH, NR4A1, NR4A2, NR4A3, DAT, etc.)	Medium	[146,148,149]
	Electrophysiology, spike and burst patterns	Low	[150,151]
	Neurotransmitter release (e.g., induced by KCl stimulation)	Low	[152,153]
Cholinergic neurons	Expression of cholinergic neuron markers (protein and gene expression) (e.g., ChAT, p75NTR, FOXG1, ISL1, LHX8, NKX2.1, ACHE, SLC5A7 (CHT1), NGFR, NTRK1 (TRKA), SLC18A3 (VACHT), etc.)	Low	[154–156]
	Electrophysiology, spike and burst patterns	Low	[154,155]
	Neurotransmitter release	Low	[155]
Astrocytes	Expression of classic astrocytic markers (e.g., Glial fibrillary acidic protein (GFAP), S100 calcium-binding protein β (S100β), N-Myc downstream-regulated gene 2 (NDRG2), Aldh1L1, GLAST and GLT1)	High	[157–159]
	Detection of calcium transients	Low	[159,160]
	Astrocytes activation (cytokines releases and morphological changes)	Medium	[161,162]
	L-glutamate or D-aspartate uptake	Medium	[12–166]
Oligodendrocytes	Expression of oligodendrocyte markers (protein and gene expression) (e.g., OLIG1, NG2, O4, O1, GalC, CNPase, etc.)	High	[167–169]
	Expression of myelin markers (protein, gene expression) (e.g., PLP1, MBP, MOG)	Medium	[167,170,171]
	`Image analysis quantification of myelin	Low	[56,170,171]
	Compact myelin sheet studies (electron microscopy)	Low	[172,173]
Microglia/Brain macrophages	Expression of microglia markers (gene expression and protein) (e.g., P2Y12, TMEM)	Medium/Low	[174–177]
	Flow cytometry assessment of CD11b and CD45 (microglia)	Medium/Low	[176,178]
	Expression of markers for brain macrophages (Iba1, Cx3cr1, Trem2, CD68)	Medium/Low	[177–179]
	Phagocytosis assay	Medium/Low	[178,180]
	Microglia activation (cytokines releases and morphological changes)	Medium/Low	[181,182]
Reproductive cells/tissues			
Spermatid	Round morphology, haploid nuclei, polarized acrosin expression	Low/medium	[183]
Sertoli cells	Extended and epithelial morphology; cytoplasmic cytoskeleton staining of KRT18, nuclear staining of SOX9; immunosuppression capability	Medium/high	[184]
Ovarian follicles	Cumulus-oocyte-complex morphology; positive staining of zona pellucida (ZP2) proteins and oocyte-specific proteins (NOBOX)	Medium	[185]

'low': available protocol(s) is(are) reliable but still at an early stage of development (i.e., lacking well developed key parameters and/or method).
'medium': available protocol(s) is(are) reliable and reproducible but mainly suitable for research;
'high': available protocol(s) is(are) reliable and reproducible and potentially

suitable for industry or regulatory use.
1) An 'endpoint' is any feature or parameter to analyse to characterize the cellular model
2) Column 'Technology readiness level' describes the degree of development of currently available cell culture protocols.

beating rate of cardiomyocytes, metabolic capacity of hepatocytes, glucose-stimulated insulin secretion of pancreatic beta cells etc.; see Tables 2–4). Hence, key morphological, biochemical and functional parameters (or endpoints) should be selected and acceptance criteria defined to characterize final cell composition of differentiated cell cultures, depending on the intended goals of the study ('fit-for-purpose' principle).

Numerous studies have been published describing protocols to obtain well-characterized hPSC-derived ectodermal, mesodermal and endodermal cell types as well as reproductive cells and tissues suitable for different purposes and different contexts of use, both for biomedical research as well as toxicity testing. One common approach in this process is to allow hPSCs to form embryoid bodies (EBs) in suspension using techniques such as ultra-low attachment culture dishes [33], AggreWell™ plates [36]. EBs can then differentiate further to form more mature cells of all three lineages, ectoderm, mesoderm, and endoderm, and be directed by activating or repressing specific signaling pathways which closely mimics the gastrulation phase of development in an embryo. It is also important to note that there is an ever-increasing number of protocols describing differentiation directly to the end cell type of interest, bypassing EB formation, including differentiation of hPSC directly to neurons, muscles, hepatocytes or pancreatic cells [37]. There are also protocols for differentiation of hPSCs to neuroprogenitor cells in monolayer by dual SMAD inhibition which bypass EB and neural rosette formation [3].

However, this large number of cell culture protocols significantly differ in their readiness level for industrial or therapeutic applications, showing variable degrees of development. In particular, some of the most common and relevant morphological, biochemical and functional endpoints can be used as acceptance criteria for the characterization of several hPSC derivatives, as shown in Tables 2–4. On the basis of expert judgment, the technology readiness level of cell culture protocols can be scored as:

- 'low' when procedure(s) is(are) well-described and reliable, although still at an early stage of development (i.e., lacking well developed key assessment parameters and/or methods);
- 'medium' when available protocol(s) is(are) reliable and reproducible but are still at the developmental stage in basic or applied research; or
- 'high' when available protocol(s) is(are) reliable and demonstrated to be reproducible and could be potentially suitable for industry or regulatory use.

It should be considered that technology in this field is rapidly evolving, with cell culture applications expanding across multidisciplinary fields. While the (non-exhaustive) list of parameters and associated readiness level scores shown in Tables 2–4 is not exhaustive and is expected to evolve with time, it serves as a basis to identify possible knowledge gaps and prioritize actions for new protocol design and implementation efforts.

4. Complexity of the applied model: 2D versus 3D

In vitro models of developmental processes are frequently used to advance mechanistic understanding of cell differentiation, migration, growth, and final maturation (both morphological and functional), all of which are impacted by their biochemical and biomechanical microenvironment [39]. Deciphering the underlying mechanisms is vital to understand in vivo processes that result in formation and function of tissues and organs and how these processes might be altered under exposure to natural or industrial chemicals or drugs (toxicity testing).

For over a century, two-dimensional (2D) cell cultures have been used as in vitro models to study cellular responses to stimulation by biophysical and biochemical cues, as well as for toxicity evaluation induced by exposure to a xenobiotic.

Cells cultured in 2D cell systems typically grow on flat plastic surfaces, sometimes treated to increase adhesive properties to enhance cell attachment and spreading. Although 2D cell culture has been the most common format for most cell culture purposes including toxicity testing, it typically generates compromised systems lacking key components of tissue architecture, cellular interaction and density. This significantly limits the possibility to replicate in vivo functionality of a given tissue or organ. Thus, 2D systems might not always be truly representative of real tissue environments, and this can increase the cost and failure rate when either developing new drug discovery platforms in clinical trials, or when carrying out risk and hazard assessment of chemicals.

Despite missing histoarchitecture and limited cell-to-cell interaction, 2D cell cultures are still used for the majority of in vitro work because they are inexpensive, well established, can typically be adapted for high throughput purposes, are amenable to comparing new results with previous studies, and are typically easier to process for analysis. All the classical endpoints used for toxicity testing were historically developed to use with monolayer cell cultures and, thus, are very well established for 2D systems. For instance, toxicity testing for the heart using monolayer 2D cultures of hPSC-cardiomyocytes is widely accepted even for regulatory purposes [40]; however, this is not always the case for hazard identification/characterization or risk assessment for kidney, brain and other organs.

Moreover, 2D cultures are widely used to gain understanding of underlying molecular mechanisms and signaling pathways where all parameters can be more readily controlled due to the simpler experimental set-ups. Monolayer 2D cultures of hPSCs differentiated into various cell types are now increasingly used for clinical and toxicological studies. For instance, hiPSC-derived pancreatic cells on platforms are being used to study gene-environment interactions that impact human β-cells and the survival of dopamine neurons [41]. hiPSC-derived neural progenitor cells, differentiated into neuronal or glial derivatives can be used for semi high-throughput toxicity testing where cellular readouts are evaluated including cell viability, immune-cytochemical quantitative expression of neuronal and glial markers or neuronal activity using microelectrode arrays (MEA) [42–45]. High throughput testing programs such as ToxCast and Tox21 have also used mainly monolayer test systems (https://www.epa.gov/chemical-research/toxcast-data-generation-overview-toxcast-assays) (https://ncats.nih.gov/tox21/projects/assays). However, the strategic plans and developments in this program are moving now towards more complex 3 dimensional (3D) organotypic cultures [46].

Much better biomimetic tissue models make 3D cell cultures more physiologically relevant than 2D cultures. 3D cultures also show a higher degree of structural complexity and retain a "steady state" (homeostasis) for longer [47] and may benefit from more complex mixed cell culture methods [48].

3D cell models present the advantage to model in vitro cell microenvironment and cyto-architecture resulting in cell/tissue physiology similar to conditions in the human body [49]. For these reasons scientific focus began to shift to 3D cell cultures more than 30 years ago, starting from the hanging drop method which [50,51] was adapted to in vitro differentiation of mouse embryonic stem cells [52]. This method allowed further improvements to a range of scientific studies including the study of embryology, reproductive/developmental toxicology, virology and genetics. However, in spite of this long history, 3D cultures only began to make rapid progress in the late 2000 s

3D cell culture techniques such as spheroids and organoids are now intensively investigated, especially in stem cell research and a broader range of techniques are currently available to generate 3D test systems.

These include the use of extracellular matrices, scaffolds, multi-layering techniques, cell aggregation by gravity, stirring or shaking approaches maintaining cells in ultra-low adherence vessels, microfluidics devices, or bioprinting. These systems have been widely reviewed [53–55] as has the use of 3D organotypic cultures for toxicity testing which was recently summarized by Matsui et al. (2021) [56]. An example of a recent success in this area is the method to generate 3D neurospheres from neural progenitor cells derived from hiPSCs or fetal primary neurons. These are already used for developmental neurotoxicity testing, to assess cell proliferation, migration, differentiation, and other key neurodevelopmental endpoints for regulatory purposes [18,19,57–62].

3D not only promotes the opportunity for better intracellular interactions and survival but also a better functionality. For example, it is hard to develop a co-culture of neurons and oligodendrocytes with active myelination, but differentiation towards myelinating oligodendrocytes can easily be achieved using 3D neurospheres, compensating for the limitation of using 2D monolayer cultures [57]. These 3D neurospheres enable robust differentiation towards both neuronal and glial cell populations, including myelination and formation of more mature neuronal network activity when compared to 2D monolayer culture [19, 57,63].

Moreover, interactions between different types of cells in these complex systems can be facilitated using microfluidics and organ-on-chip (OoC) devices [62]. The first microfluidic microphysiological systems were developed more than 15 years ago and are nowadays generally considered suitable to mimic human patho-biology/physiology because they incorporate options for microfluidic flow as in the case of blood vessels for example, thus providing alternative approaches to the use of laboratory animals in drug development and in basic and applied research [65,66]. Microfluidics systems continuously provide nutrients and cells grow (proliferate, migrate and differentiate) under more physiological conditions. Such systems have also been used for understanding the mechanics of embryonic development [67].

The widespread acceptance and use of such human relevant test systems could ultimately help reduce the reliance on the use of animal models, which have significant limitations in the accurate prediction of how drug treatments or exposure to natural or industrial chemicals and their mixtures will affect animal and human health [68].

However, there are some important issues and obstacles when working with 3D models which need consideration. As mentioned above, some types of readouts (e.g., morphological/structural, immunological, functional) and high content imaging analysis at cellular level were originally developed for monolayer cultures and now need to be adapted for 3D, which can be difficult.

It can also be challenging to distribute oxygen and other essential nutrients to all cells within the spheres/organoids. To avoid such difficulties, the size of 3D culture organoids should be optimized and controlled using scaffold-free systems, such as “hanging drop templates,” “magnetic levitation,” “magnetic 3D bioprinting”, formation of microtissues/organoids, scaffold support (e.g. hydrogel as an extracellular matrix) or vascularization [49].

It is important to note that many 3D culture platforms are still difficult to manipulate, require expertly-trained personnel to handle, are time-consuming, and are still not well standardized and have low reproducibility, thus, rendering them unsuitable for high throughput screening of drugs or chemicals. In addition, the use of hiPSC as a cellular source adds further challenges to achieve reproducibility and standardization, as discussed above. Standardization can help ensure proper characterization of 3D cultures and/or OoC devices, benchmarking using suitable series of reference compounds and ease communication among different stakeholders, for instance by agreeing on terminology and reporting methods [69]. Recently, some initiatives have been undertaken to improve standardization of 3D cultures and OoC using different platforms by introducing key quality controls (biological and technological), with the aim to improve robustness and reproducibility of these promising new technologies. In particular, the European Commission Joint Research Centre and the European Standardization Organizations CEN and CENELEC in April 2021 organized a workshop titled *"Putting Science into Standards"*, with the aim of identifying the needs and priorities for developing standards for OoC technologies, spanning selection of materials, chip design, flow rates, etc. Notably, it was concluded that performance assessment, benchmarking, interoperability and qualification of OoC technologies for different contexts of use or applicability domains would benefit from standardization [70]. Standardization will ultimately contribute to the acceleration of regulatory acceptance of these novel technological devices. The National Centre for the Advancement of Translational Science (NCATS) supported initiative toward microphysiological systems international society, calls to support global harmonization and standardization of such technologies. This should accelerate the development and move the 3D cultures and OoC towards regulatory acceptance (https://grants.nih.gov/grants/guide/notice-files/NOT-TR-20-005.html).

Clearly, the selection of a 2D versus a 3D model is likely to affect the results obtained. For example, 2D models are still more suitable to assess neuronal functionality by analysis of spontaneous electrical activity using traditional multielectrode array platform [63]. However, the fast development of new technologies, and increasing number of new bioengineered devices more suitable for electrophysiological recordings in 3D [71–74] might change that.

5. Test system validation

The principles described in Good Cell Culture Practice (GCCP 2.0) [18] give guidance to scientists as to how to obtain and record, cell and tissue culture information mainly from in vitro methods using new approaches and methods with the most advanced research and innovation test systems, such as hiPSC-derived advanced cell culture models and microphysiological systems. As defined by the FDA, “*a microphysiological system (MPS) uses microscale cell culture platform for in vitro modeling of functional features of a specific tissue or organ of human or animal origin by exposing cells to a microenvironment that mimics the physiological aspects important for their function or pathophysiological condition. MPS design may aim to provide and support cultured cells with physical (*e.g.*, temperature, pH and oxygen)/biochemical/electrical/mechanical (e.g., flow or stretch)/-structural/morphological conditions and recapitulate a set of specific properties that define a healthy or diseased organ or tissue function. MPS platforms may comprise mono-cultures, co-cultures of multiple cell types, maintenance of explants derived from tissues/organs, and/or inclusion of organoid cell formations. Organ-on-a-chip is a subset class of microphysiological systems and consists of a miniaturized physiological environment engineered to yield and/or analyze functional tissue units capable of modeling specified/targeted organ-level responses*” (https://www.fda.gov/science-research/about-science-research-fda/advancing-alternative-methods-fda).

The internationally recognized OECD guidance document on Good in Vitro Method Practice (GIVIMP) [16] is intended to support method developers and end-users working to establish new in vitro assays in academic, industry or government laboratories across all 38 OECD member countries and beyond.

In toxicology, stem cell-based in vitro methods are designed to identify potentially harmful effects of chemicals used in a variety of contexts including consumer goods, industrial processes and plant protection products. They are fast becoming key tools for a new way of doing toxicology without resorting to animal testing. Test data derived from in vitro methods are increasingly being used in combination with other information within Integrated Approaches to Testing and Assessment (IATA) to support safety decisions. However, consensus good practice is essential to ensure that in vitro data can be trusted by industry end-users and regulatory authorities for the protection of workers, consumers and the environment. Moreover, in vitro methods that undergo validation often require improvements in their design and

implementation before they can be evaluated regarding their reliability and relevance for a particular regulatory purpose. It is strongly recommended to take careful consideration of GIVIMP requirements during the development of in vitro methods as this will help improve the quality of submitted methods, accelerate their acceptability and ultimately enhance the efficiency of validation studies.

The development of GIVIMP was coordinated by the European Commission Joint Research Centre within the context of a project of the OECD Test Guidelines Program. A large number of international experts, including members of a dedicated OECD expert group and the European Union Network of Validation Laboratories (EU-NETVAL), contributed to the state-of-the-art knowledge gathered within the guidance document. GIVIMP also benefitted from a number of written commenting rounds and two expert meetings before its final endorsement by the OECD's working party for national coordinators of the test guidelines program in April 2018.

Elements described in GIVIMP that are important for the description of in vitro methods using complex stem cell and tissue-based test systems and reporting of derived results are described in detail in the GIVIMP test system chapter. Any stem cell researcher or in vitro method developer is prompted to provide information about the actual stem cells used to arrive at the measurements, the detection method, the method used for dose selection, control and reference chemicals used, specific experimental conditions, data analysis, acceptance criteria applied, validity of the data and reporting of results. It is anticipated that validation bodies and regulatory agencies will expect adherence to GIVIMP to ensure that the proposed stem cell-based method is fit for validation and, ultimately, regulatory acceptance. Regulatory bodies, validation authorities, method developers, and industry toxicologists realize the need to increase confidence in the scientific validity of novel in vitro methods with stem cell-based test systems – especially those being proposed for regulatory application [75,76]. Also, data reviewers such as companies and regulatory agencies responsible for product registrations, will have increased confidence in data generated in laboratories adhering to GIVIMP standards [77] when using stem cells. OECD issued also a specific OHT201 template (https://www.oecd.org/ehs/templates/harmonized-templates-intermediate-effects.htm) that can be completed in compliance with GCCP as part of GIVIMP when in vitro mechanistic New Approach Methodologies (NAMs) are reported. The template offers the possibility to declare that GIVIMP guidance was followed and that all elements important for the in vitro method are reported and/or documented (as listed in the template's 'helptext'). To verify that all aspects of the GIVIMP guidance are implemented including all relevant GCCP aspects [18] stem cell researchers, in vitro laboratories and routine testing facilities (e.g., national, EU and international experimental reference laboratory networks) may find it useful to consult the GIVIMP e-learning module from the European Commission https://etplas.eu/lessons/nuno-lesson/ or the GIVIMP Certification Program which provide guidance on implementation of the quality principles [78]. Good Laboratory Practice (GLP) [79] and Good Cell Culture Practice (GCCP) [18,80] are important topics to consider, but for regulatory submissions in the area of compound safety, GIVIMP serves as a comprehensive quality framework for the development or execution of in vitro methods.

6. Conclusions and future considerations

It is clear that the crucial stages at which the scientific reproducibility of hPSC-based model systems can be enhanced are the originally isolated or generated hPSCs, standardized cultures of undifferentiated cells and differentiated cultures representing in vitro assay substrates. In this paper we have outlined the key techniques for the characterization and QC of undifferentiated hPSC cell lines and reviewed the range of methods that may be applied to some representative differentiated hPSC derivatives and their technology readiness level for their application in toxicology. The importance of GCCP, its uptake and utility have been demonstrated in a recent publication by Tigges et al. [81], which provides recommendations for the implementation of GCCP in the quality control of hiPSCs used in academic research. Of course, there are new analytical developments that will continue to offer potential improvements in functional readouts for hPSC-based systems and it is vital for researchers to keep abreast of these new technologies. In particular, it is highly likely that key areas where technology will develop rapidly will be the application of 3D-cultures, micro-physiological systems and hPSC-derived organoids. It is also likely that use of these systems will reveal new biomarkers and possibilities for enhanced functional assays. However, it is important to consider that with all these dynamic scientific possibilities, it will be essential to keep the principles of GCCP and requirements of GIVMP in mind so that transition of new and exciting research methods into practical application in toxicology can be made effectively and efficiently.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Data Availability

No data was used for the research described in the article.

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