CELL & GENE THERAPY INSIGHTS

INDUCED PLURIPOTENT STEM CELLS (iPSCS)

SPOTLIGHT

EXPERT INSIGHT

Genetic stability of hPSCs and considerations for cell therapy

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Continuous advances in the isolation, reprogramming, and gene-editing, as well as their scalable and efficient culture and differentiation for commercialization, have positioned human pluripotent stem cells as an attractive starting material for cell therapy. Nonetheless, continuous culture can predispose human pluripotent stem cells to genetic alterations at the chromosomal, region, copy number, and single nucleotide levels. The detection of those alterations is challenging, as no single assay can detect all potential genetic abnormalities in human pluripotent stem cells. However, combinations of approaches can provide a clearer assessment of human pluripotent stem cell lines, and when executed at key process steps can help mitigate costly expansion of genomically-compromised cells and the potential risks that those cells may pose to patients. Encouragingly, while the appearance of some genetic alterations may be concerning, in comparison with other cellular models, human pluripotent stem cells are not inherently prone to genetic instability. Furthermore, emerging strategies to mitigate the appearance and propagation of those abnormalities have shown encouraging results. Close partnerships between human pluripotent stem cell-derived cell therapy developers and regulators will help define genetic stability risks, best practices on testing pipelines, acceptance criteria, and robust guidelines in order to deliver these transformative therapies to patients in need.

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INTRODUCTION

Human pluripotent stem cells (hPSCs) have held promise in regenerative medicine since the establishment of the first human embryonic stem cells [1]. The development of technologies to generate human induced pluripotent stem cells (hiPSCs) [2–7] marked a new era in the field, and the continuous development of ever more precise and accessible gene editing strategies have generated tremendous excitement towards the perspective of using these cells to manufacture life-saving cell

TABLE 1

Assays currently available to assess genetic instability in hPSCs.

Assay	Analyte	Target	Resolution	Scale	Estimated lowest level of mosaicism detected	Turn-around time	Cost
DNA index analysis by flow cytometry	Individual nuclei	Total DNA	Gain or loss of ≥5 chromosomes	Whole genome	2–3%	⌛	$
G-band karyotyping	Individual nuclei	Individual chromosomes and regions	5–10Mb	Whole genome	Typically, 5–15%, depending on number of cells analyzed	⌛⌛	$$
aCGH, SNP arrays	Bulk genomic DNA	Chromosome regions	10–100 kb	Whole genome	10–20%	⌛⌛⌛	$$$
FISH	Individual nuclei	Individual regions, genes	100kb–1Mb	Target sequences	5%	⌛⌛	$$
iCS-digital™ PSC 24-probe (ddPCR)	Bulk genomic DNA	Individual regions, genes, SNPs	>1bp	24 specific sequences	20%	⌛	$
Stem-Seq+™, OncoPlex (targeted NGS)	Bulk genomic DNA	Individual regions, genes, SNPs	>1bp	361, 350 genes	1–3%	⌛⌛⌛	$$$$
WES (NGS)	Bulk RNA	Individual transcripts, SNPs	>1bp	Whole transcrip-tome	1–3%	⌛⌛⌛⌛	$$$$
WGS (NGS)	Bulk genomic DNA	Individual regions, genes, SNPs	>1bp	Whole genome	1–3%	⌛⌛⌛⌛	$$$$

aCGH: array comparative genomic hybridization; FISH: fluorescence *in situ* hybridization; NGS: next-generation sequencing; ddPCR: digital-droplet PCR; SNP: single nucleotide polymorphism; WES: whole exome sequencing; WGS: whole genome sequencing.

therapies. Altogether, their virtually infinite propagation and differentiation potentials combined with novel genetic edits make them an ideal starting material with broad opportunity for treating unmet medical needs.

In spite of remarkable advances and innovations in process development tailored for large-scale hPSC culture, gene-editing, and differentiation, the cell growth and differentiation paradigms can be aggressive, fomenting a selection process favoring the outgrowth of cells that may have genetic instabilities and acquired undesirable genetic mutations.

Genetic instability has been well documented for hPSC culture and differentiation [8–24]. Furthermore, continuous propagation of hPSCs has been seen to drive genetic instability at the three fundamental levels of organization of DNA:

- Chromosomal stability, where whole chromosomes or large regions are lost or amplified;

- Copy number variations, where smaller regions containing clusters of genes and regulatory elements are amplified or lost; and
- Single nucleotide polymorphisms (SNPs), where individual nucleotides impacting the expression of specific genes are altered.

These genetic changes can alter the phenotype and function of hPSCs and/or their differentiated progenies, and therefore assessing genetic stability at all stages of the hPSC-derived cell therapy manufacturing process is critical for successful commercialization and patient delivery.

ASSESSING GENETIC INSTABILITY IN hPSCS

A number of test methods are available to assess and characterize the genetic integrity of hPSCs, summarized in Table 1. These technologies include flow cytometry [25],G-band karyotyping [26], array comparative genomic hybridization (aCGH) and SNP arrays, Fluorescence *in situ* hybridization (FISH), droplet digital (dd)PCR [27], and next-generation sequencing (NGS)-based assays [20,23,24,28]. When defining the testing strategy for genetic characterization of hPSCs the assay target and the type of genetic abnormalities detected, the assay resolution, and the assay sensitivity with respect to the level of variant mosaicism should all be carefully considered. It is important to note that currently, no single assay is able to detect all levels of genetic instability simultaneously, and at a satisfactorily low limit of detection. Nonetheless, these testing strategies can be complementary to each other when deployed at critical stages of the hPSC-derived cell therapy product manufacturing: donor cell characterization, iPSC reprogramming and seed bank generation, gene-editing of seed bank, and creation of the master and working cell banks [10,17,29,30]. Together, these tests can help build a narrative towards go/no-go decisions (Figure 1). As a practical example, G-band karyotyping can provide information on the number of chromosomes found, and on their structure (absence of duplications, translocations, etc.) but it cannot detect smaller region amplifications and deletions which can be detected by aCGH; while the latter cannot detect translocations that do not cause clear loss of heterozygosity. Similarly, G-band karyotyping will not be able to provide insights on mutations in specific genes, which can be detected by targeted, NGS-based assays, albeit the latter will not be able to detect translocations, duplications, and other larger scale rearrangements with no clear impact in individual gene sequences.

Importantly, except for whole exome sequencing (WES) and whole genome sequencing (WGS), all other assays can be provided as a service and include a report that can be used as a basis for assessing the potential pathogenicity of the findings. Although extremely rich in information, WES and WGS data typically require somewhat intensive bioinformatics analyses, adding complexity and timing requirements to the assays, however once those advanced assays and analyzes are performed, they can also be included in quality control of hPSCs.

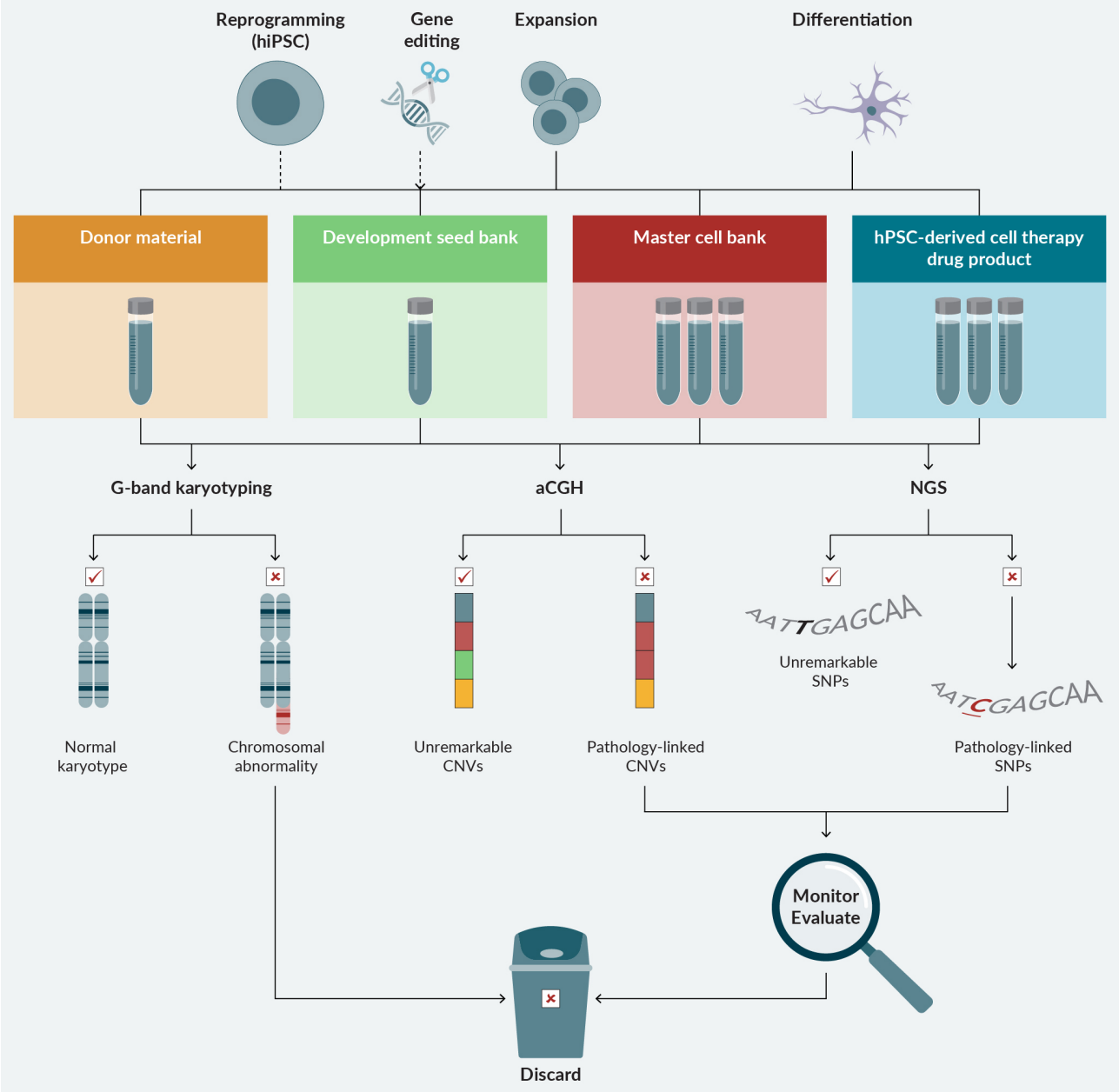
ALTERED GENETIC PROFILES IN hPSCS: WHAT DO WE KNOW?

At a high level, genetic instability can impact cells destined for therapy by disrupting the expression of key genes involved in the cell physiology, leading to potentially oncogenic, transformed phenotypes, or negatively impacting stemness and differentiation ability. Several chromosomal, regional, and gene-level abnormalities have been reported in hPSCs.

The breakthrough demonstration that the pluripotent state can be induced in differentiated cells by overexpression of a defined set of transcription factors [31] opened the avenue for generating iPSC by reprogramming a wide range of somatic cells (skin fibroblasts, peripheral blood and cord blood

FIGURE 1

An example framework of potential assays tests for assessing genetic stability throughout the hPSC-derived cell therapy workflow.



aCGH: array comparative genomic hybridization; FISH: fluorescence in situ hybridization; NGS: next-generation sequencing; ddPCR: digital-droplet PCR; SNP: single nucleotide polymorphism; WES: whole exome sequencing; WGS: whole genome sequencing.

cells, keratinocytes, urine cells and others). Considering the genetic background of the starting material, the reduced efficiency and clonogenic nature of the reprogramming process, and the complexity of epigenetic changes associated with reverting cellular identity during reprogramming (reviewed in [32]), particular attention should be paid towards careful genetic characterization of both starting material and reprogrammed material when manufacturing iPSCs. The choice of the starting material's source and donor age, need to be carefully considered, as well as the selection of the reprogramming method (non-integrating: Sendai-virus, episomal and mRNA-based versus integrating viral

vectors), in order to minimize the mutational burden of the resulting iPSC line. Several studies reported chromosomal aberrations, mutations and genetic variants associated with both parent somatic cells and the reprogramming process, in addition to culture-acquired genetic changes [33–36]. Identifying pre-existing and *de novo* acquired mutations and genetic variants is key to ensure safety of the manufactured cell therapy product.

At the chromosome level, gains of regions of the long arms of chromosomes 1, 17 and 20 or the short arm of chromosome 12, or gain of a whole chromosome (trisomy 1, trisomy 20), as well as losses of regions on chromosomes 10 and 18 are common and recurrent abnormalities in hPSCs [11,17,18,27]. Most of these large chromosomal abnormalities can be detected, with varied sensitivity, by several methods, including G-band karyotyping, ddPCR, aCGH, and FISH.

At a sub-chromosomal level, aCGH and SNP arrays have brought to light copy number variations and loss of heterozygosity in hPSCs [15–17]. Furthermore, the occurrence and propagation of those alterations seem to have a dynamic behavior across reprogramming and hPSC culture [14]. Given the complexity of the analysis and interpretation of results in these studies, alterations observed outside of genomic regions known to cause clear consequences to cell behavior must be dissected carefully, and their relevance may need to be weighed against the cell therapy niche.

At the level of individual genes, three examples of alterations that gained attention include the tumor suppressors Tumor protein 53 (TP53) [20,23,24] and B-cell lymphoma 6 (BCL6) corepressor (BCOR) [20], as well as the oncogene Bcl-2-like protein 1 (BCL2L1) [12,13,37]. Although different in nature, alterations in these three genes seem to provide selective advantage to the affected cells, as their representation increases rapidly with culture and propagation. TP53, BCOR and BCL2L1 genetic variants are typically identified through next-generation sequencing methods (targeted-NGS, WES, and WGS).

Located on chromosome 17, TP53 is a well-established tumor suppressor gene, functioning to shepherd cells that display key genetic abnormalities through apoptosis. Additionally, TP53 has been implicated in regulation of cellular stemness, differentiation and reprogramming. Noteworthy, several studies reported substantial increase in the reprogramming efficiency of somatic cells to iPSCs by inhibition or inactivation of the p53 pathway, likely through mechanisms related to promotion of cell cycle progression [38–40]. Mutations in TP53 have been detected and analyzed at length in hPSCs [8,19,22–24]. A common finding in these studies is the higher prevalence of dominant-negative TP53 mutations in hiPSCs generated from skin fibroblasts, which have been linked to exposure of parental fibroblast cells to DNA damage caused by UV radiation [20,23]. However, *de novo* TP53 mutations have been observed to occur in hPSCs that either were not derived from skin fibroblasts, or whose parental skin fibroblasts did not display detectable levels of mutations in the gene [23]. The variant allele frequencies of these mutations were generally seen to be amplified by sequential culture and through differentiation processes. Remarkably, although in a smaller number of cases, the allelic fraction of those mutations exceeded 50%, suggesting additional selective advantage resulting from the loss of heterozygosity at the TP53 locus [23]. Surprisingly, mutations in TP53 do not seem to impact the differentiation potential of affected cells towards gut epithelial cells [41] neuroepithelial cells [42] and pancreatic polyhormonal cells [43]. However, given the prominent role of TP53 in cancer, mutations in this gene have deservingly raised concerns amongst cell therapy developers [23]. Therefore, the mutational status of TP53 should be thoroughly characterized at the donor level, after reprogramming for hiPSCs, through hPSC expansion and differentiation and in the final differentiated hPSC-derived cell therapy drug product.

BCOR is a BCL-6 interacting corepressor, ubiquitously expressed, located in the X chromosome. When overexpressed, BCOR can potentiate BCL-6 repression, leading to evasion from apoptosis [44]. BCOR has been implicated in embryogenesis regulation, modulation of mesenchymal stem cell function, hematopoiesis, and lymphoid development. Somatic gene mutations of BCOR and its homolog BCORL1 have been detected in several hematologic malignancies and aplastic anemia [45]. Additionally, BCOR mutations have been linked to oculofaciocardiodental syndrome [46], highlighting its potentially complex roles in early development. Interestingly, it has been reported that blood-derived hiPSCs display lower mutational burden from UV damage compared to skin fibroblast-derived hiPSCs, but a high prevalence of acquired BCOR mutations [20]. Furthermore, BCOR mutations seem to predominantly arise *in vitro*, enabling strong selective pressure towards the altered cells. The role of BCOR mutations in hPSCs deserve further investigation: on one hand BCOR mutant cells have been reported to have impaired differentiation into ectoderm, mesoderm, and downstream hematopoietic lineages [47], while on the other hand deficiencies in forming neural stem cells and terminally differentiated neurons were observed and attributed to a bias towards mesoderm and endoderm priming [20].

BCL2L1 resides in chromosome 20q11.21, a genomic region frequently amplified in hPSCs [11–13]. BCL-XL is an anti-apoptotic protein isoform that results from the expression of BCL2L1, and which is observed to be upregulated in several human cancers [48]. Independent studies have linked the amplification and overexpression of BCL2L1 to increased resilience and growth advantage of the affected cells in hPSC cultures [12,13,19,21]. Interestingly, BCL2L1 amplification appears to favor the survival of pancreatic progenitors at crucial steps during differentiation by favorably modulating glycolysis and oxidative phosphorylation pathways [37].

MITIGATING GENETIC INSTABILITY OF hPSC-CELL THERAPIES

As more sophisticated detection methods for assessing genetic instability develop, a tighter control of starting materials and culture conditions may be able to prevent the emergence or delay the expansion of genetically abnormal cells, should they already exist below current limits of detection in starting cell lines, or develop through different culture landmarks.

Comprehensive testing of the starting material for hiPSCS will establish the genetic profile of the source material and provide an early screen for donor materials which may present abnormal genetic profiles. Alternative sources of starting material should be explored, such as bone marrow-derived mesenchymal stromal cells, which, given their internal localization in the body, are likely more protected from UV-driven TP53 mutations commonly occurring in skin fibroblasts. Given their favorable growth properties, bone marrow-derived mesenchymal stromal cells may represent a viable alternative as starting material for reprogramming to hiPSCs that would reduce the risk of UV-driven TP53 mutations.

Parallelly, evolving cell culture paradigms can be adopted to reduce the selective pressure of cells carrying alterations through culture bottlenecks including reprogramming, recovery following electroporation and low density seeding after gene editing, and adaptation to suspension culture for large scale expansion. While it may not be possible to fully prevent the appearance of genetic alterations, different strategies can be employed to delay the expansion of altered cells. These strategies may include novel and richer hPSC culture media, designed for enhanced cell tolerance of culture bottlenecks, the addition of pro-survival Rho-associated kinaseinhibitors such as Y-27632 [49] or Thiosivivin [50], or the more encompassing CEPT cocktail [51]. Other approaches, including culture under hypoxic conditions to lower oxidative stress-facilitated DNA damage [52] and the

inclusion of nucleosides into the cell culture medium to compensate for the deficiency of DNA precursors in rapidly dividing cells [53], constitute interesting avenues to be explored, and hinge upon relatively simple manipulations of hPSC cultures. Regardless of the strategy employed, comprehensive genetic testing should be performed at all critical stages in iPSC manufacturing to detect abnormalities associated with culture bottlenecks, different culture conditions and large-scale expansion.

Clustered regularly interspaced short palindromic repeat (CRISPR) technology [54] has become a central tool for gene editing in hPSCs. Further iterations of the technology enable temporal silencing of gene expression [55], as well as changes in methylation patterns [56]. Forward-looking, as more and better defined genomic hot spots impacted by hPSC culture are identified, perhaps these different modalities of CRISPR technology can be used to (i) reduce the expression of target genes that are often mutated, thus diminishing the selective advantage of mutated clones or (ii) modulate methylation patterns around fragile DNA areas to protect them from erosion and alteration.

TRANSLATION INSIGHT

hPSCs will play a central role in numerous upcoming cell therapies that aim to revolutionize treatment options and bring renewed hope for patients suffering from devastating and incurable diseases. The demonstrated safety of these cell therapies, including the genetic stability of hPSC-derived cell therapy drug products, will be crucial to their success.

Even as we conclude this manuscript, we recognize the current paucity of robust studies evaluating the implications of genetic stability of hPSCs that are statistically well-powered, while simultaneously using rigorously controlled and defined, animal component-free cell hPSC culture conditions and reagents that have a clear path towards the clinic. More work needs to be done under comparable and unified cell culture and testing paradigms to ensure translatability of results. Recent guidelines for stem cell-based research have produced a relevant framework of best practices [30], however, more work needs to be carried out to define tighter culturing and testing criteria for the safety of developing hPSC-cell therapies. At a minimum, cells should be evaluated for the presence of genetic changes when a new line is derived (by reprogramming or gene editing), when master and working cell banks are manufactured, and ideally, throughout the timespan of experiments [30].

Key to the continuing advancement of the field is higher genetic testing sensitivity, producing results that are clearer and with faster test turnaround times. Lowering the limits of detection for abnormal variants and shortening turnaround time are critical to avoid costly manufacturing of genetically abnormal cells. Next generation sequencing technologies may continue to play a prominent role here. Ideally, assays that would enable the detection of unknown mosaicism at a threshold below 1% are strongly desired, as it may be feasible to eliminate low-recurrence mutations, for example, by subcloning, with the clear caveat that this approach in itself introduces bottleneck events that may lead to similar or additional genetic alterations.

Given that cell therapy is still a relatively new treatment modality that targets several diseases for which there are no curative treatments, it will be important that therapeutic developers and regulatory bodies work closely together to more clearly define genetic stability acceptance criteria for these promising therapies. On one hand, patients need to be protected from dangerous unintended outgrowths, while on the other hand, regulations should accommodate risks deemed acceptable for patients who desperately need those therapies and have no alternative options.

Central to those discussions will continue to be evolving notions on which types of genetic instability may disqualify a cell therapy, and which gene mutations have deleterious consequences. Outlining a roadmap to

approach *de novo*, uncharacterized mutations for which patient or animal model data are not available will also be important. Closer partnership between hPSC-derived cell therapy developers, clinical geneticists, bioinformaticians, and protein engineers will be key to understanding the potential functional consequences of those mutations.

Similarly, it will also be important to factor in the role of the local niche targeted by the cell therapy. More work needs to be carried out to dissect situations where, for example, a genetic alteration impacts a gene perceived to be implicated in malignancy, however the niche or tissue where the therapy will be delivered does not favor expression of that same gene. Supporting this view are several documented examples of mutations in genes linked to cancer in specific tissues that either have no effect or cause benign conditions in other tissues [57].

Finally, while acknowledging the presence of genetic instability in hPSCs causes discomfort, the acquisition of genetic changes in cultured hPSCs is inevitable but manageable when recognized timely. It is important to acknowledge that cell culture environments are generally harsh from a biochemical perspective, with stresses thought to favor better fit, frequently altered populations. hPSCs rely on different mechanisms than somatic cells to maintain genomic fidelity, and while they do show a propensity to acquire recurrent genomic changes, when compared to other cellular models, such as cancer cell lines, these cells are not inherently genetically unstable [19], and thus, they remain an attractive starting material for cell therapies.

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