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Benvenisty, N., Draper, J.S., Gokhale, P.J. orcid.org/0000-0001-7225-4403 et al. (21 more authors) (2025) A call to action for deciphering genetic variants in human pluripotent stem cells for cell therapy. Cell Stem Cell, 32 (4). pp. 508-512. ISSN 1934-5909

https://doi.org/10.1016/j.stem.2025.03.003

A call to action for deciphering genetic variants in human pluripotent stem cells for cell therapy

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Summary

Human pluripotent stem cell (hPSC)-based therapies offer promise but pose potential risks due to culture-acquired genetic variants, some of which have been linked with cancer. An international workshop addressed these concerns, highlighting the need for improved strategies to stratify variants and chart a path towards definitive guidelines in hPSC-based therapy.

Introduction

Cell therapy using human pluripotent stem cell (hPSC)-derived cells has emerged as a transformative approach for a range of diseases. However, it is now extensively documented that the *in vitro* culture of hPSCs can lead to the emergence of a range of genetic abnormalities, from single nucleotide variants (SNVs) to large karyotypic changes, as summarized in[1]. Some of the acquired genetic aberrations that provide selective growth advantage to variant hPSCs are known to be associated with oncogenesis in cancer, raising the concern that they may also confer tumorigenic or malignant properties on either transplanted differentiated cells or residual undifferentiated hPSCs[1]. Additionally, some genetic variants were shown to affect the differentiation ability of hPSCs, with implications not only for the efficacy of cell therapy but also for the use of hPSCs in other applications, such as disease modelling[1]. Nonetheless, the significance of all acquired variants for the phenotype of hPSC-derived differentiated cells and the safety of hPSC-derived cell transplants remains unknown. This in turn impedes accurate risk-benefit assessment during cell therapy manufacture.

The most significant safety concern in cell therapy is the possibility that engrafted cells could cause tumour formation in patients. Tumours could develop from residual undifferentiated diploid hPSCs in the final cellular product, which would give rise to benign teratomas. Alternatively, stem cells or differentiated cells bearing genetic or epigenetic variants arising during the manufacture of stem cell therapeutics could become transformed and give rise to malignant tumours of a range of cell lineages (**Figure 1**). This includes both intended target cells and non-target cells that may emerge during differentiation. Despite these potential risks, the clinical translation of hPSC-derived cell therapies has shown considerable promise so far. Over 1200 patients have been transplanted with only one incidence of a tumour reported [2]. For example, Han et al. (2022) reported a tumour with lymph node metastases after transplantation of autologous hPSCs, which postoperatively were shown to contain mutations in cancer-related genes[3]. Thus, while the overall clinical experience is overwhelmingly encouraging, the rare but serious case of malignant transformation emphasizes the need for continued vigilance to ensure the long-term safety and efficacy of hPSC-based cell therapies. Cell therapy is evolving, and as new developments arise including scaleup of manufacturing protocols, identification of means to improve survival of engrafted cells, more widespread use of autologous therapies, immune cloaking, extensive genetic modifications to grafted cells, and others, and the number of patients treated increases exponentially, safety considerations will remain at the forefront.One approach to mitigating the risk of variant hPSCs is to discard

cultures or cell therapy products containing them. This strategy was employed during a phase I trial for age-related macular degeneration in Japan, which was halted upon the discovery of genetic variants not initially detected in patient's cells used for reprogramming[4]. However, this approach presupposes knowledge of which variants are actually harmful. As highlighted by Hirayama et al.[5], even the identification of *de novo* variants, such as the in-frame deletion of exon 22 in the *EP300* gene observed in their first-in-human trial of allogeneic iPSC-derived corneal endothelial cells, does not necessarily indicate a negative clinical outcome. In their study, this particular mutation was not associated with any adverse events so far. This further emphasizes the challenge of interpreting the functional consequences of identified variants and the importance of developing robust methods for assessing their potential impact.

Regulatory agencies play a critical role in evaluating the complex risk-benefit profiles of cell therapies, including a careful consideration of safety. A deeper understanding of the consequences of genetic variants and their impact on safety can inform and enhance these regulatory processes. This is particularly important given that many hPSC-based cell therapies are being developed for non-life-threatening disorders and/or are targeted to younger individuals. The use of autologous cells or engineered hypoimmunogenic cells in hPSC-derived cell therapy may further exacerbate these issues.

In response to these challenges, a Workshop funded by the UK Regenerative Medicine Platform was held in Hampshire in the UK, on May 20th-22nd 2024. Recognising the importance of interdisciplinary collaboration in addressing the complex issue of hPSC genetic variants for cell therapy, the Workshop brought together participants from different areas of science including hPSC biology, genetic stability, development, cancer, natural human variation, organ transplantation and artificial intelligence, as well as regulators, funders, and cell therapy developers from both academia and industry. This Forum article is the result of joint discussions and represents a call to action for developing improved strategies for accurately identifying and stratifying the risk of genetic variants in hPSCs and their differentiated derivatives for cell therapies.

Overcoming the challenges of variant classification: a call to action

With around 3 billion base pairs present in the human genome, and the possibility of variants occurring at all levels from SNVs to whole chromosome gains/losses, deciphering the functional consequences of variants for cell therapy presents a daunting challenge. Yet,

motivated by recent advances in various complementary strategies, we suggest that there is a way forward. We propose that the community should coordinate three primary efforts: (1) the creation of standardized registries in which variant data can be collated; (2) the development of technologies to classify and predict variants of concern; and (3) the development of technologies to mitigate the risk of variants.

A community effort to catalogue genetic variants: Towards the goal of detecting problematic variants, there is a need for centralized repositories where data regarding detected variants and the conditions in which they are found are collated across hPSC lines of diverse genetic background and sex. Standardization is a crucial consideration for these databases to enable data comparison across experiments, users, and research teams. Not only should there be well-defined parameters for the format of deposited data, but there is a need to specify the required metadata. Standardization will facilitate retrospective analysis to identify culture regimens that are associated with variant emergence through systematic reviews.

Additionally, data from preclinical studies, including assessment of variant impact on differentiated cells, and clinical settings should also be publicly available through databases or patient registries. As these databases grow, they will serve as powerful resources for developing predictive computational models, including machine learning-based approaches that can uncover patterns in variant emergence, and epidemiological studies linking variant emergence to clinical outcomes (safety and efficacy). Therefore, these databases must be designed for long-term use and accessibility, collating data across academic and industry-led research laboratories and clinical trials worldwide. This endeavour will require a centralized data management strategy that, while aspiring to FAIR (Findable, Accessible, Interoperable, Reusable) principles, acknowledges the significant challenges posed by data privacy and security regulations across jurisdictions, particularly for sensitive sequencing information. Employing strategies such as federated learning, which allows analysis to occur on decentralized datasets without directly transferring sensitive information, and secure data havens, which provide controlled environments for accessing sensitive data without compromising privacy, offer a promising path for collaborative research while upholding ethical and legal requirements.

Development of advanced assays to classify and functionally test genetic variants.

Currently, interpreting hPSC variants for cell therapy relies on a combination of approaches, but none of them are without pitfalls. *In silico* analysis leverages existing databases like COSMIC (https://cancer.sanger.ac.uk/cosmic), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and others to assess variant frequency, predicted functional impact, and reported associations with cancer or other diseases. However, the reliance on genetic analyses is problematic as databases are continuously expanding. For example, a gene may not be associated with cancer in one iteration of the database, but may be reclassified as cancer-associated, as new evidence becomes available later. Adding to the complexity of variant interpretation, cancer-associated variants have been also detected in tissues of healthy individuals suggesting that risks posed by cancer-associated variants may be dependent on additional mechanisms, such as the accumulation of further mutations in the cell, to cause transformation.

Preclinical safety studies in immunodeficient animals, particularly *in vivo* tumorigenicity assays, are critical for evaluating the safety of hPSC-derived cell products and are a regulatory requirement. These assays, typically involving orthotopic inoculation of the cell therapy product followed by long-term observation, provide essential data on potential risks, including tumorigenicity. While these models mimic aspects of the clinical scenario and are currently a cornerstone of preclinical assessment, they are not without limitations. Specifically, some models may exhibit poor sensitivity, and as such, every group is required to validate the chosen animal model, delivery route, and dose. The shortcomings of current *in vivo* assays and the pressure to reduce animal toxicity testing both mandate the development of new, complementary assays to assess tumorigenic potential and functional effects of genetic variants in cell therapeutics.

Several recent innovative technologies may provide a useful platform for building the required advanced assays for cell therapy. A notable example is a set of methods termed Multiplex Assays of Variant Effect (MAVEs), which simultaneously measure the effects of thousands of genetic variants on the phenotype of interest[6]. The high-throughput nature of this approach is enabled by modifying and assessing variants in a pooled manner, a necessary feat for achieving the required large scale of variant testing. While phenotypes explored in this context mainly centre on the growth characteristics of cells, future developments could address more complex cellular traits. In that context, it is worth highlighting technologies that promise to revolutionise *in vitro* modelling of complex human systems: organoids, assembloids, and organ-on-a-chip technology. These technologies may represent significant advances over the

current culture systems as they can more accurately model human tissues and organs. For example, a combination of organoids and organ-on-a-chip technologies has been utilised to develop a device that models human colorectal cancer[7]. When the device was populated with human colon epithelial organoids mixed in with 1% of colorectal cancer cells, cancer cells outcompeted their normal counterparts, thus mimicking the emergence of human tumours. The ability to study the emergence of tumour cells represents a powerful assay with foreseeable applications in testing of cell therapies.

Finally, leveraging machine learning models that identify patterns and make predictions based on the available datasets promises to deliver a step-change in predicting the functional effects of variants. Together, these technologies offer an avenue to developing more predictable, reliable, and faster human-specific assays. However, successful implementation will require concerted community efforts to standardize methods, identify appropriate controls, and develop effective routes for data dissemination.

Risk mitigation processes and technologies. Genetic variants in hPSCs are the outcome of both mutational events and selective forces. Thus, understanding the mechanisms underlying the processes of mutation and selection in hPSCs will offer strategies for minimising the appearance of genetically variant cells. Several studies have demonstrated the principle that by optimizing culture conditions, for example, through adding nucleosides or reducing oxygen tension, genome damage and the mutation rate in hPSCs can be reduced, as reviewed in[1]. Additionally, cell density and media composition were also shown to play a role in the selection of specific genetic changes[1]. Together, these observations highlight that optimizing culture conditions provides a crucial approach to preserving the genetic integrity of hPSC lines. Nonetheless, further research is required to devise optimal culture conditions that balance the needs of hPSC proliferation and genetic integrity. With the move to large-scale hPSC expansion in 3D, efforts should be also directed to exploring the impact of 3D culture on hPSC genome stability.

As technologies for detecting and characterizing variants mature, we should also aspire to apply established biotechnology principles to ensure the safety and quality of hPSC-derived cell therapies. The bioprocess field has established protocols for the production of materials from living sources, often involving the growth of microorganisms or mammalian cell lines (such as Chinese Hamster Ovary cells) to produce enzymes, fatty acids, glycoproteins, and other products. Here, quality-by-design principles are used to ensure robustness and

reproducibility by defining critical quality attributes (CQA), i.e. measurable features of a product that can be assessed at various manufacturing stages to ensure the product is within acceptable quality limits[8]. Leveraging emerging analytical technologies for variant detection offers the opportunity to link predictive molecular and cellular signatures of variants to the safety and quality of the final cell product. These metrics allow for a standardized approach for quality control of hPSC-derived cell products to enable their safety and efficacy. A potential approach to achieving this is the development of CQA that define "problematic" variants according to the cell therapy, such as the cell type, resident tissue, or patient context. These CQA can be monitored along the hPSC expansion and differentiation process using established and next-generation technologies for variant detection.

In addition to quality-by-design biomanufacturing, another aspirational goal is to further leverage engineering principles for risk management in cell therapy safety. For example, nuclear power plants are engineered with redundant systems to protect user and public safety, ensuring that a single system failure does not lead to catastrophic consequences. A similar approach could be explored to develop safety systems for hPSC-derived cell therapies such that the risk of variant emergence and impact on patient safety can both be predicted and mitigated by redundant technologies. Synthetic biology tools offer the potential to create "smart" cell therapies with encoded safety functions. For instance, next-generation universal hPSC lines can be genetically engineered to carry classifier circuits to report gene and microRNA expression patterns associated with variant status, allowing for these cells to be targeted for removal in the manufacturing process[9]. Additionally, "fail-safe" switches can be engineered into these cells through genetic devices that trigger the expression of suicide genes, enabling the elimination of problematic cells either during the manufacturing pipeline or after transplantation in the patient[10].

Conclusion

The appearance of genetic variants is inevitable in the development, growth, and differentiation of hPSCs for therapeutic application. However, due to limited scientific evidence on how variants impact the safety and efficacy of hPSC-based therapies, risk analysis for cell therapies will remain challenging unless we proactively address this issue.

Here, we argued that the hPSC field is ripe for confronting this challenge. Measures critical to this endeavour include generating centralized repositories for open-access sharing of variant data, as well as developing *in vitro* assays for assessing and, ultimately, predicting variant tumorigenicity. As such assays are developed and long-term clinical outcomes of variant transplantation reported, it will become possible to stratify the relative risk of genetic variants and apply this knowledge and approach to further types of variants (e.g., epigenetic and mitochondrial DNA variants). Indeed, we may come to find that some variants may even offer favourable properties, such as an enhanced engraftment efficiency, while preserving differentiation efficiency and function. By employing redundant technologies that preserve patient safety, the risk of variants can not only be enumerated but also mitigated, pushing hPSC-derived cell therapies into an era of quality-by-design biomanufacturing.

Delivering on this vision requires targeted funding opportunities to foster interdisciplinary collaborations integrating genetic analysis, functional tests, and machine learning approaches for deciphering the impact of variants in cell therapy. Additionally, training and networking initiatives will be crucial to support the community in working together to maximise the impact. Collectively, these efforts will streamline and accelerate the path for hPSC-based therapies to reach patients that need them.

Acknowledgments:

The authors acknowledge helpful discussion held at the Genetic stability of hPSCs Workshop (May 20th-22nd 2024) funded by the UK Regenerative Medicine Platform (MR/R015724/1), and at a follow-up meeting held at the ISSCR Annual Meeting in Hamburg (July 13th 2024).

Declaration of interests

Z.H. is a co-founder and majority shareholder of Regenerative Cell Therapy Consulting Ltd. D.H. is the founder and principal of Hursh Cell Therapy Consulting.

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Figures:

Figure 1. Types of tumours potentially arising from hPSCs-derived cellular therapies.