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Implications of genetic and epigenetic aberrations to the tumorigenicity of human pluripotent stem cells

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Abstract

Human pluripotent stem cells (hPSCs) hold immense promise for cell replacement therapies due to their capacity to give rise to derivatives of the three embryonic germ layers and their ability to divide indefinitely in culture. Since their first derivation less than 30 years ago, multiple hPSC-derived cell products are already in clinical trials for a range of pathologies. Nevertheless, hPSCs also possess an intrinsic tumorigenic potential and have been shown to acquire recurrent genetic and epigenetic aberrations strongly associated with cancer initiation and progression. These properties cast doubt on the safety of hPSCs and raise concerns regarding their use for transplantation. In this review, we summarize the different kinds of genetic and epigenetic abnormalities repeatedly observed in hPSCs, how they emerge, and their potential implications for the tumorigenicity of hPSC-based products. We also discuss shared and unique abnormalities found in hPSCs derived from different sources. Finally, we suggest possible methods for reducing the occurrence of these aberrations and managing their effects once they arise.

Introduction

Human pluripotent stem cells (hPSCs) include embryonic stem cells (ESCs), which are derived from preimplantation embryos [1], and induced pluripotent stem cells (iPSCs), generated by reprogramming of somatic cells [2]. These cells hold immense promise for regenerative medicine, owing to their unlimited self-renewal capacity and their ability to differentiate into cells of all three embryonic germ layers, either *in vitro* or *in vivo* [1–4]. Over the past decade, this promise has begun to materialize, with various hPSC-derived cell products advancing to clinical trials and showing encouraging early results [5].

While the intrinsic properties of hPSCs give them their unique therapeutic potential, they also include an inherent ability to form tumors *in vivo*, raising a potential barrier to the safety of hPSCs for transplantation purposes [6–8]. In this regard, extensive propagation of hPSCs in culture results in a process termed 'culture adaptation', during which selection forces lead to clonal expansion of cells with increased proliferation potential [9–12]. This process is mediated through the accumulation of both genetic and epigenetic changes, which lead to a selective advantage for cells [13–15]. Notably, many of the recurring aberrations observed in hPSCs are linked to cancer initiation and progression, emphasizing the need to understand possible implications associated with different aberrations, and the risk for their occurrence in different hPSC products designated for clinical use [16]. This necessity is especially illustrated in light of a recent report describing the emergence of a tumor with lymph node metastases following the transplantation of hPSC-derived beta cells in a diabetes patient [17].

This review summarizes current knowledge of genetic and epigenetic abnormalities in hPSCs. We discuss recurrent aberrations, the culture conditions and parameters that influence their initiation and propagation, and their relationship to tumorigenic processes, with implications for transplantation risk. We also highlight the unique, hPSC-type-dependent tendencies to acquire specific classes of abnormalities. Finally, we review strategies aimed at stabilizing hPSCs, with the goal of advancing safer hPSC-based therapies.

Intrinsic tumorigenic properties of hPSCs

One of the golden standards used to define pluripotency of hPSCs is their ability to form teratomas, benign tumors composed of derivatives from the three embryonic germ layers, once injected into immune-deficient mice [1]. This property highlights the inherent tendency of hPSCs for tumorigenesis. Indeed, hPSCs share various similarities to cancer cells [6–8,14]. These include reliance on shared signaling pathways (i.e., MAPK/ERK, PI3K/AKT, NF-kB, and TGF-β) [18], high proliferation rate [19], and telomerase activity [20]. Nevertheless, telomere dynamics differ between the two cell types, and cancer cells can maintain telomeres through recombination-based mechanisms [21]. Finally, poorly differentiated cancers were shown to utilize PSC gene regulatory networks [22].

In contrast to cancer, the tumor-forming ability of hPSCs does not solely depend on preexisting genetic or epigenetic abnormalities. Instead, it reflects their inherent pluripotent state. However, unlike the transient pluripotency observed during normal embryogenesis, hPSCs can be propagated indefinitely in vitro. This prolonged culture imposes constant selective pressures, which can drive culture adaptation. [9–12,23]. Notably, culture-adapted cells were shown to generate more aggressive tumors, often presenting an increased similarity to teratocarcinoma—the malignant form of teratoma [8,24–27].

Culture-acquired genetic aberrations in hPSCs

Types of genetic aberrations in hPSCs

Common genetic aberrations in hPSCs include alterations at the chromosomal, sub-chromosomal, and single-nucleotide levels (Figure 1). The first description of culture-acquired variants linked to adaptation was of karyotypic instability manifested as the gain of chromosome 12 and chromosome 17q [28]. This report raised high concerns regarding the safety of hPSC-based therapies, as the presence of isochromosome 12p and the gain of chromosome 17q were repeatedly seen in human embryonic carcinoma cells, which resemble hPSCs in many respects [29–31]. Moreover, testicular germ cell tumors (TGCT) often

acquire chromosome 12p material, such that it can serve as a molecular diagnostic marker for this type of tumor [32].

After identifying alterations in chromosomes 12 and 17, several other common karyotypic changes were noted. These consist of complete or partial gains in chromosomes 1, 20, and X, along with, to a lesser extent, the loss of portions of chromosomes 10 and 18 [33–36]. Accordingly, gain of chromosome material from chromosomes 1, 12, 17, and X and loss of material from chromosomes 10 and 18 were amongst the most recurrent changes observed in a study examining karyotypic imbalances in TGCT-derived cell lines [37]. Lastly, a recent analysis of over 2,600 RNA-seq samples of hPSCs and their derivatives identified that male hPSCs repeatedly lose their Y chromosome, an abnormality seen in 12% of the analyzed hPSC samples [38]. Considering that this abnormality was only observed in relatively high-passaged cells (>30 passages) and that loss of chromosome Y is suggested to serve as a cancer driver event [39], loss of Y may reflect a previously unexplored mechanism of culture adaptation. Together, these observations suggest that the *in vitro* culture adaptation of hPSCs closely mirrors the *in vivo* tumorigenicity of embryonal carcinomas and other cancers, particularly in terms of shared karyotypic abnormalities, underscoring the parallels between culture adaptation and tumorigenesis. A detailed discussion of the functional consequences of these and subsequent aberrations is provided in the sections ‘Underlying mechanisms of genetic aberration acquisition’ and 'Tumorigenic implications of genetic and epigenetic aberrations in hPSCs' below.
The development of high-resolution tools for genetic analysis, such as CGH arrays, SNP arrays, and deep sequencing-based methods, enabled the identification of smaller genetic changes associated with culture adaptation, spanning from copy number variations (CNVs) to point mutations. In this context, CNVs and loss of heterozygosity (LOH) events have been identified as common features that accumulate with extended culture and show a significant positive correlation with passage number [40,41]. Overall, hPSCs have been reported to carry a high burden of large CNVs, while exhibiting similar levels of small CNVs as somatic cells [42,43]. Markedly, several CNVs that were shown to be acquired

during prolonged culture are implicated in malignant processes. These include the amplification of regions spanning known oncogenes such as *MYC* (through a large CNV) and *PDE4DIP* [40,41], as well as deletion of known tumor suppressors, such as *HIC2* and *TP53*, or the *EP300* gene (through a small CNV for the latter two), which acetylates and stabilizes p53 [40,43,44]. In fact, the most frequently observed CNV in hPSCs is the large CNV gain of chromosome 20q11.21 [36,41,45,46], aligning with findings in tumors containing embryonal carcinoma cells, which also exhibit a gain of chromosome 20q [47–50]. In hPSCs, this amplification was shown to result in the overexpression of the gene *BCL2L1* (specifically its anti-apoptotic BCL-XL isoform) residing in this region, leading to an increased selective advantage for the cells [51,52].

At the SNV level, high-throughput exome sequencing enabled the first thorough investigation into whether culture adaptation could be driven by single-nucleotide mutations, rather than solely by large structural alterations [53]. This report identified that hPSCs repeatedly acquire and expand mutations in the tumor suppressor gene *TP53*. Alarmingly, the mutations identified were dominant negative and were among the most frequently seen in human cancers [53]. The study also found evidence for LOH events occasionally following a *TP53* mutation, further enhancing the selective advantage for mutated cells [53]. Subsequently, analysis of publicly available RNA sequencing (RNA-seq) samples of hPSCs identified cancer-related mutations in several other genes, with *TP53* being the most recurrently mutated gene [54,55]. This observation was further expanded by a more recent large-scale analysis of hPSCs and their differentiated derivatives spanning 146 cell lines, totaling over 2,200 publicly available RNA-seq samples [56]. Strikingly, this analysis found that over 20% of the samples harbored at least one Tier 1 cancer-related mutation (COSMIC database: https://cancer.sanger.ac.uk/cosmic/login), spanning 26 genes, with mutations in *TP53* appearing in 64% of the mutated samples [56]. Another recent human induced PSCs (iPSCs) analysis identified recurrent cancer-associated mutations in the *BCOR* gene acquired *in vitro* [57]. Thus, cancer-related SNV mutations

appear to serve as putative drivers for culture adaptation, with *TP53* being the strongest hit in hPSCs.

Underlying mechanisms of genetic aberration acquisition

Previous reviews have extensively discussed the mechanisms underlying the emergence and expansion of genetic variants, highlighting several important insights [15,58]. Here, we briefly summarize these findings. It has been shown that hPSCs suffer from recurrent mitotic errors, resulting from lagging chromosomes during anaphase [59,60]. This PSC feature is likely innate, considering that as many as 80% of human preimplantation embryos contain some amount of aneuploid cells, which primarily arise from mitotic errors [61]. hPSCs also present high levels of DNA damage, mainly in the form of double-strand breaks [62,63]. This damage has been strongly implicated with replication stress, which is caused by a shortened G1 phase and rapid progression through the cell cycle, and can lead to replication fork collapse [62–64].

Despite the high genomic instability observed in hPSCs, their mutational acquisition rate is relatively low compared to somatic cells [65–67]. This discrepancy has been attributed to several factors, with the predominant one being the heightened sensitivity of hPSCs to undergo apoptosis in response to DNA damage [58,68,69]. This propensity also likely reflects an inherent property of PSCs, as human blastocysts exhibit large numbers of apoptotic cells, possibly as a mechanism for removing abnormal cells from the embryo [70]. These findings may explain why pro-apoptotic pathways are repeatedly selected against during culture adaptation. The selection can occur via either loss-of-function mutations in pro-apoptotic genes such as *TP53* [53,54,56], or amplification of anti-apoptotic genes, as exemplified by the gain of chromosome 20q11.21 containing the anti-apoptotic gene *BCL2L1* [51,52]. Additional examples include the typical gain of chromosome 1q observed in hPSCs, which was attributed to the duplication of the *MDM4* gene, a negative regulator of p53, leading to apoptosis resistance [71,72]. Duplication of chromosome 17q has also been associated with amplification of the anti-apoptotic gene *BIRC5* [73]. However, this duplication was also attributed to the gain of *WNT3-WNT9B*, which affects the proliferation and not

the apoptosis levels of cells [74]. Thus, the most frequently observed genetic variants in hPSCs are enriched with apoptosis attenuation properties, implicating apoptosis as a significant bottleneck that is alleviated during culture adaptation.

Genetic aberrations in different hPSC types

The two primary sources of hPSCs are ESCs, derived from blastocyst-stage embryos [1], and iPSCs, obtained from somatic cells [2]. The differences between these two sources mean that distinct types of hPSCs have dissimilar accounts regarding the selection forces they underwent, which may be reflected in the type and frequency of abnormalities presented by the cells.

A large-scale comparison between human ESCs and iPSCs detected no significant differences in karyotypic abnormalities [34]. Nevertheless, a finer-resolution analysis did reveal differences in CNVs [42]. Specifically, both ESCs and iPSCs were shown to accumulate gene duplications during prolonged culture, but iPSC also showed recurrent deletions, which occurred mainly during the reprogramming process [42]. An important factor affecting the genetic integrity of iPSCs is the somatic mutations acquired before reprogramming. Indeed, whole genome sequencing (WGS) analysis of iPSCs revealed high levels of somatic mutations originating from their parental cells [57,75]. Notably, the mutations exhibited patterns characteristic of their tissue of origin; iPSCs derived from skin fibroblasts displayed a pronounced UV-damage signature and carried a higher overall mutational burden compared to blood-derived iPSCs, consistent with the ongoing UV exposure experienced by the skin [57,75]. Moreover, the mutations were highly clonal, suggesting that fibroblast cultures used for reprogramming are mosaic, composed of different mutants [57,75]. While blood-derived iPSCs didn't show UV-related damages, they had a higher prevalence of mutations in the *BCOR* gene [57]. Finally, a large-scale analysis of cancer-related mutations in hPSCs based on RNA-seq data did not find a significant difference in the frequency of samples showing a cancerous mutation in ESCs compared with iPSCs [56]. However, the mutations observed in iPSCs were distributed amongst a larger number of genes, suggesting that some of these mutations existed before reprogramming [56]. This report proposes that while both iPSCs and ESCs

accumulate genetic changes during culture adaptation, iPSCs often carry a mutational burden derived from their somatic cells of origin. We argue that this point needs to be taken into consideration when using iPSCs as opposed to ESCs, and when using iPSCs obtained from different tissues, such as skin fibroblasts versus blood cells. However, fully accounting for these factors remains challenging, as the exact fraction of cancer-related mutations in iPSCs that originate from parental somatic cells versus those acquired during or after reprogramming is still unknown. Furthermore, while differences in mutational signatures and burden have been observed between blood-derived and skin fibroblast-derived iPSCs, comparable analyses for iPSCs generated from other donor cell types are lacking. Addressing these gaps will be essential for accurately assessing the safety profiles of iPSCs relative to ESCs. In this context, large-scale comparisons of gene expression profiles between ESCs and iPSCs may also prove informative, as differential expression of oncogenes or tumor suppressors, even in the absence of mutation enrichment, could influence tumorigenic potential.

Epigenetic aberrations in hPSCs

It is by now accepted that culture adaptation can occur not only through the emergence of genetic variants, but also through epigenetic alterations [13]. In the context of tumorigenesis, epigenetic changes are strongly implicated in cancer initiation and progression [76–80].

Extensive characterization of the epigenetic status of cultured hPSCs converged on several types of recurrent abnormalities. These include variation in the DNA methylation levels of gene promoters and non-coding regions, loss of genomic imprinting, and erosion of X-chromosome inactivation (XCI) (Figure 1) [13,16]. This section will discuss these aberrations, their occurrence in different hPSC types, and their possible implications, considering their connection to tumorigenic processes.

Variation in the methylation of specific genes

A distinctive epigenetic feature of cancer cells is a global hypomethylation of the genome, accompanied by hypermethylation of tumor suppressor gene promoters [81–83]. In line with this feature, prolonged culturing of hPSCs was associated with hypermethylation and subsequent repression of multiple genes [84]. One of these genes, *TSPYL5*, silenced in a large fraction of hPSC lines, is also silenced in several cancers, and its expression was shown to suppress the growth of cancer cells, suggesting that its repression enhances cancer progression [84,85]. Correspondingly, silencing of *TSPYL5* in hPSCs resulted in the downregulation of differentiation-associated genes and tumor suppressor genes, together with the upregulation of pluripotency-associated transcripts [84]. In addition, DNA methylation-mediated silencing of the antioxidant *CAT* gene has also been identified as a recurrent epigenetic aberration, that is acquired in karyotypically abnormal hPSCs and embryonal carcinomas, suggesting it may play an active role during culture adaptation [86].

DNA methylation aberrations in ESCs versus iPSCs

A key distinction between ESCs and iPSCs lies in the extensive epigenetic reprogramming required for iPSCs to acquire pluripotency from a somatic origin. As a result of this process, iPSCs often exhibit recurrent DNA methylation abnormalities [87–91]. Notably, some of these changes are independent of the somatic cell of origin and persist through differentiation, compared to ESCs and their differentiated derivatives [87,89]. These aberrant methylation signatures are influenced by the specific factors used during reprogramming. For instance, distinct aberration patterns have been associated with different reprogramming cocktails [92]. In particular, some aberrantly methylated sites were shown to be enriched with MYC binding motifs, implicating the MYC factor, commonly used in the Yamanaka cocktail, as a potential contributor to these reprogramming-induced abnormalities [93]. Another signature distinguishing iPSCs from ESCs is the preservation of an epigenetic memory of their somatic cell type of origin, as manifested in their methylation landscape and differentiation potential [89,94–99]. In accordance with this phenomenon, the reprogramming efficiency was shown to be inversely correlated with the extent to which the DNA methylation

landscape of a certain somatic cell type is distinct from that of PSCs [87]. Nevertheless, epigenetic memory is primarily believed to persist transiently after the reprogramming and diminish throughout prolonged culturing [88,95,100,101], though exceptions whereby the epigenetic memory persisted were reported as well [99]. However, perhaps the most concerning abnormality distinguishing iPSCs from ESCs is the presence of reprogramming-induced epigenetic changes resembling those seen in neoplasia, suggesting that reprogramming may confer increased tumorigenic potential via epigenetic processes [102].

Loss of genomic imprinting

Genomic imprinting in mammals refers to the parent-of-origin specific monoallelic expression of a subset of genes, playing a crucial role in normal growth and development [103–105]. This epigenetic regulation is primarily established during embryonic germline development through DNA methylation and the deposition of histone modifications at imprinted differentially methylated regions (iDMRs), in a parent-of-origin specific fashion [104]. In humans, there are about 200 imprinted genes, and disruption in imprinting has been implicated in various diseases, including cancer [104,106].

Regarding cancer, several lines of evidence link tumorigenic processes to loss-of-imprinting (LOI) [106,107]. One strong connection is the increased cancer predisposition observed in imprinting syndromes caused by germline alterations at imprinted loci. For instance, Beckwith–Wiedemann syndrome, caused by alterations in the 11p15.5 imprinted region, results in increased risk of developing embryonal tumors such as Wilms tumor and rhabdomyosarcomas [107]. Another example is Prader-Willi syndrome, caused by alterations in chromosome 15q11–13 imprinted region, which leads to an increased risk of developing myeloid leukemia [106,108]. An additional line of evidence is that somatic LOI of various imprinted genes is frequently seen in different types of cancer [109–113]. Finally, widespread LOI in mice has been shown to drive cancer formation [114].

Initial characterizations of the epigenetic status of hPSCs focused on ESCs. They reported that genomic imprinting is relatively stable in these cells, despite

occasional LOI of a subset of imprinted genes, such as *IGF2* [115–117]. However, subsequent studies incorporating larger cohorts and diverse hPSC types have repeatedly observed LOI, manifested as hypermethylation (gain of methylation on the previously unmethylated allele) or hypomethylation (loss of methylation on the previously methylated allele) of iDMRs, leading to either loss of expression or biallelic expression of imprinted genes [118–123]. Of note, several characteristic features of LOI began to emerge as consistent patterns. First, once acquired, imprint loss persists during differentiation [119,122], and can also be acquired during differentiation [122]. Second, LOI is not random; some genes are more susceptible to losing imprinting than others [122]. Third, iPSCs present significantly higher levels of LOI than ESCs [120–122]. While imprint loss can originate from the somatic cell of origin, reprogramming-induced LOI was shown to be the more frequent event, possibly due to the major epigenetic reconfiguration occurring during the reprogramming procedure [122]. In total, a high fraction of ESC and iPSC samples showed LOI for at least one imprinted gene, with iPSCs presenting ~2-fold increase in LOI events compared with ESCs, exemplifying that LOI can be quite frequent for both cell types, but especially in iPSCs [122].

Lastly, a hPSC type that is particularly susceptible to LOI is naïve hPSCs. hPSCs cultured under standard conditions exhibit several features similar to the mouse post-implantation epiblast and are therefore considered to be in a "primed" state for differentiation [124,125]. Various approaches have been developed to revert hPSCs to a more “naïve” state, which resembles the preimplantation epiblast and is marked by several characteristics, including genome-wide DNA hypomethylation [125,126]. Although both naive hPSCs and pre-implantation epiblast cells display global hypomethylation, a key difference lies in the maintenance of genomic imprints: pre-implantation epiblast cells preserve imprinting despite extensive demethylation, whereas naive hPSCs tend to lose methylation at iDMRs and fail to restore it upon reversion to the primed state [127–129]. Importantly, loss of methylation leads to widespread biallelic expression of

imprinted genes, suggesting that naïve hPSCs show high levels of LOI relative to conventional ESCs and iPSCs [129].

Erosion of X-chromosome inactivation

A central epigenetic phenomenon occurring during early development in mammals is XCI, leading to random silencing of one of the two X chromosomes in female somatic cells. This process is initiated by the transcription of the long non-coding RNA - *XIST*, which subsequently coats the X chromosome that will become inactive [130]. Single-cell transcriptomics of human preimplantation embryos indicates that up to the late blastocyst, both X chromosomes are still active and transcribed [131]. Consistent with conventional hPSCs representing a more advanced, post-implantation stage, most established lines have already undergone XCI [132,133]. However, unlike *in vivo* development, XCI in hPSCs is not random, likely due to clonal expansion [132,133].

Initial examinations of the epigenetic status in hESCs revealed variation in the XCI status in different lines. In some lines, *XIST* transcripts were detected, and one of the X chromosomes seemed inactive (XaXi). Yet other lines showed no *XIST* expression [116,134]. Further investigation revealed that cells showing no *XIST* expression could be divided into two groups: cells which did not go through XCI and present two active X (XaXa; evident in a minority of cell lines), and cells which exhibit an eroded XCI state, in which part of the inactive X loses inactivation marks and becomes active (XaXe) [132,133,135–137]. Characterization of the XaXe state revealed a recurrent pattern in which regions that become activated are preferentially located toward the distal parts of the Xi, while more internal regions remain inactive [123,136,138]. Notably, the variations in XCI do not occur at late passages, but rather early after derivation of the hESC lines [133,139]. An important distinction lies between different types of hPSCs. Compared with ESCs, which show a high prevalence for erosion, iPSCs generally maintain an intact Xi derived from their somatic cell of origin [140,138,141], but erosion of XCI has also been observed in iPSCs upon prolonged culturing [142,143].

The erosion of XCI in hPSCs is of particular concern due to its connection to cancer. Specifically, an increase in the expression of X-linked genes, whether by reactivation of the Xi or loss of Xi followed by reduplication of the Xa, has been implicated in the progression of several cancers [144,145]. Additionally, the deletion of *Xist* in the blood compartment of mice led to the development of aggressive hematologic cancer with 100% penetrance, suggesting that the loss of *XIST* expression can increase the fitness of the cells [146]. These observations also align with the tendency of hPSCs and TGCTs to acquire extra copies of chromosome X, as discussed above [9]. Reinforcing this notion, hPSCs with eroded XCI show elevated expression of X-linked oncogenes, increased proliferation *in vitro*, and reduced differentiation capacity *in vivo* [142]. A crucial point to note is that, like other genetic and epigenetic abnormalities, the eroded state remains unchanged during differentiation. This indicates that hPSC-derived cells intended for therapy may carry this abnormality [133,134,140,143,147].

Tumorigenic implications of genetic and epigenetic aberrations in hPSCs

The full extent of the tumorigenic risk posed by the spectrum of genetic and epigenetic aberrations remains unresolved [148]. However, given that many aberrations recurrently associated with culture adaptation in hPSCs are strongly linked to malignancy, this risk warrants serious consideration (Table 1).

Regarding genetic variants, aberrations conferring a selective advantage in culture were shown to have other phenotypes that align with cancer. Indeed, culture-adapted hPSCs bearing aneuploidy exhibit a global gene expression signature similar to that of teratocarcinomas harboring the same karyotypic change [27]. Importantly, when transplanted into immunodeficient mice, aneuploid hPSCs form teratocarcinomas containing undifferentiated cells, whereas normal cells give rise to teratomas composed of differentiated cells [24,27]. During in vitro differentiation, some genetic abnormalities in hPSCs broadly impair differentiation potential, while others selectively affect specific lineages. A gain of chromosome 1q, for example, has been associated with reduced ability to differentiate into all three germ layers [72], whereas duplication of chromosome 20q11.21 primarily compromises neuroectodermal differentiation [149]. Yet, the

relevance of each chromosomal aberration, which enables a selective advantage in culture, to in vivo tumorigenicity is still unclear. Point mutations in cancer-related genes have also been linked to altered differentiation outcomes. Specifically, hPSC-derived neural cells harboring *TP53* mutations exhibited reduced expression of neural markers alongside sustained high levels of pluripotency markers compared to wild-type cells [56]. Similarly, mutations in *BCOR* were associated with a differentiation bias toward the mesodermal lineage [57]. Nevertheless, our ability to classify mutations as benign or potentially tumorigenic in the context of transplanted cells remains limited. At present, most potentially pathogenic mutations are identified through annotation with publicly available datasets, such as COSMIC [53,54,56]. High-throughput experimental approaches, such as saturation genome editing, offer a powerful means to expand the catalog of variants with known functional consequences [150,151]. Likewise, the recent emergence of large neural network-based models for variant-effect prediction holds great promise for identifying tumor-causing mutations, both in coding regions [152], and in the non-coding genome [153]. The latter is of particular importance; although non-coding mutations can contribute to cancer, they are often overlooked in hPSC safety assessments due to sparse annotation. One striking example is a non-coding mutation that creates a super-enhancer for the TAL1 oncogene, driving T-cell acute lymphoblastic leukemia growth [154]. The integration of these experimental and computational tools is therefore expected to substantially improve our ability to detect tumorigenic mutations and better safeguard hPSC-based products. The altered differentiation phenotypes observed in genetically adapted hPSCs, both in vivo and in vitro, raise serious concerns, suggesting that hPSC-derived therapeutic cells may harbor a small population of undifferentiated abnormal cells capable of forming malignant tumors. The current recommendations from the Food and Drug Administration (FDA) on the “safety testing of human allogeneic cells expanded for use in cell-based medical products” state that the genomic integrity of the cells should be assessed using either a sensitive cytogenetic-based method or WGS, with WGS being the preferred approach for detecting karyotypic abnormalities. The guidance also recommends that cell lines contributing to the final product be

screened for cancer-related mutations in proto-oncogenes such as *TP53*, using WGS at 50X coverage and a comprehensive comparison against a database of known cancer mutations (https://www.fda.gov/media/178113/download). The importance of such evaluation is exemplified by a clinical trial for macular degeneration that was halted after three gene deletions were identified in iPSC-derived retinal cells, which were absent from the parental fibroblasts of the patient. Notably, the mutations had no indications of being cancer-driving, yet their presence was sufficient to pause the trial [155].

Of note, the FDA's guidance regarding aberration screening focuses on genetic, not epigenetic, variants. This is likely because it is often more challenging to infer the full tumorigenic risk associated with epigenetic aberrations, along with the greater difficulty in identifying these abnormalities. While some epigenetic alterations may be benign, we argue that those aberrations strongly associated with cancer, such as those discussed above, warrant particular attention. For instance, several imprinted genes, e.g., *IGF2*, *PEG10*, *RTL1, KCNQ1OT1,* and *DLK1*, are highly sensitive LOI in hPSCs and have been implicated in the progression of various malignancies [156–163]. Therefore, not every instance of LOI should necessarily be viewed as a definitive safety concern; rather, concern may be more appropriately focused on LOI affecting genes with well-established links to cancer. Additionally, considering the connection between a gain of chromosome X dosage observed in malignancies and the highly adaptive phenotype observed in XCI eroded cells, we argue that this aberration should also be taken seriously.

An important principle emerging from the landscape of genetic and epigenetic aberrations is that no source of hPSCs is entirely immune to abnormalities or universally safe. Rather, each type of cell line presents distinct advantages and vulnerabilities. For example, as discussed above, iPSCs exhibit more stable XCI than ESCs, yet they are more prone to LOI. Similarly, while male hPSC lines do not face issues related to XCI, they are susceptible to loss of the Y chromosome. Therefore, each hPSC line must be evaluated in the context of the specific aberrations it is predisposed to acquire.

Another key consideration is the impact of different abnormalities on the differentiated derivatives of hPSCs, intended for therapy. As previously noted, various types of genetic and epigenetic aberrations can be transmitted during differentiation. This raises several important implications. First, certain aberrations may impair differentiation, resulting in undifferentiated or incompletely differentiated, culture-adapted cells in the final product—cells that could potentially give rise to malignant tumors [148]. In such cases, even if the aberrations do not lead to tumor formation, the existence of a higher fraction of partly differentiated or non-specifically differentiated cells (residual cells with identity other than the desired cell type) may compromise the therapeutic potential of the transplant. In cases where a specific aberration does not interfere with differentiation, but is transmitted to the differentiated progeny, it may still influence the tumorigenic potential of the resulting cells. For instance, post-mitotic cells, such as neurons, may be more tolerant of proliferation-enhancing abnormalities. In contrast, proliferative cell products, such as neural stem cells, may be more vulnerable to such changes. If the tumor-initiating cell is a differentiated one, the risk is for somatic tumors, rather than germ cell-like tumors that can arise from aberrant undifferentiated cells. In such cases, another factor that can influence the risk of tumorigenesis is the specific aberration in the target cell type. In that regard, somatic tumors in different tissues are distinguished through unique genetic instabilities [164], suggesting that a given mutation can lead to tumor formation in specific target cell types. In support of this notion, the higher selection towards *BCOR* mutations in blood-derived iPSCs aligns with the higher frequency of *BCOR* mutations observed in hematological malignancies compared with other cancers [57]. Overall, the effects of culture adaptation on differentiated hPSC derivatives remain poorly understood, and further characterization of cell-type-specific abnormalities will be key to developing targeted screening strategies that improve the safety of therapeutic products. In addition to these considerations, in cases where final cell products contain transient differentiation intermediates or contaminating transformed cells of heterogeneous identities, they may give rise to complex somatic tumors containing a range of cell lineages, adding another layer of complexity to the

tumors that may form [148], a risk which should also be taken into account. Optimistically, more than 1,200 patients have been transplanted with hPSC-derived cells to date, with only a single report of metastatic tumor formation [5,17]. While this suggests that tumorigenesis may be a rare event, an important caveat must be considered. The reported tumor arose following autologous transplantation of hPSC-derived beta cells, and only 12 patients have been treated with autologous cells so far [5]. Because autologous iPSCs are essentially immune-privileged, they may carry an increased risk of tumor formation due to reduced immune surveillance. Thus, this single case report becomes highly more significant when viewed as a proportion of autologous transplantations. This principle may also extend to so-called “universal” cells engineered to evade immune detection [165], warranting careful evaluation of their tumorigenic potential. Interestingly, the metastatic immature teratoma in the diabetic patient carried missense and frameshift mutations in 16 genes, two of which are related to mismatch repair [17]. In summary, while the low incidence of tumor formation originating from hPSC-derived cells reported so far is encouraging, further investigation into the potential tumorigenic risks associated with various types of aberrations is needed to fully assess the safety profile of these therapies.

Methods for the prevention and management of aberrations

Multiple strategies can be used to mitigate the effects of genetic and epigenetic aberrations, the first being the adoption of good practices that minimize their occurrence. Several methods can be proposed regarding this issue. First, as the chances for the emergence of a cell that will carry an advantageous variation increase during extended propagation, it is generally advised that cells designated for therapy are not extensively passaged beyond what is required for their utilization. Moreover, establishing an early-passage highly characterized “master cell bank”, as advised by the International Society for Stem Cell Research (ISSCR), that is separated from the working stocks of each cell line, is essential [166]. This procedure ensures that the cell line will not be discarded if a pathological variant arises in a late-passage culture.

Additional methods to mitigate the rise of harmful variants focus on lowering the mutational rate of hPSCs and attenuating the selective pressure they experience during culture. For instance, it has been shown that mutations acquired during prolonged culture of hPSCs show a strong signature of oxidative stress. Correspondingly, culturing the cells under 5% oxygen resulted in >50% reduction in their mutation rate [67]. Different cell-culture techniques were also shown to affect the integrity of the cells. In this regard, supplementation of exogenous nucleosides to the culture medium was suggested to increase the genome stability of hPSCs [63]. Furthermore, feeder-free culture conditions were implicated with distinct chromosomal aberrations [71], or even higher genetic and epigenetic instability compared to feeder-based cultures [167]. The cell passaging technique was also associated with the genetic and epigenetic integrity of hPSCs, with mechanical dissociation showing favorable outcomes compared to enzymatic dissociation [167].

Another strategy for managing the emergence of different aberrations is the selective ablation of variant cells from the culture. For example, the use of statins was shown to specifically inhibit the proliferation of hPSCs bearing duplications of chromosomes 12 and 17, but not normal hPSCs [168]. The successful and specific elimination of cells bearing a chromosome 20q11.21 duplication was demonstrated as well, using BH3 mimetics [169]. Following the differentiation of hPSCs, further steps can be taken to eliminate undifferentiated cells capable of tumor formation. One approach to achieve this goal is to sort residual undifferentiated cells out of the culture based on antibodies that target pluripotent-specific surface markers [170–173]. The use of cytotoxic antibodies, or chemicals that target hPSCs but not differentiated cells, can also be used in that context, to ablate undifferentiated cells without relying on cell sorting techniques [170,174–176].

As a final line of defense, a so-called “suicide” gene can be inserted into cells designated for transplantation, which can be used to selectively eliminate the transplanted cells in case a tumor is formed. This can be done using the herpes simplex virus thymidine kinase (HSV-tk) gene, which confers selective sensitivity

546 towards ganciclovir [177]. Creating a transcriptional link between the suicide gene
547 and a cell-division gene can further improve the system, enabling the specific
548 ablation of cells that are actively dividing [178].

549 As a cautionary note, while all these strategies seem to bear potential, further
550 assessment of their levels of efficacy is warranted before they are applied in
551 clinical contexts.

552

Conclusion

The growing number of clinical trials exploring hPSC-derived cell replacement therapies underscores the immense promise of hPSCs. However, ensuring the safety of these therapies is paramount for their successful clinical translation. While years of investigation have uncovered common genetic and epigenetic aberrations arising during hPSC culture, the full extent of their contribution to tumorigenicity and the risk associated with transplantation of aberrant cells remains unclear. It is thus vital that further efforts be directed towards unraveling the implications related to each aberration.

Nevertheless, the striking similarity between these abnormalities and those found in teratocarcinomas and other cancers, both at the molecular and phenotypic levels, strongly suggests that they warrant careful attention. Routine screening for known aberrations is therefore recommended. Until the implications of such variants are fully understood, we suggest that aberrations with established links to cancer should be monitored prior to cell transplantation.

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Table 1: Recurrent aberrations of hPSCs associated with tumorigenic processes

Category	Type of aberration	Specific aberration	Association with tumorigenicity [Reference]
Genetic aberrations	Aneuploidy	1q gain	[37]
		12p gain	[29–32]
		17q gain	[31]
		X gain	[37]
		10 loss	[37]
		18 loss	[37]
		Y loss	[39]
	CNVs	20q11 gain	[47–50]
	Point mutations	TP53, BCOR, and other cancer related genes	COSMIC database
Epigenetic aberrations	Methylation of specific genes	*TSPYL5*	[84,85]
		CAT	[86]
	Loss of Imprinting	*DLK1*	[156–158]
		IGF2	[159,160]
		KCNQ1OT1	[163]
		PEG10	[161]
		RTL1	[162]
	Erosion of X-chromosome inactivation	Loss of *XIST* expression	[146]
		Elevated dosage of X-linked genes	[144,145]

Figure legend

Figure 1: Type of recurrent genetic and epigenetic aberrations of hPSCs