The tumorigenicity of human embryonic and induced pluripotent stem cells

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Abstract | The unique abilities of human pluripotent stem cells to self-renew and to differentiate into cells of the three germ layers make them an invaluable tool for the future of regenerative medicine. However, the same properties also make them tumorigenic, and therefore hinder their clinical application. Hence, the tumorigenicity of human embryonic stem cells (HESCs) has been extensively studied. Until recently, it was assumed that human induced pluripotent stem cells (HiPSCs) would behave like their embryonic counterparts in respect to their tumorigenicity. However, a rapidly accumulating body of evidence suggests that there are important genetic and epigenetic differences between these two cell types, which seem to influence their tumorigenicity.

Teratoma
Benign tumour that is composed of differentiated tissues from all three germ layers.

Teratocarcinoma
Tumour composed of a mixture of differentiated tissues of the three germ layers. Contains foci of completely undifferentiated cells, called embryonal carcinoma cells, and is highly malignant.

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Human embryonic stem cells (HESCs) have two unique properties: self-renewal, the ability to proliferate indefinitely while maintaining their cellular identity; and pluripotency, the ability to differentiate into all of the cell types that comprise the embryo proper. These traits make HESCs promising for future regenerative medicine, but the same traits also make them tumorigenic, and consequently hinder the fulfilment of their clinical potential. Thus, HESCs could be aptly described as 'double-edged swords', as their defining characteristics make them both powerful and dangerous.

The tumorigenicity of HESCs

HESCs share cellular and molecular phenotypes with tumour cells and cancer cell lines[1–3]. Among these are rapid proliferation rate[4], lack of contact inhibition[5], a propensity for genomic instability[6,7], as well as high activity of telomerase[8], high expression of oncogenes such as *MYC*[9] and *KLF4* (REF. 10), and remarkable similarities in their overall gene expression patterns[11–13], microRNA (miRNA) signatures[14] and epigenetic status[15]. When injected into immunodeficient mice, HESCs form teratomas[5]. These tumours are so characteristic of HESCs that they have become the most stringent test for pluripotency in human cells. Indeed, treatment attempts with embryonic stem cells in animal models were shown to be fatal owing to the formation of teratoma-like tumours[16].

The above-mentioned features apply to normal diploid HESCs in their untransformed state; however, HESCs can also undergo transformation in culture. Several studies have demonstrated that culture-adapted HESCs can form more aggressive tumours, which might be classified as teratocarcinomas, the malignant counterparts of teratomas[17–20].

However, the risk of formation of teratocarcinomas on transplantation of HESC-derived cells is not limited to transformed aneuploid HESCs. Studies have shown that mouse embryonic stem cells (MESCs) with a normal karyotype form teratocarcinomas when injected into immunodeficient mice[21]. Thus, it is possible that transplanting HESCs into humans could result in malignant teratocarcinomas, rather than in benign teratomas. When injected into engrafted human fetal tissues in severe combined immunodeficient (SCID) mice, HESCs indeed generated primitive, undifferentiated tumours[22]. Given the obvious inability to directly test this in humans, this concern remains largely unsolved. However, regardless of the risk of developing malignant tumours, the formation of benign teratomas on transplantation of HESCs or HESC-derived cells into humans would also be highly alarming and unacceptable. Therefore, the tumorigenicity of HESCs is a major hurdle, which must be confronted before achievements of this field of research can be safely translated into the clinic. For recent reviews on the tumorigenicity of HESCs see REFS 2,3,20.

HiPSCs: a new source for pluripotent cells

The discovery of human induced pluripotent stem cells (HiPSCs)[23–25] has revolutionized the field of pluripotent stem cell research for two main reasons: it changed the

At a glance

- Human embryonic stem cells (HESCs) share cellular and molecular phenotypes with tumour cells and cancer cell lines. When injected into immunodeficient mice, HESCs form teratomas. The tumorigenicity of HESCs is a major hurdle, which must be confronted before the achievements from this field of research can be safely translated into the clinic.
- Sharing with HESCs their basic properties of self-renewal and pluripotency, human induced pluripotent stem cells (HiPSCs) also share their tumorigenic traits. However, HESCs and HiPSCs are not identical, and a rapidly accumulating body of work suggests considerable differences between these two pluripotent cell types.
- The transcription factors commonly used for reprogramming somatic cells into HiPSCs (*OCT4*, *SOX2*, *MYC* and krupple-like factor 4 (*KLF4*)) are highly expressed in various types of cancer. HiPSCs are commonly derived using integrating vectors, thus creating a risk for genetic alterations and for reactivation of the reprogramming factors at later stages.
- HiPSCs can acquire chromosomal aberrations, even more readily than HESCs. These can result from their somatic cells of origin, reprogramming stress and during culture adaptation. Aneuploidy of pluripotent stem cells has been suggested to increase their tumorigenicity.
- Epigenetic differences between HESCs and HiPSCs also affect their tumorigenicity. The reprogramming process is often accompanied by epigenetic alterations. The epigenetic 'memory' of the cells might also contribute to their tumorigenicity.
- Self-renewal is important for the tumorigenic traits of HESCs and HiPSCs, and cell cycle-related genes are crucial for an efficient reprogramming process. These genes are also involved in the genomic instability that characterizes pluripotent cells.
- Owing to genetic and epigenetic causes, HiPSCs are more tumorigenic than HESCs, and harbour a risk for the development of teratocarcinomas and possibly somatic tumours.
- In order to develop safe HESC- and HiPSC-based treatments, the tumorigenicity hurdle must be overcome. Three general strategies to cope with this risk have been suggested: terminal differentiation or complete elimination of residual pluripotent stem cells from culture; interfering with tumour-progression genes to prevent tumour formation from the residual pluripotent cells; and tumour detection and elimination after its initial formation in the patient's body.

perception of cellular reprogramming, showing that the plasticity of somatic cells is much greater than had previously been thought; and it offered an appealing solution to the likely immune rejection of HESC-derived cells on their transplantation into an unmatched patient, thus providing new and exciting avenues for patient-specific cell therapy. Importantly, HiPSCs also provide a possible solution to the ethical objections that have been raised against the use of HESCs, which is a highly controversial topic in many countries.

Although constituting a huge leap towards overcoming immunogenic and ethical obstacles, the translation of HiPSCs into the clinic faces the same substantial tumorigenicity problem as that of HESCs. Sharing with HESCs their basic properties of self-renewal and pluripotency, HiPSCs are doomed to share with them the other 'edge of the sword'. Indeed, HiPSCs exhibit the cellular and molecular phenotypes that make HESCs resemble cancer cells (discussed above), and form benign teratomas on injection into immunodeficient mice. However, HESCs and HiPSCs (as well as their mouse counterparts) are not identical, and rapidly accumulating evidence suggests that there are considerable differences between these two pluripotent cell types, including important aspects such as global gene expression[26], epigenetic landscape[27–29] and genomic imprinting[30]. In fact, HiPSCs are relevant for research into the tumorigenicity of pluripotent cells, as the process of cellular reprogramming of somatic cells into pluripotent cells can teach us much about the tumorigenicity of human pluripotent cells in general. As these cells are the newest and most promising type of pluripotent cells, it is crucial to specifically study the tumorigenicity of HiPSCs and to compare it to the tumorigenicity of HESCs, which are the 'gold standard' for human pluripotent cells.

Genomic imprinting
The expression of specific genes from either the maternal or the paternal allele.

CpG island shore
DNA sequence that flanks CpG islands.

Reprogramming genes and HiPSC tumorigenicity

HiPSCs were first derived by the transduction of fibroblasts with integrating viruses carrying four transcription factors: *OCT4*, *SOX2*, *MYC* and krupple-like factor 4 (*KLF4*)[24]. Although *MYC* is a well-established oncogene[31,32], the other three transcription factors are also known to be highly expressed in various types of cancer[33–40]. Indeed, one study found significant overexpression of at least one of these reprogramming genes in 18 of the 40 cancer types investigated[41]. Furthermore, in specific types of tumours, these genes were found to be associated with tumour progression and a poor prognosis[41]. Reactivation of the reprogramming factors has also been shown to predispose iPSCs to genomic instability[42]. Recently, new methods have been developed to reprogramme human somatic cells without *MYC*[25,43] or with a transformation-deficient *MYC*[44], and by combining only some of the reprogramming transcription factors with chemical inhibitors[45–48]. However, the fact remains that *OCT4*, *SOX2*, *MYC* and *KLF4* reside at the heart of the reprogramming process, stressing the potential risks of these new pluripotent cells.

Further evidence for the similarity between the reprogramming of somatic cells to iPSCs and tumorigenesis was provided in a series of papers that demonstrated how downregulation of tumour suppressors in the p53 pathway increases the efficiency of the reprogramming process and enables reprogramming with only two factors (*OCT4* and *SOX2*)[49–53] (reviewed in REF. 54). A recent report found that, in respect to their overall miRNA signature, the expression levels of miRNAs that belong to the p53 network make some HiPSC lines (and partially reprogrammed cells) more similar to cancer cell lines[14]. In addition, as telomerase activity is essential for efficient reprogramming, HiPSCs develop longer telomeres and acquire the epigenetic marks of undifferentiated cells[55,56], similar to the transformation of cancer cells[57]. Importantly, however, cellular reprogramming is not equivalent to cancerous transformation. A recent whole genome-wide study of tissue- and cancer-specific methylation of CpG island shores showed an inverse correlation between HiPSCs and cancer cells[58]. This suggests that the changes in methylation marks at CpG island shores are differentially regulated in HiPSCs and cancer cells[58].

Another rather obvious tumorigenic risk in HiPSCs is the use of integrating vectors for their derivation. Although it has been demonstrated that the viral integration is not directly linked to the reprogramming process[59], the genomic alterations created, together with the risk of reactivation of the introduced transgenes during the propagation of the undifferentiated cells or on

their differentiation, pose a considerable threat that prevents any clinical use of such genetically altered cells. Therefore, a considerable amount of effort has recently been put into producing virus-free and integration-free mouse and human iPSCs using non-integrating adenoviruses[60], expression plasmids[61,62], episomal vectors[63], piggyBac transposition[64], Cre-recombinase excisable viruses[65], direct delivery of reprogramming proteins[66,67] and synthetic modified mRNAs[68]. This rapid improvement in the technologies for the derivation of HiPSCs is expected to reduce their tumorigenicity and improve their safety, but a completely safe, simple and efficient reprogramming method has yet to be developed.

Aneuploidy in HESCs and HiPSCs

The viral integration and the reactivation of the reprogramming factors are not the only possible sources for genomic alterations in HiPSCs. Unlike HESCs, HiPSCs are derived from mature somatic cells that have undergone multiple cell divisions and have lived long enough to acquire genetic mutations. These mutations are assumed to be random and rare, but if they confer proliferative or anti-apoptotic advantages to the cells that carry them, they might be selected for during the reprogramming process, which is an inefficient process that results in HiPSCs with an abnormal genetic composition. In addition, the reprogramming process is likely to engage stress response pathways in the cells, and this might encourage the accumulation of genetic changes in the reprogrammed cells, regardless of the reprogramming method used. Although most of these genetic changes are expected to be selected against, and would therefore disappear on the first few cell divisions in culture, some might be advantageous for the cells and would therefore prevail. A recent study has provided evidence for such chromosomal aberrations, resulting either from the somatic cells of origin or from reprogramming stress, leading to HiPSCs with aberrant karyotypes at early passages[69] (FIG. 1).

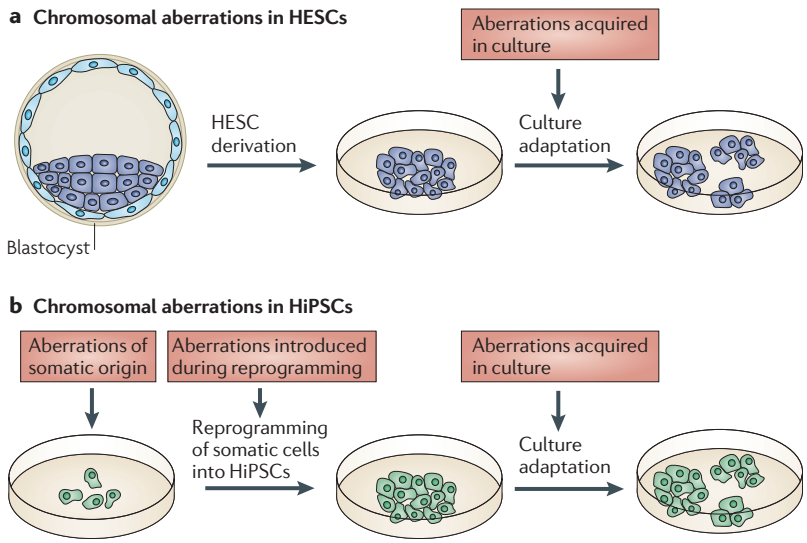

Figure 1 | **Source of chromosomal aberrations in pluripotent stem cells.** Human embryonic stem cells (HESCs) and human induced pluripotent stem cells (HiPSCs) are subjected to different selection pressures and thus might exhibit different types of chromosomal aberrations at different rates. **a** | HESCs are derived from the inner cell mass of blastocysts and mostly acquire chromosomal aberrations on prolonged growth in culture. **b** | HiPSCs are derived from somatic cells that have undergone more cell divisions and are thus more likely to be genetically abnormal. Some of these aberrations might be selected for during the reprogramming process. The reprogramming process itself is stressful for the cells and might introduce novel chromosomal aberrations, as well as select for existing ones. Finally, HiPSCs also acquire chromosomal aberrations during their prolonged passaging, and the techniques used for their culturing often differ from those used for the growth of HESCs *in vitro*.

Much like HESCs, HiPSCs can be cultured *in vitro* for long periods, and are therefore susceptible to acquiring chromosomal aberrations, in what has become known as culture adaptation (FIG. 1). Chromosomal aberrations in HESCs are not acquired randomly during culture adaptation, as some are much more frequent than others. The most common aberrations detected are gains of chromosomes 12 (especially 12p)[6,70], 17 (in particular 17q)[6,70,71], 20 (most often 20q11.21)[19,71–73] and X[6,74]. Interestingly, duplications of chromosomes 12, 17 and X are hallmarks of germ cell tumours and embryonal carcinoma cells[6,20], and the 20q11.21 region is also amplified in various cancers[75]. Culture-adapted HESCs and HiPSCs often exhibit increased proliferation capacity and decreased growth factor dependence, and they often form more aggressive malignant tumours when injected into mice[17–20]. Genes that reside in the recurrent aberrant regions have been suggested to contribute to the selective advantage that allows the aberrant cells to survive and take over the culture[6,75,76], but until recently no evidence had been provided for the role of any specific genes in this process.

HiPSCs were assumed to be similar to HESCs in regard to culture adaptation, but this issue had not been comprehensively tested until recently. HiPSCs are always karyotyped after their initial derivation, as part of the required characterization for new pluripotent cell lines, but their genomic stability after prolonged culture had not been systematically examined. One attempt to detect subkaryotypic alterations that were associated with culture adaptation of three HiPSC lines resulted in the suggestion that the genome of reprogrammed cells is both normal and highly stable even after at least 40 passages[26].

However, we have recently conducted the first large-scale analysis of aneuploidy in HiPSCs, inferring the chromosomal structure of dozens of HiPSC lines from their respective gene expression profiles[69]. Our study demonstrated the duplication of chromosome 12 to be the most common aberration in HiPSCs, and showed that this trisomy rapidly takes over the population. It also revealed that trisomy 12 is accompanied by overexpression of the important pluripotency factors *NANOG* and growth/differentiation factor 3 (*GDF3*), providing the first direct evidence for the involvement of specific genes in the process of culture adaptation[69]. It remains to be explored whether trisomy 12 also increases the tumorigenicity of HiPSCs *in vivo* (this question is still not resolved in HESCs[77,78]), and whether *NANOG* and *GDF3* have any role in this.

The genomic stability of HESCs is affected by *in vitro* environmental conditions, such as feeder cells, the make up of the culture medium, and the techniques

used for cell passaging, freezing and thawing (reviewed in REF. 75). Given the fact that HiPSCs are often more difficult to culture than HESCs, many laboratories implement slightly to moderately different techniques for the culture of HESCs and HiPSCs. This could influence the genomic instability of these cells and the selection pressures they are subjected to, further contributing to the potential difference in their tumorigenicity.

It is important to bear in mind that the genetic changes that occur during the derivation of HiPSC lines or during their adaptation in culture are not necessarily limited to noticeable aneuploidies. Gains or losses of small chromosomal regions that cannot be detected using cytogenetic analyses, as well as point mutations that cannot be detected by more sensitive DNA-based methods (such as comparative genome hybridization and copy number variation arrays), probably also occur. Indeed, a recent study demonstrated a high frequency of subchromosomal gains and deletions in HESCs and HiPSCs, and showed that deleted regions often contain tumour-suppressor genes, and that regions of chromosomal gain contain oncogenes[79]. Therefore, these minor aberrations could have major functional implications for the tumorigenicity of the HiPSCs. For example, a point mutation that increases the proliferation of the cells, that confers them with resistance to apoptosis or that hinders their differentiation capacity, might result in more aggressive teratomas. Indeed, the levels of expression of tumour-suppressor genes have been found to affect teratoma growth[80]. As advanced technologies, such as whole-genome sequencing, become more accessible, we predict that many such aberrations in HiPSCs will be revealed. The causes for genetic instability in HiPSCs, and potential ways to minimize it, have recently been summarized[81].

Epigenetic factors and HiPSC tumorigenicity

Differences in tumorigenicity between HESCs and HiPSCs do not necessarily derive solely from genetic differences, but might also be the consequence of epigenetic differences. A debate has recently taken place regarding the claims that HiPSCs and HESCs systematically differ in their global gene expression patterns and that extended culture brings them transcriptionally closer[26,82–84]. Such epigenetic differences may be relevant to the evaluation of the tumorigenicity of HiPSCs. First, these differentially expressed genes might include oncogenes and tumour suppressors that directly affect the tumorigenicity of the cells. Second, if HiPSCs become transcriptionally closer to HESCs during extended culture this would create an interesting dilemma: on the one hand, clinical use of HiPSCs would require them to be as transcriptionally similar to HESCs as possible; on the other hand, extended culture often leads to chromosomal aberrations (as described above), and thus might increase the tumorigenicity of the cells.

Consistent with the idea of differences in gene expression between HESCs and HiPSCs, a recent study compared the miRNA expression of two HiPSCs with the miRNA expression of four HESC lines, and identified ten cancer-related miRNAs that are overexpressed (> tenfold difference) in HiPSCs[85]. Another study has recently reported cancer-related epigenetic abnormalities, such as alterations in cancer-specific gene promoter DNA methylation, that arise early during reprogramming and that persist in HiPSCs[86]. Thus, accumulating data suggest that the reprogramming process is often accompanied not only by genetic abnormalities, but also by epigenetic alterations, which are expected to increase the tumorigenicity of HiPSCs.

In line with the claim that early passage HiPSCs display unique transcriptional programmes that are attenuated on continuous passaging, recent reports have shown that iPSCs retain epigenetic memory of their cells of origin, both in mouse[28,29] and in human[27,87,88]. This epigenetic memory manifests as differential gene expression and altered differentiation capacity, but it would be interesting to further explore whether it also affects the tumorigenicity of the cells, owing to one of the following possibilities: memory of cellular epigenetic transformations (that is, a direct effect on tumorigenicity) and/or memory of epigenetic characteristics that make some types of cells more vulnerable to transformation (that is, an indirect effect on tumorigenicity). The memory of cellular epigenetic transformations has already been demonstrated in a study that carried out a large-scale methylation profile of somatic donor cells and identified aberrant methylation at hundreds of sites, only some of which were reversed following reprogramming[89]. Such epigenetic memory might result in tumorigenic differences between HiPSC lines from different somatic sources. Interestingly, in mice the epigenetic memory is gradually lost during extended culture of iPSCs or during further cycles of reprogramming [28,29], and if this finding also applies to HiPSCs, it might contribute to tumorigenic differences between early and late-passage cells of the same HiPSC line.

Another important source for potential epigenetic differences between HESCs and HiPSCs is the status of genomic imprinting. Aberrant imprinting is associated with neoplasia and is evident in some types of human cancer (reviewed in REFS 90,91). Aberrant silencing or activation of imprinted genes during the reprogramming process might have implications for both their differentiation capacity and their tumorigenicity. Recent studies found variability in the expression of imprinted genes among different lines of both mouse and human iPSCs[30,92]. Although HESCs exhibit fairly stable genomic imprinting at early passages[93–95], some HiPSCs show aberrant expression of imprinted genes[30]. Furthermore, the expression state of a single imprinted gene cluster was recently shown to affect the developmental potential of mouse iPSCs and to distinguish them from MESCs[92]. Thus, it is reasonable to assume that aberrant expression of imprinted genes would also affect the tumorigenicity of some HiPSC lines.

Aberrant imprinting might also occur during the growth of pluripotent stem cells in culture, and its acquisition might therefore be defined as an epigenetic cellular adaptation. It was found that *in vitro* culture over extended periods affects the integrity of imprinted gene expression in HESCs[93,96], and it was suggested that

culturing practices result in changes of DNA methylation at differentially methylated regions (DMRs)[96]. Imprinting aberrations are also likely to occur as a result of similar culture techniques used during the prolonged culture of HiPSCs. As the imprinting in HiPSCs is less stable initially, one might anticipate that these aberrations would be even more substantial in these cells. However, the nature and severity of acquired imprinting aberrations in HiPSCs remain to be studied.

Cell cycle regulation in HESCs and HiPSCs

The remarkable self-renewal capabilities of human pluripotent stem cells require a unique cell cycle regulation, which allows them to divide independently of extrinsic mitogenic signals. HESCs and HiPSCs are characterized by abbreviated gap phases, and a high proportion of cells in the S phase and the M phase of the cell cycle[97]. The role of cell cycle regulation in pluripotency and reprogramming has been reviewed elsewhere[98].

The structure and regulation of the cell cycle influence the tumorigenicity of HESCs and HiPSCs, as self-renewal is a determinant for their tumorigenic potential. Although many of the genes that regulate pluripotency and the cell cycle are involved in maintaining the self-renewal abilities of these cells[26,99], MYC activity has been specifically shown to account for many of their cell cycle properties[98]. Other important cell cycle regulators, such as p53 and cyclin D1, were also found to be intrinsically involved in the reprogramming process[100].

Cell cycle regulation probably has an important role in the propensity of HESCs and HiPSCs to acquire chromosomal aberrations. A recent report linked the unique cell cycle of HESCs to numerical centrosomal abnormalities during mitosis[101], which might account, at least partly, for their enhanced chromosomal instability, and thus increase their tumorigenicity. Supporting this idea, another recent report found that frequently aberrant chromosomal regions in HiPSCs are enriched for cell cycle-related genes[69].

To date, no report has described the differences in cell cycle regulation between HESCs and HiPSCs, and HiPSCs have been reported to acquire the distinct cell cycle properties of HESCs during reprogramming[97]. However, the risk for reactivation of the reprogramming factors (especially *MYC*), as well as the risk for acquiring genetic abnormalities early on during propagation in culture, might make HiPSCs more vulnerable for cell cycle aberrations. Further studies are needed to determine whether such differences in cell cycle regulation sometimes exist, and whether these potential differences affect the tumorigenicity of the cells.

HiPSC tumorigenicity: are the concerns relevant?

The use of potent oncogenes for the process of reprogramming, the involvement of the p53 pathway as a barrier against this process and the epigenetic memory of HiPSCs, all raise a possible risk that probably does not exist in HESCs: namely, the risk for the development of somatic tumours (rather than the development of teratomas or teratocarcinomas). Indeed, the original generation of germline-competent mouse iPSCs resulted in various types of tumours owing to the reactivation of *Myc*[102], and many of the four-gene iPSC mice chimaeras died from cancer within the first few months of their lives[103]. Therefore, the risk for the formation of somatic tumours is not merely hypothetical.

As mentioned above, although HESCs are subjected to selection in culture, which often results in genomic instability, HiPSCs are not only subjected to the same selection pressure but also to additional selection pressures during the reprogramming process. These stronger selection pressures might increase chromosomal instability (as is evident from the appearance of chromosomal aberrations early on during their growth in culture[69]) and result in the formation of more aggressive teratomas, or even teratocarcinomas. In agreement, some reprogrammed lines fail to differentiate and form undifferentiated tumours on injection into immunodeficient mice[104]. The potential differences in the tumorigenicity of HESCs and HiPSCs, and their possible consequences for tumour formation, are summarized in TABLE 1 and shown in FIG. 2.

A study of the tumorigenicity of mouse iPSCs has recently been published[105]. The authors compared iPSCs from different origins (mouse embryonic fibroblasts (MEFs) and several adult tissues), with or without *Myc* retroviral transduction, and with or without drug selection for the expression of pluripotency genes (*Nanog* and *Fbxo15*). These various iPSCs were also differentiated into secondary neurospheres (SNS) and were examined for their teratoma-forming propensity after injection into the brains of non-obese diabetic-SCID mice. Although the teratoma-formation capability of SNS from MEF-derived iPSCs was similar to that of SNS from embryonic stem cells, SNS from iPSCs that were derived from different adult tissues varied substantially in their teratoma-forming propensity, and some of them formed more aggressive, undifferentiated teratocarcinomas. The aggressiveness of the tumours was found to correlate with the number of residual pluripotent stem cells in the SNS. Surprisingly, the use of the *Myc* retrovirus and the presence of drug selection were not found to affect the teratoma-formation capability of the iPSC-derived neurospheres[105]. Although these results highlight the potential differences in the tumorigenicity of iPSCs from different somatic sources, consistent with the epigenetic memory studies described above, the genetic composition of the iPSCs that were used in this study has not been reported. Therefore, the difference in tumorigenicity between the different lines might be a consequence of chromosomal aberrations, rather than of the tissue of origin.

A study[80] that compared the tumorigenicity of retrovirally derived and transgene-free HiPSCs observed no substantial tumorigenic differences between the two groups, as judged by blood microvessel density (MVD) within the teratomas that formed[80,106]. Interestingly, a correlation was evident between the MVD and the level of expression of p21 and p53 in the HiPSC lines. These results again suggest that the p53 pathway and the

Table 1 | **Comparison of the tumorigenicity between HESCs and HiPSCs**

Factors influencing tumorigenicity	HESCs	HiPSCs
Genetic abnormalities		
Cell of origin	ICM cells that have undergone very few divisions*	• Mature somatic cells that have undergone many cell divisions and have been more exposed to genetic and environmental mutations‡ • Might result in mutations and/or aberrations of somatic origin‡
Derivation process	A relatively minor selection pressure*	• A major selection pressure owing to forced drastic change of epigenetic landscape‡ • Might result in mutations and/or aberrations owing to reprogramming stress‡
Viral integration	Not applicable	Most of the current methods still use viral vectors for reprogramming‡
Activation of oncogenes	Not applicable	Current methods upregulate oncogenes in the reprogramming process‡
Cellular adaptation to culture	Prolonged growth in culture often results in gains of chromosomes 12, 17, 20 and X‡	Prolonged growth in culture often results in gains of chromosome 12‡
Epigenetic abnormalities		
Cell of origin	Similarity of global gene expression with some cancers (onco-fetal genes are highly expressed)*	• Similarity of global gene expression with some cancers (onco-fetal genes are highly expressed)‡ • Epigenetic memory of somatic transformations and/or of susceptible traits of the somatic tissue‡
Derivation process	No substantial epigenetic aberrations are known to occur in the process*	• Cancer-related epigenetic abnormalities arise during reprogramming‡ • Relaxation of imprinting might also occur in the process‡
Cellular adaptation to culture	Relaxation of imprinting might occur in culture*	Relaxation of imprinting might occur in culture*

HESCs, human embryonic stem cells; HiPSCs, human induced pluripotent stem cells; ICM, inner cell mass. *Medium risk of tumour generation. ‡High risk of tumour generation.

reprogramming process are linked, and that this pathway might affect the aggressiveness of HiPSC-derived teratomas[80].

More recently, direct comparisons of teratoma formation by HESCs and HiPSCs have been reported[86,107]. One study found that HiPSCs develop teratomas more efficiently and faster than HESCs, regardless of the site of injection. No differences in the composition of the teratomas were observed[107]. Although a karyotype analysis was conducted in this study, ruling out major chromosomal aberration as an explanation of the observed differences, smaller genomic aberrations that can be detected only by using higher-resolution methods, could still account for these results. Furthermore, the comparison of teratoma composition was carried out by staining for the three germ layers only, thus not precluding the possibility that HiPSC-derived teratomas contain undifferentiated foci and are more aggressive than HESC-derived teratomas. Indeed, a second study that compared teratomas that were formed by HiPSC lines with a normal karyotype with teratomas that were formed by HESC lines reported that all of the HiPSC-derived teratomas examined, although none of the HESC-derived teratomas, contained malignant characteristics (such as, focal necrosis, nuclear polymorphism, high mitotic rates and infiltration into the mouse musculature)[86]. A more comprehensive, higher-resolution comparison of teratoma formation by HESCs and HiPSCs is therefore warranted.

Coping with the tumorigenicity risks

In order to develop safe HESC- and HiPSC-derived treatments, the tumorigenicity hurdle must be overcome, and much research has been dedicated in recent years to this purpose. The various strategies that might cope with the tumorigenicity risk have been reviewed elsewhere[2,3,108], and can generally be divided into three categories[3]: terminal differentiation or complete elimination of residual pluripotent stem cells from culture; interfering with tumour-progression genes to prevent tumour formation from the residual pluripotent cells; and tumour detection and elimination on formation in the patient's body.

In view of reports suggesting that as few as several hundred pluripotent cells are sufficient to generate tumours[109,110], there is no doubt that the safest HESC- and HiPSC-derived treatments would require a 100% pure population of differentiated cells, and this can be achieved either by complete differentiation or by complete ablation of undifferentiated cells from mixed populations. Various attempts to achieve this goal have been reported[108,111], with the most recent advances being the targeted elimination of pluripotent cells by cytotoxic antibodies[112,113], and the separation of undifferentiated HESCs from a heterogeneous cell population on the basis of pluripotent-specific cell surface molecules, using magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS)[114]. However, none of the current methods results

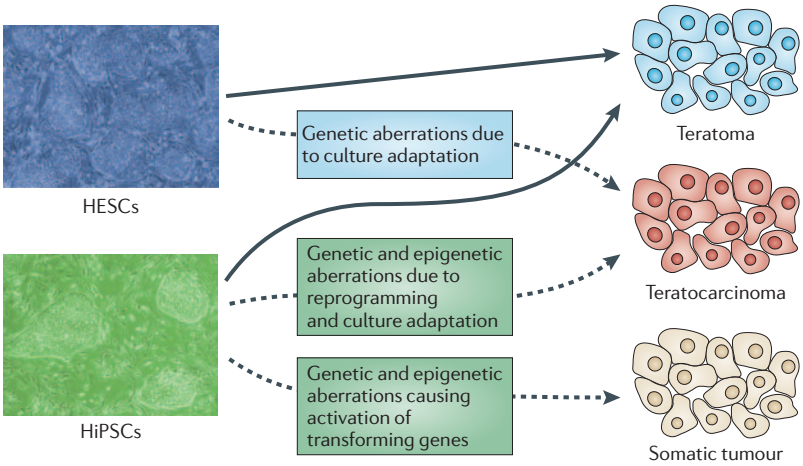

Figure 2 | **Tumour formation in pluripotent stem cells.** Residual diploid human embryonic stem cells (HESCs) that have not undergone differentiation are expected to form benign teratomas when transplanted into human patients. Although some aneuploid cells are also expected to form benign teratomas, other aneuploid cells might undergo transformation and form malignant teratocarcinomas. Residual diploid human induced pluripotent stem cells (HiPSCs), which are similar HESCs, are also expected to result in benign teratomas. However, some diploid HiPSCs that appear to be genetically normal might form teratocarcinomas owing to epigenetic transformation. This epigenetic transformation might result from epigenetic memory (tissue-of-origin traits and epigenetic alterations of somatic origin) and/or from the reprogramming process itself (such as the reactivation of oncogenes involved in reprogramming and epigenetic alterations introduced during reprogramming). Aneuploid HiPSCs might also form teratocarcinomas and genetic aberrations might occur earlier than in HESCs owing to somatic mutations, reprogramming stress, viral integration and/or culture adaptation. Unlike HESCs, diploid and aneuploid HiPSCs also harbour a risk for the formation of somatic tumours, owing to genetic and epigenetic transformation that is acquired in the somatic tissue of origin or during reprogramming. Dashed arrows indicate possible outcomes; solid arrows indicate definite outcomes. The image of HESCs is adapted from REF. 126.

in a 100% pure differentiated culture, so that complete elimination of all differentiated cells from a mixed culture might require more robust measures.

Given the current limited ability to produce a pure population of differentiated HESC or HiPSC progeny, an alternative method could be to interfere with genes that are important for teratoma formation, but are dispensable for mature tissues[20]. It was recently found that the expression of survivin (encoded by *BIRC5*), a classical onco-fetal gene with anti-apoptotic activity, is enriched in undifferentiated HESCs and their derived teratomas[115]. The ablation of survivin expression, both genetically and pharmacologically, stimulated apoptosis in cultured HESCs and in their teratomas[115]. Thus, the discovery of more teratoma-associated genes and ways to molecularly target them is a promising strategy to cope with the tumorigenicity of these cells.

A similar approach would be to interfere with genes that are essential for self-renewal in human pluripotent stem cells, thus forcing them to differentiate and decreasing their tumorigenic potential. The most obvious candidate for such an approach would be endogenous *MYC*, as several studies have recently shown that MYC represses differentiation and maintains the self-renewal of mouse and human pluripotent stem cells[116,117] (reviewed in REF. 118). The potential of *MYC* ablation for teratoma prevention seems even more promising in view of a recent report that MYC regulatory networks account for most of the transcriptional similarity between embryonic stem cells and cancer cells[119]. However, interference with *MYC* or with other pluripotency genes will inevitably compromise the pluripotency and the self-renewal of the cells, and will thus need to be restricted to the end point-differentiated cultures, in which the residual pluripotent stem cells are undesired.

The third, albeit problematic, strategy to cope with the tumorigenicity of pluripotent cells is to detect and attack the tumours once their formation has already been initiated. The genetic introduction of 'suicide' genes into HESCs is one potential way to eliminate teratomas after their formation, using specific drugs[120]. Another previously suggested idea, limited to specific diseases such as diabetes, is to make these tumours irrelevant by encapsulating the HESC-derived grafts, thus enabling their safe removal and preventing them from spreading through the patient's body[121–123]. These techniques provide another layer of defence against potential tumours, but as they do not prevent the tumour formation itself, they are doomed to remain complementary, rather than primary, coping strategies.

In principle, the same coping strategies that apply for HESCs should also apply for HiPSCs. However, the differences that might exist between HESCs and HiPSCs in their global gene expression pattern and epigenetic landscape require that every specific separation, purification or interference method described above needs to be tested on HiPSCs in order to confirm their relevance for HiPSC-based therapies. This validation is of the utmost importance given the risk that HiPSCs might generate somatic tumours. Intervening with teratoma-specific genes, for example, will not necessarily prevent the formation of somatic tumours by residual HiPSCs, as different molecular mechanisms underlie the development of different kinds of tumours.

The potential increased aggressiveness of HiPSCs, which might result in the formation of somatic tumours, aggressive teratomas or teratocarcinomas, requires the implementation of strict safety measures. Constant genetic and epigenetic safety estimations need to be routinely carried out if these cells are to be used in the clinic. In order to monitor for genetic transformations that might be associated with the reprogramming process or with the culture of HiPSCs, more sensitive measures will be needed. Although integration-, viral- and oncogene-free reprogramming is expected to decrease genetic abnormalities, a routine analysis of the DNA content of HiPSCs will nonetheless be required. High-resolution DNA analysis of regions that are prone to aberrations could complement the standard karyotype analysis; whole-genome sequencing of the cell lines might also be required in some cases. To monitor for epigenetic transformations that might arise in HiPSCs, it will be important to verify the loss of undesired epigenetic memory, as well as the normal expression levels of the imprinted genes and of the known oncogenes. This can be achieved by global and specific gene expression analyses, complemented by an analysis of epigenetic markers, such as DNA methylation, on susceptible genes.

The concerns and possibilities raised by the generation and use of HiPSCs will eventually require further clarification if optimal HiPSCs are to be defined. For example, if prolonged growth in culture results in HiPSCs acquiring chromosomal aberrations[69], but also results in an increasing similarity to their HESC counterparts[26,82] and the loss of the markers of their source of origin[28,29], a need to determine the desirable 'duration-in-culture' of HiPSCs arises. In order to do this, there is a need for systematic research that would define the optimal time-point (if such a point even exists) when the similarity to HESCs is already maximal but the epigenetic memory and the tumorigenicity are minimal. Alternatively, improving the methods of HiPSC derivation in ways that more firmly erase the epigenetic memory of the cells would help reconcile these conflicting tumorigenic forces.

Moreover, it might be that the most potent HiPSCs — the ones that appear in culture first, grow the fastest and/or resemble HESCs the most — are not the most suitable candidates for therapeutic purposes. Partially reprogrammed cells, or fully reprogrammed cells that grow more slowly in culture, might be better for clinical application (as long as they can be differentiated into the required cell type). In addition, HiPSCs produced from embryonic tissues, such as cord blood, may minimize the acquisition of genetic and epigenetic mutations, and thus may be safer than HiPSCs that are derived from adult somatic cells[124]. This might also be true for HiPSCs that are derived from stem cells. These cells have been reported to require the transduction of only one (for neural stem cells[125]) or two (for cord blood stem cells[124]) reprogramming factors, and consequently decrease the risk for the reactivation of these oncogenes. Last, differences in the genetic backgrounds and epigenetic signatures between individual HiPSC lines might make specific lines more tumorigenic than others, regardless of their cellular origin, derivation process and cell culture conditions. The tumorigenicity of the cells is therefore expected to have a more central role in defining the optimal HiPSC lines for regenerative medicine.

Future perspectives

Although much effort has been put into trying to generate integration- and virus-free HiPSCs, and into developing new and improved coping strategies with the tumorigenicity of pluripotent stem cells, most of the mechanistic biological research in the field currently focuses on the generation of HiPSCs that are as similar as possible to HESCs, and on studying the emerging differences between these two cell types. As the reprogramming field matures and moves towards the clinic, more biological research on the tumorigenicity of HiPSCs, its sources and its consequences, should be forthcoming. Once we better understand the biolgy of cellular reprogramming and the biological differences between HESCs and HiPSCs, we should be able to generate HiPSCs that are only functionally similar to HESCs but are less tumorigenic and therefore safer for clinical application.

Although the data reviewed here might suggest that HiPSCs are more tumorigenic than HESCs, their somatic origin might in fact turn out to be advantageous in this regard. If we succeed in reprogramming cells so that they acquire only desirable aspects of cellular potency and can differentiate *in vitro* into functional cells of the type required, but at the same time lack the ability to form tumours *in vivo*, this might enable us to disentangle the Gordian knot that links pluripotency and tumorigenicity. As our control over the epigenetic landscape of the cell increases, this dream might not be as far from realization as it was just a few years ago.

Note added in proof

While this article was in the press, three important articles that further stress the genetic and epigenetic differences between human embryonic stem cells (HESCs) and human induced pluripotent stem cells (HiPSCs) were published. Using high-resolution genetic approaches, two of these papers reported copy number variations (CNVs)[127] and protein-coding point mutations[128] that arise during the reprogramming process. The point mutations were found to be enriched in cancer-related genes[128]. In the third paper, a whole-genome profiling of DNA methylation was carried out at single-base resolution, revealing somatic epigenetic memory and aberrant reprogramming of DNA methylation in HiPSCs[129]. Together, these papers suggest genetic and epigenetic abnormalities that distinguish HiPSCs from HESCs, and strongly support the idea, which was raised in this Review, that HiPSCs are likely to be more tumorigenic than HESCs.

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The authors declare no competing financial interests.